Bioprinting of Human Neural Tissues Using a Sustainable Marine Tunicate-Derived Bioink for Translational Medicine Applications

Soja Saghar Soman¹, Mano Govindraj¹, Noura Al Hashimi¹, Jiarui Zhou¹,², Sanjairaj Vijayavenkataraman¹,²*

¹The Vijay Lab, Division of Engineering, New York University Abu Dhabi, Abu Dhabi, UAE
²Department of Mechanical and Aerospace Engineering, Tandon School of Engineering, New York University, Brooklyn, USA

Abstract: Bioprinting of nervous tissue is a major challenge in the bioprinting field due to its soft consistency and complex architecture. The first step in efficient neural bioprinting is the design and optimization of printable bioinks which favor the growth and differentiation of neural tissues by providing the mechanophysiological properties of the native tissue microenvironment. However, till date, limited studies have been conducted to make tissue specific bioinks. Here, we report a novel bioink formulation specifically designed for bioprinting and differentiation of neural stem cells (NSCs) to peripheral neurons, using a marine tunicate-derived hydrogel and Matrigel. The formulation resulted in seamless bioprinting of NSCs with minimal processing time from bioink preparation to in vitro culture. The tissues exhibited excellent post-printing viability and cell proliferation along with a precise peripheral nerve morphology on in vitro differentiation. The cultured tissues showed significant cell recovery after subjecting to a freeze-thaw cycle of −80 to 37°C, indicating the suitability of the method for developing tissues compatible for long-term storage and transportation for clinical use. The study provides a robust method to use a sustainable bioink for three-dimensional bioprinting of neural tissues for translational medicine applications.

Keywords: 3D bioprinting; Neural stem cells; Peripheral neurons; Sustainable bioink; Extrusion bioprinting

1. Introduction

Our understanding of the nervous system disorders and its therapeutic developments majorly depends on the animal models and two-dimensional cell culture systems. Most of these traditional models cannot address the questions that pertain to species variations, sensitivity, and complexity of the human nervous system. These limitations demand a more realistic in vitro human model to study the nervous system. Biomaterials engineering, three-dimensional (3D) biofabrication, and stem cell technology can help design innovative tissue systems that can be used to model the physiology and pathobiology of human nervous system. Peripheral nervous system is a complex network of elongated nerves running throughout the body. Injury to peripheral nerves is a very common neurological disease that is generally caused by direct mechanical trauma or degeneration. The self-repairing ability of peripheral nerves is limited and nerve injury can lead to life-long disability. 3D bioprinting of peripheral nerves is a promising technology to engineer peripheral nerve tissues for treatment as well as disease modeling. Stem cell technology combined with bioprinting offer important tools to make viable peripheral nerve conduits and nerve tissues[1-3].

3D bioprinting requires the use of biocompatible bioinks, which must be optimized to favor the differentiation and growth of specific cell types for the formation of target tissues. The viscoelastic properties of the bioink can be tuned for printing specific tissue types...
as well as to support specific cell populations\textsuperscript{[4]}. Induced pluripotent stem cells (iPSCs) and iPSC-derived stem cells are important cell sources for tissue bioprinting, as these cells can be differentiated to cells of choice when cultured in specific media. The printed tissue can be used for regenerative medicine applications to make tissue transplants such as peripheral nerve conduits, brain patches, and for neurodegenerative disease modeling\textsuperscript{[5]}. Specific genetic line iPSCs derived from patients, are a powerful tool to study diseases such as Parkinson’s disease, Alzheimer’s disease, and cancer. In the 3D bioprinting field, it has been presumed that the soft tissues such as brain and nerves require much optimization as they are difficult to bioprint, compared to the hard tissues, due to the finer variations in the viscoelastic properties of the hydrogels. Many recent research papers have reported the necessary conditions for 3D bioprinting neural tissues using soft hydrogel-based bioinks\textsuperscript{[6]}. Researchers successfully bioprinted brain-mimicking tissues using primary cortical neurons mixed in a gellan gum-based bioink modified with the RGD peptide\textsuperscript{[7]}. A recent work attempted to bioprint a model spinal cord using human iPSC-derived neural stem cells (NSCs) suspended in an alginate-based bioink\textsuperscript{[8]}. However, most of these studies brought up the difficulty in proliferation of NSCs in traditionally-used hydrogels\textsuperscript{[9]}. The advent of 3D bioprinting and tissue engineering has opened up a new discipline to precisely develop human organ systems in vitro. Essentially, 3D bioprinting helps to biofabricate compatible biomaterials into desirable shapes designed with a software. Most of the bioprinted neural tissues have been generated using extrusion-based methods, laser-assisted printing, inkjet printing, drop-on-demand method, microfluidic printing technology, and point-dispensing printing method\textsuperscript{[10-12]}. The most common method used for bioprinting neural tissue is extrusion bioprinting. In this type of bioprinting, one or more types of neural cells were mixed and suspended in a compatible hydrogel, and extruded in a layer-by-layer fashion according to a digital design, assisted by pressure, to form a tissue construct\textsuperscript{[12-15]}. The choice of cells, the formulation of cell-specific bioinks, and optimized printing parameters are the most important topics in bioprinting\textsuperscript{[16]}. It is considered difficult to optimize printing conditions for the soft tissues, due to their mechanosensitive nature\textsuperscript{[7]}. Compared to other types of cells, stem cells are more sensitive to sheer stress generated by the bioprinting process\textsuperscript{[17]}. Hence, it is essential to formulate bioinks and optimize printing methods that can protect the cells from the sheer stress and provide an ideal tissue microenvironment for the cell growth and differentiation\textsuperscript{[18]}. When it comes to peripheral neurons (PNs), the bioink should allow outgrowth of neurites and axons within the printed construct\textsuperscript{[19,20]}. An ideal bioink provides smooth flow through the nozzles without any clogging that will reduce the total printing time and cellular stress.

The use of cellulose-rich sea squirts (other species of tunicates) for developing cardiac patches was explored recently, mainly due to its conductive nature and fiber orientation\textsuperscript{[21]}. We hypothesized that the tunicate dECM would support neural tissue engineering as they also are conductive tissues like cardiac patches\textsuperscript{[22]}. Previously, our laboratory has reported the biocompatibility of tunicate-derived hydrogels for bioprinting mouse embryonic fibroblasts (MEFs) and as a wound dressing material\textsuperscript{[23]}. This work is an advancement to use the marine tunicate-based bioink to 3D bioprint NSCs and its differentiation into PNs. The cytocompatibility of the marine tunicate dECM scaffolds was evaluated by culturing and differentiation of the human iPSC-derived NSCs into PN. Further, a bioink using the tunicate dECM powder and Matrigel was formulated and optimized for bioprinting of NSCs that differentiated in vitro into PNs. The bioink formulation and bioprinting parameters were optimized for bioprinting NSCs that proved to be efficient in providing a conducive tissue microenvironment for the PN differentiation. Lattice-shaped neural tissue constructs were bioprinted in a dish and their cellular properties and cold storage potential were characterized. The neural tissue cultures and constructs were analyzed for cell viability, cell proliferation, and cell differentiation as PNs (Figure 1). The current work expands the scope of bioprinting by adopting a novel sustainable bioink for bioprinting of human NSCs and its differentiation into PN for regenerative medicine applications and disease modeling.

2. Materials and methods

2.1. Cell culture

iPSC-derived normal human NSCs were purchased from AddexBio, San Diego, USA (Catalogue number P0005048). The cell culture plates were coated with Matrigel and 1 × 10\textsuperscript{6} cells were seeded onto one well of a six-well plate. The cells attached on the plates in 24 – 48 h. NSCs were cultured in 5% CO\textsubscript{2} at 37°C with alternate day media changes using NSC Growth Medium (Catalogue number C0013-09, AddexBio).

2.2. Decellularization of tunicate extracellular matrix (dECM) scaffold

Fresh tunicates (Polyclynum constellatum, NCBI Accession number MW990087) were collected from the Zayed Port, Abu Dhabi, United Arab Emirates. The samples were thoroughly washed with deionized water. The outer rough layer of the tunicates was removed
using a sterile surgical knife and the whole hydrogel-like tunic tissue was separated into a culture dish. The tunic tissue was cleaned using deionized water at room temperature few times, before being cut into required dimensions. The tunic tissue pieces were stirred in decellularization buffer consisting of 10 mM Tris, 1 mM of ethylenediamine tetra acetic acid (EDTA), 0.2% v/v of Triton X-100, and 1.5% of sodium dodecyl sulfate, at a pH of 7.5 for 48 h. The buffer was changed every 2 h until 10 h. Detailed description on the tunicate material and the decellularization process are described by Govindharaj et al.\textsuperscript{[23]} Cellular debris was removed by washing with deionized water after 10 h. The decellularized tissue pieces were cut into dimensions of 1 cm × 1 cm × ~0.1 cm for NSC seeding for cytocompatibility, proliferation, and differentiation studies.

2.3. Culture and differentiation of NSCs on tunicate dECM scaffolds

The scaffolds were sterilized for 1 h in UV irradiation in a biosafety cabinet. The sterilized scaffolds were washed 3 times with prewarmed phosphate-buffered saline (PBS) and 1 time with the NSC medium. Confluent cultures of NSCs were harvested using accutase enzyme and washed in the NSC media. The cells were counted and concentrated to 3 × 10^6 cells in 30 μL volume of NSC medium and seeded on to the dECM scaffolds placed in the wells of 24-well plate. The plates were incubated in a 5% CO\textsubscript{2} incubator at 37°C. After 4 h of incubation, 0.5 mL of NSC media was added. The culture media was changed after 24 h and then every alternate day. After 3 days of culture, the NSC medium was replaced with the PN induction medium. The PN media was composed of neurobasal media (Thermo fisher scientific) supplemented with 1× non-essential amino acids, 1× GlutaMAX\textsuperscript{TM} (Sigma), 1X N2, 1 X B27 (Thermo fisher scientific), 20 ng/ml EGF (Sigma), 20 ng/ml bFGF, 10 ng/ml nerve growth factor-β, and 25 μM Y27632 (Merck Millipore) for differentiation of NSCs to PN. Media changes were performed once every 3 days for 2 weeks\textsuperscript{[24]}. The cells were cultured in the PN induction medium for another 12 days and checked for the NSC to PN differentiation using specific markers at day 3 and day 12.

2.4. Cell viability in the tunicate dECM scaffolds

The cell viability and proliferation in the dECM tunicate scaffolds were analyzed on day 3, 7, and 12. The dECM tunicate scaffolds with cells and without cells were stained with Calcein AM and Ethidium homodimer1 (Invitrogen LIIVE/DEAD\textsuperscript{TM} Viability/Cytotoxicity Kit, for Mammalian Cells, catalogue number L3224). Before staining, the cells were washed with prewarmed physiological saline. The cells in the dECM tunicate scaffolds were stained with 500 μL of 2M Calcein AM and 4M Ethidium homodimer1 working solution for 45 min at room temperature. After the incubation, the dECM tunicate scaffolds were lifted from the wells and mounted on a clean slide followed by confocal imaging using a Leica SP8 confocal laser scanning microscope. In this staining
method, live cells are distinguished by the presence of ubiquitous intracellular esterase activity, which is determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant Calcein AM to the intensely fluorescent Calcein. The polyanionic dye Calcein is well retained within live cells, producing an intense uniform fluorescence in live cells. Ethidium homodimer-1 dye enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence on binding to nucleic acids, thereby producing a bright red fluorescence in dead cells.

2.5. Cell proliferation on tunicate dECM scaffolds

AlamarBlue assay (AlamarBlue HS Cell Viability Reagent, Invitrogen, Catalogue number A50101) was used as a measurement for the determination of cell viability and proliferation. Cell growth was analyzed at different time points: 3, 7, and 12 days. Scaffolds were incubated with 10 µL of AlamarBlue solution per each 100 µL (1:10 ratio) of media and incubated for 4 h. The AlamarBlue reaction mix was collected in a 96-well plate and the absorbance was measured at a wavelength of 570 nm with a reference wavelength of 600 nm using a microplate reader (Epoch, BioTek). The percentage reduction of the AlamarBlue reagent, which is linear measurement of the viable cells in the culture was calculated using the online AlamarBlue colorimetric calculator (Biorad).

2.6. mRNA expression of PN markers

The RNA from the dECM cultured nerve cells and bioprinted nerve tissues were isolated using Qiaquick RNA extraction kit (Qiagen) according to the manufacturer’s instructions. The extracted RNA was quantified using Nanodrop ND-1000 spectrophotometer (Nanodrop technologies, Wilmington, DE). 1 µg of mRNA was reverse transcribed into cDNA using Superscript Vilo IV cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time quantitative PCR reactions were carried out in triplicates with 500 ng cDNA template per reaction using SYBR master mix (Thermo Fisher Scientific) in a StepOnePlus Real-Time PCR System (Applied Biosystems). mRNA of neural markers – (i) a pan-neuronal marker which is TUBB3 (β3 tubulin), (ii) two PN specific markers, namely, peripherin (PRPH) and neurofilament heavy polypeptide (NEFH), and (iii) a stemness marker HNK1 were analyzed in the day 7 and day 12 induced samples. The sequences of the forward and reverse primers of genes analyzed were adapted from Vijayavenkataraman et al.[25]. The target gene expression was normalized to the house keeping gene GAPDH. The results were expressed as relative mRNA expression compared to the day 3 samples.

2.7. Immunocytochemistry of PN protein marker

The NSC seeded scaffolds or neural tissue constructs were fixed with 4% paraformaldehyde (Sigma) for 10 min at room temperature. Fixed cells were permeabilized using 0.1% TritonX-100 in PBS (Sigma) for 15 min, washed thrice with 0.05% Tween-20/PBS (Sigma), and blocked with 1% bovine serum albumin for 1 h to avoid non-specific binding. Subsequently, the cells were incubated with Rabbit Anti-Neurofilament heavy polypeptide (NEFH) antibody (1:50 in 1% BSA in PBS, ab8135, Abcam) at room temperature for 1 h. The scaffolds or constructs were washed with PBS for 3 times and incubated with fluorescent labeled secondary antibody Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488, ab150077, Abcam) for 1 h at room temperature, then counterstained with 1 µg/mL of nuclear stain, DAPI (4’,6-diamidino-2-phenylindole, Sigma). The images were taken with a Leica SP8 confocal laser scanning microscope and analyzed.

2.8. Scanning electron microscopy (SEM)

After day 3 of culturing of NSCs on the tunicate dECM scaffolds, the cell-loaded scaffolds appeared impermeable to light; hence, SEM was carried out to get a clear picture of the cell growth and differentiation. For SEM, the cultured scaffolds were fixed in 4% paraformaldehyde for 1 h at room temperature and dehydrated with serial concentration of ethanol ranging from 50%, 70%, and 100%, then frozen in critical point dryer. The scaffolds were, then, coated with gold and imaged using a SEM (Qanta, Thermo Fisher Scientific).

2.9. Design of tissue constructs

Scaffolds were designed and fabricated using RegenHU 3D Discovery printer BioCAD software (RegenHU, Switzerland). They were designed in a square 8 mm × 8 mm grid pattern with a line spacing of 2 mm and a total thickness of 0.5 mm, with each layer being 0.5 mm thick comprising vertical and horizontal struts. Using a built-in software well plate editor, the toolpath was calibrated to print the constructs in a 24-well plate format. The tool path was generated and saved as an. iso file in the BioCAD software.

2.10. Bioink preparation

For making the bioink, the decellularized tissue pieces were frozen at −80°C overnight and lyophilized using a Christ Alpha 1-2 LD Lyophilizer for 48 h. The lyophilized scaffolds were sterilized with ethanol and UV radiation before making the tunicate powder. For making the powder, the dECM tissues were sliced into smaller sections and immersed in liquid nitrogen (~5 mL) in a mortar. The frozen tissue
sections were pulverized into fine powder using a mortar and a pestle. After lyophilization, the ECM powder was mixed with pepsin enzyme in a ratio of 10:1 w/w/100 mL 0.01 N HCl. The solution was digested for 48 h at room temperature under constant stirring using a magnetic stir bar and plate until the solution becomes viscous with no visibly undigested granules. Then, 10 mg/mL of digested ECM solution was aliquoted and frozen at −80°C to terminate pepsin digestion. Further, the digested ECM solution was mixed well and dialyzed against water at 4°C for 72 h. Finally, the obtained ECM powder was freeze-dried and lyophilized for further use. All the buffer components and chemicals used for ECM powder preparation were from Sigma-Aldrich, USA. The tunicate powder was sterilized using UV irradiation for 2 h before preparing the base hydrogel. The base hydrogel for bioprinting was prepared by slowly adding NSC media to 100 mg of tunicate powder to make a final volume of 1 mL. The hydrogel concentration was optimized for NSC bioprinting by adding different concentrations of Matrigel (Matrigel hESC-qualified Matrix, Catalogue number 354277, Corning) starting from 50%, 35%, 31%, and 26%. The higher concentrations of Matrigel made the hydrogel more viscous and did not allow printing. About 26% of the Matrigel in the base hydrogel was found to be optimal and facilitated smooth printing of the cell-free scaffolds. Therefore, this formulation was used to make the bioink for cell printing. A bioink containing 26% Matrigel, 10% tunicate powder, and 0.1 mL of the NSC suspension containing 7.58 × 10^6 cells were formulated (a total volume of 1 mL), which could print 20 tissue constructs in a 24-well plate. Each of the constructs consumed ~50 µL of the bioink, with ~4 × 10^6 NSCs. Matrigel was kept at 4°C before being added to the media, as the pure Matrigel solidifies in higher temperatures. The preparation of bioinks was carried out in a biosafety cabinet at room temperature within 15 min before printing.

2.11. Rheology and printability

ElastoSens Bio 2 (Rheometer) was used to justify the rheology properties of the hydrogel as well as the printability of layered structures under room temperature. A minimum volume of 4mL of hydrogel was used for the test. The test lasted a total of 120 min. The test was performed under room temperature (22°C) to mimic the 3D bioprinting condition. About 10% tunicate hydrogel and 26% Matrigel in NSC media were used to make the hydrogel. Shear storage modulus and shear loss modulus over time were obtained. The characterized hydrogel was used to print lattice structures with the layers ranging from one to ten.

2.12. 3D bioprinting

RegenHU 3D discovery bioprinter inside a biosafety cabinet at room temperature were used for bioprinting. The printer and the biosafety cabinets were sterilized under UV light for 1 h before printing. Bioink containing NSCs were loaded in a 3 mL sterile syringe and connected to the air pressure supply. A needle with 0.51 mm inner diameter was used for the printing (Needle DD-135N ID=0.51/G21 L=25.4, RegenHU, Switzerland). Print parameters were adjusted to obtain continuous flow rate and smooth hydrogel fibers with minimal spreading. A feed rate of 2 mm/s and pressure of 0.3-0.4 MPa were used; the total print time was under 30 min per one 24-well plate. The printability of the bioink was assessed by switching on the pressure and the filament formation at the tip of the needle. The needle diameter, pneumatic pressure, and nozzle moving speed were optimized to deliver continuous extrusion of the bioink in the designated well of the well plate.

2.13. Cross-linking of the printed tissue constructs and tissue culture

For optimization of printing and cross-linking, the cell-free control hydrogel filaments were immersed in a cross-linking solution and PBS to check the strength of the filament formation. Immersion in 250 mM of CaCl_2 could make a smooth filament of cross-linked hydrogel at room temperature. We have added the blue stain Alcian blue to the control hydrogels for better visibility. The same concentration of the CaCl_2 solution was used to crosslink the cell containing tissue constructs after bioprinting. The cross-linker solution was removed after 5 – 10 min of incubation at room temperature and the constructs were washed with prewarmed PBS. After washing with PBS, the cell-laden constructs were incubated in nutrient-rich NSC media containing 10% fetal bovine serum at 37°C in 5% CO_2. The nutrient rich media was changed to NSC medium after 15 min.

2.14. PN differentiation of bioprinted tissue constructs

Bioprinted constructs were initially cultured in the NSC media for 5 days; once the cells adapted to the new 3D environment, they were induced with PN media for differentiation to PN. The tissue constructs were analyzed for cell viability and cell proliferation as described for the dECM scaffolds. The tissue constructs were analyzed for PN-specific marker expression by immunofluorescence and qPCR with the same procedure used for the dECM scaffolds. SEM was done to see the tissue construct morphology after culturing and differentiation.

2.15. Viability and proliferation of the freeze-thawed dECM-grown and bioprinted PN

On day 12 of induction, the differentiated PN grown on the dECM scaffolds and bioprinted tissues were washed
twice using the fresh prewarmed PN media. Then, the tissues were immersed in 1:1 ratio of PN media and cell freezing media (Embryomax freezing media, Catalogue number ES002D, EMD Millipore), incubated at room temperature for 10 min. The 24-well plate containing tissues were frozen directly at −80°C. After 1 week of freezing, the plates were taken out and kept at 37°C for 15 min. The thawed freezing media was removed and the tissues were washed twice in 0.5 mL of prewarmed fresh PN media. The tissues were further cultured in the PN media for a week with alternate day media changes. The cell viability and proliferation were assessed by AlamarBlue assay and live-dead staining.

2.16. Statistical analysis

Results were analyzed statistically. All graphical data represent the mean ± standard deviation of at least three independent experiments. Differences between treatments were tested using the two-tailed Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and *****P < 0.00001 were considered statistically significant in all cases.

3. Results and discussion

3.1. NSC culture and PN differentiation in the tunicate dECM scaffolds

To design a cell-specific bioink for neural tissues, the key factors considered in this study were the type of cells and the choice of biomaterials used. The tunicate tissue majorly composed of biocompatible and biodegradable cellulose[23,20]. The decellularization process of the tunicate tissue could yield clean, transparent looking scaffolds with natural pores, in which we hypothesized would aid in the cell adherence and proliferation. Since the stem cells are sensitive to many intrinsic and extrinsic factors for growth such as matrix coating and cell seeding density, the cytocompatibility of these scaffolds was initially tested using MEFs[23]. The scaffolds offered good cytocompatibility and growth of MEFs. In this work, NSCs were seeded on the scaffolds without any matrix coating (usually NSCs are seeded on a Matrigel-coated surface) at a high seeding density and neural induction was given on day 3 of the culture. The NSCs were attached to the tunicate dECM scaffolds by 24–48 h after seeding and started the colony formation. The scaffolds became impermeable to light by day 3 (Figure 2A-C). Hence, further imaging was carried out using SEM. On day 3 of PN induction (Figure 2D), the electron micrograph of the NSCs on the scaffolds still showed the fibroblast-like morphology, which changed to a more rounded cell appearance by day 7 (Figure 2E) with distinct colony formation, filling in the natural pores of the scaffolds. By day 12 of neuronal induction, the cells showed clear morphology of peripheral neuronal fibers, which formed a network of PN with elongated neural outgrowth throughout the surface of the scaffolds (Figure 2F). We hypothesize that these morphology changes might be due to the mechanical surface cues the cells experience from the surface of the dECM scaffold.

Cell viability of NSCs seeded on tunicate dECM scaffolds was analyzed on days 3, 7, and 12 of culture. Live-dead staining was used to see the viable cells on scaffolds and colorimetric AlamarBlue assay was used for the quantification of cell proliferation. The live-dead assay of cells grown on the tunicate dECM scaffolds showed an increase in the number of green florescent cells over time and proliferation of thread-like structures by day 12 (Figure 2J-L). AlamarBlue assay showed significant cell proliferation on day 12 compared to day 3 (Figure 2N). Between day 3 and day 7, the proliferation was not significant, probably because the cells are adapting to the new culture environment along with the pressure of cellular differentiation to PN (Figure 2N). These experiments proved that the tunicate dECM scaffolds provide a very conducive tissue microenvironment for the growth of different types of cells, including NSCs.

The immunofluorescence staining of the cells in different stages of the induction showed expression of PN marker, NEFH by day 12, which indicated the differentiation of the NSCs to PN on the scaffolds with the induction media. The day 3 controls did not show NEFH expression (Figure 2G-I). qPCR was performed to analyze the mRNA expression of the PN markers on day 3 and day 12 of PN induction. The relative mRNA expression of NEFH and PRPH expression increased four to five-fold on day 12 of the PN induction, compared to the day 3 samples, while the HNK1 gene expression was remarkably reduced (Figure 2M). HNK1 is a stem cell marker, the reduction of this gene indicates the differentiation of stem cells to PNs. Day 12 neurons also showed slightly increased levels of the pan neuronal marker TUBB3 (Figure 2M). The gene expression was normalized to house-keeping gene GAPDH (Figure 2M).

3.2. Bioink formulation, characterization, cross-linking, and bioprinting

The base hydrogel for bioprinting was optimized before adding the cell component. The tunicate hydrogel and different concentrations of Matrigel in the NSC media were used for making the base hydrogel. Matrigel is a matrix protein which polymerizes at physiological temperature; therefore, aliquots of Matrigel were stored at 4°C before mixing with NSC media. High concentrations of Matrigel (>30%) in the base hydrogel caused clogging of the needle and did not extrude from the nozzle due to increased viscosity and rapid solidification within the
Figure 2. NSC to PN differentiation in dECM tunicate scaffolds. (A-C) The NSC-loaded dECM tunicate scaffolds under the light microscope: (A) dECM tunicate scaffold without cells, (B) scaffold just after cell loading, and the cells appear rounded and floated over the scaffold, and (C) cells attached to the scaffolds on day 3 of seeding. The scaffolds appeared impermeable to light by day 3 of seeding (scale bars = 125 µm). (D-F) SEM images of the NSC-loaded tunicate dECM scaffolds: (D) Day 3 of NSC culture on the scaffolds showing fibroblast-like morphology, (E) the NSCs changed to more rounded cell appearance by day 7, and (F) formation of extended peripheral neuron fibers by day 12 of PN induction (scale bars = 50 µm). (G) The cell-free control scaffold with DAPI and NEFH staining. (H) DAPI and NEFH staining on day 3 of PN induction. (I) Expression of NEFH protein on day 12 in the induced NSCs-loaded scaffolds indicate the differentiation of the NSCs to PN (scale bars = 100 µm). (J-L) Live-dead staining on cells grown on the tunicate dECM scaffolds showed an increase in number of green fluorescent cells over time and proliferation of thread-like structures by day 12 (scale bars = 100 µm). (M) Gene expression studies using qPCR experiments; the relative mRNA expression of NEFH and PRPH were increased to four- to five-fold on day 12 of the PN induction, compared to the day 3 samples, while the stemness marker HNK1 expression was remarkably reduced, indicating the differentiation of NSCs to PN. Day 12 neurons also showed increased levels of the pan neuronal marker TUBB3. The gene expression was normalized to housekeeping gene GAPDH. Data reported as mean ± SD (n = 3; *P < 0.05, **P < 0.01, ***P < 0.001, by two-tailed Student’s t-test between test and control samples). (N) AlamarBlue cell proliferation assay showed significant cell proliferation on day 12 compared to day 3, P < 0.05.
needle. Even when the printing pressure was increased from 0.45 MPa to 0.65 MPa, no extrusion happened. At a lower Matrigel concentration (26%), the hydrogel extruded smoothly and the lattices of cell-free scaffolds were printed in <1 min/scaffold. The shear modulus of the hydrogel formulation was analyzed and found to have a steady increase in shear storage modulus across the monitored time in ambient temperature (22°C), while the shear loss modulus was more or less steady indicating the more viscous nature of the hydrogel (Figure 3K).

The optimized hydrogel was tested for its printability by printing multilayered lattice structures. We printed one layer to ten layers. The single layer became more shrunken after the crosslinking, while the two-layer structure looked stable and more refined after crosslinking. The five- and ten-layer structures became a blob as it got polymerized. The three-layer structure also lacked the clear lattice structure, in which we suspect may impede with the imaging. Hence, we proceeded further experiments with two layered structures for bioprinting.
and nerve differentiation studies (Figure 3M). NSCs were added to the optimized hydrogel formulation to make the bioink for neural tissue bioprinting. The formulation followed the same seamless pattern as that of the cell-free printing and we could bioprint quickly at room temperature.

Cross-linking of the printed constructs was optimized using the combinations of cell-free hydrogel controls. Alcian blue was added to the hydrogel to give clear visibility in the liquid interphase of the cross-linking solution. The optimized formulation showed quick and stable crosslinking while treated with the ionic crosslinker CaCl₂. The filament formulation was consistent on printing within a 250 mM CaCl₂ solution when compared to the PBS (Figure 3A-F). On addition of Matrigel, hydrogel with Matrigel showed consistent droplet formation and downward flow compared to the hydrogel without Matrigel (Figure 3G and H).

For the bioprinting experiments, the tissue constructs were designed and fabricated using RegenHU 3D Discovery printer (Figure 3I). The toolpath for the tissue construct was also generated (Figure 3J). The design facilitated the deposition of the bioink in a layer-by-layer fashion with the dimensions of the tissue construct being 8 mm × 8 mm × 1 mm. The pore size of the lattice was measured as ~900 μm using the digital camera images (Figure 3K). The bioprinted lattice showed uniformity in the strut size and the pore size as measured in the bioprinted tissue constructs (Figure 3L). This study used 0.51 mm diameter needle and the number of layers was set as two, which gives an approximate height of the construct as 1 mm. The chosen dimensions facilitated the mounting of the tissues in a glass slide for imaging and for the scaling up of the tissue production using specific quantities of the bioink.

Cell-laden constructs were printed after adding the NSCs to the optimized hydrogel, cross-linked post-printing and cultured in vitro. The cross-linked tissue constructs stayed soft enough to allow the cellular activities such as adherence, migration, proliferation, and differentiation and at the same time possessed enough post-printing structural stability and stiffness to form a nerve tissue throughout the in vitro culture period. The method was scaled-up to automate the printing process of printing tissue constructs in 24-well culture plates, expanding the scope of bioprinting to develop disease-in-dish models and for making human tissues for regenerative medicine applications (Figure 3N).

### 3.3. NSC proliferation and PN differentiation

The neural growth and building of neural network from the stem cells in vitro require the guidance of axons in an efficient and long-lasting manner. Our experiments proved that the formulated bioink provide ideal conditions for the 3D neural outgrowth[1]. Many different factors influence cell differentiation and axon protrusion in vitro. Here, we modified the neural tissue environment using both physical and chemical stimuli. The physical environment was modified by optimizing the bioink composition by adding specific concentration of Matrigel. Initial experiments of NSC differentiation on dECM scaffolds (Figure 2) did not require Matrigel coating for the cell growth and differentiation. However, when the bioinks were formulated without Matrigel for bioprinting, the NSCs did not grow well, contrary to the expectations (Figure 4D-F). To provide a more cell-friendly environment, Matrigel was then added to the dECM bioink. Matrigel is a reconstituted basement membrane derived from extracts of Engelbreth-Holm-Swarm mouse tumors. The tumor basement membrane consists of a thin layer of ECM sheets that are primarily made up of type IV collagen, entactin, heparan sulfate proteoglycans, laminin, and growth factors to support cell growth[28,29]. Matrigel closely resembles the complex extracellular environment of the basement membrane, where cells adhere during tissue formation. Both the live-dead staining (Figure 4A-C) and AlamarBlue cell proliferation assay (Figure 4K) showed highly significant cell growth by day 7 and day 12 post-printing in the Matrigel-containing bioink. Figure 4J shows the live-dead staining image of the whole construct. Figure 4G-I shows the live-dead staining of control hydrogels (without any cells). From these results, it can be inferred that the addition of Matrigel to the tunicate dECM bioink aids better cell encapsulation and favor enhanced cell adhesion and growth[30,31]. While the dECM scaffold, without any matrix coating, favored cell adhesion, growth, and differentiation, it could be probably due to its inherent porous structure as the NSCs were directly seeded onto the scaffold, without subjecting the cells to any undue stress (as with bioprinting). We hypothesize that the addition of Matrigel might have help to resist the cellular stress generated by the bioprinting procedure, which was evident from the better cell proliferation in the Matrigel containing bioink. The bioprinted tissue showed more cell proliferation compared to the dECM cultured cells due to the presence of Matrigel and also due to better exchange of nutrients in all parts of the construct than the dECM scaffolds.

With 3D bioprinting, it is a difficult task to find the bioink formulations that are printable with good post-printing structural stability and at the same time provide the physicochemical cues to meet the biological needs of the cells for differentiation, as these characters of the bioinks are mutually exclusive with many hydrogels[30-32]. Most of the high shape fidelity bioinks are highly viscous and pose difficulty in printing due to nozzle clogging. There were difficulties in extruding our bioink with a high percentage of Matrigel as a bioink component. Matrigel containing bioink required more care and optimization.
as it contributed to the temperature and time sensitivity during printing. At room temperature, high percentage Matrigel (>30%) bioink solidified faster in the needle, resulting in clogged nozzles. This delayed the whole printing process and undesirable printing outcomes, as the cells experience more stress during the printing process. Hence, it is important to develop easy-to-use simple formulations which will work well in ambient temperatures without causing any printing delay. The formulation of 10% tunicate hydrogel and 26% Matrigel showed consistent seamless printability of NSCs at room temperature. The preparation of bioink took ~15 min (until loading the bioink cartridge onto the printer) and the printing of each scaffold took ~1 min. The total time required to print a 24-well plate was approximately ~24 min, which is optimal for stem cell bioprinting.

In the bioprinted tissue constructs, the NSCs started to proliferate by 3–5 days post-printing. Cell viability post-printing was confirmed by DAPI staining on day 1 (Figure 5A). On day 5, once the cells appeared settled to grow, PN induction media was added. The immunofluorescence staining of the cells on different stages of the induction showed expression of PN marker NEFH by day 12, indicating the differentiation of the NSCs to PN inside the tissue constructs (Figure 5C), while the day 3 sample did not stain for NEFH (Figure 5B). By day 12 of neuronal induction, the cells showed clear morphology of peripheral neuronal fibers, which formed a network of PN in the tissue constructs. SEM images of the bioprinted constructs on day 3 (Figure 5D-F), day 7 (Figure 5G-I), and day 12 (Figure 5J-L) clearly shows the changes in cell morphology due to PN differentiation. The scanning electron micrograph of the PN cells showed typical PN morphology protruding on the surface of the tissue construct. The direction of the cells appeared perpendicular to the direction of the printing (Figure 5L). This observation requires more detailed investigation in the future, to know the physiological determinants of the directionality of the nerve formation, which can possibly give valuable information for PN injury repair. The quantitative polymerase chain reaction was performed to analyze the mRNA expression of the PN markers on day 3 and day 12 of PN induction (Figure 5M). The mRNA expression of PN markers PRPH and NEFH was upregulated on day 12 of PN induction compared to day 3. The stemness marker HNK1 was significantly downregulated and the change in the pan neural marker TUBB3 was non-significant (Figure 5M). The mRNA profile (Figure 5M) gives clear clues on the PN differentiation.

3.4. Cold storage compatibility of the bioprinted tissues

One of the ultimate aims of tissue bioprinting is future regenerative medicine applications. This requires short- or long-term storage of tissues and tissue transportation in ultra-low temperatures. A freeze-thaw study was conducted to analyze the storage potential of the bioprinted tissues (Figure 6). The ability of the bioprinted and dECM scaffold tissues for its cold shock recovery was analyzed after keeping them frozen for a week in a freezing
Figure 5. NSC to PN differentiation in bioprinted neural tissue constructs. (A) DAPI staining on day 1 post-printing. (B) DAPI staining and NEFH immunofluorescence staining on day 3 of the neural induction showed no NEFH expression. (C) DAPI staining and NEFH immunofluorescence staining on day 12 of the neural induction showed high NEFH expression, indicating PN differentiation. Scale bars = 100 µm. (D-F) SEM images of the bioprinted tissue constructs on day 3. (G-I) SEM images of the bioprinted tissue constructs on day 7. (J-L) SEM images of the differentiated neural tissue construct on day 12 showed remarkable neural cell morphology and neural filament formation (yellow arrows), compared to day 3 and day 7 post-induction. The direction of neural filament formed were perpendicular to the printing direction (blue arrows), which requires further investigation. (M) mRNA expression of PN markers PRPH and NEFH were upregulated on day 12 of PN induction compared to day 3. The stemness marker HNK1 was significantly downregulated and the change in the pan neural marker TUBB3 was non-significant (NS). Data are expressed as mean±SD (n = 3; *P < 0.05, ***P < 0.001, by two-tailed Student’s t-test between test and control samples).
media at −80°C. The cold shock recovery of the tissues was assessed using cell viability and cell proliferation assays. The cells showed initial slow recovery after the cold shock, but recovered and proliferated on culturing them further for a week. The bioprinted tissues showed better cell proliferation compared to the dECM scaffold-tissue. The results are encouraging to explore further on the storage and transportation of bioprinted tissues for translational medicine applications.

4. Conclusions

We demonstrated seamless bioprinting and differentiation of NSCs to PN using a custom-designed bioink for neural tissues. We optimized the bioprinting workflow at room temperature, which makes it easy to handle and quick to print. The printed tissue constructs maintain the soft-tissue consistency required for the nervous tissue throughout the culture period and exhibited high cell viability and proliferation. On induction, the bioink aided the formation of peripheral nerve tissues with well-formed neurites. The cultured tissues showed significant recovery from cold shock at −80°C, which holds promise in using this method to develop tissues for clinical use. Moreover, our bioprocessing method efficiently uses an untapped source of biomaterial to formulate tissue-specific bioinks. The development of sustainable bioinks from marine invasive tunicates would open up new avenues for scaling up the hydrogel-based soft-tissue bioprinting for translational medicine applications.

Acknowledgment

This research was partially carried out using the Core Technology Platforms resources at New York University Abu Dhabi.

Funding

The research was funded by the NYUAD startup grant for Sanjairaj Vijayavenkataraman.

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Conceptualization, methodology, validation, formal analysis, investigation, visualization, writing--original draft: Soja Saghar Soman
Methodology, formal analysis, validation: Mano Govindharaj
Methodology, formal analysis, visualization: Noura Al Hashimi
Methodology, visualization: Jiarui Zhou
Conceptualization, fund acquisition, project administration, visualization, writing – review and editing, supervision: Sanjairaj Vijayavenkataraman

References

1. Qiu B, Bessler N, Figler K, et al., 2020, Bioprinting Neural Systems to Model Central Nervous System Diseases. Adv Funct Mater, 30:1910250.
https://doi.org/10.1002/adfm.201910250.
2. Soman SS, Vijayavenkataraman S, 2020, Perspectives on 3D Bioprinting of Peripheral Nerve Conduits. Int J Mol Sci, 21:5792.
https://doi.org/10.3390/ijms21165792
3. Yu X, Zhang T, Li Y, 2020, 3D Printing and Bioprinting Nerve Conduits for Neural Tissue Engineering. Polymers (Basel), 12:1637.
https://doi.org/10.3390/polym12081637
4. Gao F, Xu Z, Liang Q, et al., 2019, Osteochondral Regeneration with 3D-Printed Biodegradable High-Strength Supramolecular Polymer Reinforced-Gelatin Hydrogel Scaffolds. Adv Sci (Weinh), 6:1900867.
https://doi.org/10.1002/advs.201900867
5. Soman SS, Vijayavenkataraman S, 2020, Applications of 3D Bioprinted-Induced Pluripotent Stem Cells in Healthcare. Int J Bioprint, 6:280. https://doi.org/10.18063/ijb.v6i4.280

6. Srubar WV 3rd, 2021, Engineered Living Materials: Taxonomies and Emerging Trends. Trends Biotechnol, 39:574-83. https://doi.org/10.1016/j.tibtech.2020.10.009

7. Lozano R, Stevens L, Thompson BC, et al., 2015, 3D printing of layered brain-like structures using peptide modified gellan gum substrates. Biomaterials, 67:264-73. https://doi.org/10.1016/j.biomaterials.2015.07.022

8. Joung D, Truong V, Neitzke CC, et al., 2018, 3D Printed Stem-Cell Derived Neural Progenitors Generate Spinal Cord Scaffolds. Adv Funct Mater, 28:1801850. https://doi.org/10.1002/adfm.201801850

9. Madhusudanan P, Raju G, Shankarappa S, 2020, Hydrogel systems and their role in neural tissue engineering. J R Soc Interface, 17:20190505. https://doi.org/10.1098/rsif.2019.0505

10. Bsoul A, Pan S, Cretu E, et al., 2016, Design, microfabrication, and characterization of a moulded PDMS/SU-8 inkjet dispenser for a Lab-on-a-Printer platform technology with disposable microfluidic chip. Lab Chip, 16:3351-61. https://doi.org/10.1039/c6lc00636a

11. Park S, Kim D, Park S, et al., 2018, Nanopatterned Scaffolds for Neural Tissue Engineering and Regenerative Medicine. Adv Exp Med Biol, 1078:421-43. https://doi.org/10.1007/978-981-13-0950-2_22

12. Shaqour B, Aizawa J, Guarch-Pérez C, et al., 2021, Coupling Additive Manufacturing with Hot Melt Extrusion Technologies to Validate a Ventilator-Associated Pneumonia Mouse Model. Pharmaceutics, 13:772. https://doi.org/10.3390/pharmaceutics13060772

13. Levato R, Jungst T, Scheuring RG, et al., 2020, From Shape to Function: The Next Step in Bioprinting. Adv Mater, 32:e1906423. https://doi.org/10.1002/adma.201906423

14. Moroni L, Burdick JA, Highley C, et al., 2018, Biofabrication strategies for 3D in vitro models and regenerative medicine. Nat Rev Mater, 3:21-37. https://doi.org/10.1038/s41578-018-0006-y

15. Ouyang L, Highley CB, Rodell CB, et al., 2016, 3D Printing of Shear-Thinning Hyaluronic Acid Hydrogels with Secondary Cross-Linking. ACS Biomater Sci Eng, 2:1743-51. https://doi.org/10.1021/acsbiomaterials.6b00158

16. Assuncao-Silva RC, Gomes ED, Sousa N, et al., 2015, Hydrogels and Cell Based Therapies in Spinal Cord Injury Regeneration. Stem Cells Int, 2015:948040. https://doi.org/10.1155/2015/948040

17. Stolberg S, McColonkey KE, 2009, Can shear stress direct stem cell fate? Biotechnol Prog, 25:10-9. https://doi.org/10.1002/btp.124

18. Li C, Ouyang L, Armstrong JP, et al., 2021, Advances in the Fabrication of Biomaterials for Gradient Tissue Engineering. Trends Biotechnol, 39:150-64. https://doi.org/10.1016/j.tibtech.2020.06.005

19. De Santis MM, Alsafadi HN, Tas S, et al., 2021, Extracellular-Matrix-Reinforced Bioinks for 3D Bioprinting Human Tissue. Adv Mater, 33:e2005476. https://doi.org/10.1002/adma.202005476

20. Echeverria Molina MI, Malollari KG, Komvopoulos K, 2021, Design Challenges in Polymeric Scaffolds for Tissue Engineering. Front Bioeng Biotechnol, 9:617141. https://doi.org/10.3389/fbioe.2021.617141

21. He Y, Hou H, Wang S, et al., 2021, From waste of marine culture to natural patch in cardiac tissue engineering. Bioact Mater, 6:2000-10. https://doi.org/10.1016/j.bioactmat.2020.12.011

22. Dunlop MJ, Clemons C, Reiner R, et al., 2020, Towards the scalable isolation of cellulose nanocrystals from tunicates. Sci Rep, 10:19090. https://doi.org/10.1038/s41598-020-76144-9

23. Govindharaj M, Al Hashemi NS, Soman SS, et al., 2022, Bioprinting of bioactive tissue scaffolds from ecologically-destructive fouling tunicates. J Clean Prod, 330:129923. https://doi.org/10.1016/j.jclepro.2021.129923

24. Zhu Q, Li M, Yan C, et al., 2017, Directed Differentiation of Human Embryonic Stem Cells to Neural Crest Stem Cells, Functional Peripheral Neurons, and Corneal Keratocytes. Biotechnol J, 12:67. https://doi.org/10.1002/biot.201700067

25. Vijayavenkataraman S, Kannan S, Cao T, et al., 2019, 3D-Printed PCL/PPy Conductive Scaffolds as Three-Dimensional Porous Nerve Guide Conduits (NGCs) for Peripheral Nerve Injury Repair. Front Bioeng Biotechnol, 7:266. https://doi.org/10.3389/fbioe.2019.00266

26. Athukoralalage SS, Balu R, Dutta NK, et al., 2019, 3D Bioprinted Nanocellulose-Based Hydrogels for Tissue Engineering Applications: A Brief Review. Polymers (Basel), 11:898. https://doi.org/10.3390/polym11050898

27. Altman GH, Horan RL, Martin I, et al., 2016, Design, microfabrication, and characterization of a moulded PDMS/SU-8 inkjet dispenser for a Lab-on-a-Printer platform technology with disposable microfluidic chip. Lab Chip, 16:3351-61. https://doi.org/10.1039/c6lc00636a

28. Athukoralalage SS, Balu R, Dutta NK, et al., 2019, 3D Bioprinted Nanocellulose-Based Hydrogels for Tissue Engineering Applications: A Brief Review. Polymers (Basel), 11:898. https://doi.org/10.3390/polym11050898

29. Altman GH, Horan RL, Martin I, et al., 2016, Design, microfabrication, and characterization of a moulded PDMS/SU-8 inkjet dispenser for a Lab-on-a-Printer platform technology with disposable microfluidic chip. Lab Chip, 16:3351-61. https://doi.org/10.1039/c6lc00636a
28. Duarte Campos DF, Lindsay CD, Roth JG, et al., 2020, Bioprinting Cell- and Spheroid-Laden Protein-Engineered Hydrogels as Tissue-on-Chip Platforms. *Front Bioeng Biotechnol*, 8:374. https://doi.org/10.3389/fbioe.2020.00374
29. Saldin LT, Cramer MC, Velankar SS, et al., 2017, Extracellular matrix hydrogels from decellularized tissues: Structure and function. *Acta Biomater.*, 49:1-15. https://doi.org/10.1016/j.actbio.2016.11.068
30. Baena JM, Jiménez G, López-Ruiz E, et al., 2019, Volume-by-volume bioprinting of chondrocytes-alginate bioinks in high temperature thermoplastic scaffolds for cartilage regeneration. *Exp Biol Med (Maywood)*, 244:13-21. https://doi.org/10.1177/1535370218821128
31. Sharma R, Smits IP, De La Vega L, et al., 2020, 3D Bioprinting Pluripotent Stem Cell Derived Neural Tissues Using a Novel Fibrin Bioink Containing Drug Releasing Microspheres. *Front Bioeng Biotechnol*, 8:57. https://doi.org/10.3389/fbioe.2020.00057
32. Skylar-Scott MA, Uzel SG, Nam LL, et al., 2019, Biomanufacturing of organ-specific tissues with high cellular density and embedded vascular channels. *Sci Adv*, 5:eaaw2459. https://doi.org/10.1126/sciadv.aaw2459
33. Bernal PN, Delrot P, Loterie D, et al., 2019, Volumetric Bioprinting of Complex Living-Tissue Constructs within Seconds. *Adv Mater*, 31:e1904209. https://doi.org/10.1002/adma.201904209
34. Keshavarz M, Wales DJ, Seichepine F, et al., 2020, Induced neural stem cell differentiation on a drawn fiber scaffold-toward peripheral nerve regeneration. *Biomed Mater*, 15:055011. https://doi.org/10.1088/1748-605X/ab8d12
35. Wang J, Kong X, Li Q, et al., 2021, The spatial arrangement of cells in a 3D-printed biomimetic spinal cord promotes directional differentiation and repairs the motor function after spinal cord injury. *Biofabrication*, 13:ac0c5f. https://doi.org/10.1088/1758-5090/ac0c5f
36. Wen Z, Zheng QJ, 2006, Directional guidance of nerve growth cones. *Curr Opin Neurobiol*, 16:52-8. https://doi.org/10.1016/j.conb.2005.12.005
37. Murphy SV, De Coppi P, Atala A, 2020, Opportunities and challenges of translational 3D bioprinting. *Nat Biomed Eng*, 4:370-80. https://doi.org/10.1038/s41551-019-0471-7
38. Vijayavenkataraman S, Van WC, Lu WF, et al., 2018, 3D bioprinting of tissues and organs for regenerative medicine. *Adv Drug Deliv Rev*, 132:296-332. https://doi.org/10.1016/j.addr.2018.07.004

**Publisher’s note**

Whioce Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.