Developing Bioinks for 3D Bioprinting Motor Neuron Progenitor Cells

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Abstract

In the United States, there are more than 12,000 new cases of spinal cord injury each year[1]. No effective treatment is available for these injuries except high-expense lifetime care. Here we introduce a fibrinogen-based bioink for the purpose of 3D bioprinting motor neuron progenitor cells (MNPCs) into hydrogel scaffolds as a potential treatment for major spinal cord injury. While other bioinks are commercially available, such as gelatin methylacrylate (GELMA) and Matrigel, a murine Engelbreth-Holm-Swarm (EHS) sarcoma extract, these bioinks are toxic to MNPCs and are not fully defined, respectively. For this reason, a fully defined bioink that is not toxic to MNPCs must be developed. The results suggest that the bioink developed for 3D bioprinting supports viability and differentiation of MNPCs, but the printing process that MNPCs go through while being extruded into hydrogel scaffolds must further be optimized to increase MNPC viability and differentiation.

Introduction

Major human spinal cord injury currently has no effective treatment. Societal costs are very large, as well as the personal loss sustained by the patients and their families. In the United States, there are more than 12,000 new cases of spinal cord injury each year[1]. Young adults are primarily affected, and the estimated lifetime cost of a cervical spinal cord injury in a 25-year-

old is approximately four million dollars[2]. Since there are currently no effective therapies for spinal cord injury, any new treatments would have a huge impact on public health.

After a spinal cord injury, a lesion forms at the site of injury which inhibits the regeneration of axons (Figure 1)[3]. Injection of neural progenitor cells into the injured spinal cord has shown to result in short-term functional recovery, but poor cell survival (1.2%) and spatial orientation due to immune rejection were also a result (Figure 2)[4–8]. Drs. Ann Parr (Neurosurgery) and Michael McAlpine (Mechanical Engineering) at the University of Minnesota currently have a collaborative grant to develop a 3D bioprinted, customizable spinal cord scaffold loaded with MNPCs and oligodendrocyte progenitor cells (OPCs) that can be inserted in this lesion as a potential treatment for spinal cord injury. These bioprinted scaffolds have the capability to direct the growth, migration, and differentiation of MNPC/OPCs within the spinal cord.

Bioinks are the materials that are used to produce the matrix of the 3D printed tissue. The lab has previously tried a commercial bioink, gelatin methacrylate (GELMA), to print MNPC/OPCs, but had very low post-printing cell viability. The lab hypothesized that GELMA performed poorly with MNPC/OPCs due to free radicals being produced in the photo-polymerization step of 3D bioprinting [9]. Photo-polymerization is the process of changing the properties of the bioink when exposing it to ultraviolet or visible light while printing. The lab currently uses Matrigel, a murine Engelbreth-Holm-Swarm (EHS) sarcoma extract composed of poorly controlled mixtures of growth factors and basement membrane proteins. Batch to batch variation of Matrigel leads to inconsistent 3D bioprinting settings and thus, a fully defined bioink that results in high cell viability, growth, migration, and differentiation of MNPC/OPCs within the spinal cord must be developed. This would be the first step in creating a clinically relevant

scaffold through new advances in custom 3D bioprinting technology.

Recent work by Jennifer A. Lewis at Harvard University and Anthony Atala at Wake Forrest has shown that a fibrinogen-based bioink might be a good substitute to GELMA and Matrigel because a fibrinogen-based bioink can be fully defined and requires no photo-initiator as a means of cross-polymerization[10,11]. Fibrinogen is a natural protein that is responsible for blood clotting within the body. Fibrinogen is polymerized to a fibrin matrix with the addition of thrombin, the enzyme assisting fibrinogen in the blood clotting process. Because the main components of these fibrinogen-based bioinks are naturally found in vertebrates, it is believed that a fibrinogen-based bioink more closely resembles conditions within the body, and may be more optimal for cell viability, growth, migration, and differentiation.

Materials and Methods

Cell Differentiation and Viability. MNPCs were differentiated from human induced pluripotent stem cells (hiPSCs) using a protocol develop by the lab of Drs. Ann Parr and James Dutton within the Stem Cell Institute at the University of Minnesota. The lab uses a hiPSC system using defined media and a recombinant substrate that employs cell dissociation with a hypertonic citrate solution which eliminates variability during hiPSC cell expansion and provides a simple cGMP-compliant technique for hiPSC cultivation that is appropriate in both research and commercial applications[12]. The cell expansion begins with a neural induction by SMAD inhibition using LDN. Neural progenitor expansion is then performed using retinoic acid (RA), LDN193189, and CHIR99021. MNPC expansion occurs when the neural progenitors are switched to RA and smoothened agonist (SAG). Finally, maturation occurs when replacing the RA/SAG components with a number of neural growth factors including neurotrophic factor 3, brain-derived neurotrophic factor, and

glial-derived neurotrophic factor (Figure 4). All live dead staining was performed using the Molecular Probes™ LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells. Calcein AM was used as the "live" component of the kit and Ethidium homodimer-1 was used as the "dead" component of the kit.

Scaffold Materials. The primary scaffold was a Poly (ethylene glycol) diacrylate (PEGDA) hydrogel mixture consisting of 20% PEGDA (700MW), 5.5% polyethylene oxide (1 million MW), 0.5% Lithium-acylphosphinate (LAP), and 10% glycerol. LAP was used as a photo initiator and functions to crosslink the scaffold when cured using 405 nm light. Glycerol was used for preventing liquid evaporation and for optimizing viscosity of the mixture. Figure 5 depicts the molecular structure and use of the acrylate groups and backbone. Double acrylate groups provide photo-curable properties and the backbone provides slow in vivo degradation. Photo-curing allows for mechanically strong hydrogels to be formed, creating an effective scaffold for 3D modeling. Surface tension of the PEG chains are similar to those of cellular membranes causing PEGDA to be an inefficient material for cells to sit [13].

Fibrinogen-based Bioink Preparation and Composition. The fibrinogen-based bioink as a cell-laden bioink was a mixture of gelatin from porcine skin (G2500), fibrinogen from bovine plasma (F8630), hyaluronic acid sodium salt from *Streptococcus equi* (53747) and $CaCl_2$. Solutions of fibrinogen, gelatin, and $CaCl_2$ were prepared prior to the printing process and diluted to the proper volume using N2B27 or DMEM immediately before printing. A 15 wt/vol% gelatin solution (type A; 300 bloom from porcine skin) was produced by warming in DPBS (1x Dulbecco's PBS without calcium and magnesium to 70°C and stirring for 12 h, and then the pH was adjusted to 7.5 using 1M NaOH. The gelatin solution was then sterile filtered and stored at

4°C. A fibrinogen solution (50 mg/mL) was produced by dissolving fibrinogen in sterile DPBS without calcium and magnesium. The solution was held at 37°C without stirring until completely dissolved and then sterile filtered and stored at -20°C until use. Stock $CaCl_2$ (250mM) was created by dissolving $CaCl_2$ powder in DPBS without calcium and magnesium. HA was dissolved in N2B27 or DMEM at 37°C by stirring until completely dissolved, prior to the addition of the gelatin, fibrinogen, and $CaCl_2$ solutions. The final concentrations for each component were 10 mg/mL fibrinogen, 7.5 wt/vol% gelatin, 2.5 mM $CaCl_2$, and 3 mg/mL HA.

2D and 3D printing process. A Fisnar industrial robotic dispenser was used for the 3D printing of the PEGDA scaffold as well as the fibrinogen-based bioink. The Fisnar printer allowed for four possible materials to be loaded into standard size syringes, along with tip gauges ranging from 27 -33 GA, and mounted to the dispenser. The material in each syringe was independently extruded through application of a pressure regulator. The regulator allowed for pressures ranging from 0 to 100 psi to be used for extrusion-based 3D printing [14].
Two of its four possible heads were utilized for simultaneous scaffold and cell printing. One head was loaded with PEGDA and the other with fibrinogen-based bioink loaded with MNPC/OPCs (Figure 6).

Two materials can be printed simultaneously allowing (i) optimization of printing time to reduce cell death due to long exposures to room temperature, and (ii) precise placements of the cells in the designed scaffold. The scaffolds were printed in the –x to +x direction using a metal tip with an inner diameter of 200 μm. The PEGDA was extruded using a pressure of 22.5 psi, printed at room temperature, and cured real-time using 405 nm light.

The fibrinogen-based bioink was then printed in-between the channel walls using continuous droplets with varying pressures between 0.5 psi and 1.5 psi. The fibrinogen-based

bioink was printed using a metal tip with an inner diameter of 100 μm and was uniformly heated to 37 °C through the use of a jacket to prevent solidification of the bioink. The jacket consisted of the metal film, insulating tape, metal wiring, and cover tape. A DC power supply was used to apply current to the wires.

After printing, the scaffolds were polymerized with a thrombin/transglutaminase (TG) solution for 15-20 minutes. Thrombin from bovine plasma (T4648) and TG (MooGloo) are responsible for the cross-linking of the fibrinogen-gelatin bioink. The polymerization solution was then replaced with N2B27 medium plus three neurotrophic growth factors. Solutions of thrombin and TG were prepared prior to the printing process. The TG solution (60 mg/mL) was created by dissolving lyophilized powder (Moo Glue) in DPBS without calcium and magnesium and stirring at 37°C until completely dissolved, sterile filtered and stored at -20°C. To create the thrombin solution, lyophilized thrombin was reconstituted at 100 U/mL using DPBS and stored at -20°C. Prior to use, the TG solution was added to the thrombin solution in order to achieve a final concentration of 0.2 wt%.

Results and Discussion

Optimization of bioink. The first step in the optimization of the fibrinogen-based bioink was to find a composition which promoted viability and differentiation of MNPCs in a 2D culture before moving to a 3D printed scaffold. Initially, a bioink previously produced by Atala *et al.* [10] resulted in average MNPC viability of 66.07% at 24 hours and 81.54% at 48 hours. A second scaffold, previously produced by Lewis *et al.* [11], resulted in average MNPC viability of 73.09% at 24 hours and 85.71% at 48 hours (Figure 7). Moving forward, the Lewis *et al.* bioink composition, containing a composition previously described, was used. The Lewis *et al.* original

bioink did not contain HA, but HA was added to provide consistency while printing and preventing jamming of the printing tip in addition to decreasing shear forces present while printing [10].

Printing Viability. After a fibrinogen-based bioink was developed that resulted in optimal cell viability and differentiation in a 2D culture prior to the printing process, it had to be determined whether or not the fibrinogen-based bioink also resulted in optimal viability and differentiation in a 2D culture after the printing process had been performed. Initially, all stock components of the fibrinogen-based bioink were diluted in DPBS to the final concentrations needed. When MNPCs were suspended in this bioink, printed through the nozzle and plated 3D into silicon scaffolds, resulting live/dead stains showed large aggregates of dead MNPCs with no projecting axons present, very clearly indicating that the MNPCs were not in an optimal environment, when compared to a Matrigel suspension (Figure 8). It was hypothesized that unlike Matrigel, the fibrinogen-based bioink resulted in poor viability and differentiation because it has no nutrients to support the MNPCs during the printing process. The printing process itself is very long and tedious. MNPCs are starved of nutrients for upwards of 1.0 to 1.5 hours. Matrigel is able to provide nutrients during this printing process, but as stated earlier, Matrigel is not consistent from batch to batch and for that reason, a fibrinogen-based bioink is more optimal.

In order to provide nutrients/growth factors to the MNPCs during the printing process itself, a change was made to the fibrinogen-based bioink. Instead of diluting out the bioink components with DPBS, two different variations of the bioink were produced—one using the basal media DMEM as a dilution solution and the second using the differentiation media, N2B27 as a dilution solution. Both modified fibrinogen bioinks contained 29% of either DMEM or N2B27 in the final modified fibrinogen-based bioink concentration.

When MNPCs were suspended in this modified fibrinogen-based bioink, printed through the extrusion tip and plated 2D, resulting live/dead stains showed viabilities of 70.9%, 67.38%, and 19.43% for the modified bioink containing 29% DMEM at time points of 2 hours, 24 hours, and 48 hours respectively. Live/dead stains showed viabilities of 75.9%, 55.2% and 18.3% for the modified bioink containing 29% N2B27 at time points of 2 hours, 24 hours, and 48 hours respectively (Figure 9). Based on this data, it is assumed that it is not the fibrinogen-based bioink that is resulting in low cell viability and differentiation, but the stress and amount of time that the MNPC/OPCs go without proper nutrients during the printing process.

Moving forward, optimization of the MNPC/OPC printing process will help with viability and differentiation when printing with this fibrinogen-based bioink. Efficiency in the time of printing itself, the time that scaffolds are out of media after printing is complete, and transportation of scaffolds from the printing location to the cell culture/incubation location are all aspects that must be optimized in order to make a fibrinogen-based bioink viable for printing MNPC/OPCs in a fully defined bioink.

In addition to the problem of the printing process, there is also the question of fibrinolysis. When cells start to produce plasmin, the fibrin crosslinked bioink will begin to degrade. Stoddard *et al.* has found that the use of epsilon-aminocaproic acid (EACA) is a useful for preventing plasmin-based fibrinolysis. Additionally, it was found that EACA does not hinder differentiation of human bone marrow-derived mesenchymal stem cells[15], which may indicate that MNPC/OPCs derived from hiPSCs would also be able to successfully differentiate in the presence of EACA. If these barriers facing the use of fibrinogen as a bioink for 3D bioprinting are overcome, it would be a step in the right direction for development of an effective treatment for major human spinal cord injury.

Conclusion

In conclusion, a fibrinogen-based bioink was developed based on previous literature that is capable of promoting viability and differentiation of MNPCs when plated before the printing process. However, when plating MNPCs after the printing process, viability and differentiation are greatly reduced, indicating that the printing process itself is adversely affecting the viability and differentiation of these cells. Moving forward, the 3D bioprinting process will continue to be optimized in order to promote increased MNPCs viability and differentiation. The plasmin-based degradation of the polymerized fibrin scaffold will also be analyzed to ensure structural integrity for the length of time needed. This optimization process will help to develop a useable bioink for 3D bioprinting MNPCs for spinal cord injury repair.

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Figures

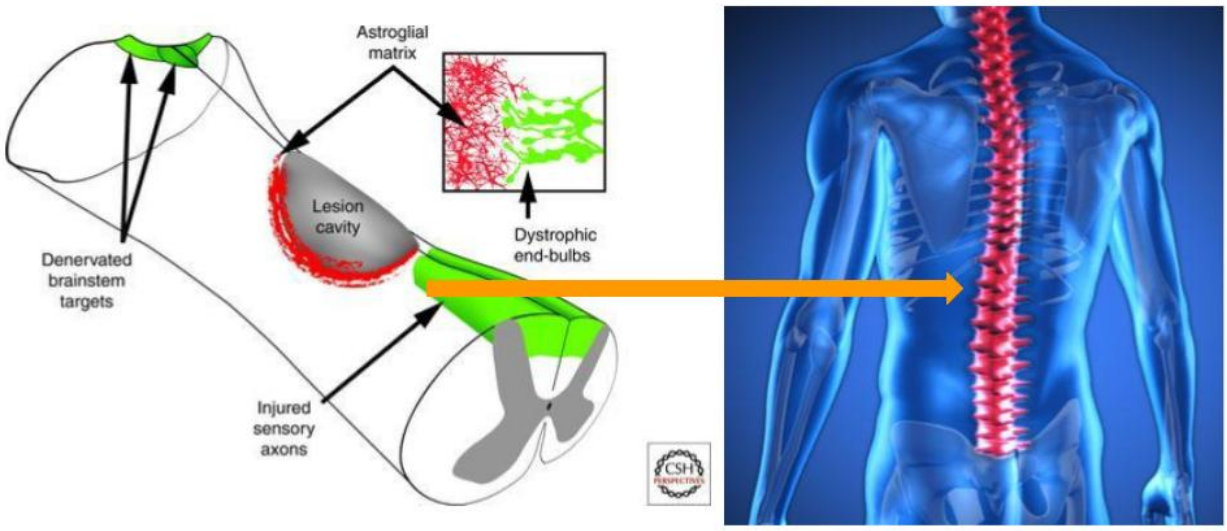

Figure 1. Anatomy of the spinal cord. The spinal cord connects the majority of the PNS to the brain. The spinal cord also sends sensory signals to the brain and performs reflexive movements[16,17].

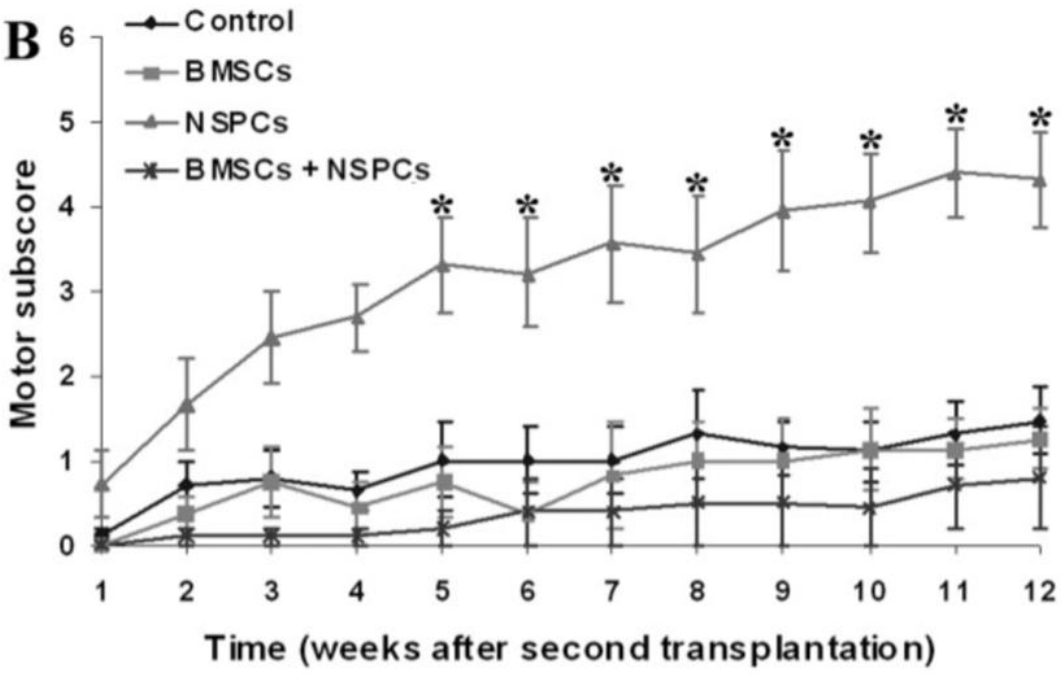

Figure 2. Motor sub-score performance in rats with spinal cord injury after the injection of neural progenitor cells into the injured spinal cord [8].

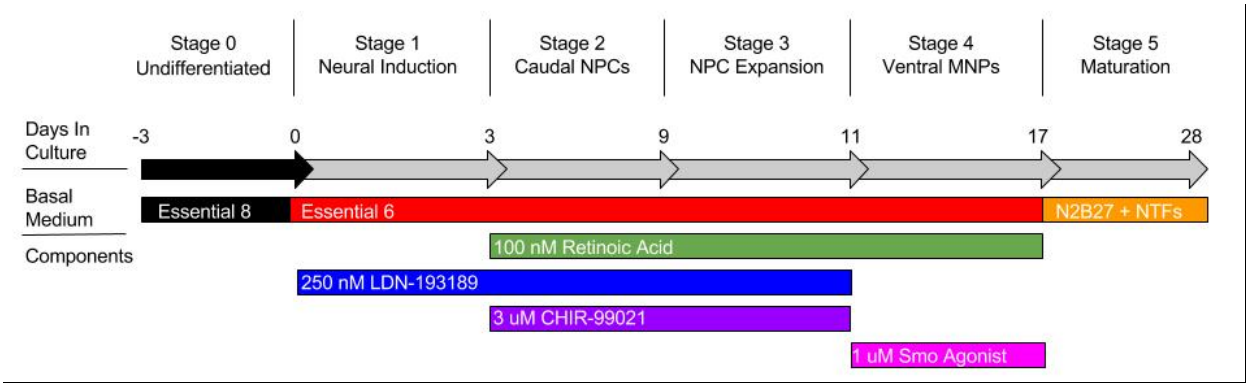

Figure 3. c-GMP Compliant Differentiation of Motor Neuron Progenitor Cells and Motor Neurons.

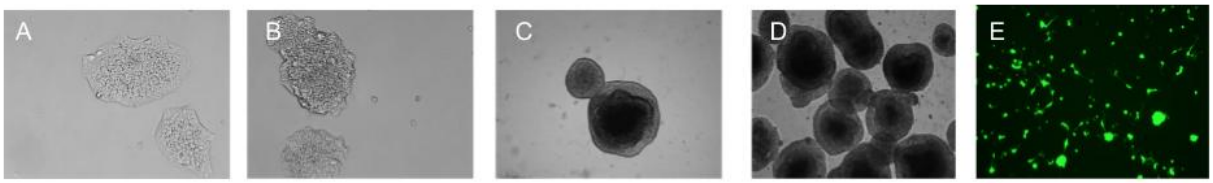

Figure 4. Visualization of c-GMP Compliant Differentiation of Motor Neuron Progenitor Cells and Motor Neurons. (A) Day zero: human induced pluripotent stem cells. (B) Day three: neural induction of hiPSCs. (C) Day 12: ventral motor neuron progenitors. (D) Day 12: ventral motor neuron progenitor buds. (E) Day 18: mature motor neurons stained with Calcein AM live/dead stain.

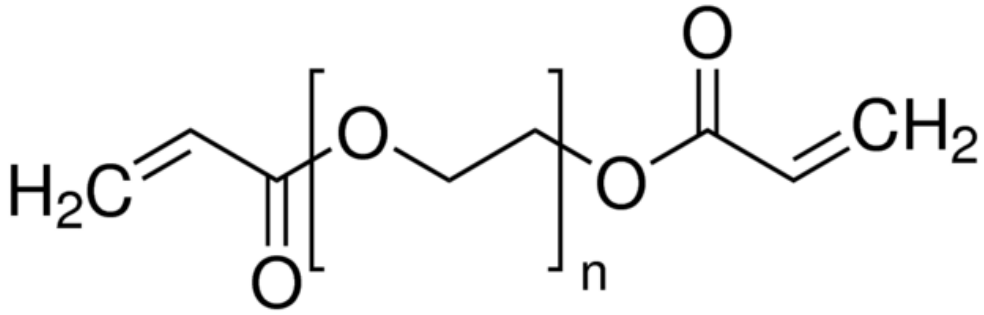

Figure 5. Poly (ethylene glycol) diacrylate (PEGDA) molecular structure [13,18].

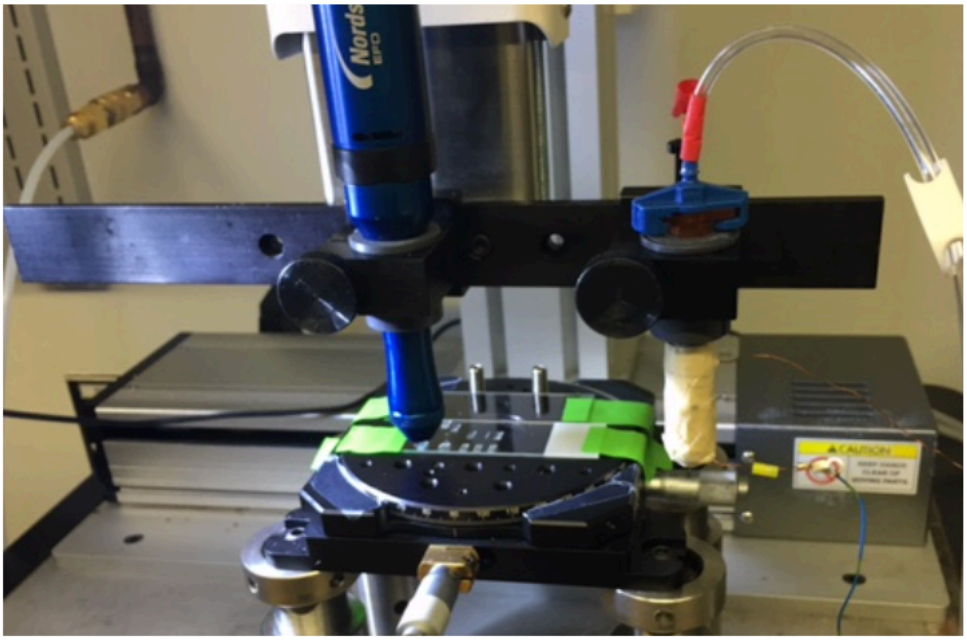

Figure 6. Fisnar industrial robotic dispenser with dual dispensing syringes.

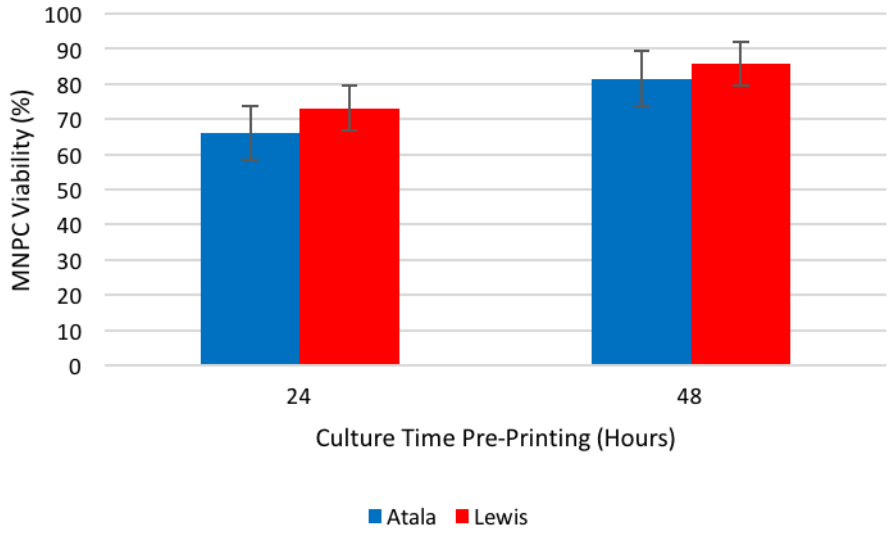

Figure 7. MNPC 2D culture viability pre-printing process.

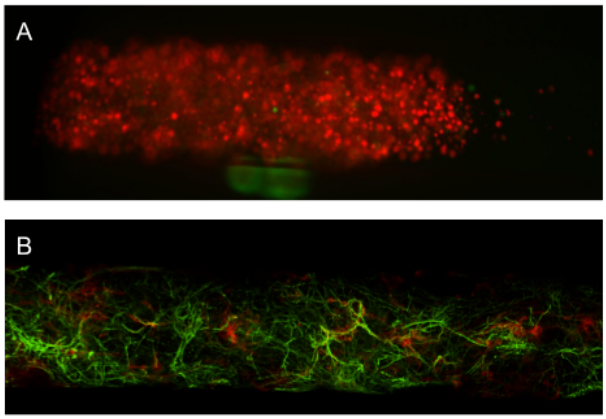

Figure 8. (A) Live/dead stain of MNPCs in fibrinogen-based bioink. (B) Confocal 2D max projection antibody stain of MNPC/OPCs in Matrigel. The green (488) channel was stained with Beta-3-tubulin, a marker for motor neurons. The red (555) channel contained OPCs which are mCherry GFP naturally.

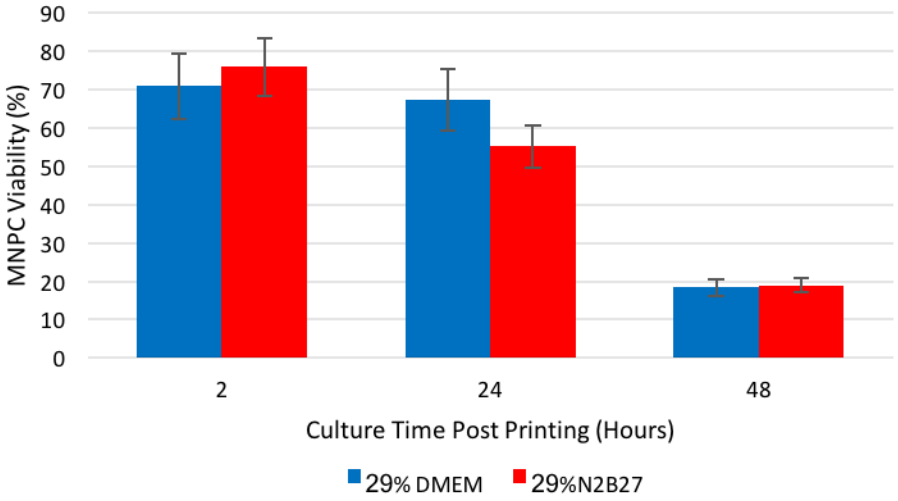

Figure 9. MNPC 2D culture viability post-printing process in modified fibrinogen-based bioinks.

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