Development of Novel 3D Printed Scaffolds with Core-shell Nanoparticles for Nerve Regeneration

Se-Jun Lee, Wei Zhu, Lanier Heyburn, Margaret Nowicki, Brent Harris and Lijie Grace Zhang

Abstract— A traumatic injury of peripheral nerves is serious clinical problem that may lead to major loss of nerve function, affecting quality of patient’s life. Currently nerve autograft is widely used to reconstruct the nerve gap. However, such surgical procedure suffers from many disadvantages including donor site morbidity and limited availability. In order to address these issues, neural tissue engineering has focused on the development of synthetic nerve scaffolds to support bridging a larger gap and improving nerve generation. For this purpose, we fabricated a novel 3D biomimetic scaffold, which has tunable porous structure and embedded core-shell nanoparticles with sustained neurogenic factor delivery system, using stereolithography based 3D printing and co-axial electrospraying techniques. Our results showed that scaffolds with larger porosity significantly improve PC-12 neural cell adhesion compared to ones with smaller porosity. Furthermore, scaffolds embedded with bovine serum albumin containing nanoparticles showed an enhancement in cell proliferation relative to bared control scaffolds. More importantly, confocal microscopy images illustrated that the scaffold with nerve growth factor (NGF) nanoparticles greatly increased the length of neurites and directed neurite extension of PC-12 cells along the fiber. In addition, the 3D printed nanocomposite scaffolds also improved the average neurite length of primary cortical neurons. The results in this study demonstrate the potential of this 3D printed scaffold in improving neural cell function and nerve growth.

Index Terms— core-shell nanoparticles, drug delivery, nerve regeneration, 3D printing

I. INTRODUCTION

NEURAL tissue is degenerative in nature after injury and has notoriously poor self-regenerating capacity. In most cases, artificial intervention is necessary once injuries occur to promote adequate healing. Currently, transplantation of autograft is the most commonly used method to bridge the lesion site [1], [2]. However, there are some innate drawbacks to autologous transplantation. For instance, the use of autografts often leads to pain and donor site morbidity. Also reconnecting the proximal and distal stump of the injured nerve is very difficult due to size inconsistency between donor nerve on autograft [3], [4]. Alternatively, neural tissue engineering is being explored in an effort to develop artificial nerve scaffolds to overcome these limitations.

Neural tissue engineering primarily focuses on the development of artificial nerve scaffolds to replace the damaged tissues and eventually restore nerve function. The fundamental strategies for neural tissue engineering involves integration of three-dimensional biomimetic scaffolds and various neural cells to mimic natural extra-cellular matrices (ECM) in nervous system [1], [5]-[8]. Ideally, the neural scaffold should meet several requirements before implanting to the injured nerve. First, the scaffold should act as substrate to allow neural cell adhesion, migration, and proliferation. Second, the scaffold should be biocompatible and biodegradable with very low toxicity. Additionally, the surface of scaffold should be highly porous to allow sufficient gas and nutrient exchange [9]. Designing scaffolds with nano to micro topological features having sustained release of growth factors is also highly desirable to closely interact with the nanostructured ECM [10]-[12]. The continual supply of exogenous growth factor from the implanted scaffold is also critical to support axon regeneration [1].

When designing a degradable drug delivery system for cells and tissue, it is important to consider that bioactive proteins and growth factors are delivered for prolonged time periods and in a controlled manner [13]. Therapeutic drugs with short half-life circulation limit healing potential for comprehensive tissue repair. An effective neurogenic factor delivery system can successfully facilitate neurite outgrowth and tissue regeneration [14]. Thus, in this study, we fabricated biodegradable core-shell nanoparticles for sustained release of bioactive factors to enhance nerve regeneration. Specifically, a co-axial electrospraying technique was successfully implemented to produce poly lactic-co-glycolic acid (PLGA) nanoparticles encapsulating bovine serum albumin (BSA) and nerve growth factors (NGF). This process allows two immiscible solutions to electrospray through two separate channels into one nozzle. The co-axial electrospray method holds several advantages over the conventional double emulsion method. It is preferred for preparing bioactive factors and genes, because it eliminates emulsion steps with exposures to harmful organic solvents [13], [15], [16]. In addition, coaxial electrospray has the potential to encapsulate...
reagents with much higher efficiency compared to the double-emulsion process [13], [15]-[17]. NGF is a neurotrophic protein that is essential in the development and survival of neural cells. Numerous studies have reported that the NGF loaded neural scaffold enhances neurite extension [18]-[21]. We chose PLGA as a carrier material to encapsulate the bioactive factors in our study. Many studies have demonstrated that PLGA is a promising candidate for controlled therapeutic delivery due to its excellent biocompatibility, mechanical properties, and biodegradability [13], [22].

Biodegradable and biocompatible scaffolds can be fabricated by various methods such as electron beam lithography, photolithography, electrospinning and 3D printing [5], [23]-[25]. Amongst them, 3D printing techniques have drawn great interest because they can prepare scaffolds with highly controlled spatial architecture to meet the customized requirements. Recent works employed direct laser polymerization techniques to fabricate a custom-made 3D microstructured scaffold with biocompatible resins such as hybrid organic-inorganic materials [26], PLGA [27] or the use of ECM proteins [28]. The biocompatibility of the laser-formed scaffold was sustained throughout the long-term in vivo study [26]. Another research group combined fused filament 3D printing with direct laser writing ablation to fabricate 3D microporous structures with grooves and microholes [29]. In addition, several studies have used 3D printing techniques to print aligned and multi-layered nerve scaffold. For example, Evangelista et al successfully used a commercial stereolithography (SL) system to fabricate 3D, single-lumen polymer based nerve conduit to promote peripheral nerve regeneration [30]. SL is a laser-based 3D printing system capable of fabricating aligned micro-macro size 3D constructs via a layer by layer assembly method. In addition, SL printing can use biocompatible and biodegradable polymers such as poly (ethylene glycol) diacrylate (PEG-DA) to fabricate porous scaffolds that are adequate for cells and biomolecules. While SL printing has numerous advantages, it is not capable of achieving nano-scale architecture. As previously discussed, nano-features are highly desirable for nerve scaffolds to effectively mimic native nanostructured extracellular matrix. To meet this need, we integrated the above core-shell nanoparticles on SL printed nerve scaffolds for the first time achieving nano-topology and sustained bioactive molecule delivery for enhancing nerve regeneration. First, square grid geometry was chosen as the base pattern to create a 3D porous scaffold with internal pores and channels. More complex three-dimensional structure such as a tubular network will be examined in future study. In addition, the printed scaffolds were thoroughly characterized and evaluated using two types of neural cells, rat pheochromocytoma cells (PC-12) and primary rat cortical neurons, in vitro.

II. METHOD

A. Nanoparticle synthesis and characterization

Core-shell nanoparticles were fabricated by an electrospraying method (Figure 1A) using a core-shell needle with 0.254 mm inner and 0.698 mm outer diameters. The core solution was comprised of 1.0% (w/v) BSA in distilled water. PLGA dissolved in acetone at a concentration of 2.5% (w/v) served as the shell solution. The two solutions were fed at a flow rate of 2.5 mL/h through a single syringe. A 9 ~ 15 kV voltage was applied to obtain stable cone jet mode of nanofluids. The nanoparticles were collected in water in a petri dish about 8-10 cm beneath the needle tip. Scanning electron microscope (SEM, Zeiss NVision 40 FIB) and transmission electron microscope (TEM, JEOL 1200 EX) were used to examine the morphology of BSA encapsulated nanoparticles. Lyophilized nanoparticles were resuspended in distilled water, dried overnight on a copper grid, and coated with gold prior to imaging.

B. Evaluation of nanoparticles

PLGA core-shell nanoparticles were evaluated for encapsulation rate, loading efficacy, and release profile following methods outlined in our previous study [14]. BSA embedded nanoparticles were collected in ultra pure water followed by centrifuging, freezing and lyophilizing for testing. The BSA solution was also sprayed on the PEG-DA scaffolds directly for the control group. The encapsulation rate of nanoparticles was calculated by BCA Protein Assay Reagent kit (Pierce Biotechnology). Briefly, 10 mg of lyophilized
nanoparticles were dissolved in 1 mL of 0.1 M NaOH solution. Following overnight incubation at 4 °C, the antigen concentration was determined using a micro-BCA protein assay kit according to the manufacturer's instructions for 96-microwell plates (Corning Inc., Corning, NY). The absorbance of the samples was measured at 562 nm using a microplate reader. Blank PLGA nanoparticles were used as the control. The encapsulation efficacy was calculated by dividing the mass of BSA encapsulated in the spheres by the total mass of BSA used in sphere fabrication, as shown in the equation below.

\[
Encapsulation\ efficacy = \frac{BSA\ encapsulated\ in\ nanoparticles}{Total\ weight\ of\ BSA} \times 100\%
\]

The loading efficacy was determined by the following equation:

\[
Loading\ efficacy = \frac{BSA\ encapsulated\ in\ nanoparticles}{Total\ weight\ of\ nanoparticles} \times 100\%
\]

In order to evaluate BSA release from neural scaffolds \textit{in vitro}, 0.5% BSA nanoparticles were 3D printed into a scaffold with 68% porosity (printing details are in next section, the porosity is selected based on the results of our PC-12 adhesion and proliferation studies to be described next). The same printed scaffolds with aqueous sprayed BSA served as controls. Both of the samples were separately placed in 96-microwell plates (Corning Inc., Corning, NY). The assay kit according to the manufacturer's instructions for concentration was determined using a micro-BCA protein solution. Following overnight incubation at 4 °C, the antigen nanoparticles were dissolved in 1 mL of 0.1 M NaOH solution. The supernatant was collected and identical fresh buffer was added. Supernatant was analyzed by BCA Protein Assay Reagents kit (Pierce Biotechnology) and the BSA accumulative release was quantified. Each sample was prepared in triplicate.

C. 3D neural scaffold fabrication

A SL based 3D printing was developed on the existing Printrbot® rapid prototyping platform for addictive manufacturing of photocrosslinkable hydrogel scaffolds. The 3D printer consists of a movable Z-stage and a 110 µm fiber optic-coupled solid-state UV (355 nm) laser (MarketTech, Scotts Valley, CA) mounted on an X-Y toolhead for three-axis motion. The laser beam was focused to a spot diameter of 190 ± 50 µm with an energy output of ~ 20 µJ at 15 kHz. Specifically, the printing configuration was controlled by the Prinifier control software package. The laser frequency was optimized in the 7-10 kHz range and the printing speed was set to 25 mm/s.

Scaffolds geometries were designed with small, medium, and large square pores, corresponding to 44%, 56%, and 68% porosity, using computer-aided design (CAD) software. Printable hydrogel inks were composed of 60 wt% poly (ethylene glycol) (PEG, Mn 300), 40 wt% poly (ethylene glycol) diacrylate (PEG-DA, Mn 700) and photoinitiator (Irgacure 819) (0.5 wt% of PEG-DA amount). For the nanoparticle-embedded scaffold fabrication, lyophilized nanoparticles were blended with printable solution by ultrasonication in concentrations of 0.1% (w/v), 0.5% (w/v), and 1% (w/v), respectively. Figure 1 shows the overall design.

D. 3D printed nanocomposite scaffold fabrication

SL printed scaffolds were imaged via SEM to assess their morphology and channel size. All samples were sputter coated with gold for 8 s (sputter set point 80 mTorr Vacuum pressure) and visualized with a Zeiss SigmaVP SEM. Tensile mechanical testing was conducted with MTS Criterion Model 42 under a constant crosshead rate of 1 mm/min. All printed scaffolds were cut into 10 mm by 40 mm rectangular sheets and clamped on each end by mechanical grips. The linear portion of the resulting tensile stress-strain curve was used to calculate Young's modulus. A Raman spectrometer (Horiba Scientific) was used to analyze the 3D printed nanocomposite scaffolds at a 532.06 nm line of Ar+ laser for excitation over a range of 0 - 4000 cm⁻¹. Experiments were conducted at 25 °C and 45% humidity.

The contact angles of the nanocomposite scaffolds were measured by a drop shape analyzer (DSA4, Krüss) equipped with a camera to determine surface hydrophilicity of the scaffolds. Briefly, circular samples with 8 mm diameters were placed on glass slides. 1.0 µL of ultra pure water was pumped automatically on the samples' surface using a syringe. Temporal images immediately after the droplet fell down were selected from videos for contact angle measurement. All experiments were conducted in ambient conditions and were performed at least three times per group.

E. Neural cell culture and growth studies

PC-12 cells (ATCC) were utilized to evaluate the neural cell response on printed nerve scaffolds. PC-12 cells were cultured in RPMI 1640 (ATCC), a high glucose media, supplemented with 10% horse serum (ATCC), 5% fetal bovine serum (FBS, ATCC), 1% L-glutamine (Sigma-Aldrich), and 1% penicillin-streptomycin (10,000 U/mL penicillin and 10,000 µg/mL Streptomycin, Gibco). Cells were cultured on collagen coated flasks (Becton, Dickinson and Company) under a humidified atmosphere with 5% CO₂ at 37°C. Medium was changed every two days. SL printed scaffolds were punched into small circle samples with 8 mm diameter. The samples were then sterilized by UV for 15 minutes, rinse twice using PBS, and pre-wetted in poly-l-lysine at 37 °C before seeding cells. PC-12 cells were seeded at a density of 30,000 per scaffold for 4 hour cell attachment and 2, 4 and 6 day proliferation studies. At each prescribed time, cell seeded scaffolds were transferred to a new well plate. Cell numbers were quantified via Alamar Blue assay (Promega) and analyzed using a Thermo Scientific Multikan GO Spectrophotometer at 570 nm and 600 nm wavelength light after adding Alamar Blue solution for 4 hours at 37 °C. The adhesion and proliferation studies were repeated three times with three replicates per group, totaling 9 samples per group.

Cortical neuronal cultures were harvested from rat embryonic cortices. All protocols involving animals were approved by the Georgetown University Institutional Animal Use and Care Committee, and were in compliance with the...
standards stated in the Committee on Care and the Use of Laboratory Animals of the Institution of Laboratory Resources DH EW Publication. Briefly, cortices from embryonic day 18 (E18) Sprague-Dawley rat embryos (Charles River, Wilmington, MA) were dissected from their meninges and blood vessel in calcium-free Hank’s buffered saline solution (HBSS). All cortices were then minced and dissociated using sterile fine-tipped forceps and scissors. The minced tissues were incubated in Trypzean for 3 minutes. Following the removal of Trypzean, PBS containing of 40uL/mg DNAse was added to the tissue and tissues were triturated through a 5 mL pipet. The supernatant was then gently centrifuged at 300 g for 10 min at room temperature. The cell pellet was resuspended in Neurobasal media (Gibco) with 1% B 27 supplement (Gibco), 0.5 mM glutamine and 50 ng/ml NGF until immunocytochemistry.

F. Immunocytochemistry of PC-12 cells and primary cortical neurons

For differentiation studies, PC-12 cells and primary cortical neurons were cultured in respective standard medium with 50 ng/mL NGF on large porosity scaffold, with and without nanoparticles, and NGF sprayed scaffold for 7 (PC-12) and 8 (primary neuron) days. All scaffolds were coated with laminin for 4 hours in order to enhance cellular attachment. At specified time, samples were rinsed with PBS and fixed with 10% formalin for 10 minutes at room temperature. The cells were further permeabilized with 0.1% Triton X-100 in PBS for 5 minutes followed by blocking with PBS containing 1% BSA. Diluted primary antibodies, rabbit anti-MAP2 antibody (1:500; Abcam) and mouse anti-TuJ1 (1:1,000, Covance), were gently added into scaffolds and incubated at room temperature in a moist environment to prevent drying. This was followed by second antibodies incubation with Alexa Fluor 488 (Abcam) goat anti-rabbit and Alexa Fluor 594 goat anti-mouse (Life technologies) at room temperature. The cell nuclei were stained by 10 µg/mL 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Life technologies). Laser scanning confocal microscopy (LSCM 710, Zeiss) was employed to visualize and monitor the 3D neural cell growth and neurite extension of cells.

G. Cell and neurite length counting

Neuronal cells and neurite length were quantified with image analysis software (ImageJ; National Institutes of Health, Bethesda, MD) and the NeuriteTracer plugin. At least four areas from 10× objective were randomly selected for analysis on each sample (2 samples/group). A total of 207 to 391 cells/group were observed with nuclear staining (DAPI) and the portion of neuronal cells, that is, TUJ-1 positive cells, were calculated. The NeuriteTracer plugin of the neuronal marker TUJ-1 to quantify the neuronal differentiation. The total neurite length was defined as the total measured length of all the neurites in a field. The average neurite length is calculated by dividing the total neurite length by the total neurite count. Similarly, the average total neurite length per cell was calculated by dividing the total measured lengths of all neuritis in the field by the total cell count. Finally, the average length of the longest neurite per cell was calculated.

H. Statistical analysis

All quantitative data are expressed as average ± standard error of the mean. Numerical data were analyzed via student’s t-test to determine differences amongst the groups. Statistical significance was considered at p < 0.05.

III. RESULTS

A. Nanoparticle characterization in vitro sustained BSA release

Figures 2A and B show the SEM and TEM images of PLGA core-shell nanoparticles. Our previous study examined the uniform structures of electrosprayed core-shell PLGA nanoparticles [14]. The nanoparticles have a nanostructured spherical shape and homogeneous diameter distribution around 81 to 327 nm with average diameter of 170 nm. The results showed that the electrosprayed core-shell PLGA nanoparticles have a high encapsulation rate (80.45%) and loading efficacy (41.25%).

The results of the in vitro cumulative release study demonstrated that scaffolds embedded with BSA-PLGA nanoparticles presented much lower initial burst release than bare BSA directly sprayed on scaffold (Figure 2C). In the initial 10 hours, almost full release was observed for the samples directly sprayed with BSA. In contrast, around 60% of BSA was released from the core-shell nanoparticles at the same time point. The cumulative drug release of nanoparticles was observed to be significantly lower than the other group in 24 hours (p<0.05). Comparably, the drug release rate from NGF-PLGA nanoparticles in the scaffold was consistently lower than that of bare NGF directly sprayed on the scaffold over a 7 day release period (Figure 2C). Around 47% of total NGF was released from the samples embedded with NGF-PLGA nanoparticles, whereas bare NGF sprayed on scaffold released close to 80% of its total contents in the

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initial 10 hours.

B. 3D printed nanocomposite scaffold characterization

Figures 3A-F represent the CAD models of scaffolds with different porosity designed by commercially available 3D software. All scaffolds were designed with square patterns forming highly aligned channels. The photo images show that all scaffolds (Figure 3G-I) are printed as depicted in CAD models (Figures 3A-C). The scaffolds with nanoparticles (Figures 3J-L) were also successfully printed while maintaining the size and scale of the aligned channels. In general, the scaffolds with nanoparticles exhibited more turbid color due to the incorporation of PLGA.

Figure 4 displays the SEM morphology of printed scaffolds with porosity percentages of 44%, 56% and 68%, respectively (Figures 4A-C). It reveals that the SL-based printer can produce uniformly oriented scaffolds with micrometer resolution channels. It was also observed that the pores were completely interconnected throughout the whole structure. In the present study the scaffold with 68% porosity was further evaluated with incorporation of different concentrations of BSA nanoparticles. The cell attachment study, which will be discussed in more detail in the next section, determined that scaffolds with 68% porosity attracted more PC-12 cells than scaffolds with other porosity. The increased concentration of BSA nanoparticles of 0.1%, 0.5% and 1.0% results in a slightly thicker channel with rougher surfaces (Figures 4D-F). Despite decrease of roughness with increasing BSA nanoparticle concentration, they all remain rougher than scaffolds without nanoparticles, as illustrated by high magnification SEM images (Figures 4G-I). Nonetheless, they were still able to maintain the same consistent geometry compared to the control group.

Figure 5 shows nanotopological features on the surface of 3D printed scaffold with BSA nanoparticles. By modifying a surface with nanoparticle coating, we can create nanotopography to a three-dimensional scaffold. BSA-PLGA nanoparticles appear in the form of a grayish white powder on the surface of the scaffold at 3K magnification (Figure 5A). When the magnification was increased to 55K (Figure 5B), the nanoparticle having sphere morphology was clearly visible on the surface of the scaffold.

Surface Raman spectroscopy characterization of the SL printed scaffolds with and without PLGA nanoparticles was carried out to characterize the functional groups present on the scaffolds (Figures 6A and 6B). Representative Raman spectra
of PEG-DA were observed on both scaffolds. These include typical double-bond carbon and oxygen stretching vibration $\nu(\text{C}=\text{O})$ that appeared at 1727 cm$^{-1}$ and double-bond carbon and carbon $\nu(\text{C}=$ C) was observed at around 1600 cm$^{-1}$ [31]. In addition to the characteristic peaks of PEG-DA (Figure 6A), BSA nanoparticle loaded scaffolds showed a new peak at around 3400 cm$^{-1}$ which corresponds to overlapping absorption of hydroxyl group $\nu(\text{O-H})$ from PLGA and amine group $\nu(\text{N-H})$ from NH of BSA [32].

**TABLE I**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Contact Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large porosity scaffold</td>
<td>35.8 ± 5.1</td>
</tr>
<tr>
<td>0.1% BSA nanoparticle scaffold</td>
<td>49.1 ± 8.4</td>
</tr>
<tr>
<td>0.5% BSA nanoparticle scaffold</td>
<td>54.5 ± 9.6</td>
</tr>
<tr>
<td>1.0% BSA nanoparticle scaffold</td>
<td>20.6 ± 6.9*</td>
</tr>
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</table>

Surface wettability of scaffolds by contact angle analyzer (DSA4 Kruss). Data are mean ± standard error of the mean; n=4. *p<0.05 when compared to large porosity scaffold without BSA loaded nanoparticles.

Table 1 shows the contact angle of large porosity scaffolds with and without different concentrations of BSA nanoparticles. The presence of 0.1% and 0.5% PLGA nanoparticles did not change the contact angle significantly compared to the control group. The contact angle was significantly decreased from 35.4 ± 5.1° to 20.6 ± 6.9° after incorporation of 1.0% PLGA nanoparticles onto the large porosity scaffold. These indicate that the presence of PLGA nanoparticles on the scaffolds can improve hydrophilicity, which may contribute to better cell adhesion. However, it should be noted that all scaffolds with and without nanoparticles maintained a relatively high wettability.

**TABLE II**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Young's modulus (MPa)</th>
<th>Ultimate tensile strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large porosity scaffold</td>
<td>0.35 ± 0.06</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>0.5% BSA nanoparticle scaffold</td>
<td>1.01 ± 0.11*</td>
<td>0.63 ± 0.06*</td>
</tr>
</tbody>
</table>

Tensile testing of large porosity scaffolds by mechanical test system (MTS Criterion Model 42). Data are mean ± standard error of the mean; n=4. *p<0.05 when compared to large porosity scaffold without BSA loaded nanoparticles.

For tensile mechanical testing, all scaffolds were cut into 10 mm by 40 mm rectangular sheets and clamped on each end by mechanical grips. The Young's modulus of the large porosity scaffolds with and without PLGA nanoparticles were 1.01 ± 0.11 MPa and 0.35 ± 0.06 MPa, respectively (Table 2). The addition of BSA-PLGA nanoparticles significantly increased the Young's modulus by 188% over the plain large porosity scaffold without the nanoparticles. Similarly, the use of nanoparticles increased the ultimate tensile strength by 250% over the plain scaffold. The obvious increases in Young's modulus and ultimate tensile strength are mainly because of the incorporation of BSA-PLGA nanoparticles.

Fig. 7. PC-12 cell adhesion on 3D printed scaffolds with various porosity after 4 hour culture. Data are mean ± standard error of the mean; N=9. *p<0.05 when compared to all other scaffolds.

**C. PC-12 cell growth and differentiation studies**

The in vitro cell-scaffold interaction studies were performed using PC-12 cells first. Figure 7 revealed that the PC-12 cells adhere well on all scaffolds. The cell adhesion on scaffolds with large porosity, 68%, was significantly higher than any other group. Specifically, the large porosity scaffold increased the attached cell number by 65% and 43% over the medium (56% porosity) and small (44% porosity) porosity scaffolds respectively. Based on the cell attachment studies shown in Figure 7, the large porosity scaffold was selected for further cell evaluation. The cell proliferation on large porosity scaffold with 0.5% BSA encapsulated PLGA nanoparticles is significantly higher than any other group after 4 and 6 days (Figure 8).

Fig. 8. Enhanced PC-12 cell proliferation in 3D printed scaffolds with BSA/PLGA nanoparticles after 6 days culture. Data are mean ± standard error of the mean, N=9; *p<0.05 when compared to scaffolds with 0.1% and 1% BSA nanoparticles at day 2; **p<0.01 when compared to all other scaffolds at day 4; ***p<0.05 when compared to all other scaffolds at day 6.

scaffold with 0.5% BSA encapsulated PLGA nanoparticles is significantly higher than any other group after 4 and 6 days (Figure 8). Particularly, the scaffold with 0.5% BSA nanoparticles significantly enhanced the proliferation by 36% over the control scaffold. In contrast to the control, the PLGA core-shell nanoparticles are expected to decrease initial burst release of BSA and keep a slow and steady release over the entire proliferation study to promote PC-12 cell proliferation.
Figure 9 shows the confocal micrographs of PC-12 cells cultured on large porosity scaffolds, with and without nanoparticles, and NGF directly sprayed scaffold for 7 days. Various scaffolds show positive evidence of PC-12 cell attachment and growth. All scaffolds supported the outgrowth and extension of neurites. Two neuronal markers, TuJ1 and MAP2, that reveal the early and late stages of neuronal differentiation [33] were used to image the neural differentiation of PC-12 cells. PC-12 neurite extension was enhanced on scaffolds with nanoparticles. More importantly, neurites oriented preferentially in the direction of the scaffold pattern (Figure 9C). Meanwhile, axons were randomly extended out when cultured on bare control scaffolds and NGF sprayed scaffolds (Figures 9A and B).

Figure 10. Confocal microscopy images of PC-12 cell neurite growth on various 3D printed scaffolds at day 7. (A-C) Staining of DAPI to detect nuclei. (D-I) Double staining of MAP2 and TuJ1 to detect neurite outgrowth of PC-12 cells on various scaffolds after 7 days of culture. (J-L) Merged PC-12 cell images. (M-O) The associated neurite outgrowth was traced automatically by NeuriteTracer. Scale bar = 50 μm.

Figure 11. Quantification of neurite length of PC-12 cells on 3D printed scaffolds after 7 days culture. Neurite length was analyzed using ImageJ software and NeuriteTracer. (A) Total neurite length. (B) Average neurite length is the total neurite length divided by total neurite count. (C) Average total length of neurites per cell. (D) Average length of the longest neurite per cell. Data are mean ± standard error of the mean, n=4, *p<0.05 when compared to all other groups and **p<0.05 when compared to control at day 7.
In addition, neurite outgrowth on all scaffolds was quantified by Image J and NeuriteTracer (Figure 10&11). The use of bioactive nanoparticles in printed scaffolds greatly enhanced neurite outgrowth. The NGF nanoparticle incorporated scaffolds significantly improved total neurite length and average neurite length compared to any other group at Day 7 (Figures 11A-B). Similar trends were noted when looking at just the longest neurite per cell in each condition (Figure 11D). The scaffold with NGF nanoparticles increased the average length of the longest neurites by 84% over the bare control scaffold. The average total length of neurites per PC-12 cell was not statistically significant among 3D scaffolds (Figure 11C).

D. Measurement of neurite outgrowth in primary cortical neurons

Figure 12 shows the confocal micrographs of primary cortical neurons cultured in Neurobasal medium with NGF and B27 supplement for 8 days on different large porosity scaffolds. Moderate neurite extension, indicating good attachment, was observed on various 3D printed scaffolds. All scaffolds have similar cell growth density, even after 8 days. Consequently, the primary cortical neurons benefited from the incorporated NGF nanoparticles. Compared to the NGF directly sprayed scaffolds, the scaffolds with NGF nanoparticles significantly increased the total neurite length and presented a higher of 29% (Figure 13A). However, both the control and the scaffolds with NGF nanoparticles exhibited similar total neurite lengths after 8 days of culture. Similarly, the scaffold incorporated with NGF nanoparticles promoted the average neurite length by 35% and 21% over the control and NGF sprayed group respectively (Figure 13B). When regarding the longest neurite length per cell in each condition, the effect of incorporated NGF nanoparticles was more apparent. The NGF nanoparticle incorporated scaffold increased the average length of the longest neurites by 54% over the control group (Figure 13D). The quantitative analyses of average total length of neurites per cell showed no statistically significant difference among the groups (Figure 13C).

IV. DISCUSSION

A. 3D printing aligned porous scaffolds for neural regeneration

Despite progress in the study of peripheral nerve injury and regeneration over the past decade, full functional recovery is hindered by the lack of reliable biological or synthetic nerve constructs for bridging nerve defects [34]. Recently, SL printing systems have successfully demonstrated their capacity for printing porous 3D scaffolds for neural tissue repair [35]. The SL printed scaffolds are ideal for nerve repair applications due to the tunability in mechanical properties and biodegradation through polymer selection suitable for specific implants. Particularly, multi porous microarchitectural features of hydrogel scaffolds have shown a superior capacity in directing neurite outgrowth and extension, and successfully
connecting targeted injured tissues [3]. The pore interconnectivity allows for cell attachment, proliferation, and nutrient diffusion for cell survival [36], [37]. The extent of ECM secretion was also improved by increasing the pore size [36]. Many studies have reported that higher porosity and wide pore diameters are ideal for nerve conduits to allow the cells to infiltrate into the 3D scaffold and to enhance the growth rate [3], [38]-[42]. Specifically, Rutkowski et al. observed maximum growth rate at a porosity of 75% for Schwann cell-seeded conduits [42]. Our cell attachment studies confirmed that scaffolds with larger porosity performed significantly better than ones with smaller porosity.

The scaffold topography is known to greatly influence cell attachment, proliferation and differentiation [43], [44]. Hoffman-Kim et al. reported microgrooved topographies can support neural cell alignment [45]. Our 3D printed hydrogel scaffolds in this study exhibit involvement of the important aligned structure cues as illustrated by SEM micrographs and PC-12 cell confocal microscopy images. It is believed that the 3D configuration of aligned porous scaffolds can provide topographical directional cues for axonal regeneration across long nerve gaps. In our study, enhanced neurite outgrowth and oriented extension in PC-12 cells and primary neurons were observed on scaffolds with NGF nanoparticles (Figures 9-13). The micro-scale channels probably provided a groove-like geography for directing cell body and neurite extension. Compared to the neurites on the bare scaffold, the neurites on nanoparticle incorporated scaffolds exhibited aligned distribution with less branching. The reduced branching of neurites can prevent the formation of neuritoma, leading to a better neural repair [46]. Overall, our NGF nanoparticle embedded scaffolds promoted neurite outgrowth and extension along the microporous channels, which can enhance axonal regeneration and synapse formation at the injured site.

B. Sustained nano drug delivery in neural tissue engineering

A localized and controlled drug delivery from scaffold is very important for promoting nerve regeneration. Proper nerve regeneration only occurs when sufficient amounts of neurotrophins and other growth factors are present at the injured area. NGF, and other neurotrophic factors, have a short half-life, and nerve regeneration occurs over periods of weeks, so the use of slow release delivery system is vital [47].

Both nanoparticle and microparticle drug delivery systems are very common methods in neural tissue engineering. However many studies have demonstrated that nanoparticles have a number of advantages over microparticles [48]. Due to its smaller size, nanoparticles can access a wider cellular area, which may provide sustained delivery of therapeutic agents for difficult-to-treat diseases like brain tumors [49]. In addition, nanoparticles have better encapsulation rates and prolonged drug release compared to that of microparticles [50]. In our study, the novel PLGA nanoparticles fabricated by coaxial electrospraying present a desirable drug release behavior. The developed nanocomposite scaffold can incorporate a variety of materials inside of the nanoparticles such as bioactive proteins, NGF, or other neurotrophins. The BSA release profile of our designed core-shell nanoparticle embedded scaffolds presented a lower initial burst and longer release compared to bare BSA directly sprayed on scaffolds. The high encapsulation and loading efficacies, as well as sustained drug release profile over the period of 10 days, make these core-shell PLGA nanoparticles a promising delivery system for the controlled release of various neurotrophic factors in neural regeneration.

More importantly, we, for the first time, integrated the PLGA core-shell nanoparticles with the SL printed neural scaffold, which altered the surface and physical properties of the scaffold and provided the favorable nano- and micro-environment for improved PC-12 cell proliferation. Since natural ECM of neural tissue has many nanostructured features, the substrate with nano-topographical cues can elicit a more enhanced effect on cellular attachment and morphology [51]. In addition, the enhanced hydrophilicity and mechanical properties that originated from the integration of nanoparticles also distinguished our designed neural scaffold from more traditional designs. Surface wettability of biomaterial scaffolds is believed to be related to mediating cell adhesion, proliferation and differentiation on substrates [52]. A more hydrophilic surface can contribute to more specific protein adsorption and higher cell adhesion in the initial period of cell culture [53]. Our results (Table 1) showed that the involvement of PLGA nanoparticles can decrease the contact angle which may partly contribute to better cytocompatibility properties of nanocomposite scaffolds than controls. Furthermore, the PLGA core-shell nanoparticles can provide an altered surface chemistry (Figures 4D-F) and topography (Figures 5B) on large porosity scaffolds that may further improve neural function. It has been found that cell behavior depends strongly on nanoroughness of the material surface. Zamani F. et al found that nanometer surface roughness on electrospun fibers promoted attachment and proliferation of A-172 nerve cell growth rate by 50% in comparison with smooth electrospun scaffolds [54].

C. Enhanced neurite outgrowth on 3D nanocomposite neural scaffolds

The neurite outgrowth on PC-12 cells and primary cortical neurons was assessed by four different methods: (1) total neurite length, (2) average length of neurites (3) average length of total neuritis per neural cell and (4) average length of the longest neuritis per neural cell (Figures 11 and 13). Overall, it was found that 3D scaffolds with NGF nanoparticles greatly increased the neurite outgrowth of PC-12 cells and primary cortical neurons (Figures 11 and 13). As we discussed earlier, the incorporation of nanoparticles enhanced the nanoroughness of the scaffold and delivered bioactive molecules over a sustained period. However the molecular mechanism for the effect of incorporated nanoparticles on neurite outgrowth is not well understood. It appears that both PC-12 cells and primary cortical neurons attach well to the laminin-coated scaffolds, regardless of the use of nanoparticles. But the addition of nanoparticles to the scaffolds increased the average length of neurites and decreased the number of neurite branching. This explains why PC-12 cells and primary cortical neurons on NGF nanoparticle incorporated scaffolds had higher average length of neurites (Figures 11B and 13B) and the longest neurite length per cell
(Figures 11D and 13D) than both NGF sprayed and control scaffolds. In contrast, the average total neurite length per PC-12 cell and primary cortical neuron (Figures 11C and 13C) was not significantly different among the groups. This is due to the varied cell density observed on each scaffold. Compared to the scaffolds with NGF sprayed scaffolds, NGF nanoparticle incorporated scaffolds also produce a relatively high number of total neurite length for PC-12 and primary neuron cells (Figures 11A and 13A).

V. CONCLUSION

A series of novel nerve scaffolds, with controlled porous structure and a sustained bioactive factor nanosphere delivery system, were successfully fabricated via a table-top SL 3D printing method. Scaffolds with larger porosity significantly improve PC-12 cell adhesion compared to ones with smaller porosity. More importantly, greatly improved PC-12 and primary cortical neuron cell functions were observed on the 3D printed neural scaffolds with bioactive factor loaded nanospheres indicating its great potential for peripheral nerve or central nerve system defect repair and regeneration.

REFERENCES

[23] C. M. O’Brien et al., “Three-dimensional printing of...


