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Original Article

Interactions of neural-like cells with 3D-printed polycaprolactone with different inner diameters for neural regeneration

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KEYWORDS
Neural regeneration;
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Abstract *Background/purpose:* Peripheral neural regeneration is an interesting and challenging field. The aim of this study was to investigate the interactions of neural-like PC12 cells and Poly-D-Lysine (PDL)-coated 3D-printed polycaprolactone (PCL) scaffolds with different inner diameters of half tubular array (HTA) (0, 200, 300, and 400 μm), respectively.
Materials and methods: This study used the fused deposition modeling (FDM) technique with 3D-printing to fabricate the thermoplastic polymer. Scaffold properties were measured by mechanical testing, and coating quality was observed under a scanning electron microscope (SEM). PC12 cell biocompatibility was examined by an MTT assay. Cell differentiation was evaluated by immunofluorescence staining.
Results: The cell viability of PC12 cells on PDL-coated PCL scaffolds with a 200-μm inner diameter of HTA was shown with significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) than other PCL groups at all experimental dates. The SEM observation showed that PDL-coated PCL scaffolds with 200-μm inner diameters of HTA promoted cell adhesion. An immunofluorescence staining of PC12 cells on the PDL-coated PCL scaffold with a 200-μm inner diameter of the HTA group showed that it stimulated PC12 cells for neurite formation much better than the other groups.

A PDL-coated PCL scaffold with a 200-μm inner diameter of HTA can promote the growth and differentiation of PC12 cells better than other groups. It indicated that PDL-coated PCL scaffolds with a 200-μm inner diameter HTA can be used for further neural regeneration application.

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Introduction

Neural regeneration is an interesting issue; nerve defects were caused by trauma, and degenerative disease is becoming an important health challenge due to inadequate natural regeneration.[1] Neural tissue engineering is a rapidly growing field to not only improve the disadvantages of traditional methods but also find new therapeutic methods to regenerate neural tissue according to 3D-printing technology.[2] In this study, we used the fused deposition modeling (FDM) platform, which is one of the most widely used 3D-printing techniques, in an additive manner, such as layer by layer, to fabricate the thermoplastic, bioresorbable polymer.[3] Moreover, it is extensively adopted to produce scaffolds for tissue engineering and drug delivery devices due to polycaprolactone's (PCL) suitable mechanical properties and superior biocompatibility. However, PCL has slow biodegradability, and in order to increase the rate of PCL degradation as well as increase mechanical strength, it is often integrated with other hydrolytically degradable polymers.[4,5] Furthermore, PCL polymers have a semi-crystalline morphology; they are insoluble in water, alcohols, and diethyl ether.[6]

Previous studies showed that PCL scaffolds can be used for the creation of neural regeneration in vitro. Based on these results, PCL scaffolds promoted adipose-derived stem cell (ADSC) differentiation in vitro.[7]

In addition, PCL is a hydrophobic, biodegradable polymer that has poor cellular adhesion and is surface modified with artificially synthesized polymers. Poly-lysine is an extensively used bioactive polymer for cell adhesion and, with its high ability to absorb water, is known to attract neurons and promote neurite outgrowth. In other words, Poly-D-lysine (PDL) chemically synthesizes amino acid chains that are also resistant to enzymatic degradation.[8—11]

The purpose of this study was to develop PDL-modified 3D-printed PCL scaffolds with different inner diameters of half tubular arrays (HTA) for neural regeneration.

Materials and methods

Fabrication of 3D-printed polycaprolactone scaffolds with different inner diameters of half tubular array

Architectural design of 3D-printed scaffolds was conducted using the software Schetchup Make 2016 (Trimble Inc., Sunnyvale, CA, USA). The 3D structures were exported to STL files and sliced by Ultimaker Cura 2.7 (Ultimaker B.V., Utrecht, The Netherlands) to create a g-code for 3D printing. The print speed was 20 mm/s; the layer height and the scaffold wall thickness of HTA were 50 and 250 μm, respectively. PCL scaffolds were designed for neuronal regeneration with different inner diameters of HTA (0, 200, 300, and 400 μm). An Ultimaker 2.0 Plus 3D printer equipped with a 250 μm copper nozzle and PCL filament (Facilan™ PCL 100, Haarlem, The Netherlands) of 2.85 mm diameter was employed to carry out 3D printing. In the process of printing, the build plate temperature and the nozzle temperature were set at 30 °C and 180 °C, respectively. 3D design of PCL scaffold and fabricated PCL scaffolds were observed by SEM (scanning electron microscope) (Fig.1).

Mechanical testing

The tensile properties of the PCL scaffolds with 0-μ m inner diameter of HTA were analyzed per month, stored in PBS for 6 months, and at each time point (n = 6—8) as outlined previously. Samples were placed in phosphate-buffered saline (PBS) and incubated at 370 °C in a humidified atmosphere containing 5 % CO_2. Every month, 6—8 samples were sprayed with 75 % alcohol and dried at room temperature for 1 day. Subsequently, gripped within the tensile grips of an Instron 5566 (Instron Co., Norwood, MA, USA) a providing 40 mm in length with 3 mm in width, every sample thickness was measured. Stress-strain curves were obtained from each sample, and using the sample's cross-sectional area, the tensile strength, elongation, and Young's modulus were calculated.

Cell culture

The PC12 cell is one of the most commonly used in neuroscience research. Traditional PC12 cells were grown in suspension. PC12 cells were purchased from the Bioresource Collection and Research Center, Taiwan. The cells were cultured in RPMI-1640 medium supplemented with 10 % horse serum, 5 % fetal bovine serum, and 1 % penicillin/streptomycin solution (all from Gibco-BRL, Grand Island, NY, USA). Cells reach up to 80—90 % confluency, then are pelleted by centrifugation at 900 rpm for 4 min. PC12 cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 and the culture medium was replaced every 2—3 days.

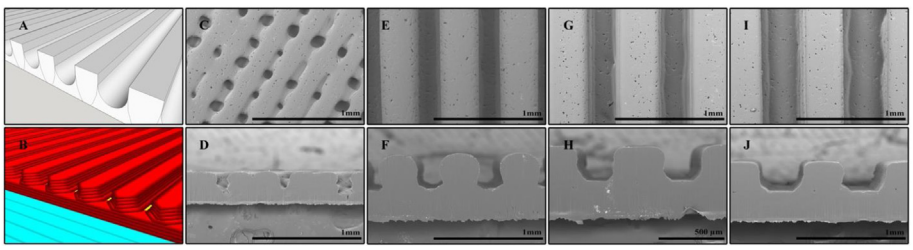

Fig. 1 3D design of PCL scaffold with a 200-μ m inner diameter of HTA (A). 3D-slicing of PCL scaffold (B). Top view of 3D-printed PCL scaffold with 0, 200, 300, and 400 μ m inner diameters of HTA (C.E.G.I). Cross-section of 3D-printed PCL scaffold with 0, 200, 300, and 400 μ m inner diameter of HTA (D.F.H.J). PCL: Polycaprolactone; HTA: Half tubular array.

Surface coating

Scaffolds were previously prepared and placed in 24-well tissue culture polystyrene plates (1.9 cm^2, Sigma-Aldrich, St. Louis, MO, USA). An O-silicon rubber ring was placed on each of the experimental scaffolds in the plate well to prevent floating. The scaffolds and silicon rubber rings were sterilized by dipping in 75 % ethanol, adding UV light overnight, and then rinsing with phosphate buffered saline (PBS). 3D-printed PCL scaffolds of varying inner diameter sizes were pre-coated with 0.1 mg/ml Poly-D-Lysine (PDL, Gibco) solutions, added to the 24-well plate in the volume needed to cover the surface of the scaffold, and incubated at 37 °C for 30 min. After that, the solutions were aspirated and washed with PBS once.

Cell adhesion and growth morphology analysis

Cell adhesion and growth morphology analyses were performed using SEM (scanning electron microscopy). PC12 cells were grown on PDL coated TCPS (for negative control) and 3D-printed PCL scaffolds with different inner diameters, such as 0, 200, 300 and 400 μm HTA. Cells with 5×10^4 in density were used for cell adhesion and morphology analysis after being cultured on days 1, 4, and 7. Eventually, PC12 cells were cultured on PCL scaffolds and then fixed with methanol for 40 min at 4 °C. After fixation, all samples were washed twice in PBS solution for 15 min, dehydrated in sequentially increasing ethanol concentrations of 50 %, 60 %, 70 %, 80 %, and 100 % for 10 min in each step, and then dried in the freezer and vacuumed to fully remove the ethanol. Finally, the samples were coated with a slightly thick layer of platinum and observed under an SEM (TM3000 Tabletop Microscope, Hitachi, Schaumburg, IL, USA).

Cell viability assay

PC12 cells were cultured on 3D-printed PCL scaffolds with various inner diameters of HTA (0, 200, 300, and 400 μm, respectively) and cultured for 1, 4, and 7 days at a density of 1×10^4 cells in 1 ml culture medium. Cell viability was evaluated by using the MTT assay with thiazolyl blue tetrazolium bromide (Thermo Fisher Scientific, Haverhill, MA, USA). The concentration of MTT reagent, about 5 mg/ml, was added to each sample in a 24-well plate and incubated for 3 h at 37 °C with 5 % CO_2 to allow the formation of MTT formazan. The resulting formazan crystals were dissolved with dimethyl sulfoxide (DMSO, Sigma-Aldrich). The optical density of the formazan solution was read on an ELISA plate reader (Synergy™ HT, BioTek, Winooski, VT, USA) at 570 nm. The absorbance was proportional to the number of cells attached to the scaffold surface.

Neural differentiation and immunofluorescence assay

3D-printed PCL scaffolds were coated and disinfected, cells with a density of 1×10^5 were seeded, and differentiation medium was added with serum reduced Opti-MEM medium (Gibco) with 0.5 % FBS and 50 ng/ml Nerve Growth Factor 2.5S (NGF 2.5S, EMD Millipore, Temecula, CA, USA), and renewed every 2−3 days, up to the experimental date for neuronal differentiation of PC12 cells to occur.

Neural differentiative potentials of PC12 cells were evaluated by measuring the expression of neuron-associated proteins such as Glial Fibrillary Acidic protein (GFAP), beta-III tubulin on PDL-coated 3D-printed PCL scaffolds with different inner diameters, such as 0, 200, 300 and 400 μm of HTA. After 14 days of cell culture (cell density was 2×10^4 cell in 1 ml), the induction medium was aspirated and washed with PBS with 0.1 % tween 20 solutions for 3 times. The cell-scaffold constructs were then fixed in 4 % paraformaldehyde with 0.1 % triton-X 100 for 30 min at 4 °C. The nonspecific binding was blocked by incubating with 5 % bovine serum albumin (BSA, Sigma, Marlborough, MA, USA) in PBS for 1 h at RT on the shaker at 50 rpm. Subsequently, the samples were stained with primary antibody. Mouse monoclonal antibody beta-III tubulin (Abcam, Cambridge, UK) at a dilution of 1:200 and glial fibrillary acidic protein antibody (EMD Millipore) (1:200) were added at 37 °C for 30 min and then washed with PBS three times, followed by Goat anti-mouse Alexa Flour 594 antibody and Goat anti-mouse Alexa Flour 488 conjugated secondary antibody (Thermo Fisher Scientific) were used to visualize the signal for 1 h at room temperature with a new 5 % BSA blocking solution and washed with PBS. The nuclear DNA was stained with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 15 min at RT and washed with PBS two times. Immediately, the immunocytochemical-stained cells were visualized by a confocal fluorescent microscope (ZEISS Axiovert 200, Oberkochen, Germany) and protein expressions of induced cells were observed.

Statistical analysis

The data were expressed as means and standard deviations. An unpaired *t*-test was used to compare parameter differences within different groups between different time points. A *P*-value below 0.05 was identified as statistically significant.

Results

Tensile strength

An overall increase in PCL scaffold stiffness was obtained for degraded samples compared to that of the first month following storage in PBS. The tensile strength of the samples was measured per month during storage in PBS for 6 months. The PCL samples (month-1) demonstrated the highest strength value (12.6230 ± 0.8110). The tensile strength steadily decreased from month 1 to month 4. The tensile strength value reduction was evident significantly during months 5 and 6. The lowest tensile strength value was (8.0470 ± 0.3360 MPa) after 6 months, and the final reduction was up to 36.3 % after 6 months of storage in PBS (Fig. 2A). Our result shows that the PCL scaffold has degradation ability in vitro.

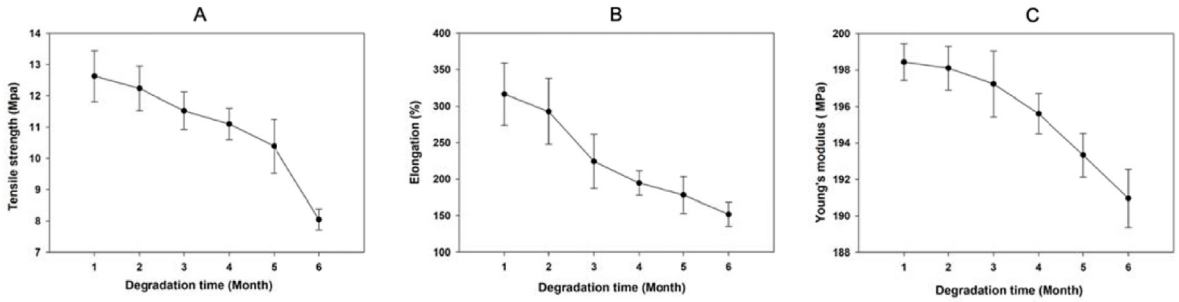

Fig. 2 The mechanical properties of a PCL (Polycaprolactone) scaffold with a 0 inner diameter of HTA (Half tubular array) follow hydrolytic degradation in phosphate buffered saline at 370 °C for 6 months. The mechanical properties were measured every month. The tensile strength steadily decreased from month 1 to month 4, and the value reduction was more evident and significant during months 5 and 6 (A). The maximum strain percentage decreased significantly during month 3, and the degradation rate decreased gradually up to 6 months (B). Young's modulus reduction with time dependence was observed without statistical significance (C). n = 6–8, $^{*}P < 0.05$.

Elongation

Scaffolds stored in PBS for up to 180 days demonstrated a significant decrease in maximum strain over time. The maximum strain of PCL scaffolds at the first month was (316.1970 ± 42.788 %), and after storage for 180 days in wet conditions, the maximum strain percentage decreased significantly during month 3, afterward decreasing gradually, with a final strain of (151.747 ± 16.489 %). After incubation for 6 months, the reduction was 52 % at the last time point (Fig. 2B).

Young's modulus

Time zero Young's modulus of PCL scaffolds was 198.4250 ± 1.00 MPa, and during the hydrolytic degradation, a gradual reduction was observed. Overall, from month 1 to month 6, degradation increased homogeneity but was not significant. Young's modulus, which suffered the lowest stiffness, was 190.950 ± 1.60 MPa after 6 months of storage, a 3.765 % drop from the last time point (Fig. 2C).

Cell adhesion and growth morphology analysis

SEM investigation showed the results of PC12 cell adhesion and growth morphology on the PDL-coated PCL scaffolds with various inner diameters of HTA and TCPS. Cells were attached well, such as by cell-to-cell adhesion and clumps, to both PDL-coated PCL scaffolds and TCPS at day 7. However, clumped cells were more prevalent on the control group PCL scaffold with a 0-μm inner diameter of HTA at all experimental dates and on the PCL scaffold with a 200-μm inner diameter of HTA at day 7; the shape was round and ovoid on the PCL scaffolds, with a flat shape on the TCPS at all experimental dates (Fig. 3).

Cell viability

PC12 cells were cultured on the TCPS and PCL scaffolds with various inner diameters of HTA, respectively. Subsequently, cell viability was measured after 1, 4, and 7 days of culture. The optical absorbance of PC12 cells was observed at OD 570 nm and increased dramatically in all groups at days 1, 4,

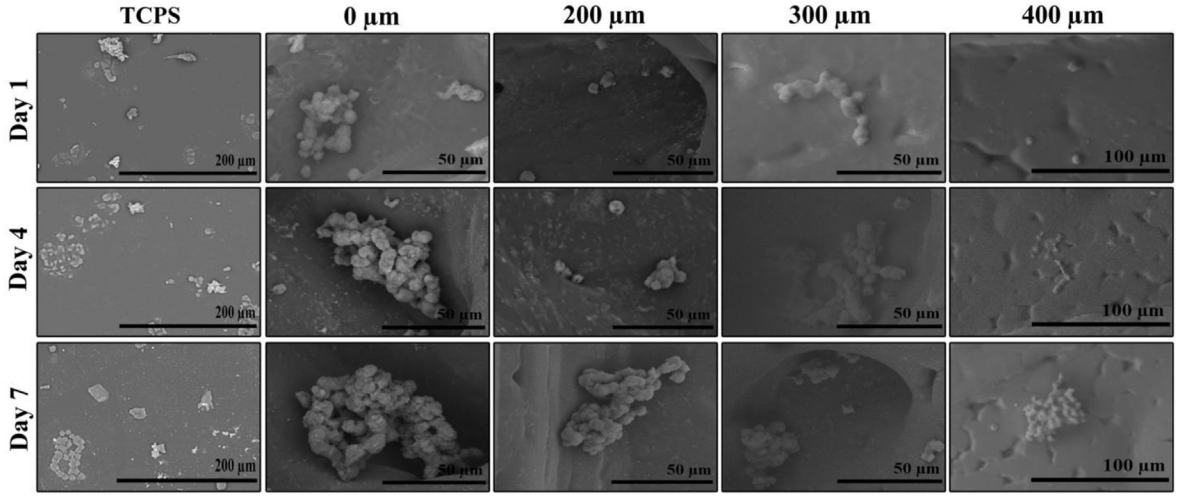

Fig. 3 SEM analysis for PC12 cell growth morphology and attachment on the PDL (Poly-D-Lysine)-coated TCPS (Tissue culture polystyrene) and PCL (Polycaprolactone) scaffolds with 0, 200, 300, and 400 μm inner diameters of HTA (Half tubular array) at days 1, 4, and 7.

and 7. However, on the PCL scaffold with a 200 μm inner diameter of HTA, cell growth was the best, with a statistically significant difference in experiment dates (Fig. 4).

Cell differentiation and immunofluorescence staining

Under the induction of PC12 differentiation, PC12 cell-neurites extended and could be observed visually on PDL-coated TCPS surfaces on culture days 7 and 14 by a light microscope. Cells were single or clumped, and their shape was flat, round, and ovoid. Even cells with long axons and some dendrites were determined (Fig. 5).

According to the immunofluorescence staining (Fig. 6), we observed that cells were single and flat on the PDL-coated PCL scaffolds with different inner diameters of HTA. Even cells with long axons and some dendrites were determined on the TCPS. Axons are only indicated on the PDL-coated PCL scaffolds with 200 and 300 μm inner diameters of HTA after neural induction at day 14. However, cells and axons were few to reach out on PDL-coated PCL scaffolds with 300 μm inner diameters of HTA and several axons were determined on the PDL-coated PCL scaffolds with a 200 μm inner diameter of HTA. PC12 cell neurite outgrowth was evaluated by measuring the expression of neuronal tubulin-associated proteins such as beta-III

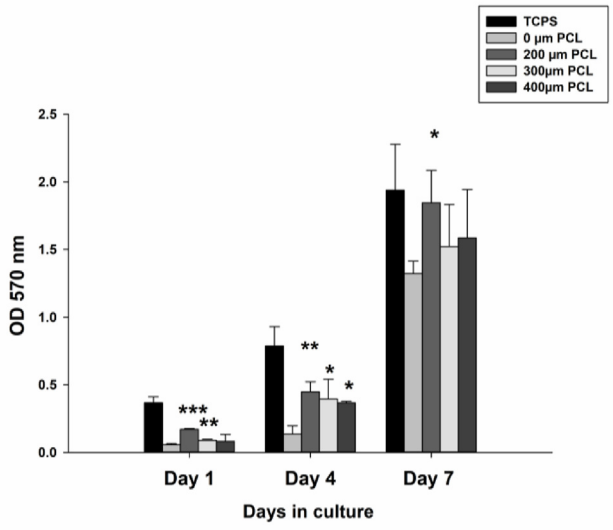

Fig. 4 MTT assay of PC12 cells on PCL scaffolds with different inner diameters (0, 200, 300, and 400 μm) of HTA, respectively, 1, 4, and 7 days after cell seeding (n = 4). The data are presented as the mean and standard deviation. An asterisk denotes significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) in formazan absorbance compared to the PCL scaffolds with a 0 μm inner diameter of HTA. PCL: Polycaprolactone; HTA: Half tubular array.

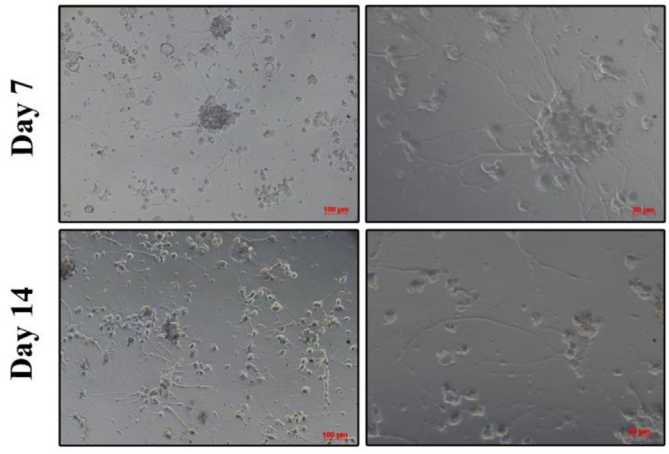

Fig. 5 NGF induced PC12 cell differentiation on the PDL coated TCPS after 7 and 14 days respectively. Magnification (10X and 20X). NGF: Nerve growth factor; PDL: Poly-D-Lysine; TCPS: Tissue culture polystyrene.

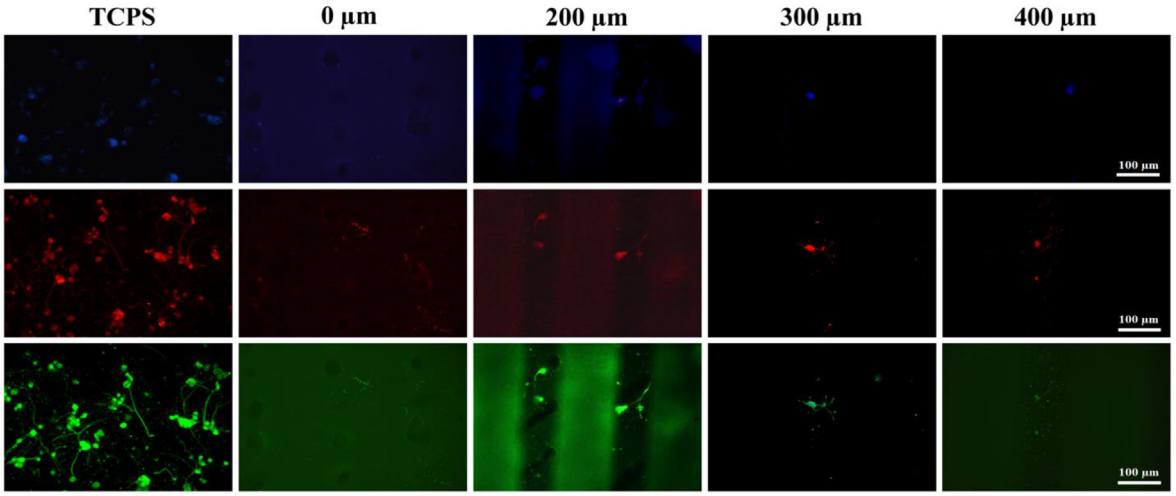

Fig. 6 Immunofluorescence staining images of PC12 cells after incubation for 14 days (Magnification 10x). Beta-III tubulin (red) and GFAP (green) neural markers were expressed within the PC12 on PCL scaffolds with 200 and 300 μm inner diameters of HTA and TCPS. DAPI (blue) was used for cell nuclei mark. GFAP: anti-glial fibrillary acidic protein; PCL: Polycaprolactone; HTA: Half tubular array; TCPS: Tissue culture polystyrene; DAPI: 4′,6-diamidino-2-phenylindole.

tubulin, anti-glial fibrillary acidic protein (GFAP) on PDL-coated PCL scaffolds with different inner diameters of HTA.

Tubulin is a major component of the cytoskeleton and comes in five different forms: alpha, beta, gamma, delta and epsilon. Alpha and beta tubulins form heterodimers, which multimerize to form a microtubule filament. A variety of beta tubulin isoforms, including beta-I, beta-II, beta-III, beta-IV, beta-V, beta-VI, and beta-VIII have been identified and expressed in mammalian tissues. Additionally, beta-III is a neuron-specific cytoskeletal protein. GFAP is a class III intermediate filament protein of astrocytes. Furthermore, results of beta-III tubulin and GFAP antibody immunofluorescence staining indicated that PC12 cells differentiated into neuron-like structures and astrocytes-like cells, respectively.

We found that induced PC12 cells for 14 days adhered not much to 3D-printed PDL-coated PCL scaffolds. In addition, cells are not determined on the PCL scaffold with 0 μm inner diameters of HTA, and the characteristics of neural processes weren't as obvious as they were in the PCL scaffold with 400 inner diameters of HTA. In regards to immunofluorescence staining, we observed clear both beta-III tubulin and GFAP markers for neural-like cells that after the development of axons.

Discussion

The peripheral nervous system (PNS) and the central nervous system (CNS) are the two divisions of the nervous system. Sensory and motor neurons, which transmit and receive information from every area of the body to and from the central nervous system, make up the PNS. The peripheral nervous system also includes cranial nerves, which originate in the brain or brain stem and innervate parts of the head and neck. Facial cranial nerves innervate the muscles in the face that control facial expressions. A peripheral neuron is made up of a cell body that carries out extensions, termed axons that are vital for targeting distant tissues and organs. Known as fascicles, these axons are grouped together in bundles and covered in myelin sheath membranes formed by Schwann cells. Because nerve bundles have a complicated anatomical structure and a degenerative process. Strategies for nerve regeneration and repair for critical defects have frequently failed to restore adequate nerve function. Current applications include nerve conduits constructed of biocompatible polymers including collagen, polycaprolactone (PCL), and polyglycolic acid (PGA). All the same, current conduits are limited and only be used for defects of a few centimeters and primarily function as structural support clotting factor concentrators between the proximal and distal stumps.[12] The proper regeneration of axons into endo-neural tubes that guide them back to their original target organs is a crucial requirement for a complete functional recovery. However, regenerating axons experience a notable delay at the site of injury and proceed in a staggered manner (staggered axonal regeneration) toward the distal nerve stumps from the injury site. In fact, the regeneration of axons crosses slowly before they enter the distal nerve stumps. The axons regrow at a slow transport rate of 1–3 mm/d only when they reach the distal stumps.[13] End-to-end microsurgery is commonly regarded as a successful therapeutic approach for peripheral nerve with a defect gap that is less than 5 mm. However, in cases with chronic peripheral nerve defect with a large defect gap higher that 5 mm, the body's capacity for self-regeneration is severely limited, necessitating the use of an efficient implant to bridge the nerve gap and promote the function of axonal recovery. There is a need for an alternative non-invasive treatment because donor tissue is limited.[14] Based on those facts, we have designed PCL tube scaffolds with an inner diameter of HTA to support the direction of the regenerating axon climb to distal stumps.

In this study, we have developed 3D-printed PCL scaffolds with different small inner diameters of HTA (0, 200, 300, and 400 μm) for use with FDM printer. PCL is an

implantable polymer material that is biocompatible, biodegradable, and well-elastic, with a wide application in the tissue engineering field. Hence, PCL was used in this study for PC12 cell interactions. PCL is a semicrystalline hydrophobic polyester with a slow degradation profile of about 2—4 years in vivo. However, the material is capable of being fully degradable.[15]

For constructing a scaffold for tissue engineering, degradation ability and degradation mechanism are important factors. Chemical treatment, the molecular weight of the polymer, and the morphology and structure of both the polymer and the scaffold are important factors. For example, not only scaffold porosity and chemical composition of the copolymers and crystallinity affect the rate of degradation, but also the polymer release of acidic breakdown molecules causes auto-catalysis to occur, which consequently increases the rate of degradation. It depends, before the polymer system loses weight, there is an accumulation in the amorphous phase of carboxylic acid end groups, which autocatalytically accelerate the hydrolysis.[16—18] We performed in vitro degradation studies at six time points (Months 1, 2, 3, 4, 5, and 6). The degradation rate of PCL increased monthly. Biomaterial biomimetics is the primal concept in the design of neuronal tissue engineering scaffolds. The better biomimetic character of the scaffold is good for the cell's environment, and the cells can behave as they did in their aboriginal tissue microenvironment. Hence, biomaterials with morphological and mechanical properties closer to the aboriginal tissue are expected to result in advanced tissue engineering. Our in vitro degradation results demonstrated that before PCL scaffolds were incubated, the initial tensile strength was (13.01 $\pm$ 0.7), which is close to the aboriginal peripheral nerve properties (6.78—11.7 MPa).[19,20]

During the degradation experiment, the mechanical properties of PCL scaffolds decreased continuously, and the tensile strength decreased slowly from the first month to the fourth month. Degradation was significantly increased in the fifth and sixth months. The sample with higher degradation had reached low values after 6 months (8.047 $\pm$ 0.3 MPa), and the degradation rate increased to 36,3 % compared to the first month (Fig. 2A). The first-month maximum strain of PCL scaffold was (316.1970 $\pm$ 42.788 %), and after storage for 6 months in PBS in vitro, the final low value was (151.747 $\pm$ 16.4 %), and the degradation rate increased up to 52 % after 6 months (Fig. 2B). Young's modulus of the PCL scaffold decreased and dropped to 3.765 % at the last time point (Fig. 2C). Previous studies demonstrated that PCL matrices can be used to increase the degradation rate; however, they must keep the elastic modulus stable during the degradation process.[21]

Furthermore, suspended PC12 cells were adhered to hydrophobic, biodegradable polymer PCL after being coated with PDL. Because PDL is the cationic polymer that enhances electrostatic force between anionic cell surfaces, serum-free and serum-reduced medium cultures can be dramatically improved by coating the culture surface with a polymer. SEM results showed that PC12 cells adhered well to PDL-coated PCL scaffolds, and their shape was round and ovoid on culture day 1,4, and 7; the cells were more clustered on culture day 4 and 7. However, the PC12 cell shape was flat on the PDL-coated TCPS surface (Fig. 3).

In the assay of cell viability, since the base number of seed cells was low and they were well adapted to the microenvironment, proliferation increased significantly at all experiment dates. However, PC12 cell proliferation was significantly higher at various days on the PDL-coated PCL scaffold with a 200 μm inner diameter of HTA than in other groups (Fig. 4).

PC12 cells were derived from rat pheochromocytomas and are an immortalized cell line similar to the primary culture of fetal neurons. PC12 cells have been widely used as a neuronal model in vitro.[22] There are two collections of PC12 cell lines: PC12 adherent and PC12 suspension. PC12 cell suspension up to about 10—30 passages, the morphology of the cells was changed. Changes in passages during the study can cause errors in the results. It has been shown that PC12 cells cultured for 16 passages lose their differentiation ability.[23] Our study shows that PC12 cells are starting to differentiate around 2—3 days following NGF treatment. As mentioned above, the suspension PC12 cells cannot adhere to the plastic surface; therefore, it is required to increase the surface of the differentiation vessels.

After strong adhesion of suspension PC12 cells that were also treated with 50 ng/ml NGF for stimulating PC12 cell differentiation. NGF is a trophic factor that promotes several activities, including signaling, apoptosis, and neuronal differentiation.[24] Following NGF treatment, PC12 cells obtain sympathetic neuron-like phenotypic properties, which include the discontinuation of cell proliferation.[25] Being a neural-like cell line, PC12 could be subcultured, allowing experiments with repeatability; also, PC12 cells respond to NGF in a cell cycle-specific manner, and the p53 gene plays a role in NGF-mediated differentiation by inducing cell cycle arrest.[26,27]

NGF is a polypeptide neurotrophic factor that activates the trombomyosin receptor kinase A (TrKA) to alleviate cell transition from proliferation to differentiation.[28] In addition, NGF affects biochemical, electrophysiological, and morphological changes in PC12 cells by flattening and growing neurites, making them similar to the sympathetic nerves.[29,30] PC12 cells were in trouble with the traditional culture methods with limited neurite outgrowth, a high proliferation rate, a low differentiation rate, short neurite length, a low adhesion rate, and a lack of synapse-like structures.

In order to make PC12 cells more similar to neurons, the Opti-MEM medium containing 0.5 % FBS and 1 % HS with 50 ng/ml NGF is regulated, and cells can show higher adhesiveness, slower proliferation, increased differentiation, and longer neurites. Therefore, we used serum-reduced Opti-MEM medium to prevent those disadvantages of traditional culture methods and get better neuronal differentiation.[31] Our neuronal differentiation result showed that 50 ng/ml NGF-induced neuronal differentiation with serum-reduced Opti-MEM promoted neuronal outgrowth on the TCPS (Fig. 5). After the differentiation result on the TCPS, an immunofluorescence assay was performed. Beta-III tubulin is an early marker of neuronal differentiation, and GFAP is a specific marker of astrocytes.

Cell seeding with reduced cell density and beta-III tubulin and GFAP staining of PC12 cell outgrowth are shown in Fig. 6. The results indicate that the scaffolds support neuronal differentiation. However, several axons were determined on the PDL-coated PCL scaffolds with a 200 μm inner diameter of HTA.

Conclusion

In the current study, 3D-printed PCL scaffolds with different inner diameters of HTA coated by PDL were used, and scaffolds with a 200 μm inner diameter of HTA could induce the growth and differentiation of PC12 cells better than scaffolds with 0, 300, or 400 μm inner diameters of HTA. The results of our study indicate that PDL-coated PCL scaffolds with a 200 μm inner diameter of HTA can be used for further neural regeneration applications.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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