doi: 10.22100/ijhs.v3i2.236
Original Article

IJHS 2017;3(2):29-32
ijhs.shmu.ac.ir

IJHS
International Journal of Health Studies

Poly-lactic Acid/Gelatin Nanofiber (PLA/GTNF) Conduits Containing Platelet-Rich Plasma for Peripheral Nerve Regeneration

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Received: 3 May 2017
Accepted: 1 June 2017

Abstract

Background: *Polymeric scaffolds have achieved immense importance in the field of nerve tissue engineering.*
Methods: *In the present study, the combination of thermally induced phase separation (TIPS) and electrospinning methods were used to fabricate poly- (lactic acid)/gelatin nanofiber/PRP-scaffolds. Several physical and mechanical tests (weight loss measurement, surface wettability, porosity, microstructure observation via SEM photography, mechanical tests such as tensile strength, and Young modulus) and cellular assays (MTT assay and DAPI staining) were explored to assess the scaffolds capability to serve as neural guidance conduit. In this study, we hypothesized that conduits enriched with PRP may provide a better regenerative environment for nerve tissue repair.*
Results: *This study suggests that GTNF/PRP incorporated scaffolds revealed better biological and physical properties than PLA only scaffolds.*
Conclusions: *Results indicate that when GTNF/PRP is incorporated into the PLA scaffolds, resultant mechanical properties, porosity and cell attachment, and viability in vitro were better than pure PLA.*

Keywords: Thermally induced phase separation (TIPS), Poly-lactic acid/gelatin Nanofiber (PLA/GTNF), Platelet rich plasma (PRP).
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Please cite this paper as: Farzamfar S, Esmailpour F, Rahmati M, Vaez A, Mirzaii M, Garmabi B, Shayannia A, Ebrahimi E,Vahedi H, Salehi M. Poly-lactic Acid/Gelatin Nanofiber (PLA/GTNF) Conduits Containing Platelet-Rich Plasma for Peripheral Nerve Regeneration. Int J Health Stud 2017;3(2):29-32.

Introduction

Peripheral nerve injury interferes with the brain's ability to transfer massages to the functional organs and tissues.[1] The peripheral nervous system has an inherent capability to partly regenerate after injury.[2] This process is disrupted when the injury size exceeds the critical size.[3] Nerve autografts are considered as the gold standard to treat peripheral nerve injuries;[4] however, their several drawbacks hamper their clinical usage.[5] Therefore, alternative solutions are highly recommended. Tissue engineering seeks to regenerate the defective tissue using a combination of biomaterials, cells, and growth factors.[6,7] Tissue engineered neural guidance conduits (NGCs) are potent in enhancing the gap that can be bridged in peripheral nerve injury.[8] Several materials are available for the fabrication of the NGCs. Among these, PLA has achieved immense attention due to its biodegradability; biocompatibility, and efficient mechanical properties.[9] Furthermore, gelatin Nanofibers (GNFs) have accelerated the NGCs capability to foster more robust tissue regeneration.[10] Recently, enrichment of NGCs channel with growth factors has resulted in a promising functional recovery after injury.[11] Platelet-rich plasma (PRP) is a potent product in treating peripheral nerve injury.[12] Due to the high concentration of growth factors in PRP, it has been used successfully in peripheral nerve regeneration.[13] The present study aimed to assess the therapeutic effects of PLA/GNF NGCs comprising PRP in a rat model of peripheral nerve injury.

Materials and Methods

In this study, we used Kajikawa et al.'s technique for making PRP. Six donor rats were euthanized and their complete blood was extracted via decapitation. To prevent clotting, the blood samples were supplemented with 3.8% sodium citrate (Sigma, St Louis, Missouri). These samples were centrifuged at 1500 rpm for 10 min. The supernatant containing the platelet-rich layer was separated from the red blood cell layer. After centrifugation at 3000 rpm for 10 min, a cell pellet was created. A maximum value of the supernatant was discarded, leaving the cell pellet. The pellet was then suspended in the residual supernatant. 20-µL aliquots of the PRP solution were prepared and preserved overnight at 4 °C. Before using, the platelets were activated with 3.33 µL 10% CaCl2 (Sigma) and 3.33 µL 300 IU thrombin (Sigma).

Gelatin solution (gelatin powder (bovine skin, type B)) was prepared by dissolving 40 g of gelatin powder in 100 cc aqueous acetic acid/water (ratio, 75:25, %v/v) at room temperature. For electrospinning, the gelatin solution containing 100 µL PRP was transferred into a 10 ml syringe with 18 gauge metal needles connecting to a positive high voltage source set to 20 kV. Tip to mandrel distance was set to 15 cm. The syringe was placed into a feeding pump (SP1000, Fanavaran Nano-Meghyas, Iran) with 0.4 ml/h feeding rate. The extruded solution was collected at room temperature on an aluminum foil. Turning rate of the drum was 500–550 rpm. Furthermore, the fabricated scaffolds were transferred to liquid nitrogen tank and were preserved for 24 h. Instantly after removing the fibers from the tank; they were crushed and preserved at 4 °C. Eventually, the crushed nanofibers were

crosslinked by the vapor of 10% glutaraldehyde for 12 h. The crosslinked nanofibers were preserved in the desiccator for 7 days.

PLA with a total concentration of 6% (w/v) was dissolved in 1, 4-dioxane for 24 h. Furthermore, GTNFs containing PRP were dispersed in the PLA solution with a weight ratio of 70:30 (70% PLA, 30% GTNFs). The resulting solution was then transferred to −20 °C and was frozen for about 6 h. The samples were then immediately transferred to a freeze dryer at −77 °C (121550PMMA, Christ, Spain) for 72 h. The dry scaffolds were collected after freeze-drying. These scaffolds were cut using water jet device in the shape of NGCs of 14 mm length, with an outer diameter of 3 mm and inner diameter of 1 mm.

Primary rat SCs were harvested from sciatic nerves of 1 day-old Wistar rats (School of Pharmacy of Tehran University of Medical Sciences, Tehran, Iran) using modified Brockes method.[14,15] The cells were cultured in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12; Gibco, Grand Island, USA) and fetal bovine serum (FBS; Gibco, Grand Island, USA), 100 unit/ml of penicillin and 100 mg/ml of streptomycin in a humidified incubator with 5% CO2 and 37 °C temperature. The cell medium was changed every 48 h. Before cell seeding, the scaffolds were sterilized by ultra violet light irradiation for 60 min. Third passage cells were seeded at a density of 7×103 per scaffold.

The morphology of the conduits was observed by scanning electron microscope (SEM; DSM 960A, Zeiss, Germany) at 15 kV by coating with gold for 130 s using a sputter coater (SCD 004, Balzers, Germany).

The surface wettability of the scaffolds was evaluated with the sessile drop method using a static contact angle measuring system (G10, KRUSS and Germany). Our data revealed that PLLA scaffolds.

For in vitro degradation experiment, the samples were immersed in 10 mL PBS. The initial weight of the samples was measured after drying in vacuum for 2 days. At every instance, the samples were retrieved and were dried to gain a constant weight. By using the following equation, weight loss of conduits was calculated: Weight loss (%) = (W0−W1)/W0×100

Where W0 is the initial weight of samples and W1 is the dry weight after removing samples from the media.[16]

Liquid displacement technique was used to evaluate the porosity of the scaffolds, by the following equation; [17]

Porosity (%) = (V1-V3)/ (V2-V3)

Where V1 is the initial volume of ethanol (96%), V2 is the volume after scaffold immersion, and V3 is the volume of ethanol after scaffold removal (after 10 min).

Mechanical properties (tensile strength and Young's modulus) were measured using an Instron 5566 universal testing machine (Instron, MA) at a strain rate of 10 mm/min. Each test was performed at least thrice.

The proliferation of the seeded cells was assessed using MTT assay after 48 h of incubation. The cell medium was discarded and 0.01 mL of 5 mg/mL MTT in PBS plus 0.09 mL fresh media was added. Furthermore, the cells were incubated at 37 °C for 4 h. After the formation of purple formazan crystals, 0.1 mL DMSO was added to dissolve them. The supernatant absorption was read at 570 nm using a microplate reader (Stat fax-2100, awareness technology Inc, palm city, FL, USA). Cells cultured on tissue culture plates without scaffolds served as negative control. The mean of the triplicate wells for each specimen was reported.[17]

The cell attachment on the conduit was investigated using fluorescence microscope with an optical filter of 360 and 460 nm excitation and emission wavelengths, respectively. After 48 h of cell seeding, cells were stained with 4, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA). Cells were fixed with 2.5% glutaraldehyde and were then incubated with 0.2% 100× to enhance the dye penetration. DAPI solution (concentration1:1000) was added to the cell conduit constructs and incubated for 5 min at room temperature. In order to remove the unbound DAPI, conduits were washed thrice with PBS.[16]

Data were expressed as the mean ± standard deviation. The results were statistically analyzed by SPSS-21 software using the student t-test. In all evaluations, $P<0.05$ was considered as statistically significant.

Results

The SEM images illustrated that the fabrication method used in this study led to the porous structure of the PLA/GTNF/PRP scaffolds (figure 1). This scaffold structure may facilitate the metabolite diffusion through the wall of the conduits, which is essential for optimal nerve regeneration.

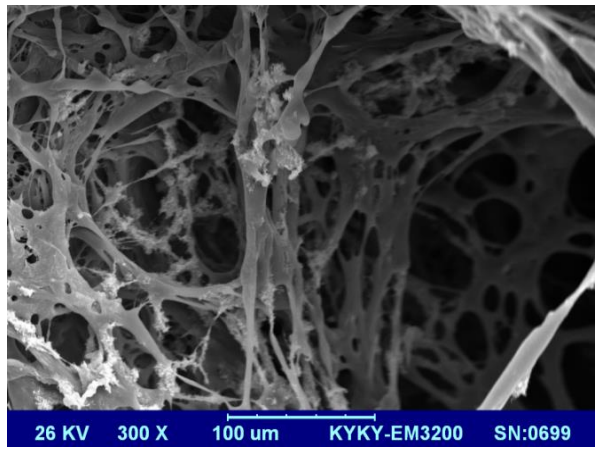

Figure 1. Representative SEM micrograph of PLA/ GTNF-PRP scaffolds

The surface wettability of the scaffolds was evaluated with Zisman method (table 1). PLA scaffolds revealed a contact angle of 119±0.56º, whereas scaffolds incorporated with GTNF/PRP indicated a lower contact angle of 79.5±0.71º.

Table 1. Surface wettability of the scaffolds was measured with Zisman method

Samples	Contact angle (°)
PLA	119±0.56
PLA/Gelatin containing PRP	79.5±0.71

The weight loss was measured for 2 months (table 2). During this period, PBS solution was exchanged with a fresh solution every week. At predetermined time intervals, the scaffolds were removed from the media, washed with distilled

water, dried under vacuum to a constant weight, and their weights were measured. The results revealed that weight loss for the scaffolds containing gelatin nanofibers was faster than the PLA only scaffold.

Table 2. Weight loss measurement of the scaffolds

Samples	Weight loss (WL) % 30 day	Weight loss (WL) % 60 day
PLA	1.7	8.2
PLA/Gelatin containing PRP	14.6	23.5

Liquid displacement method was used to evaluate the porosity of the samples. As presented in table 3, the porosity of the prepared conduits is sufficiently higher (>80%) in order to use them for tissue engineering.[18] The PLA conduit revealed higher porosity (90.5%) compared to the PLA/GTNF/PRP (89.6%).

Table 3. Porosity of the conduits

Samples	Porosity (%)
PLA	90.5 %
PLA/Gelatin containing PRP	89.6 %

As presented in table 4, the scaffolds fabricated in this study indicate adequate mechanical properties to serve as a promising neural guidance conduit. The results revealed that the incorporation of GNF/PRP accelerated the tensile strength and Young's modulus values of the PLA conduits.

Table 4. Mechanical properties of the conduits

Samples	Tensile strength (MPa)	Young's modulus (MPa)
PLA	1.88±0.32	3.98±0.44
PLA/Gelatin containing PRP	3.27±0.65	4.25±0.31

Cell viability was evaluated using MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assay 48 h after cell seeding (figure 2). The GTNF/PRP incorporated scaffolds revealed more cell viability than the PLA only scaffolds.

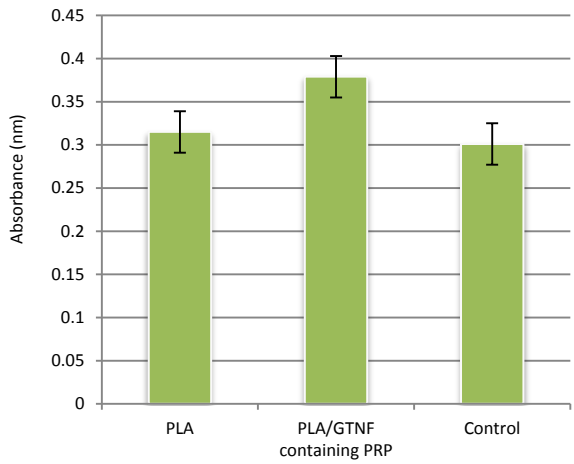

Figure 2. MTT assay 48 h after cell seeding

Fluorescence microscopy confirmed the attachment of cells on PLA and PLA/GTNF/PRP scaffolds as presented in figure 3(a) and 3(b). As illustrated, the number of cells attached to PLA/GTNF/PRP scaffolds was higher than PLA only conduits.

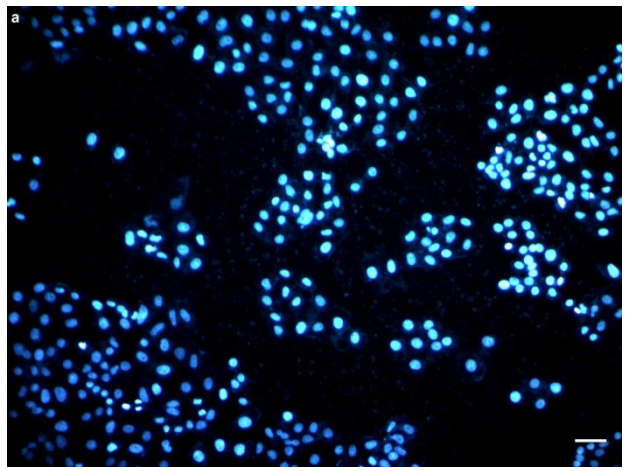

Figure 3a. DAPI staining: attachment of cells on PLA conduit

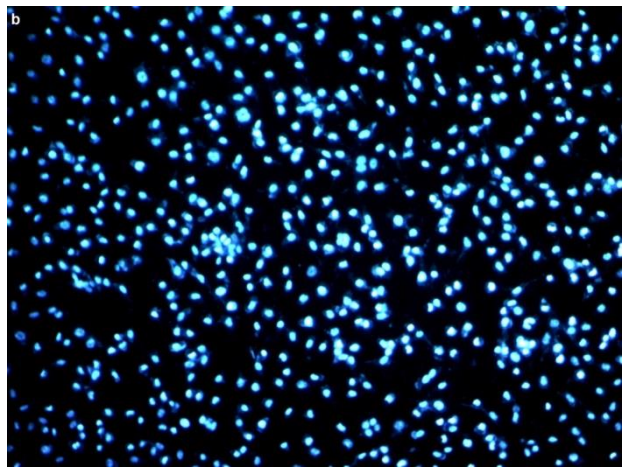

Figure 3b: DAPI staining: attachment of cells on PLA/GTNF-PRP conduit

Discussion

In traumatic nerve injuries with large gaps, autografts and allografts are considered; however, these solutions face certain limitations. In this study, we demonstrated that composite scaffolds fabricated from GTNFs comprising PRP and PLA could enhance favorable cellular functions. SEM images revealed that conduits comprised porous structure with good interconnectivity of the pores, which proves beneficial for the metabolite diffusion resulting in a better regenerative environment. Contact angle measurement indicated significant difference between the fabricate scaffolds. The methyl groups (CH3) present in PLA structure make this polymer highly hydrophobic, whereas amino (NH2) and hydroxyl (COOH) groups in gelatin impart a relatively high hydrophilicity to the gelatin-based scaffolds. Therefore, the addition of gelatin to PLA decreased its hydrophobicity by the means of its molecular chains making it more appropriate for interacting with the biological systems. PLA, due to its greater hydrophobicity, degrades slowly. We reported that scaffolds modified with GTNF/PRP have a significantly higher

degradation rate compared with PLA only scaffolds at both time intervals. The greater hydrophilicity of gelatin facilitates water permeation and accelerates scaffolds degradation. Mechanical strength of conduits is an important property as poor mechanical strength can cause the conduits to collapse when implanted or to rupture during suturing. Tensile strength measurement indicated that PLA only conduits revealed a significantly lower tensile strength compared to PLA/GTNF/PRP scaffolds. In vitro biocompatibility studies reported that cells grown on PLA scaffolds revealed a significantly lower proliferation rate compared to the scaffolds modified with GTNF/PRP; this may occur as the cytocompatible and cationic nature of GTNF and growth factor released by platelets enhances the cell attachment and proliferation when compared with PLA only scaffold. DAPI staining images indicated that a higher number of cells attached on PLA/GTNF/PRP scaffolds compared with PLA scaffolds. Cell attachment sites present in GTNF structure could explain this. In the present study, we hypothesized that PRP may enhance the PLA/GTNF conduits capability to foster more robust nerve regeneration. Growth factors released by this gel can prevent apoptosis, accelerate cell proliferation, and attract undifferentiated cells to injury site that will eventually repair the tissue.

In this research, the electrospinning and TIPS methods were collectively used to fabricate the conduits of pure PLA and PLA/GTNF/PRP (70:30). Our results revealed that when GTNF/PRP is incorporated into PLA scaffolds, resultant mechanical properties, porosity and cell attachment, and viability in vitro provided better results than pure PLA. Addition of GTNF/PRP into PLA scaffolds ameliorated the tensile strength, Young's modulus result, hydrophilicity, cell viability, cell attachment, and degradation rate of the conduits.

Acknowledgement

We wish to express our appreciation to Dr. Gholamreza savari for his attribution in this study.

Conflict of Interest

The authors declare no financial conflicts of interest.

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