



Increased neuritogenesis on ternary nanofiber matrices of PLCL and laminin decorated with black phosphorus

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ABSTRACT

Recently, the composites of varying biomaterials and two-dimensional (2D) nanomaterials have been increasingly developed in the field of tissue engineering and regeneration owing to their excellent physicochemical and mechanical properties. In particular, the combination of biocompatible polymers and 2D nanomaterials can endow the nanocomposite with novel biofunctional properties while maintaining the inherent character of every single material. These nanocomposites are known to have the potential for improvement of cellular behaviors such as cell adhesion, proliferation, and differentiation. In this study, poly(L-lactide-co-ε-caprolactone) (PLCL) and laminin (Lam) nanofibers functionalized with black phosphorus (BP) were successfully fabricated to increase the neuritogenesis of HT22 hippocampal neuronal cells using an electrospinning process. The ternary nanofiber matrices of PLCL/Lam/BP were found to be suitable for supporting the attachment and proliferation of hippocampal neurons. Furthermore, these PLCL/Lam/BP nanofiber matrices could not only promote neurite outgrowth and alignment but also increase the neuritogenesis of hippocampal neurons by providing optimal microenvironments for neuronal differentiation. The underlying mechanism of this phenomenon was confirmed by determining the expression levels of genes related to neurogenesis. In conclusion, these results suggest that BP-functionalized composite nanofibers could be a promising candidate as a scaffold for neural tissue engineering and regeneration.

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Introduction

Nerve tissue is a sophisticated and highly differentiated system that is often damaged by traumatic injuries, degenerative diseases, tumor ablation, or iatrogenic injuries of surgery. A slight nerve injury can spontaneously heal, but it is difficult to regenerate severe nerve damage, requiring external supports [1]. Conventional strategies have adopted end-to-side nerve coaptation for clinical reconstruction through allograft, xenograft, and autograft [2–5]. This therapeutic approach is quite limited because of the limited supply, immune reaction, and ineffectiveness. Therefore, many studies have focused on the development of artificial

scaffolds for neuronal regeneration with tissue engineering approaches [6–10]. Artificial scaffolds can mimic the natural extracellular matrix (ECM) and induce desirable cellular behaviors. Several techniques such as electrospinning and 3D printing have been introduced to fabricate artificial scaffolds [11–14]. Among them, electrospinning was highlighted to fabricate an aligned fibrous matrix structure. It is known that the phenotype and destiny of cells are highly associated with mechanical and topological cues [15,16]. The electrospun nanofibrous matrix can topologically modulate cells and induce several biological effects such as differentiation of stem cells into specific lineage or maturation of anisotropic tissues (e.g., skeletal muscles, cardiac muscles, and neurons) [16–20]. Especially in neuronal cell engineering, aligned nanofibers can provide axon guidance to neuronal cells and enhance the neurogenic differentiation. Synthetic polymers such as polylactides, polycaprolactones, and poly(lactic-co-glycolic acid) are popularly used as the backbone of nanofiber-based scaffold due to their easiness to handle,

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biocompatibility, and controllable biodegradability [21–23]. Furthermore, various kinds of biomolecules are incorporated into the nanofiber matrix, such as proteins, peptides, inorganic materials, and chemokines, to fabricate biofunctional scaffolds.

The development of functional composite scaffolds enables them to possess more favorable properties while maintaining the intrinsic nature of a single material. Hence, we incorporated two biofunctional cues, laminin (Lam) and black phosphorus nanoparticles (BPNPs) into poly(L-lactide-co-ε-caprolactone) (PLCL). In terms of neuronal tissue engineering, PLCL polymers have several advantages, such as biocompatibility, long-term degradability, suitable mechanical property, and non-immunogenic property. PLCL has been widely used in biomedical fields because of these superior physicochemical and biological properties. However, their lack of cell adhesion sites and biofunctionality mainly limited their application in the tissue engineering field [24]. Lam is a plentiful glycoprotein in the basement membrane of nerve tissues, which comprises a multi-domain, cross-link-shaped structure consisting of three polypeptide chains ($\alpha 1$, $\beta 1$, and $\beta 2$) [25]. It is known that Lam in the nerve tissue membrane gives signals such as axonal guidance and neurite outgrowth, and controls cellular behaviors such as adhesion, migration, proliferation, and differentiation [23,26,27]. Therefore, the incorporation of Lam can endow physiological similarity to nanofiber scaffolds that promote neuronal cell growth and neurite outgrowth.

Recently, BP, also known as phosphorene, has attracted great attention as one of the most prospective two-dimensional (2D) nanomaterials in biomedical fields such as bioimaging, biosensing, and nanomedicine, due to its fascinating physicochemical properties and electrical conductivity as well as excellent biocompatibility [28–30]. Especially, the degradation product of BP in the physiological environment is known to be nontoxic phosphate anions, which are required for skeletal development and osteogenesis as well as early regeneration of peripheral nerves [31–33]. Furthermore, due to the high conductivity of BP, it can assist the cell-cell interaction and recovery of peripheral nerve, which plays an important role in the regeneration of the neuronal system [34]. These properties make BP an attractive 2D nanomaterial for tissue engineering and regeneration. In this study, PLCL and Lam nanofibers functionalized with BP (PLCL/Lam/BP)

were fabricated by electrospinning to investigate whether or not they can be used as neural tissue engineering scaffolds (Fig. 1). The physicochemical properties of PLCL/Lam/BP nanofibers were characterized by atomic force microscopy (AFM), scanning electron microscopy (SEM), water contact angle, and Raman spectroscopy. The cellular behaviors of HT22 hippocampal neuronal cells on the nanofiber matrices were also investigated. Moreover, the underlying mechanism for the morphological changes in neuronal cells was examined by determining the expression levels of genes related to neurogenesis by using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Materials and methods

Preparation and morphological characterizations of BPNPs

BPNPs were prepared using liquid exfoliation procedure from the bulk BP materials as described elsewhere [35–37]. In brief, 0.4 g of BPNPs was dispersed in 100 mL distilled water and subjected to ultrasound sonication for 30 min. The resulting BPNPs were maintained at room temperature for 10 min. The ultra-sonication procedure was repeated three times to produce BPNPs decanting a 10 mL of BP supernatant from the previous solution. The BPNPs were dried in the open air for 24 h and agitated by ultra-sonication for 15 min before its use for analysis. The morphologies of BPNPs were observed by high-resolution transmission electron microscopy (HT-TEM; JEM-3010, JEOL, Japan). The samples for TEM analysis were prepared by drop-casting a dilute solution of BPNP onto carbon-coated copper grids. BPNPs were imaged by atomic force microscopy (AFM, XE-100, Park Systems) with a silicon cantilever in non-contact mode. The images with $10\ \mu\text{m} \times 10\ \mu\text{m}$ or $45\ \mu\text{m} \times 45\ \mu\text{m}$ scan sizes consisted of 512×512 points of height data and a scan rate of 1.0 Hz/line. Surface data from AFM were further processed with XEI software (Park Systems).

Fabrications of ternary nanofibers of PLCL, Lam, and BP

PLCL (75:25, molecular weight = 220 kDa) was purchased from BMG Inc. (Kyoto, Japan). Lam was purchased from Gibco

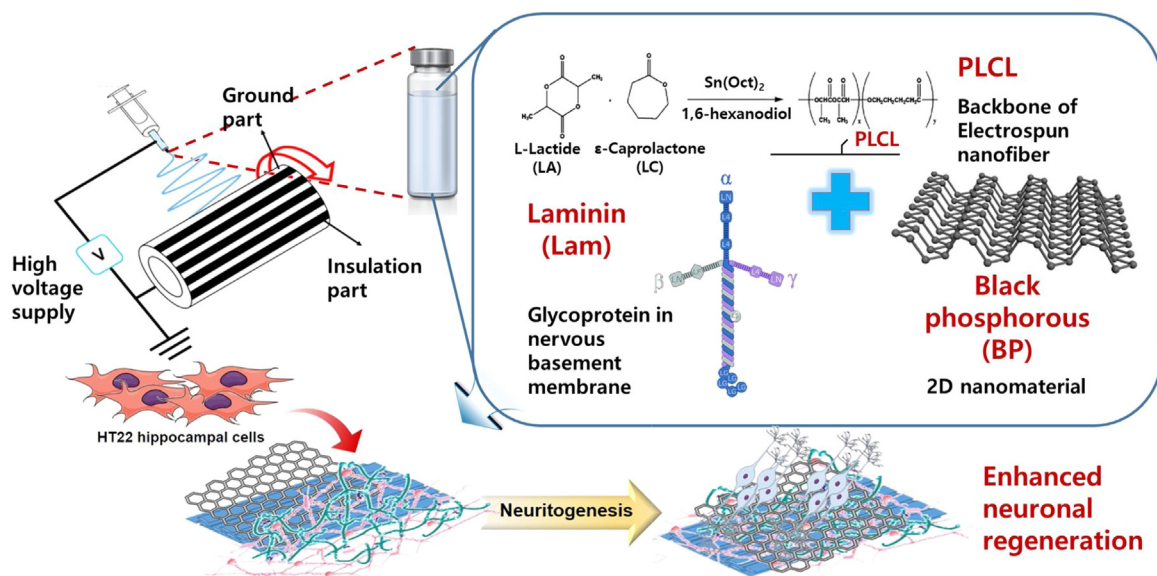


Fig. 1. Schematic diagram of PLCL/Lam/BP nanofiber matrices. PLCL/Lam/BP nanofibers were fabricated by electrospinning. PLCL has suitable mechanical property, biodegradability, and controllable degradation rate. Incorporated Lam and BP have the potential to control cellular behaviors. HT22 hippocampal neuronal cells were seeded onto the prepared PLCL/Lam/BP nanofiber matrices and substantially induced neuritogenesis after several days of culture.

(Singapore). Ternary nanofibers composed of PLCL, Lam, and BP were fabricated by electrospinning. Briefly, PLCL with a concentration of 10 wt.% was prepared by dissolving in HFIP (1,1,1,3,3,3-hexafluoro-2-propanol, Sigma-Aldrich, St. Louis, MO) with stirring for 12 h. Then, Lam was added to a completely dissolved PLCL solution with a ratio of 94 (PLCL):6 (Lam). Lastly, BPNPs were added to the PLCL/Lam solution at 10 µg/mL. Each solution of PLCL, PLCL/Lam, and PLCL/Lam/BP was loaded into a respective syringe (HSW, Tuttlingen, Germany) with a 25-gauge stainless-steel needle. The working distance between the needle and collector was set at 15 cm with a flow rate of 0.5 mL/h, while a voltage of 18 kV (DC high voltage power supply, NanoNC, Seoul, Korea) and rotation of 2000 rpm were applied. Each type of nanofiber was fabricated followed by slow-vacuum drying at room temperature overnight. Prior to use, the prepared nanofibers were cut into disks with 9 mm diameter and exposed under ultraviolet light for sterilization.

Characterizations of PLCL/Lam/BP nanofibers

The surface morphology of nanofibers was observed with scanning electron microscopy (SEM, TESCAN VEGA3, Brno, Czech Republic) at an accelerated voltage of 20 kV. The nanofibers were imaged by AFM. The images were analyzed using the same method as mentioned above. Raman spectra of nanofibers were recorded using a Raman spectroscope (Ramboss 500i, Dong Woo Optron Co., Gwangju, Korea) equipped with a charge-coupled device (CCD) camera (iDusDV420A-OE Andor Technology, Belfast, Ireland) and a precise motorized stage (SGSP 20–85, Sigma Koki Co., Ltd., Tokyo, Japan). A radially polarized solid-state laser of 532 nm (LasNova 50, LASOS, Jena, Germany) was focused onto the sample using a water immersion objective lens ($\times 60$ magnification, numerical aperture of 1.2 UPlanSApo, Olympus, Tokyo, Japan) and resolved with a monochromator (Monora500i, Dong Woo Optron Co., Gwangju, Korea). 50 mW laser power at 532 nm attenuated by using a neutral density (ND) filter of 50% transmittance at the objective was used for all measurements. Water contact angles of the nanofibers were measured with the sessile drop method using an OCA 10 goniometer (DataPhysics, Filderstadt, Germany). Then, 10 µL of distilled water was dropped onto the surface of the nanofiber and the images were captured and the contact angle was calculated by an optical system. Fiber orientation was quantified by FFT mode of ImageJ software (National Institutes of Health, Bethesda, MD) based on SEM images. The average fiber diameters were calibrated based on SEM images.

Cell culture and conditions

HT-22 mouse hippocampal neuronal cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Welgene, Daegu, Korea) with high glucose concentration containing 10% fetal bovine serum (Welgene) and 1% antibiotic-antimycotic solution (Abs, 10,000 units of penicillin, 25 µg/mL of amphotericin B and 10 mg of streptomycin, Sigma-Aldrich) at 37 °C in a humid incubator with 5% CO₂. Cells were detached from the flask at 70% confluency using trypsin-EDTA and seeded at a density of 1×10^4 cells/well on PLCL, PLCL/Lam, and PLCL/Lam/BP. All samples were incubated in the same conditions with cell culture for evaluated initial cell attachment and proliferation.

Cell proliferation and differentiation assays

The initial cell attachment and proliferation were evaluated by a Cell Counting Kit-8 assay (CCK-8, Dojindo, Kumamoto, Japan). The HT-22 mouse hippocampal neuronal cells were seeded on PLCL, PLCL/Lam, and PLCL/Lam/BP nanofiber matrices and incubated for 6 h to 7 days (6 h for initial cell attachment and 1, 3, and 7 days for

proliferation measurement). The CCK-8 assay was used according to the manufacturer's protocol. Briefly, the CCK-8 solution, mixed with growth media was dispensed into each sample and incubated for 2 h at 37 °C in the dark. After the completion of the incubation period, the supernatant was collected and transferred into a new 96-well plate. The absorbance was measured at 450 nm with SpectraMax 340 ELISA Reader (Molecular Device Co., Sunnyvale, CA, USA).

For the differentiation study, HT-22 mouse hippocampal neuronal cells were seeded on each nanofiber matrix for 3 days, the culture medium was changed from growth medium to differentiation medium; neurobasal medium (Gibco) containing Abs, L-glutamine (Gibco), B-27 supplement (Gibco), N2 supplement (Gibco), epidermal growth factor (PeproTech, Houston, TX) and basic fibroblast growth factor (PeproTech).

Immunofluorescence staining

The nuclei, F-actins, and neurofilaments of HT-22 hippocampal neuronal cells were confirmed by the immunofluorescence staining. Cell morphology was observed in the growth medium, which was replaced with the differentiation medium to observe the neurofilaments. After 7 days, cells were fixed with a 3.7% formaldehyde solution (Sigma-Aldrich) for 10 min and treated in 0.1% Triton X-100 (Sigma-Aldrich) for 5 min, followed by blocking with a 5% bovine serum albumin (BSA, GenDEPOT, Barker, TX) solution for 30 min. Subsequently, the cells were incubated with tetramethylrhodamine isothiocyanate (1:40 in 2% BSA solution; Molecular Probes, Eugene, OR) for 30 min in the dark at room temperature. The neurofilaments were immunostained with anti-neurofilament heavy polypeptide antibody (Abcam, Cambridge, MA), followed by a secondary goat anti-rabbit IgG heavy and light chains (Abcam) conjugated with fluorescein isothiocyanate. The nuclei were counterstained with a 4',6-diamidino-2-phenylindole (1 µM, Sigma-Aldrich) solution in DPBS (Sigma-Aldrich). The fluorescence images were confirmed using a confocal laser scanning microscope (CLSM, Zeiss, Oberkochen, Germany) and a custom-built two-photon laser fluorescence microscope (TPLM) as described elsewhere [18,20,38].

RNA isolation and real-time qRT-PCR

To evaluate the mRNA expression level according to different surface conditions, HT-22 mouse hippocampal neuronal cells were seeded on each nanofiber matrix for 3 days, the culture medium was changed from growth medium to differentiation medium. After 7 days, cells were dissociated with 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA) by mild pipetting. Total RNA was extracted from the detached cells using TRIzol reagent (Invitrogen) and an RNeasy Mini Kit (Qiagen, Grand Island, NY). SuperScript III First-Strand cDNA Synthesis System (Invitrogen) was further used to synthesize first-strand cDNA from total RNA according to the manufacturer's instructions. The mRNA expression of specific genes was then determined by real-time qRT-PCR using the total first-strand cDNA as the template and Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). The sequences of the primers for NeuN, DCX, MAP2, Nestin, and 18S rRNA are shown in Table 1. The 18S rRNA expression level was used as an endogenous normalizer and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All variables were tested in three independent cultures for each experiment, which was repeated twice ($n=6$). The quantitative data are expressed as the mean $\pm$ standard deviation (SD). The data

Table 1
Primers for each gene.

Gene	Primer
18s	Forward 5'- CGGCGACGACCCATTGCAAC Reward 3'- GAATCGAACCTGATTCCTCCGTC
MAP2	Forward 5'- GAGAAACGTTCTTCCTCCC Reward 3'- GTGTGGAGGTGCCACTTTT
NeuN	Forward 5'- CACCACTCTCTTGCTCCGTTTGC Reward 3'- GGCTGAGCATATCTGTAAGCTGC
DCX	Forward 5'- ATGCAGTTGTCCCTCCATTTC Reward 3'- ATGCCACCAAGTTGTCATCA
Nestin	Forward 5'- AGAGGACCAGGTGCTTGAGA Reward 3'- TCCTCTGCGTCTTCAAACCT

were tested for the homogeneity of the variances using Levene's test, prior to statistical analysis. Statistical comparisons were carried out using a one-way analysis of variance (ANOVA; SAS Institute Inc., Cary, NC), followed by a Bonferroni test for multiple comparisons. A p value <0.05 was considered significant.

Results

Morphology and cytotoxicity of BPNPs

The surface morphology of BPNPs was examined by TEM (Fig. 2(a)). The prepared BPNPs showed spherical morphology and uniform size in tens of nm with a mean diameter of 57.60 ± 6.43 nm (Fig. S1). Fig. 2(b) shows representative AFM images and a height profile of the BPNPs. BPNPs have a few nm heights when measured in the form of as-deposited sheets. The height profiles of BPNPs showed the range of nm, which corresponds to several layers of BP. Furthermore, the Raman spectral data revealed distinct A_g^1 , A_g^2 , and B_{2g} bands appearing in phosphorene (Fig. S2), which are similar to the Raman spectral results of BP [35–37]. These results indicate that BPNPs are well distributed with a circular and multilayered form in the range of a few nm.

The cytotoxicity of BPNPs was evaluated to optimize the concentration of incorporation into nanofibers of BPNPs. We examined the cytotoxicity of BPNPs in HT22 hippocampal neuronal

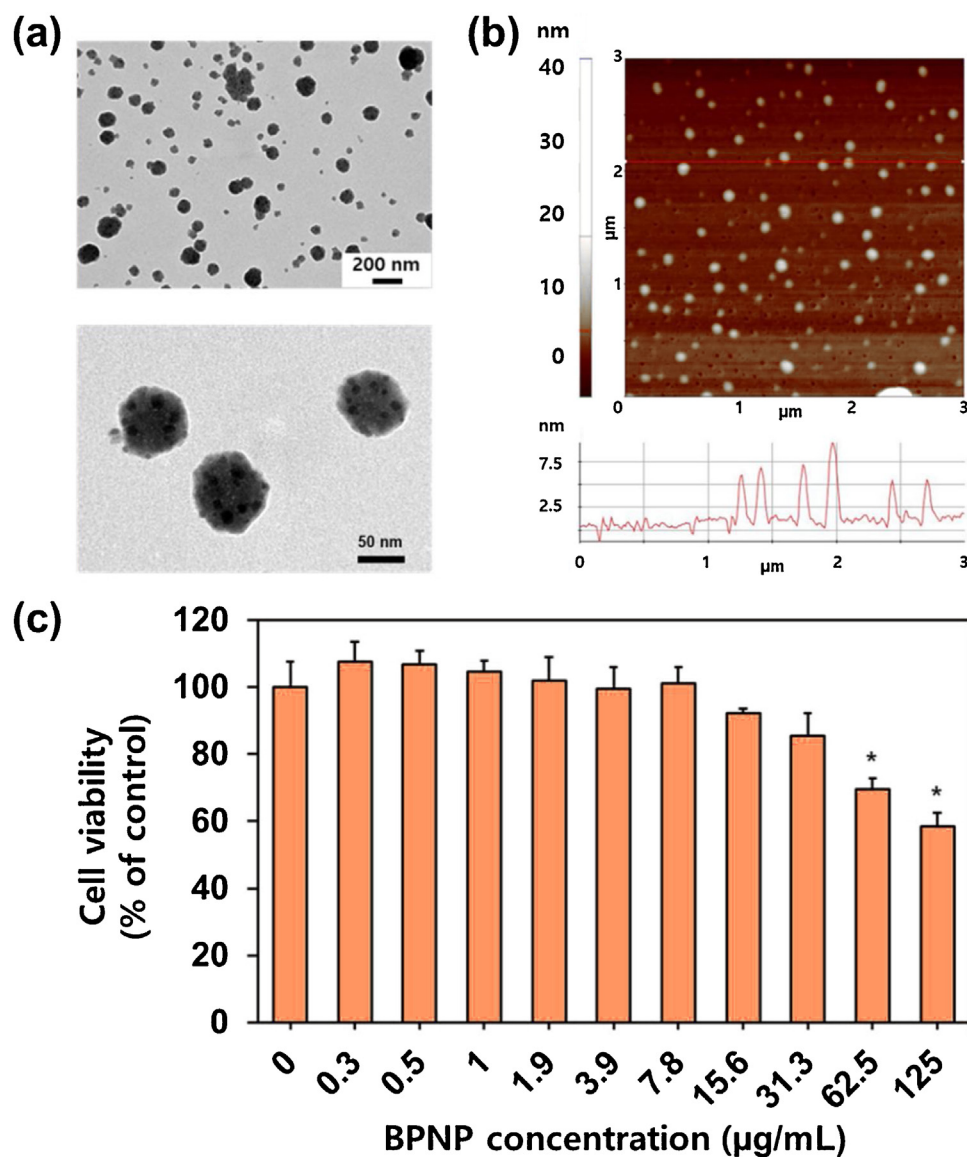


Fig. 2. Morphology and cytotoxicity of BPNPs. (a) TEM and (b) AFM images with a height profile of BPNPs. (c) Viability of HT22 hippocampal neuronal cells treated with different concentrations of BPNPs. All images shown in this figure are representative of six independent experiments with similar results. The data of (c) are expressed as the mean $\pm$ SD ($n=6$). An asterisk (*) denotes a significant difference compared to the other concentrations, $p < 0.05$.

cells. A decreasing trend in cell viability was observed in a dose-dependent manner when the cells were treated with BPNPs, as shown in Fig. 2(c). High cell viability was maintained at concentrations lower than $31.3 \mu\text{g/mL}$ (81% viable) after 24 h in all conditions. However, cells were affected by the toxicity of BPNPs over the concentrations of $62.5 \mu\text{g/mL}$. It is known that the size of BPNPs is closely related to the degree of their cytotoxicity, which is mediated by the generation of reactive oxygen species (ROS) and the disruption of the cell membranes [39]. In this study, few-layered BPNPs were used with tens of nm diameter and showed a significant ($p < 0.05$) decrease of the cell viability only at relatively higher concentrations of $\geq 62.5 \mu\text{g/mL}$ ($\approx 70\%$ viable or lower). Our previous study demonstrated that BP nanosheets with hundreds of nm diameter showed increased ROS generation and membrane damage to I-929 fibroblasts at concentrations of $\geq 62.5 \mu\text{g/mL}$ ($\approx 40\%$ viable or lower) [40]. On the other hand, another study presented that nm to μm range of ribbon- and stone-like non-layered BP particles showed higher cytotoxicity ($\approx 50\%$ viable) against A549 human lung carcinoma epithelial cells even at the concentration of $25 \mu\text{g/mL}$ [41]. Overall, BPNPs used in this study exhibited cytocompatibility because they are relatively small sized and layered sheets morphology, which might reduce size-dependent membrane damage, even considering different types of cells were tested.

Physicochemical characteristics of PLCL/Lam/BP nanofibers

The surface topography and roughness of the nanofibers were measured using AFM (Fig. 3(a)). 3D images of AFM show that the surface of the nanofibers containing Lam and BP was rough compared to the pristine PLCL nanofibers, which is expected to promote cell attachment and proliferation. Moreover, the surface roughness was measured to be less than $2 \mu\text{m}$, and the surface of the nanofibers was found to be smooth. PLCL-based nanofibers had a nanometer-scale diameter and no beads, suggesting that the nanofibers were fabricated successfully as scaffolds. SEM images were observed as a continuous and three-dimensional network structure with interconnected pores that were similar to the natural ECM (Fig. 3(b)). Moreover, alignment and average fiber diameter of each nanofiber were quantified (Fig. 3(c) and 3(d)). PLCL, PLCL/Lam and PLCL/Lam/BP nanofibers showed aligned structure with nano-sized diameter, which can potentially promote cellular alignment and axonal extension.

Water contact angle measurements showed that all nanofibers had a hydrophobic surface (Fig. 4(a)). As Lam and BPNPs were incorporated into the nanofibers, the contact angle showed a tendency to decrease, indicating that Lam and BPNPs increased the hydrophilicity of nanofibers that is helpful to cell adhesion and growth [36,42]. In addition, UV treatment to nanofibers was found to increase surface hydrophilicity [42]. Therefore, all nanofiber matrices were UV-treated prior to an *in vitro* study, which could be helpful for sterilization.

On the other hand, Raman spectra of PLCL, PLCL/Lam, and PLCL/Lam/BP nanofibers showed the characteristic peaks of each material (Fig. 4(b)). Because the PLCL is a copolymer of poly(lactic acid) (PLA) and poly(caprolactone) (PCL), peaks for pristine PLA and PCL were observed $39,40$. The peaks at 873 cm^{-1} ($\nu \text{ C}=\text{COO}$), 1042 cm^{-1} ($\nu \text{ C}-\text{CH}_2$), 1092 cm^{-1} ($\nu \text{ C}-\text{O}-\text{C}$), 1128 cm^{-1} ($\gamma \text{ CH}_3$), and 1452 cm^{-1} ($\delta \text{ CH}_3$) are assigned to the semicrystalline phase of pristine PLA [43]. In the pristine PCL, the peaks at 1730 and 1736 cm^{-1} correspond to the amorphous phase, and the peak at 1720 cm^{-1} is assigned to the crystalline phase [44]. Other peaks at 1470 – 1415 cm^{-1} , 1303 – 1281 cm^{-1} , 1107 – 1033 cm^{-1} , and 912 cm^{-1} reveal the crystalline phase of pristine PLCL [45]. In particular, the $\text{C}=\text{O}$ stretching vibration is assigned around 1700 cm^{-1} [44]. The PLCL peaks are present in all nanofibers. Raman spectra of the BP in the PLCL/Lam/BP nanofibers peak have revealed that the three prominent peaks appeared at 354 cm^{-1} , 433 cm^{-1} , and 462 cm^{-1} can be attributed to A_g (out-of-plane mode), B_{2g} and A_{2g} (in-plane modes), respectively, which well agreed with those of the neat BP (Fig. S2) [46–48]. All these results demonstrate that the nanofibers were well prepared, and Lam and BP have been successfully incorporated into PLCL nanofibers.

Cellular behaviors on PLCL/Lam/BP nanofibers

HT22 hippocampal neuronal cells were cultured on the PLCL, PLCL/Lam, and PLCL/Lam/BP nanofiber matrices to assess the initial attachment and proliferation. The initial attachment of HT22 hippocampal neuronal cells was not hindered, showing that all the PLCL-based nanofiber matrices supported neuronal cell adhesion and growth (Fig. 5(a)). As shown in Fig. 5(b), the cells on nanofiber matrices showed a normally proliferating tendency during the culture period. It was found that the proliferation was slightly changed from 1 to 3 days. On the contrary, the proliferation of cells on PLCL/Lam/BP nanofiber matrices at 7 days significantly

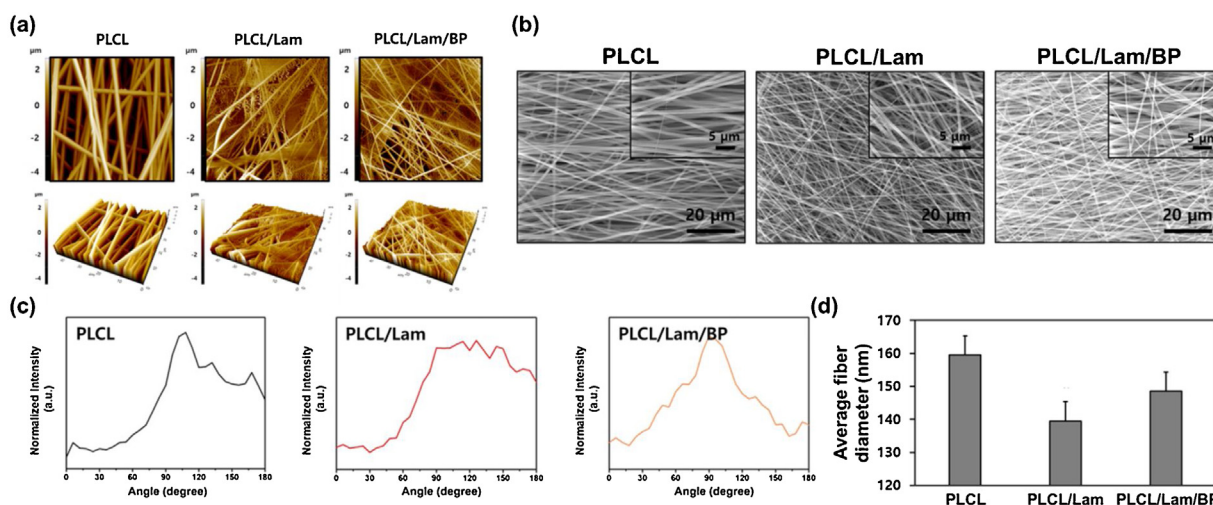


Fig. 3. Morphological characterizations of prepared nanofibers. (a) AFM, (b) SEM, (c) fiber orientation, and (d) average fiber diameter of PLCL, PLCL/Lam, and PLCL/Lam/BP nanofibers. All images shown in this figure are representative of six independent experiments with similar results. The data of (d) are expressed as the mean $\pm$ SD ($n = 6$).

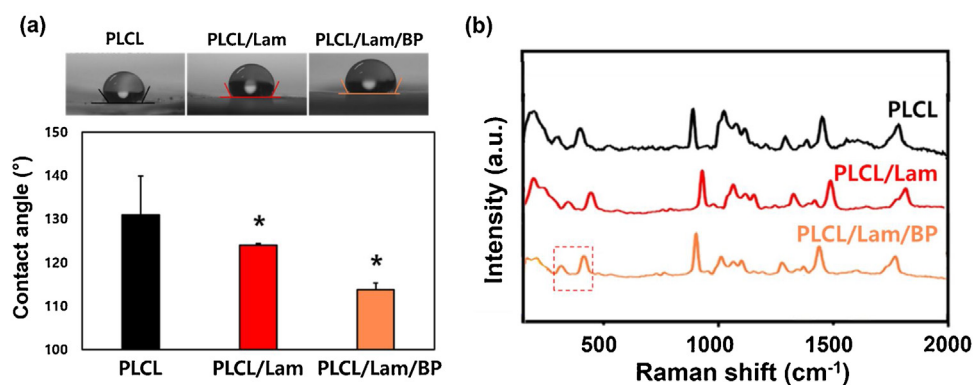


Fig. 4. Physicochemical properties of prepared nanofibers. (a) Contact angle and (b) Raman spectra of PLCL, PLCL/Lam, and PLCL/Lam/BP nanofibers. All images shown in this figure are representative of six independent experiments with similar results. The data of (a) are expressed as the mean $\pm$ SD ($n=6$). An asterisk (*) denotes a significant difference compared to the PLCL group, $p < 0.05$.

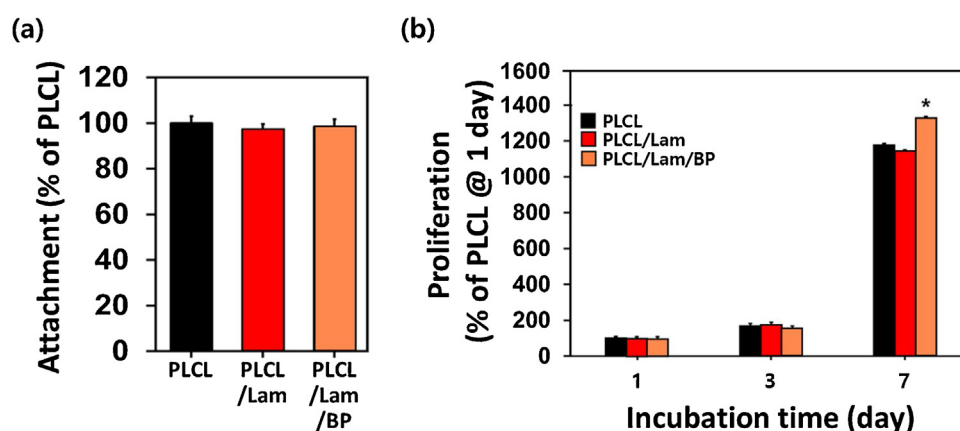


Fig. 5. (a) Cell adhesion and (b) proliferation of HT22 hippocampal neuronal cells on PLCL, PLCL/Lam, and PLCL/Lam/BP nanofiber matrices. The data are expressed as the mean $\pm$ SD ($n=6$). An asterisk (*) denotes a significant difference compared to the other groups, $p < 0.05$.

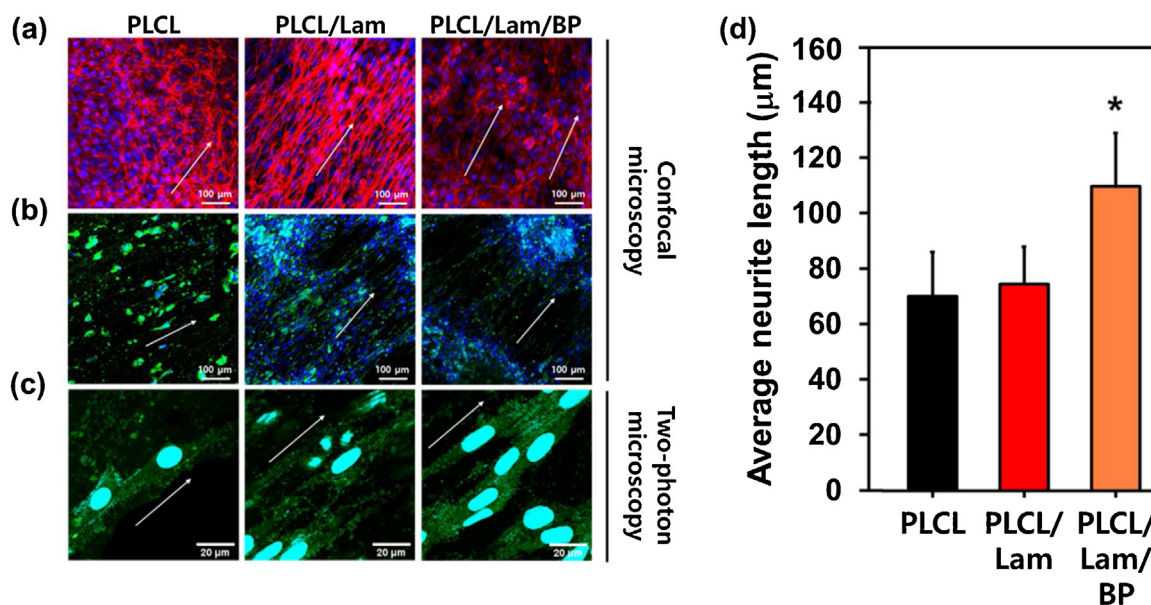


Fig. 6. Immunocytochemical analysis. (a) and (b) confocal microscopic images of HT22 hippocampal neuronal cells on PLCL, PLCL/Lam, and PLCL/Lam/BP nanofiber matrices. (c) Two-photon microscopic images. (d) Average neurite length. Fluorescence of (a)–(c) denotes the nucleus (blue), f-actin (red), and neurofilaments heavy chain (green), respectively. Arrows in (a)–(c) indicate the fiber-aligned orientation. All images shown in this figure are representative of six independent experiments with similar results. The data of (d) are expressed as the mean $\pm$ SD ($n=6$). An asterisk (*) denotes a significant difference compared to the other groups, $p < 0.05$.

increased compared to other groups, which is considered to be mediated by the positive influence of BP on cell proliferation. This phenomenon can be explained in part by a previous study reporting that nanofiber scaffolds blended with collagen type I could facilitate proliferation or differentiation functions of hematopoietic stem cells through providing a topographically favorable cell microenvironment [49]. We confirmed that BP was dose- and time-dependent by increasing cytotoxicity in proportion to concentration and exposure time, which was the BP attributed to oxidation and membrane destruction. This was probably ascribed to the continuous release of BP with the production of phosphate, which was reported to enhance cell proliferation at a high extracellular concentration [50,51].

The morphologies of HT22 hippocampal neuronal cells on each nanofiber matrix were identified using CLSM (Fig. 6(a)). On PLCL-based nanofiber matrices, HT22 hippocampal neuronal cells were appreciatively grown and well-aligned on the matrix surface along the direction of nanofibers (white arrows). Therefore, the nanofiber matrices can assist cell proliferation and provide guidelines during cell growth and spreading out, which may also affect the direction of neurite outgrowth and alignment. Hence, the neurogenic differentiation of HT22 hippocampal neuronal cells on the PLCL-based nanofiber matrices was investigated. The HT22 hippocampal neuronal cells on the PLCL-based nanofiber matrices were cultured in a growth medium for 3 days and then replaced with a differentiation medium to induce the neurite outgrowth. The differences in neurite outgrowth in each group are shown in Fig. 6(b)–(d). The differences in neurite outgrowth within each nanofiber matrix were observed by CLSM (Fig. 6(b)) and TPLM (Fig. 6(c)). The neurite of cells has directionality in all nanofiber matrices. On the PLCL nanofiber matrices, the cell population was markedly reduced and short neurites were observed compared with PLCL/Lam and PLCL/Lam/BP. On PLCL/Lam/BP nanofiber matrices, the cells were identified as having the most independent and longest neurites, and showed aggregated morphology and extended neurites. These results correlate with the earlier studies that the laminin promoted factors for the differentiation and

neurite outgrowth [52–54]. Laminin assumes a critical role in neuronal cell growth and axonal extension; therefore, polymer-based matrices modified with laminin are employed for supporting neuronal cellular behaviors [55,56].

mRNA expression levels of neuronal markers on PLCL/Lam/BP nanofibers

To elucidate the biochemical mechanisms associated with increased neurite production on PLCL/Lam/BP nanofiber matrices, the expression of neuronal development-related genes such as DCX, NeuN, MAP2, and Nestin at the gene level was observed using qRT-PCR. Doublecortin which is encoded by the *DCX* gene is a well-known marker for early nervous system development [57,58]. NeuN, which serves as a pre-mRNA alternative splicing regulator, is a well-established marker for the development of post-mitotic neurons and highly conserved among different species [59,60]. The interaction of microtubules with actin induces cell migration and nerve growth cone movement [61]. The MAP2 is known to be necessary for neuronal growth from the amount of evidence [62,63]. Neuroepithelial stem cell protein, known as Nestin, is a cytoskeletal protein in neural stem cells in the developing or adult brain [64]. By observing the expression of these genes related to neurogenesis at the RNA level, it is possible to infer the relationship between morphological changes and the biochemical mechanism of cells.

The mRNA expression level of *DCX* in HT22 hippocampal neuronal cells cultured on PLCL/Lam/BP nanofiber matrices was about 2 and 1.7 times higher than those on PLCL and PLCL/Lam matrices, respectively (Fig. 7(a)). NeuN mRNA on PLCL/Lam/BP nanofiber matrices was significantly increased compared to PLCL matrices, but expressed at a level similar to that on PLCL/Lam matrices (Fig. 7(b)). It has been reported that NeuN is mostly observed in mature neurons, whereas *DCX* has been used as an established marker for nascent neurons [65,66]. From these results, it can be inferred that neuronal growth induced by BP might be affected in the early stages. As shown in Fig. 7(c), it was

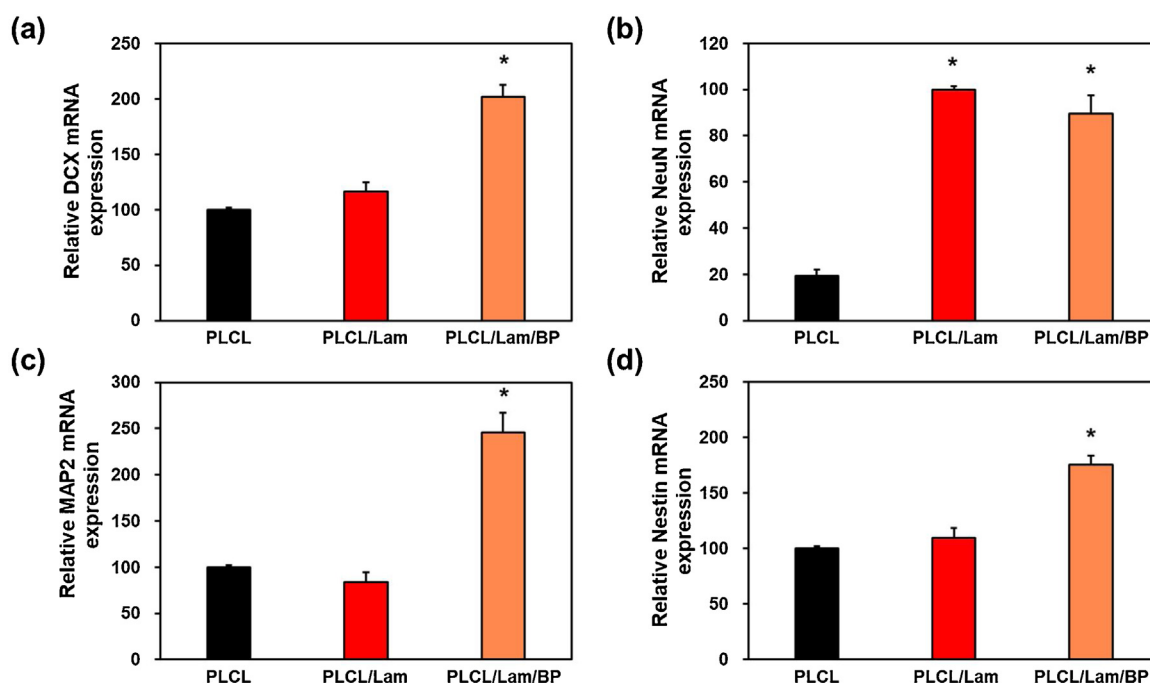


Fig. 7. (a)–(d) mRNA expression levels of neuronal markers such as *DCX*, NeuN, MAP2, and Nestin in HT22 hippocampal neuronal cells cultured on PLCL, PLCL/Lam and PLCL/Lam/BP nanofiber matrices. All data are presented in relative amount of mRNA expressed on the PLCL matrix and expressed as the mean $\pm$ SD ($n = 6$). An asterisk (*) denotes a significant difference compared to the other groups, $p < 0.05$.

revealed that the expression of MAP2 increased nearly 2.5-fold in HT22 cells cultured on PLCL/Lam/BP nanofiber matrices compared to PLCL matrices. Under the same conditions, Nestin also showed 75% increased expression compared to PLCL matrices (Fig. 7(d)). MAP2 is known as one of the representative neuron-specific MAPs, which is associated with the actin cytoskeleton [62]. Nestin is an intermediate filament protein, expressed primarily in progenitor cells that develop and regenerate tissue [67]. Cell division is achieved by breaking down the cytoplasmic and nuclear compartments and dividing them into daughter cells through reconstruction. This extensive remodeling is regulated by cytoskeleton protein such as microtubules, intermediate filaments, and actin microfilaments [67]. In particular, genes used as markers for neuronal development in this study can be developed into structural proteins expressing microfilament or cytoskeleton. Arguably, BP exerted some influence on the biosynthesis of structural proteins in the early stages of neural tissue development.

Discussion

During the past decade, 2D nanomaterials have attracted remarkable scientific attention owing to their excellent biocompatibility as well as extraordinary physicochemical, mechanical, and electrical properties. In particular, recent seminal studies reported that some 2D nanomaterials, such as graphene, boron nanosheets, transition metal dichalcogenides, antimonene, and MXene can be exploited as a promising nanoplatform with potential in the field of bio-photonic nanomedicine such as photothermal and photodynamic therapy, drug delivery, bioimaging, and biosensing [68–72]. Among these emerging 2D nanomaterials, BP is a layered structure of phosphorus elements and has been increasingly used for bio-applications since its discovery in 2014 [29]. Besides the well-known advantages of BP such as biocompatibility and biodegradability [73], BP has been shown to have the intrinsic unique properties including electron-transfer capacity, puckered lattice configuration, and long blood circulation time, which could offer prospective nanoplatforms for a wide range of biomedical applications [74–77]. However, some 2D nanomaterials have their shortcoming, which hinders their clinical applications [78]. Graphene displays extremely high carrier mobility, but it has no bandgap, which limits its applications in biosensing and bioimaging [79]. Despite possessing a finite bandgap, the low carrier mobility of transition metal dichalcogenides prevents their practical applications in the clinic [80]. In contrast, BP is shown to possess a well-balanced performance, i.e. moderate carrier mobility and direct and tunable bandgap [78]. Due to the balance of these features, BP is frequently employed in biosensing and bioimaging fields.

BP, with the unique puckered 2D sheet structure, possesses a much larger surface-area-to-volume ratio than other 2D materials, which results in extremely high drug-loading capacity [81]. Moreover, BP displays negligible elemental toxicity and outstanding biodegradability in the biological environment which distinguishes it from other common 2D nanomaterials [78]. BP is completely degradable *in vivo*, and the products, such as phosphite ions, phosphate ions, and other P_xO_y , are nontoxic and can readily exhibit renal filtration [31,82]. Also, BP is the most stable and the least reactive allotrope in phosphorus while commonly existing white phosphorus has poor stability that is easy to oxidize and spontaneously combust leading high toxicity [83]. On the other hand, it was shown that from blow-spun poly(lactide-co-glycolide) nanofibers containing 0.66, 1, and 2 mg/mL of BP, around 17% (from 2 mg/mL) to 70% (from 1 mg/mL) of BP were released after 7 days of incubation, while about 33% (from 1 mg/mL) to 78% (from 2 mg/mL) of BP were maintained in the fibers [84]. However, the PLCL/

Lam/BP nanofibers prepared in this study contain only 10 $\mu\text{g/mL}$ of BP. Although the polymer type, the spinning method, and the initial BP contents were different from the previous study, the amount of BP released from the nanofibers of the present study is expected to be extremely too small to induce cytotoxicity. There are still a lot of problems that need to be solved to achieve the requirements of clinical applications of BP, such as stability, *in vivo* toxicity, excretion, etc. [78]. To realize the clinical use of BP, it is necessary to accomplish more detailed toxicological studies along with the study on the exact mechanism of action of BP. In addition, it is of paramount importance to optimize the amount of BP to be incorporated into the fibers and reduce it as much as possible because the dissolved BP from the nanofiber matrices may adversely affect the cell viability. Although potential toxicity such as cytotoxicity, immunogenicity, and clearance pathway should be further studied, our previous studies demonstrated that BP was cytocompatible enough to promote cellular adhesion, proliferation, and differentiation in certain concentration range [36,37].

In the tissue engineering field, BP has been mainly exploited for bone regeneration owing to its composition of single phosphorus elements that are similar to the inorganic component of natural bone [85,86]. Meanwhile, a recent study reported that BP has been shown to support neural regeneration by targeting copper ions that cause neurons to degenerate in the blood–brain barrier [87]. Furthermore, the photothermal characteristic of BP endows extraordinary permeability to the blood–brain barrier, therefore, potentially can be used as a drug delivery carrier to the central nervous system. High electrical conductivity derived from many pairs of the electron in phosphorus atoms within the puckered layer of BP and supply of phosphorus ions which is vital for the moderation of cell signaling might contribute to the enhanced neuronal maturation [85]. Although there are few studies on how BP induce the maturation and neurite outgrowth of HT22 cells, significantly enhanced neuritogenesis on PLCL/Lam/BP nanofiber matrices was observed. It is suggested that the extraordinary electrical conductivity of BPNPs derived from the specific puckered structure enhanced cell signaling, hence induced cytoskeleton rearrangement and stretched shape of the cell body and nucleus. Moreover, BPNPs continuously release phosphate, an important neurogenic differentiation signal facilitator designed to stimulate neurite outgrowth [88].

Conclusions

This study focused on the neurite outgrowth induced on ternary nanofiber matrices of PLCL and Lam decorated with BP. From the physicochemical characterizations, it was confirmed that the optimal combination of Lam and BP was incorporated into PLCL nanofibers and resultantly ternary nanofiber matrices were successfully prepared by electrospinning process. When the PLCL-based nanofiber matrices with a hydrophobic property were incorporated with Lam and BP, they showed significantly improved cell growth due to synergistic effects of the intrinsic hydrophilic properties of Lam and the bioactive role of BP. Such an increment in the surface hydrophilicity of matrices can enhance cellular behaviors, such as initial attachment, proliferation, and differentiation, as well as the interactions between cells and substrates. In addition, it was confirmed that the cell proliferation and neurite outgrowth were remarkably enhanced on the ternary nanofiber matrices of PLCL/Lam/BP. The underlying mechanism of this phenomenon was confirmed by determining the expression levels of genes related to neurogenesis. The enhanced expression levels of several neuronal markers on the PLCL/Lam/BP nanofiber matrices imply that the morphological changes including neurite outgrowth are due to the increased expression of structural proteins at an RNA level. Taking all the results into consideration, it is suggested that

the PLCL/Lam/BP nanofibers can be exploited to craft a range of strategies for the development of novel scaffolds to accelerate neural regeneration because these BP-incorporated fiber matrices have the potentials to stimulate neuritogenesis.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jiec.2020.09.009>.

References

- [1] S.G. Klebanoff, J.L. Singer, H. Wilensky, *Psychol. Bull.* 51 (1954) 1.
- [2] A. Hazari, M. Wiberg, G. Johansson-Ruden, C. Green, G. Terenghi, *Br. J. Plast. Surg.* 52 (1999) 653.
- [3] S.E. Mackinnon, V.B. Doolabh, C.B. Novak, E.P. Trulock, *Plast. Reconstr. Surg.* 107 (2001) 1419.
- [4] C.J. Coombs, E.W. Ek, T. Wu, H. Cleland, M.K. Leung, *J. Plast. Reconstr. Aesthet. Surg.* 62 (2009) 1580.
- [5] H. Jia, Y. Wang, X. Tong, G. Liu, Q. Li, L. Zhang, X. Sun, *Synapse* 66 (2012) 256.
- [6] L. Ghasemi-Mobarakeh, M.P. Prabhakaran, M. Morshed, M.H. Nasr-Esfahani, S. Ramakrishna, *Biomaterials* 29 (2008) 4532.
- [7] L. Ghasemi-Mobarakeh, M.P. Prabhakaran, M. Morshed, M.H. Nasr-Esfahani, S. Ramakrishna, *Tissue Eng. A* 15 (2009) 3605.
- [8] S.W. Hong, J.H. Lee, S.H. Kang, E.Y. Hwang, Y.S. Hwang, M.H. Lee, D.W. Han, J.C. Park, *Biomed. Res. Int.* 2014 (2014) 212149.
- [9] E.J. Lee, J.H. Lee, Y.C. Shin, D.G. Hwang, J.S. Kim, O.S. Jin, L. Jin, S.W. Hong, D.W. Han, *Biomater. Res.* 18 (2014) 18.
- [10] M. Gajendiran, J. Choi, S.J. Kim, K. Kim, H. Shin, H.J. Koo, K. Kim, *J. Ind. Eng. Chem.* 51 (2017) 12.
- [11] Z. Meng, Y. Wang, C. Ma, W. Zheng, L. Li, Y. Zheng, *Mater. Sci. Eng. C* 30 (2010) 1204.
- [12] E.Y. Heo, N.R. Ko, M.S. Bae, S.J. Lee, B.J. Choi, J.H. Kim, H.K. Kim, S.A. Park, I.K. Kwon, *J. Ind. Eng. Chem.* 45 (2017) 61.
- [13] M. Patel, H.J. Hong, W.G. Koh, *J. Ind. Eng. Chem.* 80 (2019) 729.
- [14] C. Wang, W. Huang, Y. Zhou, L. He, Z. Chen, X. He, S. Tian, J. Liao, B. Lu, Y. Wei, M. Wang, *Bioact. Mater.* 5 (2020) 82.
- [15] M.M. Moghaddam, S. Bonakdar, M.R. Shariatpanahi, M.A. Shokrgozar, S. Faghihi, *Curr. Stem Cell Res. Ther.* 14 (2019) 268.
- [16] P. Roach, T. Parker, N. Gadegaard, M. Alexander, *Surf. Sci. Rep.* 65 (2010) 145.
- [17] Y.C. Shin, J.H. Lee, M.J. Kim, S.W. Hong, B. Kim, J.K. Hyun, Y.S. Choi, J.C. Park, D.W. Han, *J. Biol. Eng.* 9 (2015) 22.
- [18] Y.C. Shin, C. Kim, S.J. Song, S. Jun, C.S. Kim, S.W. Hong, S.H. Hyon, D.W. Han, J.W. Oh, *Nanotheranostics* 2 (2018) 144.
- [19] J. Joshi, D. Brennan, V. Beachley, C.R. Kothapalli, *J. Biomed. Mater. Res. A* 106 (2018) 3303.
- [20] M.P. Prabhakaran, J.R. Venugopal, S. Ramakrishna, *Biomaterials* 30 (2009) 4996.
- [21] C. Eriskens, D.M. Kalyon, H. Wang, *Biomaterials* 29 (2008) 4065.
- [22] Y.C. Shin, J.H. Lee, L. Jin, M.J. Kim, Y.J. Kim, J.K. Hyun, T.G. Jung, S.W. Hong, D.W. Han, *J. Nanobiotechnol.* 13 (2015) 21.
- [23] S.J. Song, Y.C. Shin, S.E. Kim, I.K. Kwon, J.-H. Lee, S.H. Hyon, D.W. Han, B. Kim, *Sci. Rep.* 8 (2018) 5570.
- [24] F. Yang, R. Murugan, S. Wang, S. Ramakrishna, *Biomaterials* 26 (2005) 2603.
- [25] M. Sasaki, S. Kato, K. Kohno, G.R. Martin, Y. Yamada, *Proc. Natl. Acad. Sci. USA* 84 (1987) 935.
- [26] N. Zhu, M. Li, Y. Guan, D. Schreyer, X. Chen, *Biofabrication* 2 (2010) 045002.
- [27] R.A. Neal, S.S. Tholpady, P.L. Foley, N. Swami, R.C. Ogle, E.A. Botchwey, *J. Biomed. Mater. Res. A* 100 (2012) 406.
- [28] Y. Li, Z. Liu, Y. Hou, G. Yang, X. Fei, H. Zhao, Y. Guo, C. Su, Z. Wang, H. Zhong, Z. Zhuang, Z. Guo, *ACS Appl. Mater. Interfaces* 9 (2017) 25098.
- [29] J.R. Choi, K.W. Yong, J.Y. Choi, A. Nilghaz, Y. Lin, J. Xu, X. Lu, *Theranostics* 8 (2018) 1005.
- [30] M. Qiu, W.X. Ren, T. Jeong, M. Won, G.Y. Park, D.K. Sang, L.P. Liu, H. Zhang, J.S. Kim, *Chem. Soc. Rev.* 47 (2018) 5588.
- [31] Y. Qian, W.E. Yuan, Y. Cheng, Y. Yang, X. Qu, C. Fan, *Nano Lett.* 19 (2019) 8990.
- [32] J. Shao, H. Xie, H. Huang, Z. Li, Z. Sun, Y. Xu, Q. Xiao, X.F. Yu, Y. Zhao, H. Zhang, H. Wang, P.K. Chu, *Nat. Commun.* 7 (2016) 12967.
- [33] Y.P. Kim, G.S. Lee, J.W. Kim, M.S. Kim, H.S. Ahn, J.Y. Lim, H.W. Kim, Y.J. Son, J.C. Knowles, J.K. Hyun, *J. Tissue. Eng. Regen. Med.* 9 (2015) 236.
- [34] Z. Wang, J. Zhao, W. Tang, L. Hu, X. Chen, Y. Su, C. Zou, J. Wang, W.W. Lu, W. Zhen, R. Zhang, D. Yang, S. Peng, *Small* 15 (2019) e1901560.
- [35] H.U. Lee, S.Y. Park, S.C. Lee, S. Choi, S. Seo, H. Kim, J. Won, K. Choi, K.S. Kang, H.G. Park, *Small* 12 (2016) 214.
- [36] Y.B. Lee, S.J. Song, Y.C. Shin, Y.J. Jung, B. Kim, M.S. Kang, I.K. Kwon, S.H. Hyon, H. U. Lee, S.H. Jung, D. Lim, D.W. Han, *J. Ind. Eng. Chem.* 80C (2019) 802.
- [37] S.J. Song, I.S. Raja, Y.B. Lee, M.S. Kang, H.J. Seo, H.U. Lee, D.W. Han, *Biomater. Res.* 23 (2019) 23.
- [38] M.J. Kim, Y.C. Shin, J.H. Lee, S.W. Jun, C.-S. Kim, Y. Lee, J.-C. Park, S.-H. Lee, K.D. Park, D.-W. Han, *Biomater. Res.* 20 (2016) 2.
- [39] N.M. Latiff, W.Z. Teo, Z. Sofer, A.C. Fisher, M. Pumera, *Chem. Eur. J.* 21 (2015) 13991.
- [40] S.J. Song, Y.C. Shin, H.U. Lee, B. Kim, D.W. Han, D. Lim, *Nanomaterials* 8 (2018) 408.
- [41] N.M. Latiff, C.C. Mayorga-Martinez, Z. Sofer, A.C. Fisher, M. Pumera, *Appl. Mater. Today* 13 (2018) 310.
- [42] M. Ferrari, F. Cirisano, M.C. Morán, *Colloids Interfaces* 3 (2019) 48.
- [43] K.K. Gupta, N. Pal, P.K. Mishra, P. Srivastava, S. Mohanty, P. Maiti, *J. Biomed. Mater. Res. A* 102 (2014) 2600.
- [44] S.I. Jeong, I.D. Jun, M.J. Choi, Y.C. Nho, Y.M. Lee, H. Shin, *Macromol. Biosci.* 8 (2008) 627.
- [45] S.H. Huang, T.-T. Hsu, T.-H. Huang, C.-Y. Lin, M.-Y. Shie, *J. Dent. Sci.* 12 (2017) 33.
- [46] D. Chimene, D.L. Alge, A.K. Gaharwar, *Adv. Mater.* 27 (2015) 7261.
- [47] O. Akhavan, E. Ghaderi, E. Abouei, S. Hatamie, E. Ghasemi, *Carbon* 66 (2014) 395.
- [48] J.U. Lee, M. Kim, H. Cheong, *Appl. Microscopy* 45 (2015) 126.
- [49] K. Ma, C.K. Chan, S. Liao, W.Y. Hwang, Q. Feng, S. Ramakrishna, *Biomaterials* 29 (2008) 2096.
- [50] M.C. Roussanne, M. Lieberherr, J. Souberbielle, E. Sarfati, T. Drüeke, A. Bourdeau, *Eur. J. Clin. Invest.* 31 (2001) 610.
- [51] X. Zhang, Z. Zhang, S. Zhang, D. Li, W. Ma, C. Ma, F. Wu, Q. Zhao, Q. Yan, B. Xing, *Small* 13 (2017) 1701210.
- [52] A.D. Lander, D.K. Fujii, L.F. Reichardt, *Proc. Natl. Acad. Sci. USA* 82 (1985) 2183.
- [53] D. Edgar, R. Timpl, H. Thoenen, *EMBO J.* 3 (1984) 1463.
- [54] D. Hantaz-Ambroise, M. Vigny, J. Koenig, *J. Neurosci. Res.* 7 (1987) 2293.
- [55] L. He, S. Tang, M.P. Prabhakaran, S. Liao, L. Tian, Y. Zhang, W. Xue, S. Ramakrishna, *Macromol. Biosci.* 13 (2013) 1601.
- [56] N.E. Zander, T.P. Beebe Jr., *Biointerphases* 9 (2014) 011003.
- [57] G. Kronenberg, K. Reuter, B. Steiner, M.D. Brandt, S. Jessberger, M. Yamaguchi, G. Kempermann, *J. Comp. Neurol.* 467 (2003) 455.
- [58] T. Toda, S.L. Parylak, S.B. Linker, F.H. Gage, *Mol. Psychiatry* 24 (2019) 67.
- [59] S. Maxeiner, A. Glassmann, H.-T. Kao, K. Schilling, *Histochem. Cell Biol.* 141 (2014) 43.
- [60] H.Y. Wang, P.F. Hsieh, D.F. Huang, P.S. Chin, C.H. Chou, C.C. Tung, S.Y. Chen, L.J. Lee, S.S.F. Gau, H.S. Huang, *Sci. Rep.* 5 (2015) 1.
- [61] A.A. Ayanlaja, Y. Xiong, Y. Gao, G. Ji, C. Tang, Z. Abdikani Abdullah, D. Gao, *Front. Mol. Neurosci.* 10 (2017) 199.
- [62] A. Caceres, J. Mautino, K.S. Kosik, *Neuron* 9 (1992) 607.
- [63] J.H. Dinsmore, F. Solomon, *Cell* 64 (1991) 817.
- [64] A. Bernal, L. Arranz, *Cell. Mol. Life Sci.* 75 (2018) 2177.
- [65] S. Zimatkina, O. Karnyushko, *Neurosci. Behav. Physiol.* 47 (2017) 122.
- [66] K. Jin, M. Minami, J.Q. Lan, X.O. Mao, S. Batteur, R.P. Simon, D.A. Greenberg, *Proc. Natl. Acad. Sci. USA* 98 (2001) 4710.
- [67] S.R. Clarke, A.K. Shetty, J.L. Bradley, D.A. Turner, *Neuroreport* 5 (1994) 1885.
- [68] W. Tao, X. Ji, X. Xu, M.A. Islam, Z. Li, S. Chen, P.E. Saw, H. Zhang, Z. Bharwani, Z. Guo, *Angew. Chem. Int. Ed.* 129 (2017) 12058.
- [69] X. Ji, N. Kong, J. Wang, W. Li, Y. Xiao, S.T. Gan, Y. Zhang, Y. Li, X. Song, Q. Xiong, *Adv. Mater.* 30 (2018) 1803031.
- [70] C. Xing, S. Chen, X. Liang, Q. Liu, M. Qu, Q. Zou, J. Li, H. Tan, L. Liu, D. Fan, H. Zhang, *ACS Appl. Mater. Interfaces* 10 (2018) 27631.
- [71] V. Urbanová, M. Pumera, *Nanoscale* 11 (2019) 15770.
- [72] S. Luo, X. Yang, L. Zhou, J. Li, H. Chen, *J. Mater. Chem. B* 8 (2020) 2974.
- [73] M. Wang, T. Fan, Y. Zhou, H. Zhang, L. Mei, *Adv. Funct. Mater.* 29 (2019) 1808306.
- [74] M. Qiu, A. Singh, D. Wang, J. Qu, M. Swihart, H. Zhang, P.N. Prasad, *NanoToday* 25 (2019) 135.
- [75] M. Tatullo, F. Genovese, E. Aiello, M. Amantea, I. Makeeva, B. Zavan, S. Rengo, L. Fortunato, *Materials* 12 (2019) 2301.
- [76] G. Qu, T. Xia, W. Zhou, X. Zhang, H. Zhang, L. Hu, J. Shi, X.F. Yu, G. Jiang, *Chem. Rev.* 120 (2020) 2288.
- [77] Z. Xie, T. Fan, J. An, W. Choi, Y. Duo, Y. Ge, B. Zhang, G. Nie, N. Xie, T. Zheng, Y. Chen, H. Zhang, J.S. Kim, *Chem. Soc. Rev.* (2020), doi:<http://dx.doi.org/10.1039/d0cs00215a>.
- [78] T. Fan, Y. Zhou, M. Qiu, H. Zhang, *J. Innov. Opt. Health Sci.* 11 (2018) 1830003.
- [79] R. Balog, B. Jørgensen, L. Nilsson, M. Andersen, E. Rienks, M. Bianchi, M. Fanetti, E. Lægsgaard, A. Baraldi, S. Lizzit, *Nat. Mater.* 9 (2010) 315.
- [80] B. Liu, L. Chen, G. Liu, A.N. Abbas, M. Fathi, C. Zhou, *ACS Nano* 8 (2014) 5304.
- [81] W. Chen, J. Ouyang, H. Liu, M. Chen, K. Zeng, J. Sheng, Z. Liu, Y. Han, L. Wang, J. Li, L. Deng, Y.N. Liu, S. Guo, *Adv. Mater.* 29 (2017) 1603864.
- [82] T. Guo, Y. Wu, Y. Lin, X. Xu, H. Lian, G. Huang, J.Z. Liu, X. Wu, H.H. Yang, *Small* 14 (2018) 1702815.
- [83] L.N. Al Barqouni, S.I. Skaik, N.R.A. Shaban, N. Barqouni, *Lancet* 376 (2010) 68.

- [84] N. Kamyar, R.D. Greenhalgh, T.R. Nascimento, E.S. Medeiros, P.D. Matthews, L.P. Nogueira, H.J. Haugen, D.J. Lewis, J.J. Blaker, *ACS Appl. Nano Mater.* 1 (2018) 4190.
- [85] L. Cheng, Z. Cai, J. Zhao, F. Wang, M. Lu, L. Deng, W. Cui, *Bioact. Mater.* 5 (2020) 1026.
- [86] X. Wang, J. Shao, M. Abd El Raouf, H. Xie, H. Huang, H. Wang, P.K. Chu, X.-F. Yu, Y. Yang, A.M. Abdel-Aal, *Biomaterials* 179 (2018) 164.
- [87] W. Chen, J. Olnnuyang, X. Yi, Y. Xu, C. Niu, W. Zhang, L. Wang, J. Sheng, L. Deng, Y.N. Liu, *Adv. Mater.* 30 (2018) 1703458.
- [88] D. Vaudry, P. Stork, P. Lazarovici, L. Eiden, *Science* 296 (2002) 1648.