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Research Article

Assessment of Morphology, Activity, and Infiltration of Astrocytes on Marine EPS-Imbedded Electrospun PCL Nanofiber

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Tissue engineering using a biomaterial including bioactive compounds has been researched as a way to restore injured neural systems. Extracellular polymeric substances (EPS) extracted from marine seaweeds have been known to produce positive effects on physiological activities in human tissues. In this study, an electrospun nanofiber containing brown seaweed EPS was proven to be a candidate biomaterial for neural tissue engineering. Glial fibrillary acidic protein (GFAP) as a specific marker protein increased in the astrocytes cultured on the polycaprolactone (PCL) nanofiber containing EPS (EPS-PCL nanofiber), compared with PCL nanofiber. The upregulation of GFAP indicates that the EPS-PCL nanofiber induced astrocyte activation, which supports physiological agents favorable to restore injured neural tissue. Astrocytes could infiltrate into the EPS-PCL nanofiber mat without toxicity, comparable to PCL nanofiber. These results imply that EPS-PCL nanofiber could be a useful biomaterial to regulate astrocyte activity at a molecular level and could be considered as a novel therapeutic material for neural tissue engineering.

1. Introduction

Regarding central nervous system (CNS) injuries, astrocytes are one of many glial cells that are important in supporting neural systems to operate correctly [1, 2]. Following some reports, astrocytes are regarded as curative agents in therapy of CNS injuries [3], while they are also known to interfere with neural regeneration [4, 5]. The mechanism to explain the astrocyte effect on neuronal systems is connected to various growth factors, cytokines, proteoglycans, and other tissues [6–12]. Especially, GFAP is an important biomarker to determine the state of astrocytes, which is involved in the CNS injury response and plays a critical role in neural tissue organization. For example, GFAP has been known as an essential protein for construction of cerebral white matter and normal myelination [13].

Three typical ways have been used to treat CNS injuries: drug delivery, cell therapy, and tissue engineering. Medication has been administered mainly in a solution or liposome vesicle to the injured site or in the blood stream [14]. Various bioactive substances from natural resources have been tried in studies of neurological disorders [15]. Seaweed-originated polysaccharides also have demonstrated abilities to regulate neurons and glial cells [16]. However, a recent development of drug delivery systems using biomaterials for drug control release has encountered several problems such as drug instability, side effects, and low controllability. In the case of cell therapy including stem cells, injected cells must reside correctly in the target injured tissue and follow-up events such as survival, differentiation, and curing activities must be understood and controlled. Unfortunately an injected cell's fate in an implanted state is rarely controllable. Therefore, as in drug delivery, cell therapy has also been actively administered in conjunction with biomaterials in order to better control the injected cell's fate while benefiting the injured nerve tissue. Namely, a neural tissue engineering approach is highlighted in terms of control of drugs and cell therapy within the injured tissue.

Biomaterials for neural tissue engineering should be thin, flexible, porous, biocompatible, biodegradable, compliant,

neuroinductive, and neuroconductive. Various types of scaffolds have been developed including nanofibers, sintered matrices, nanofoams, hydrogels, and nanotubes. Among these, hydrogels and nanofibers have recently been underscored for their peculiar advantages [17, 18]. They show potential to facilitate nerve regeneration by providing ECM-like environments and structures favorable to cells and tissues. Hydrogels can be easily injected into the cavity of the injured tissue with low tension but may need frequent changing and are hard to sterilize. However, nanofibers are relatively easy to sterilize and can also guide axonal growth direction even though injection into a tissue is not easy compared to hydrogels.

Nanofibers with polysaccharides for neuron cells have been actively researched. However, glial cells have been rarely studied in relation to nanofiber, especially, at a molecular level, even though glial cells are highly involved in subsequent events following CNS injury. Therefore, this work was to ascertain the availability of brown seaweed-derived EPS incorporated into polycaprolactone (EPS-PCL) nanofiber as a therapeutic nanomedicine in CNS injuries by observing GFAP expression and astrocyte infiltration into the nanofiber mat.

2. Materials and Methods

2.1. Extraction of EPS and Fabrication of Electrospun Nanofiber Mats. EPS derived from brown seaweed, *Undaria pinnatifida*, were obtained based on the hot water extraction method of Fujiki et al. [19]. Briefly, a dried brown seaweed was ground to small pieces, mixed in distilled water at 100 g/L, and autoclaved at 121°C for 30 min. Then, the pieces of seaweed were removed by filtering. EPS were precipitated by an excess of ethanol and obtained by centrifugation at 14,000 ×g for 20 min. The precipitated EPS were dissolved in 0.5 mM EDTA solution and dialyzed by dialysis membrane to remove salt. Dialyzed EPS were lyophilized to make a powder form. Finally the obtained EPS sample was stored at −20°C until use. To prepare polymer solution for electrospinning, 15% (wt/v) of PCL (Mn 80,000; Sigma-Aldrich, USA) was dissolved in mixture of tetrahydrofuran (THF, Junsei, Japan) and N,N-dimethylformamide (DMF, Junsei, Japan) with a ratio of 7 : 3 (v/v). A PCL solution containing EPS from *Undaria pinnatifida* was prepared by adding 0.5% (wt/v) EPS in a PCL polymer solution. Electrospun nanofiber mats were fabricated using an electrospinning apparatus [20] consisting of a syringe pump (KDS100, KD Scientific, USA), high voltage supply, and a flatted collector. Two kinds of solution were electrospun with the following conditions. Flow rate of solution was at 2 mL/hr. The distance from needle tip to aluminum collector was 16 cm under a 14 kV. A sample of the electrospun nanofiber mats was stored in vacuum to remove the organic solvent completely and placed under UV light to ensure sterility before cell culturing.

2.2. Characterization of Electrospun Nanofiber. The morphology of electrospun nanofiber was examined using field emission scanning emission microscope (FE-SEM, Hitachi S-4200, Japan). In order to obtain SEM images, nanofiber mats were coated by a Pt sputter. Each image was taken under an accelerating voltage of 10 kV. The diameter of electrospun nanofiber was determined from SEM images using Image J (National Institutes of Health, USA). 200 spots of diameter were measured to calculate average and standard deviations for statistical analysis. The hydrophobicity of PCL and EPS-PCL nanofiber mats was determined by contact angle analyzer (S-EO Phoenix touch, Korea) using 3 μL distilled water as probe liquid on 5 spots of each sample. To observe the pattern of EPS composition of EPS-PCL nanofiber, transmission electron microscopy (TEM, Hitachi H-7600, Japan) was operated at 80 kV. Nanofibers for TEM images were electrospun on copper grids.

2.3. Isolation of Primary Astrocytes. Primary astrocytes were isolated from the cerebra of 1 day old *Sprague-Dawley* rats (SAMTACO, Korea) as previously described [21]. The cerebra tissues extracted from neonatal rats were minced using a Pasteur pipette and then filtered through a 0.7 mm cell strainer. The cells in filtrate were counted using a hemocytometer and 2×10^7 cells were seeded on a T-75 flask (SPL, Korea). After incubation for 7 days in an incubator with 5% CO_2 at 37°C, the cells were shaken at 200 rpm for 24 hrs. Nonadherent cells were removed by centrifugating the supernatant and then the cultured medium was fed back to cells in the T-75 flask with fresh medium with a ratio of 2 : 8. Once mixed cells go through all of the above process, only astrocytes will be remained.

2.4. Western Blot for GFAP Analysis. The expression of GFAP was measured by a western blot. GFAP are typical intermediate filaments derived from activated astrocytes in the CNS. Astrocytes were cultured for 5 days on tissue culture polystyrene (TCPS), PCL, and EPS-PCL nanofibers, followed by washing with PBS and adding RIPA lysis buffer. The harvested total protein was quantified by a BCA protein assay kit. For electrophoresis, the quantified protein was loaded into a 12% acrylamide gel and separated by its molecular weight. The resultant SDS-PAGE gel was transferred to a PVDF membrane, followed by incubation for 6 hrs in TBS with 10% skim milk to block non-specific protein binding at 4°C. Primary antibody (rabbit anti-GFAP polyclonal IgG antibodies, ab7260, Abcam) was diluted with TBS supplemented with 10% skim milk at a ratio of 1 : 50000 and then reacted on the transferred membrane for 4 h at 4°C. After washing three times with TBST for 5 min, secondary antibody (goat anti-rabbit IgG-HRP, sc-2004, Santa Cruz), which was diluted with TBS supplemented with 10% skim milk, was reacted for 1 h at room temperature. The membrane was washed three times with TBST for 5 min, and finally, the reaction parts with ECL solution were detected on the film. These development steps were performed in a dark room.

2.5. Immunostaining of GFAP of Astrocytes Cultured on Nanofiber. Electrospun nanofibers were blocked for 1 hour using 1% BSA in PBST. After blocking, the cells were soaked in the PBST with 1% BSA and rabbit anti-GFAP polyclonal

IgG antibodies (1 : 5000, ab7260, Abcam) overnight at 4°C. The sample was washed with PBS three times each for 5 min and then reacted with goat anti-rabbit secondary antibody-Texas Red (1 : 5000, ab6800, Abcam) dissolved in PBST with 1% BSA for 1 hr at room temperature. Afterwards, the samples were washed with PBS three times for 5 min. To ascertain each cell, we used Hoechst 33342 which stained all cell nuclei for 1 min. Double stained samples were observed under confocal laser scanning microscope (LSM 510 META, USA).

2.6. Evaluation of the Infiltration of Astrocytes. For a cross-section assay to observe infiltration of cells, nanofibers should be thick enough for cell migration. For a cross-section assay, collected nanofibers must be over 200 nm. Primary astrocytes were seeded at 3×10^4 cells per well on a 24-well plate. 5 days after astrocytes were seeded into the nanofibers, the culture media were removed and the nanofibers were washed with chilled PBS (pH 7.4), treated with 4% paraformaldehyde at RT for 20 min to fix the astrocytes cultured on the nanofiber, and washed twice with PBS. They were then dehydrated by ethanol/deionized water (10%, 20%, and 30%) in order. This astrocyte-cultured mat measured at $1 \times 1\,\text{cm}^2$ was cut out and rapidly frozen at −20°C with OCT (optimal cutting temperature) compounds (Tissue-Tek, Europe). Next, these were sectioned at 50 μm widths, attached to coating slide glasses (SUPER FROST, MATSUNAMI, Japan), and washed by PBS (pH 7.4) 2 times. After that, H&E staining was performed to confirm astrocyte infiltration.

3. Results

3.1. Characterization of EPS-PCL Nanofiber. Images of PCL and EPS-containing PCL nanofibers were taken using SEM and TEM to characterize morphology and confirm the existence of EPS. Comparing Figures 1(a) with 1(b), the EPS particle was confirmed to be incorporated into the EPS-PCL nanofiber. During extracting EPS, proteins and alginic acid were removed by heating and precipitation, respectively. Small molecules were also eliminated by dialysis step. Thus, following a reference [22], the isolated EPS is thought to consist of laminarin and fucoidan as main components. Fucoidan is a sulfated polysaccharide which is stained by alcian blue. In order to ascertain its presence and distribution, the EPS-PCL nanofiber was stained by alcian blue. As seen in Figure 1(b), sulfated polysaccharides can be seen to be homogeneously distributed over the EPS-PCL nanofiber. Both nanofibers exhibited similar cylindrical morphologies and diameter distribution (Figures 1(c)–1(f)). PCL and EPS-PCL nanofibers had a thickness of 560.56 ± 107.66 and 551.41 ± 107.65 nm in diameter, respectively. Contact angle was measured to investigate the effect of EPS incorporation on hydrophilicity of EPS-PCL nanofiber (Figures 1(c) and 1(d)). PCL and EPS-PCL nanofibers showed 129.29 ± 2.45° and 135.27 ± 2.04° of contact angle, respectively. Topography and surface energy both can influence contact angle cooperatively. However, since topography is similar to both nanofibers, EPS can be considered to affect the increased hydrophilicity of EPS-PCL nanofiber.

3.2. GFAP Expression of Astrocytes on EPS-PCL Nanofiber. In order to assess whether EPS activates astrocytes, the expression level of GFAP in astrocytes was measured on PCL and EPS-PCL nanofibers by western blot. As shown in Figure 2, GFAP was more expressed on EPS-PCL nanofiber than on PCL nanofiber. It means that although there is less expression than on TCPS, where two-dimensionally cultured conditions were simulated as an injury [23], EPS, as well as the dimensional similarity of nanofiber to ECM, induced the astrocytes to become more activated.

3.3. Morphology of Astrocytes on PCL and EPS-PCL Nanofiber Mats. SEM images were taken to observe cell morphologies and evaluate adhesion of astrocytes on the nanofiber (Figure 3). The appearance of cells in a particular environment is known to have a certain shape (e.g., polygonal shape in normal brain and stellate shape in injury environment). Upon larger magnification, astrocytes are shown to adhere tightly to three-dimensional nanofibers, regardless of existence of EPS. But, there is no significant difference in morphologies between PCL and EPS-PCL nanofibers.

In order to investigate GFAP pattern inside astrocytes on nanofibers, astrocyte morphologies immunostained with GFAP were compared between PCL nanofiber and EPS-PCL nanofiber (Figure 4). But, cells in this research did not form a regular morphology. Astrocytes on PCL and EPS-PCL nanofibers both express GFAP correctly but significant differences were not detected in the confocal images.

3.4. Infiltration Effect of Astrocytes Cultured on Nanofiber. A thick nanofiber on which astrocytes were cultured for 5 days was cross-sectionalized by using Cryostat (Leica CM-1800, USA) so that astrocytes infiltration can be observed (Figure 5). The cross-section was stained with hematoxylin and eosin (H&E) to find astrocyte positions in the nanofiber. First of all, infiltration of astrocytes tested the case of culture on PCL nanofiber as control. It was easy and has no cytotoxicity (data not shown). As in Figure 5, it was found that the cells infiltrated deeper into EPS-PCL nanofiber. That is because, regardless of the depth, there seemed to be more pores to offset the compression pressure than expected. This means that media and nutrients could be provided easily, which is such an important fact to use biomaterials in tissue engineering; cells should be alive in the implanted scaffold. Both the figures suggested a potential that the topography of the nanofiber was able to induce cell infiltration.

4. Discussion

As well as supporting the neuronal physical structure, astrocytes help neural plasticity and synapse-formation [24, 25]. In addition, in an acute CNS injury, astrocytes have been reported to contribute to neuroregeneration by regulating immunity, reinstating homeostasis, affording neurotrophic factors, reconstructing the damaged lesion, and protecting spared neural tissue [26]. For these reasons, astrocytes have widely been studied as a curing agent for CNS injuries. The objective of this research was to investigate whether EPS-PCL

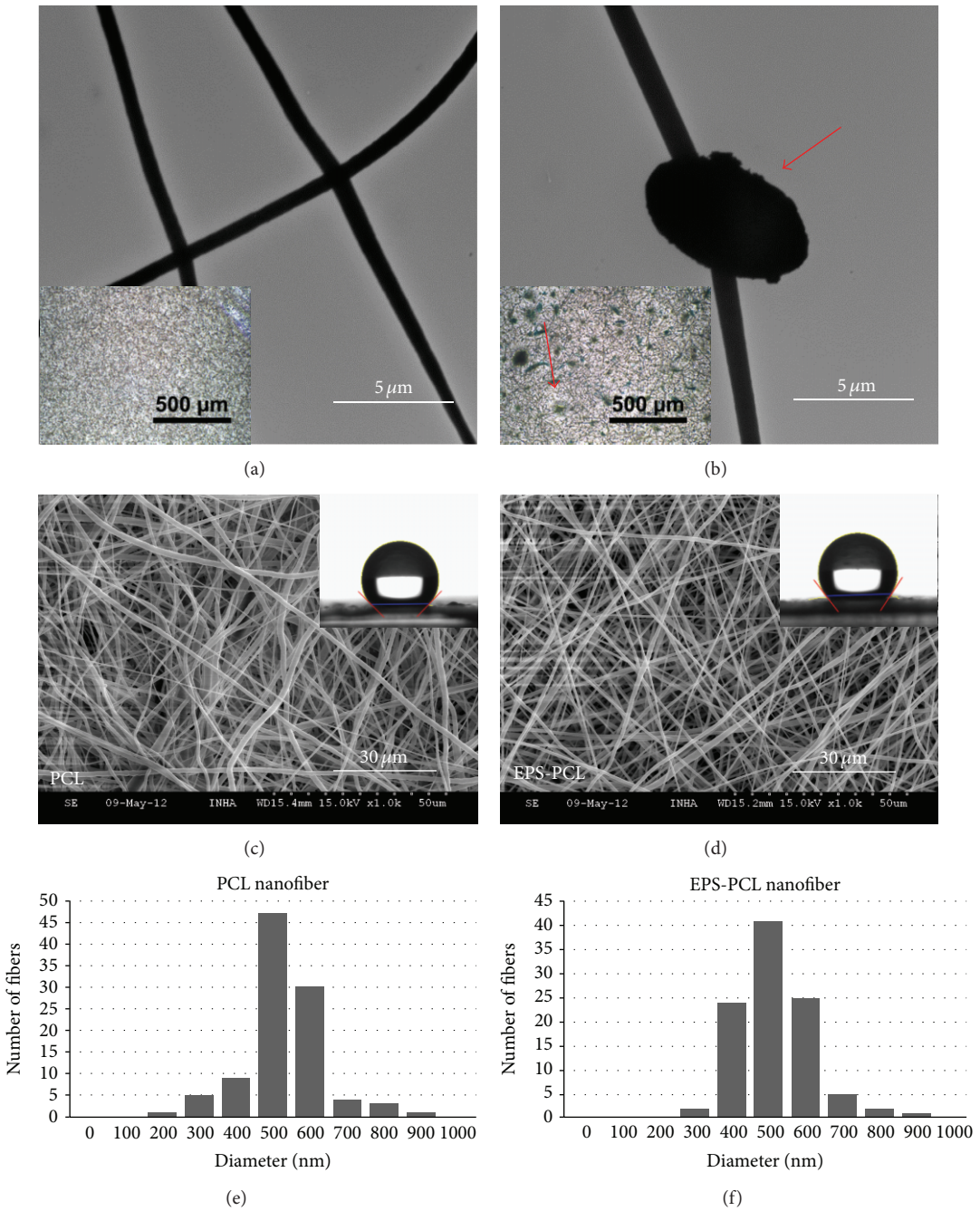


Figure 1

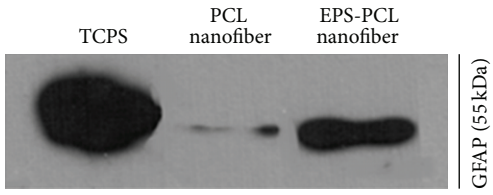
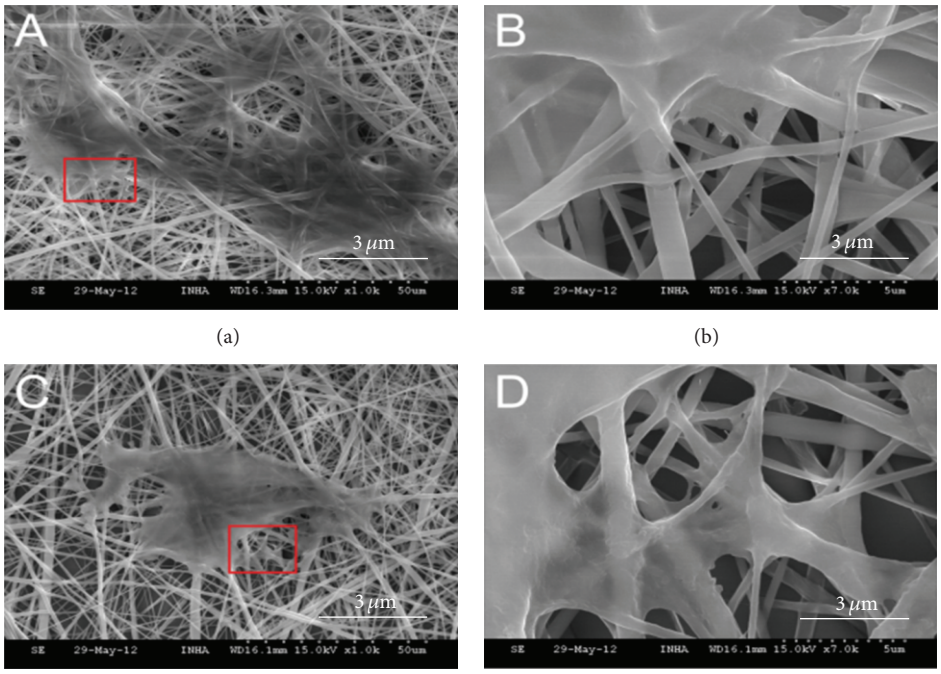


Figure 2

nanofiber is a favorable scaffold for astrocytic tissue engineering in CNS injuries by observing molecular behaviors, morphological change, and infiltration of astrocytes on EPS-PCL nanofiber.

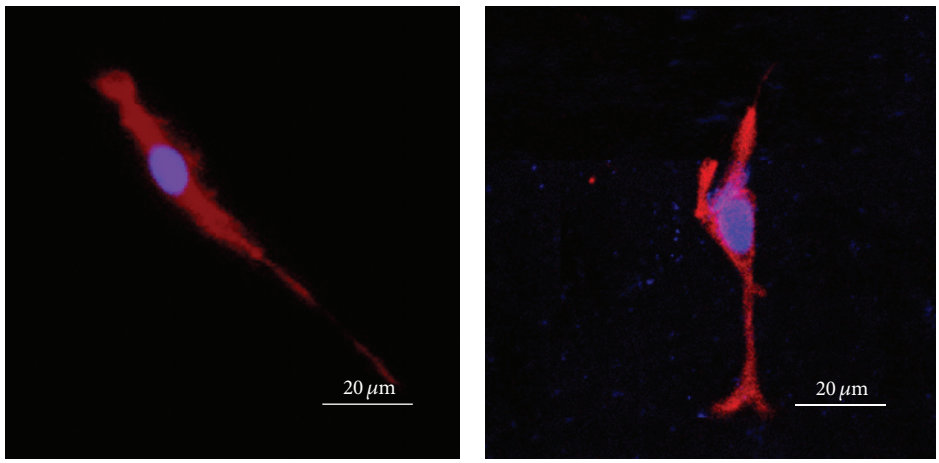
EPS-PCL nanofiber may promote biochemical effects to astrocytes. In this work, we at most tried to investigate the biochemical effects of EPS-PCL nanofiber. In this sense, it is important to make PCL and EPS-PCL nanofibers gain similar morphologies so that morphological variations would rarely influence astrocyte activity. Thus, in order to minimize structural variations between PCL and EPS-PCL nanofibers, we optimized electrospinning conditions to make similar diameters in both nanofibers. Meaning, there was almost no topography difference between PCL and EPS-PCL nanofibers.



(a) (b)

(c) (d)

Figure 3



(a) (b)

Figure 4

Polysaccharide which is extracted by hot water extraction and filtration methods is mainly composed of laminarin and fucoidan. Fucoidan is a sulfated polysaccharide which can be stained by alcian blue. Taking a look at alcian blue in Figure 1(b), the sulfated polysaccharide of EPS was stained within the PCL nanofiber. The distribution of EPS was sporadic over the PCL nanofiber mat. That is because solubility differences between EPS and PCL make them immiscible. Choosing an appropriate solution such as an ionic liquid, EPS and PCL polymers could be homogeneously miscible. However, for this experiment, the existence of EPS in the PCL nanofiber can be assured to be a sufficient condition to investigate its effect on astrocyte activity. By adding EPS into PCL nanofiber, the surface energy of the EPS-PCL nanofiber increased because EPS is composed of macromolecules with charges. However, aside from this hydrophilicity, the fiber diameter and morphology were set to be similar between both nanofibers.

GFAP is a biomarker that represents astrocyte activation. In order to maintain hypertrophic processes of cells, astrocytes upregulate GFAP levels in an activated state [27, 28]. Once upregulated, GFAP-activated astrocytes produce neurotrophic factors to help neural tissue protection and regeneration [29–31]. Astrocytes cultured on EPS-PCL nanofiber were activated with higher expression of GFAP in our study. This indicates that EPS were a factor in influencing astrocyte

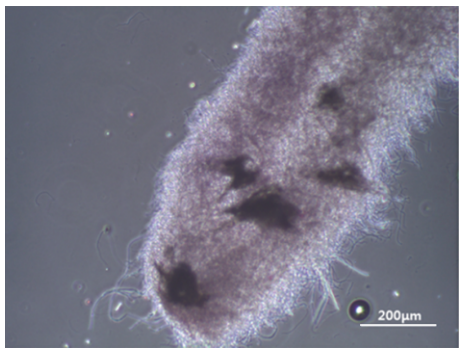


Figure 5

activity. In our previous study, while EPS were added in the culture medium, astrocyte viability was influenced (data not shown). In this sense, EPS molecules could be released from the immersed nanofiber and they contributed to a biochemical effect to astrocyte activities.

Astrocytes become reactive when hypoxia, ischemia, or seizures induce brain tissue damage [32]. It has been known that astrocytes *in vivo* took specific shapes depending on the circumstances. Resting astrocytes transform their morphologies into a stellate shape when they are activated [33]. But astrocytes on PCL and EPS-PCL nanofibers appeared as irregular morphologies from Figure 4. Topography, surface chemistry, and biochemical properties of a cell culture substrate are main causes to determine the adhesion and morphological change of a cell. From the experimental result of astrocyte morphology, surface chemistry and biochemical effects did not have a huge impact on astrocyte morphology, but similar topography instead predominates in astrocyte morphology, considering the similar and irregular shapes of astrocytes regardless of EPS incorporation. Moreover, as reported in previous research from our group, morphological difference of astrocytes was not shown on PCL nanofiber or a biochemical material such as *spirulina* added PCL nanofiber [34].

One of the important factors for materials to be used in tissue engineering is whether cells would be able to infiltrate into the materials such as electrospun nanofibers. It was demonstrated that cells on both PCL and EPS-PCL nanofibers infiltrated into the nanofibers with no significant differences in the level of infiltration. Considering astrocytes infiltrated and through EPS-PCL nanofiber pores and maintained alive state, EPS incorporated into PCL nanofiber is told not to give cytotoxicity to 3D growth of astrocytes through the nanofiber mat. In order for a scaffold to be used in neural tissue engineering, it should help cells grow and survive in a 3-dimensional environment, promoting axonal regrowth and guidance and reestablishment of a functional neural network [23]. Since EPS-PCL nanofiber did not show cytotoxic effect to astrocyte infiltration and viability in the 3D environment of the nanofiber, it is told to be adequate as a scaffold in astrocyte tissue engineering.

When the artificially constructed astrocyte tissue is implanted into a CNS injury, it must support neuronal regeneration. For this purpose, astrocytes first should show good viability, proliferation, and activated markers when seeded on the scaffold. In this sense, EPS-PCL nanofiber is a good candidate to support neuronal protection and regeneration judging from the experimental results previously described. In addition, following that laminarin and fucoidan are both already known to modulate immune responses of microglia [35, 36], PCL nanofiber containing laminarin and fucoidan could be a potential biomaterial for neural tissue engineering that can promote astrocyte survival and activation favorable to axonal regeneration and guidance while suppressing inflammation events by the other cells such as microglia.

5. Conclusions

There have been several studies about nanofibers and polysaccharides applied to astrocytes, but cooperative effects of nanofibers containing polysaccharides on astrocytes have rarely been researched, and in particular, molecular studies have not yet been reported. In this research, EPS derived from marine brown seaweed, *Undaria pinnatifida*, were studied over astrocyte activation in view of the topography and biochemical effect of the nanofiber. Cellular morphologies were similar irrespective of the presence of EPS in the nanofiber. This is presumed to be because similar topographies, PCL and EPS-PCL nanofibers, predominate astrocyte morphology. GFAP expression was higher on EPS-PCL nanofiber, which means astrocytes were more activated on EPS-PCL nanofiber. Finally, astrocytes could easily infiltrate into EPS-PCL nanofiber from the seeding surface to the lower part of the fiber mats without toxicity.

Biomaterials must facilitate astrocytes to express sufficient neurotrophic factors to constitute a favorable environment for neural regeneration when applied to the injured CNS and also to have a structure through which astrocytes can easily infiltrate. Considering the experimental results of astrocyte activation and infiltration described above as well as the fact that laminarin and fucoidan modulate inflammatory events of microglia and protect axons in an Alzheimer's environment [35], EPS-PCL nanofiber could be thought to be a suitable material for neural tissue engineering. However, in order to verify the effectiveness of EPS-PCL nanofiber, further studies are needed about expression levels of other neurotrophic factors, cytokines and also biological studies *ex vivo* or *in vivo*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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