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The behaviour of neural stem cells on polyhydroxyalkanoate nanofiber scaffolds

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ABSTRACT

Polyhydroxyalkanoates (PHA) have demonstrated their potentials as medical implant biomaterials. Neural stem cells (NSCs) grown on/in PHA scaffolds may be useful for repairing central nervous system (CNS) injury. To investigate this possibility, nanofiber matrices (scaffolds) prepared from several PHA via a novel phase separation process were studied to mimic natural extracellular matrix (ECM), and rat-derived NSCs grown in the PHA matrices were characterized regarding their in vitro differentiation behaviors. All three PHA materials including poly(3-hydroxybutyrate) (PHB), copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate (P3HB4HB), and copolymer of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) supported NSC growth and differentiation both on their 2D films and 3D matrices. Among three PHA nanofiber matrices, PHBHHx one showed the strongest potentials to promote NSC differentiation into neurons which is beneficial for CNS repair. Compared to the 2D films, 3D nanofiber matrices appeared to be more suitable for NSC attachment, synaptic outgrowth and synaptogenesis. It was suggested that PHBHHx nanofiber scaffolds (matrices) that promote NSC growth and differentiation, can be developed for treating central nervous system injury.

1. Introduction

In many cases adult central nerve system (CNS) is unable to regenerate to repair its functions following pathological trauma or disease [1–3]. For instance, spinal cord injury (SCI) often leads to permanent paralysis of motor functions and loss of sensation below the site of spinal injury, due to the inability of the adult spinal fibers to regenerate once injured. To achieve axonal regeneration following CNS injury, there are several important issues to be considered: 1) scar tissue formation; 2) phagocytosis-induced tissue gaps; 3) inhibitory factors for axonal growth in the mature mammalian CNS; and 4) failure of many adult neurons to initiate axonal extension [1–4].

Recent studies indicate that neural stem cells (NSCs) possess great potential as an important therapeutic tool to treat a number of CNS disorders [5–7]. The source of NSC seeds can be obtained not only from embryonic and adult brain tissues but also from animal or human embryonic stem (ES) cells [5,8]. These cells are able to proliferate in vitro through many passages without losing their multipotentiality, and differentiate into astrocytes, neurons or oligodendrocytes if properly induced [5]. Several previous reports demonstrated the benefits of fine-tuning extrinsic signals such as soluble growth factors and cell–cell contacts for regulating the proliferation and differentiation of NSCs [6–8]. Materials without cytotoxicity as extracellular matrix (ECM) substitute have also beneficial influence on NSCs functions [4]. In addition, nanotechnology-based CNS regeneration therapy has already become one of the exciting new areas in the nerve tissue engineering [3,4].

Polyhydroxyalkanoates (PHA) have been demonstrated to be a family of biopolymers with good biodegradability and non-cytotoxicity [9,10]. Some interests have focused on developing PHA application as bio-implant materials including nerve tissue engineering materials. Bian et al. developed PHA copolymer PHBHHx consisting of 3-hydroxybutyrate and 3-hydroxyhexanoate into nerve conduits with non-toxicity, good nerve regeneration, and strong mechanical properties, they found the PHBHHx based nerve conduits to be able to repair in vivo peripheral nerve damage [11]. Novikova et al. demonstrated that PHB scaffold seeded with Schwann cells can promote spinal cord repair [12]. Although these artificial matrices showed certain advantages, the dimensions of

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the matrix fibers and their pore sizes were still far greater than the nanoscale realm of actual ECM [13–15]. To mimic the real microenvironment of ECM for NSC growth and differentiation, novel nanofiber matrices based on PHA polymers were prepared via a novel phase separation process, various degrees of successes were achieved [10,13].

This paper aimed to evaluate the possibility of applying PHA nanofiber matrices to promote neural growth and differentiation. To do this, nanofiber scaffolds (matrices) based on three commercially available PHA materials were prepared and their influences on NSCs survival, proliferation and differentiation studied. The most suitable material that can promote NSC differentiation into neurons and axonal elongation while inhibiting glial scar formation, was to be selected as a material for further study leading to the repairment of CNS injury.

2. Materials and methods

2.1. Materials

Adult female Sprague–Dawley rats were purchased from Animal Experiment Center, Shantou University. Poly lactic acid (PLA) was obtained from Natureworks (USA). PHB, PHBV (5.7 mol% HV) and PHBHHx (6 mol% 3HHx) were kindly donated by the Microbiology Laboratory of Tsinghua University (Beijing, China). P3HB4HB (5 mol% 4HB) was as an experimental gift from Tianjin Green Bioscience Co. Ltd. (China). All the above PHA had molecular weight over 300,000 Dalton.

2.2. Fabrication of PHA matrices

2.2.1. Preparation of films based on PHA

Films of pure PLA, PHB, PHBV, P3HB4HB, and PHBHHx were prepared by solution casting method as previously described [16]. Briefly, 1 g of material was dissolved in 50 ml of chloroform under vigorous agitation for 60 min at 60 °C. Following evaporation of chloroform, films of ~50 μm in thickness were formed. The thickness was measured by Vernier calipers and confirmed by SEM. Because both sides of the film prepared using this method showed different surface roughness, the rougher side was arbitrarily chosen for cell cultures. Before the cultivation, all films were sterilized by immersing in 75% (v/v) ethanol aqueous solution for 2 h, followed by ultraviolet radiation for 1 h, then by in PBS overnight [17].

2.2.2. Preparation of PHA 3D matrices

PHA materials were dissolved in chloroform at 60 °C. Ten ml PHA chloroform solution was mixed with 2.5 ml dioxane, followed by sonication for 20 min. The mixture was incubated at 4 °C until gel formation. The gels were immersed into water for 1 day, followed by 1-h incubation at −80 °C [12]. Subsequently the gel was placed in a freeze-dryer to remove residual solvents. Prior to the application in cell cultures, those scaffolds or matrices were dealt with the same method of PHA films as described above.

2.3. Isolation and culture of cortical neural stem cells

Neural stem cells (NSCs) were cultured as previously described [18]. Briefly, the neocortices of E13–15 rat embryos were dissected, cut into small pieces and mechanically triturated in cold physiological buffered saline (PBS). The dissociated cells were collected by centrifugation for 10 min at 1000 rpm, and re-suspended in a serum-free medium containing serum-free Dulbecco's Modified Eagle's Medium/F12 medium (DMEM/F12, Gibco) supplemented with 2% B27 (Gibco), 20 ng/ml EGF (Peprotech) and 20 ng/ml b-FGF (Peprotech). The number of viable cells was counted by trypan blue exclusion assay in a hemocytometer. Cells were plated on un-treated Petri dishes in the culture medium, and incubated with 95% air/5% CO_2 (Thermo Electron Corporation, USA) at 37 °C. The culture medium was changed every four days. After seven days, mechanically dissociated NSCs and undissociated neurospheres were re-plated on a new culture flask at a density of 10^5–10^6 cells/ml with the fresh culture medium containing the same concentration of b-FGF. These single cells proliferated into spherical cellular aggregates after 2–3 days [19]. The procedure of subculture (serum-free medium) was repeated again and cells were collected for studying the behaviour of NSCs grown on the PHA films and in 3D nanofiber scaffolds.

2.4. Immunocytochemistry

Immunostaining was performed on cultured cells on 96-well polystyrene plates. Cells were fixed in ice-cold 4% paraformaldehyde for 20 min at room temperature and washed twice in PBS. After incubation for 10 min with 0.3% Triton-X-100, the cells were further incubated with 10% goat serum for another 15 min, followed by primary antibody incubation in PBS (anti-β-tubulin III IgG at 1:100, chemicon; or anti-GFAP 1:80 sigma) for 2 h at 37 °C or overnight at 4 °C. Following three washing processes with PBS, FITC- or CY3-conjugated secondary antibodies (1:100, sigma) were added at room temperature for an hour. After another thrice washes with PBS, all nuclei were stained with DAPI for 10 min at room temperature. Images were taken under a fluorescence microscope equipped with a charge-coupled device camera (Eclipse TE 2000; Nikon).

2.5. Scanning electron microscopy (SEM) examination

After cultivation for 7 days, films or scaffolds grown with cells were washed twice with PBS, and cells were fixed with 2.5% glutaraldehyde at room temperature for 2 h or under 4 °C overnight. Fixed films were dehydrated by ethanol in an increasing concentration gradient, followed by lyophilization. The PHA matrices were mounted on aluminum stumps, and then coated with gold in a sputtering device for 1.5 min at 10 mA and examined under a scanning electron microscope (SEM, JSM-6360LA, JEOL, Japan).

2.6. Growth characterization of NSCs on different PHA matrices

The growth assay of NSCs was performed in 96-well polystyrene plates. The PHB, PHBHHx, and PHB4HB nanofiber scaffolds were cut to fit into the size of the 96-well plates. The NSC spherical aggregates were planted into the scaffolds and the scaffolds were placed into each well of the 96-well plates, added with 200 μl differentiation medium (DMEM/F12, B27 with 10% FBS). After incubation for 3 days, cells were stained with Fluo-4 AM and their morphology observed under laser scanning confocal microscope (LSCM 510 Meta, Zeiss, Germany).

2.7. Cell viability assay

The NSCs (1×10^4 cells/well) were seeded on each film placed in the 24-well plates. Cells cultivated in the same wells without films were used as a control. Plates were incubated in the DMEM/F12 medium containing 10% FBS at 37 °C in a 5% CO_2 incubator for 7 days, and the cell viability was studied using the CCK-8 assays according to the manufacturer's instructions. Briefly, 450 μl serum-free culture medium and 50 μl CCK-8 solutions were added to each sample. After incubation at 37 °C for 3 h, the optical density (OD) was measured at 450 nm using a plate spectrophotometer (MK3, Thermo, USA). Six parallel replicates were read for each sample.

2.8. Western blotting analysis

Cells were plated on different scaffolds as described previously. Upon removing culture media by aspiration, cells were washed twice with PBS and lysed in RIPA buffer containing PMSF, EDTA, pepstatinA, leupeptin and aprotinin. Lysates were centrifuged for 5 min at 15,000 rpm, and supernatants were collected and total proteins measured by a Bradford protein assay kit. Pre-stained protein standards (Biolabs, USA) were used as loading controls. Equal amounts of proteins were loaded on 12.5% SDS-polyacrylamide gel. After electrophoresis, gels were transferred to PVDF membranes, which were blocked at room temperature for 1 h in Western blocking buffer (Beyotime), and incubated with primary antibodies at 4 °C overnight. Primary antibodies used were mouse antibodies specific for the GAPDH (1:1000, Beyotime), β-tubulin III (1:1000, Sigma), rabbit antibodies specific for glial fibrillary acidic protein, GFAP (1:100, Sigma). After rinsing, blots were incubated in PBST with peroxidase conjugated second antibodies (goat anti-mouse IgG and goat anti-rabbit IgG, all at 1:1000 content) under room temperature for 1 h, and washed three times for 10 min in wash solution. The peroxidase reaction was visualized by an enhanced chemiluminescence (ECL) method, and the images analyzed with Image Station 2000R (Kodak, Japan) [20,21].

2.9. Statistical analysis

All data were presented as the mean values plus standard deviation (SD) of six parallel studies. Statistical comparisons were performed using Student's *t*-test and carried out by GraphPad Prism5 (Graph-Pad, La Jolla, CA).

3. Results

3.1. Characterization of NSC spherical aggregates

Cortical cells were isolated from pregnant SD rat embryos on 12.5–15.0 days. Most of the isolated cells proliferated and formed clusters of small round cells that eventually grew into floating spherical aggregates (Fig. 1A). When cultivated in a serum-free B27 medium supplemented with EGF and b-FGF, these spheres were

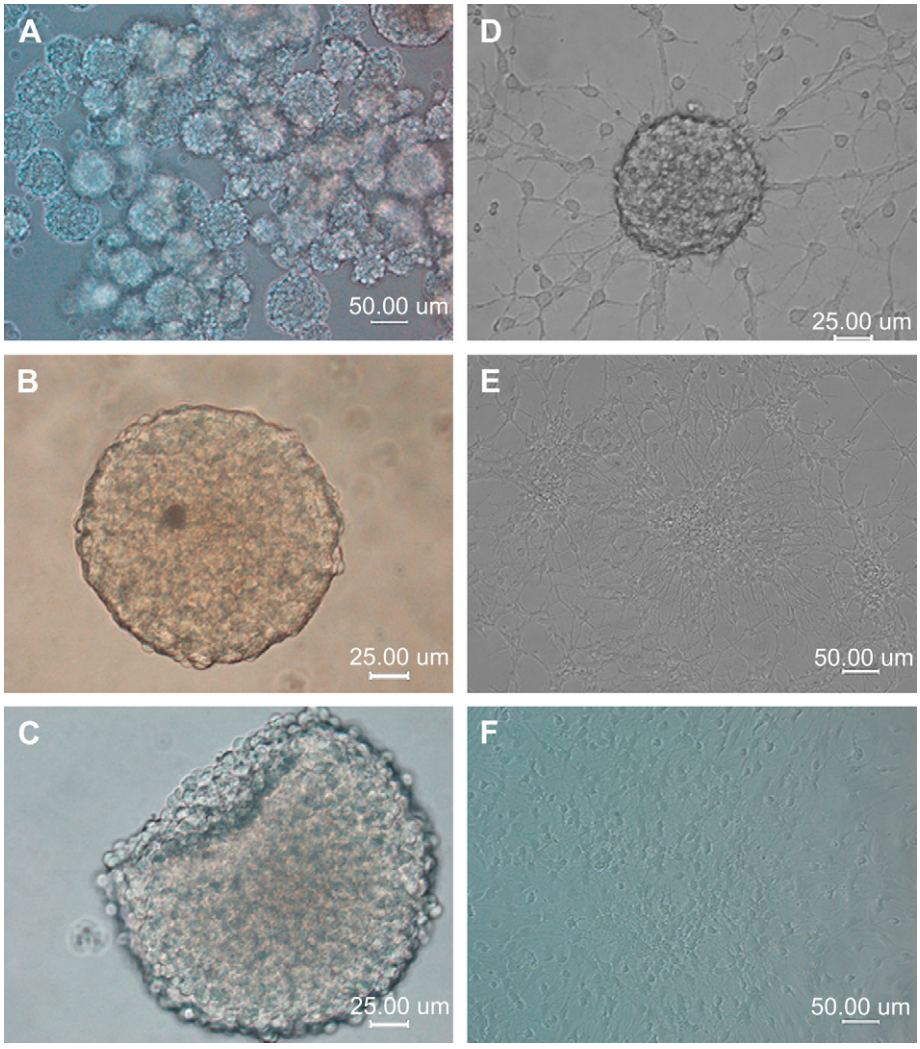

Fig. 1. Characterization of NSC spherical aggregates. (A) NSC spheres at primary passage; (B) A single sphere at primary passage 2; (C) A blow-up view of the internal structure of an NSC sphere; (D) Growth of an NSC sphere plated on PLL and cultured for 4 days; (E) 7 days under serum-free culture medium. (F) NSC sphere was plated on culture plate for 7 days in 10% FBS culture media. A, E, F scale bar = 50 μm; B, C, D scale bar = 25 μm.

passaged by mechanical dissociation once every 7 days. These cells proliferated and grew into new spheres (Fig. 1B and C), and also migrated out from the spheres after being plated on a cell culture plate coated with poly-L-lysine (PLL) or containing fetal bovine serum (FBS) culture medium (Fig. 1D–F).

Immunocytochemical characterization was performed on spheres on a PPL-coated cell culture plate. After 7-day cultivation in serum-free culture medium these spheres were stained positive for nestin (Fig. 2A), most of them either expressed GFAP (Fig. 2C) or β-tubulin III (Fig. 2D), implying that the cells were multipotential and mostly generated into either GFAP-positive astrocytes or β-tubulin III positive neurons. It was concluded that those NSC spheres had the capacity to divide and differentiate into neurons and astrocytes, respectively (Fig. 2F). Thus, we considered the isolated brain cells as the neural stem cells (NSC) and the spheres as NSC spheres.

3.2. Behaviors of NSCs on various PHA or PLA films

The SEM images (Fig. 3A2–E2) for NSCs cultured for 7 days on different PHA films and PLA film under 10% FBS conditions showed that NSCs could grow on various films with normal elongation, although PHA films (PHB, PHBV, PHBHHx and PHB4HB films) and PLA films showed different surface morphology and surface roughness under the SEM. These results suggested that both PHA and PLA materials possessed ability to support growth of NSCs.

3.3. Cell viability study

NSC adhesion and viability on different films were studied using CCK-8 assays after 10 days of cultivation. Data (Fig. 6) show that NSCs grown on the PHBHHx and PHB4HB films had higher cell viability than those on the PLA and PHB films. The highest viability of cells was observed with the PHBHHx films, suggesting that PHBHHx film provided a better growth environment compared with that of other films.

3.4. Morphology of PHA nanofiber scaffolds

Nanofiber scaffolds made of PHB, PHBHHx, PHB4HB were respectively studied by SEM. Three-dimensional interconnected fibrous networks were observed in these matrices with averaged fiber diameters in the range of 50–500 nm, which are similar to the dimension of major ECM component collagen (Fig. 5).

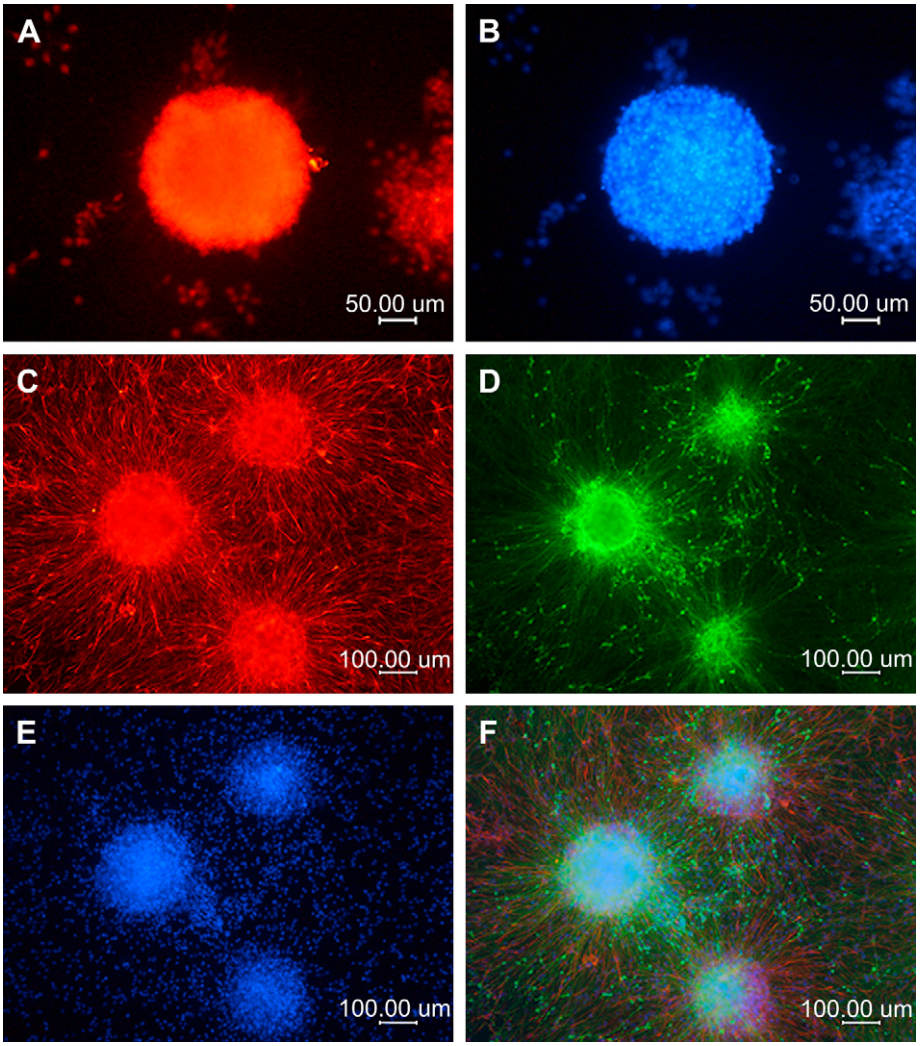

Fig. 2. Immunofluorescence staining of a typical NSC sphere showed that the cells were (A) nestin$^+$, counterstained with (B) DAPI$^+$. NSC spheres were cultured on PLL-coated substrates in serum-free medium after 7 days and were double immunostained for (C) GFAP$^+$, (D) β-tubulin III$^+$ and counterstained with (E) DAPI. It is obvious in the merged image (F). Scale bar for A, B = 50 μm; for C, D, E, F = 100 μm.

3.5. Growth characterization of NSCs on different PHA nanofiber scaffolds

In order to observe the morphology of NSCs grown on PHA nanofiber scaffolds, single cells were dissociated from NSC spheres and then plated on various nanofiber scaffolds. On the 3rd day, the cells were stained by Fluo-4 AM to be observed under LSCM. As shown in Fig. 8, the NSCs grown on various PHA nanofiber scaffolds with 10% FBS medium conditions had established good connection through neurites on the 3rd day. This is especially prominent for the case with PHBHHx nanofiber scaffold.

3.6. Cell viability on PHA nanofiber scaffolds

NSCs viability on different nanofiber scaffolds was similarly studied using CCK-8 assay. The results showed that cells grown on PHBHHx nanofiber scaffold had higher viability than those on other materials (Fig. 6D). Compared with cells on the films, three-dimensional nanofibers better promoted cell viability.

3.7. Effects of PHA nanofiber scaffolds on NSC differentiation

The effect of different PHA nanofiber scaffolds on NSCs differentiation was examined by the expression of β-tubulin III proteins of the NSC spheres. Western blotting analysis showed higher expression of β-tubulin III in NSCs grown on PHBHHx nanofiber scaffold than on PHB and PHB4HB scaffolds (Fig. 7), indicating that PHBHHx nanofiber scaffold either promoted NSC growth (hence resulting in more neuron-like cells expressing β-tubulin III) or promote NSCs to differentiate favorably into neuron-like cells. More neural differentiation is beneficial for establishing neurites connection during the repair process for spinal cord injury [4].

3.8. SEM study of NSCs growth on PHBHHx nanofiber scaffold

Under the SEM examination, NSCs cultivated on PHBHHx nanofiber scaffold developed extended processes and plausible neurite connections after 7 days of cultivation (Fig. 8), implying good compatibility with PHBHHx materials.

4. Discussion

Treatment of CNS diseases or damages has been a research focus of all times. However, there is not an effective solution to better address this focus until recently [22]. It is known that the ECM environment has strong influence on the physiological status of CNS, especially under pathological conditions [4]. To overcome this

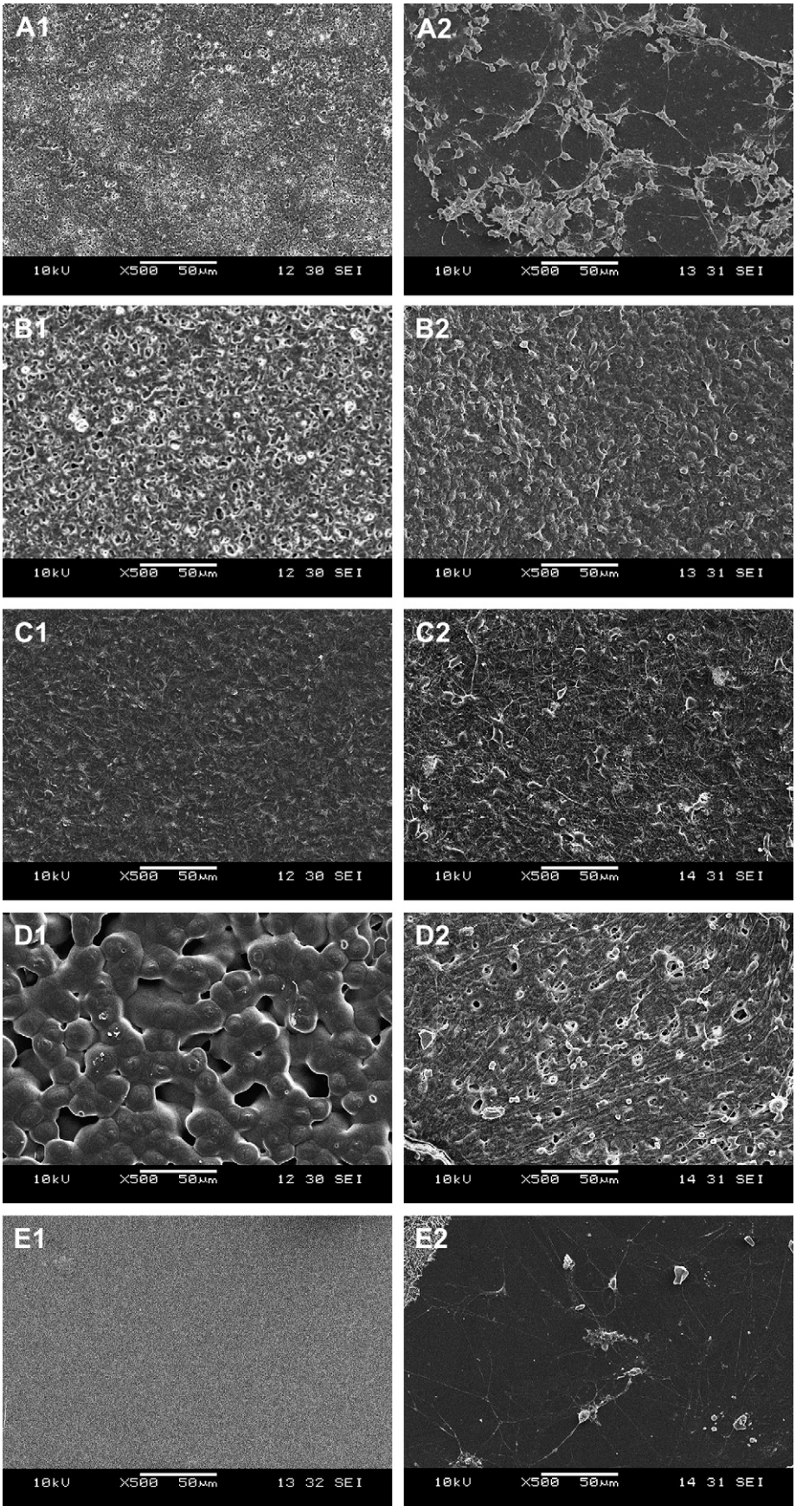

Fig. 3. SEM images of (A) PHB, (B) PHBV, (C) PHB4HB, (D) PHBHHx and (E) PLA films without NSCs (A1–E1) and with NSCs cultured for 7 days (A2–E2), ×500.

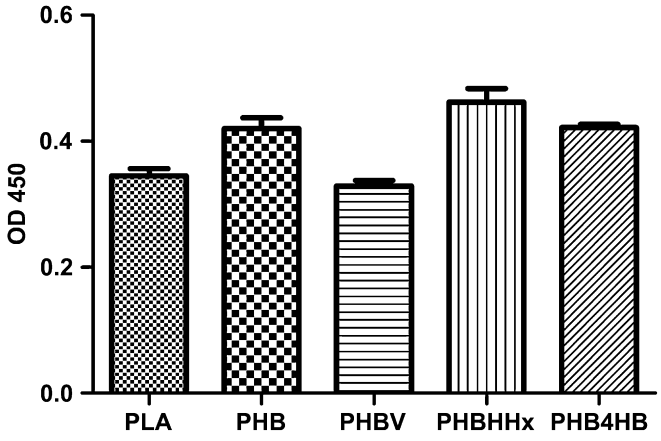

Fig. 4. CCK-8 assay of NSCs cultured on PLA, PHB, PHBV, PHBHHx and PHB4HB films after incubation for 10 days (n = 6).

problem, neural tissue engineering has been applied to restore post-injury CNS functions [3]. Neural stem cells were used as main seed cells in nerve regeneration because of the potential therapeutic effect for repairing various CNS disorders [23]. NSCs isolated from both the CNS and peripheral nervous system (PNS) have been shown capable of differentiation into neurons, astrocytes and oligodendrocytes [5,24]. Additionally, advanced nano-based polymers are being considered to be used as a substrate biomaterial for nerve engineering due to the simple fact that the nanostructure mimics more closely the ECM dimensions, thus useful for promoting cell attachment, migration and proliferation [13].

Polyhydroxyalkanoates (PHA), especially PHB, PHBV, PHBHHx and PHB4HB, had been repeatedly demonstrated with a good biodegradability and without cytotoxicity in vitro and in vivo [10,25–28]. Many interests have focused on using PHA materials for tissue engineering applications [10,25]. The SEM images of PHA surface showed that films made of PLA, PHBV, PHBHHx and PHB4HB had different surface roughness and topology (Fig. 3A1–E1). PHA films, especially PHBHHx film, possessed the most notable roughness and unique topology while PLA film showed smooth surface among all the tested materials. Moreover, NSC was still able to grow on PHA and PLA materials with satisfactory viability (Fig. 3A2–E2), and with better cellular proliferation on various PHA films than on PLA film.

A porous structure with highly interconnected spaces is a desirable property for scaffold implants, as such property will provide an interconnecting mechanic support for cell attachment and migration, and hence may promote proliferation [15,29].

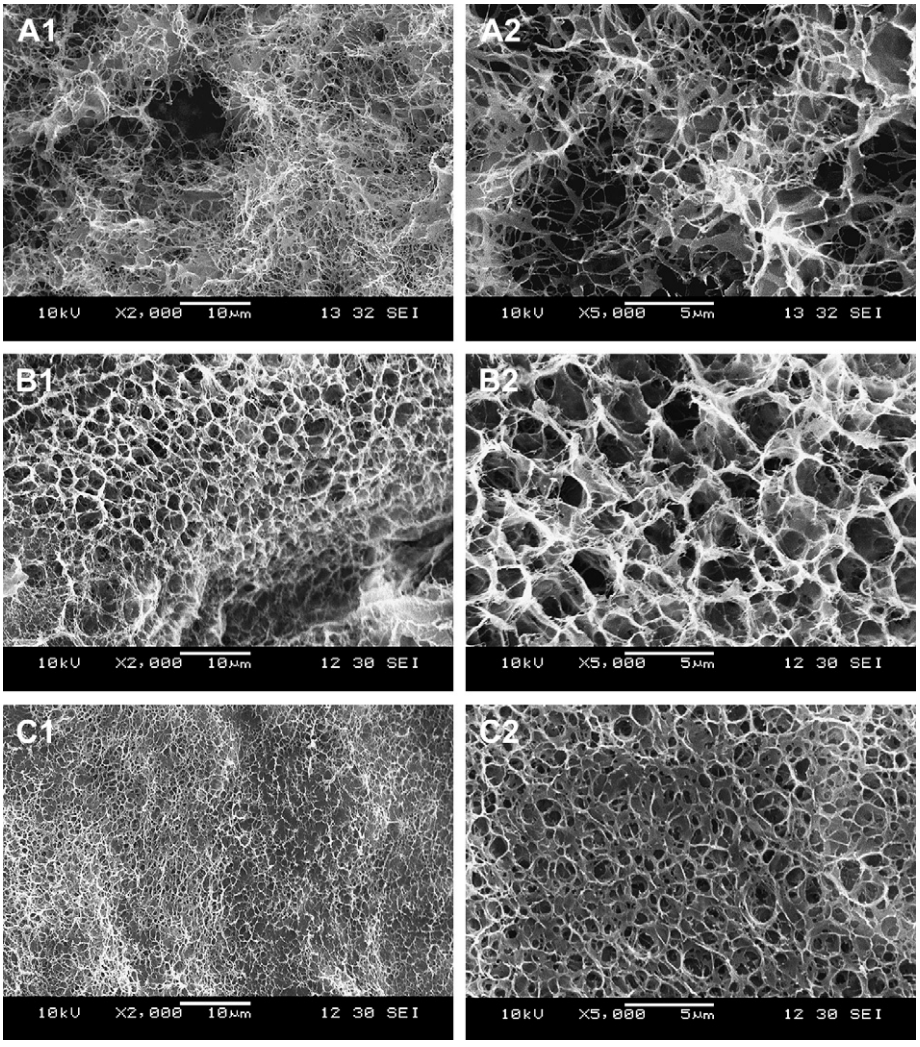

Fig. 5. SEM images of (A) PHB, (B) PHBHHx, (C) PHB4HB nanofiber scaffolds. Magnification for: (A1–C1): ×2000; (A2–C2): ×5000.

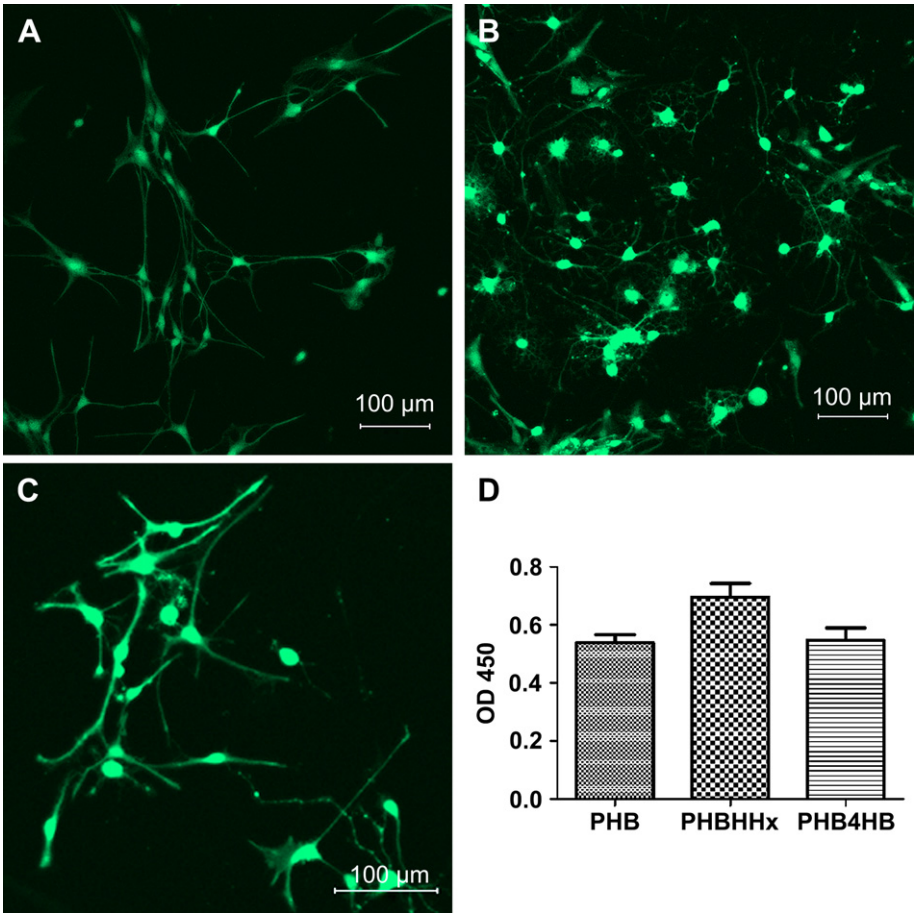

Fig. 6. Study of morphology of NSCs grown on (A) PHB, (B) PHBHHx, (C) PHB4HB nanofiber scaffolds using LSCM, and (D) CCK-8 assay of the viability of NSCs grown on different PHA nanofiber scaffolds for 10 days (n = 6).

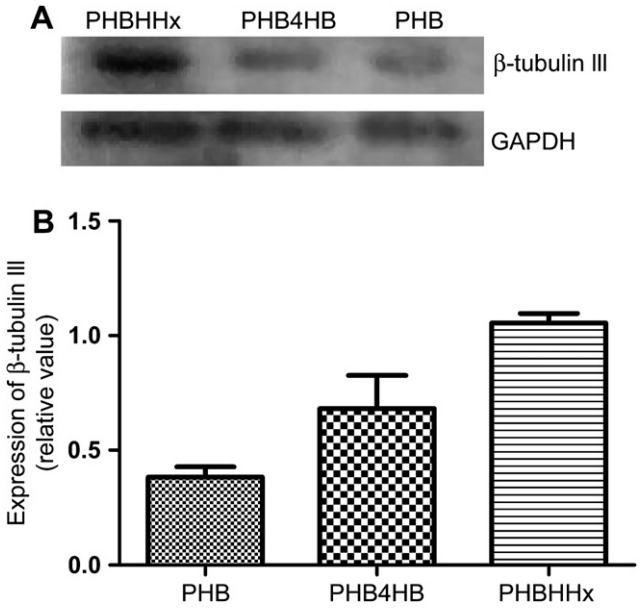

Fig. 7. Expression levels of β-tubulin III protein of NSCs grown on different PHA nanofiber scaffolds. A: Western blotting analysis; B: The relative protein density (relative value: protein density of β-tubulin III protein/protein density of GAPDH protein) ± standard error of the mean (n = 3) are plotted.

The SEM studies on PHB, PHBHHx and PHB4HB nanofiber scaffolds showed that these PHA matrices were made of continuous fibrous networks that constituted highly interconnected porous structures (Fig. 5). The diameters for these empty space ranged from 50 to 500 nm, which are similar to the natural ECM structure [13]. Because the mechanical properties of PHA nanofiber scaffolds can be regulated by the length of PHA chains, various materials of different PHA chain strengths can be easily fabricated. Compared with nanofibers of PLA and self-assembly peptides, these innovative PHA materials overcome insufficient mechanical strength often seen in medical applications of tissue engineering [12].

SEM analyses found that neurites were able to penetrate into the interior of PHBHHx nanofiber scaffold to establish close connection between cells (Fig. 8), suggesting that the nanofiber scaffold made of PHBHHx could provide mechanical support or even a positive cue to guide the neurite outgrowth.

According to the results of CCK-8 assay (Fig. 4), the viability of NSCs on PHA films, especially PHBHHx films, was much higher than on PLA films. This result can mainly be attributed to the difference of surface roughness and surface hydrophilicity. This is consistent with the previous results demonstrating that non-cytotoxic PHBHHx had better cell growth supporting ability compared with other PHA and PLA biomaterials did [30]. It is therefore suggested that the PHBHHx film used as a cell matrix might be more suitable for NSC survival in a long-term culture. The viabilities of NSCs on PHA nanofiber scaffolds were significantly higher than those on any other flat films, as nanofiber scaffolds with a 3D nanostructure are advantageous for cells to

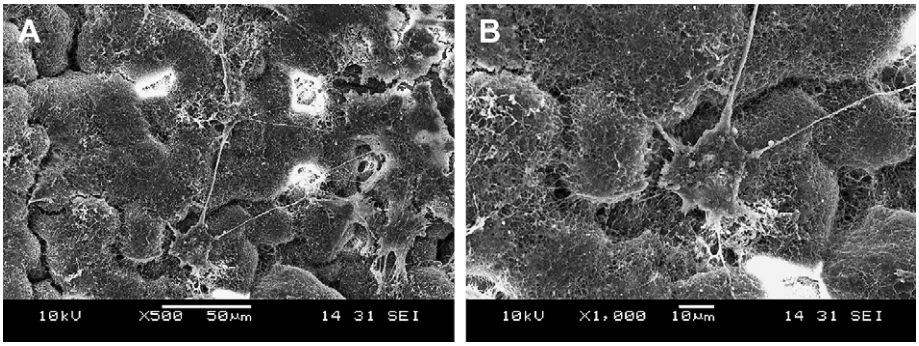

Fig. 8. SEM study of NSCs grown on the PHBHHx nanofiber scaffold cultured for 7 days: magnification for A: ×500; and for B: ×1000.

absorb nutrients, to receive signals and discard wastes. The results of CCK-8 assay were supportive of SEM results as found in a previous study [13], strongly suggesting that nanofiber scaffolds made of PHA, in particular PHBHHx, provided a suitable microenvironment for NSC growth and differentiation.

The images of LSCM showed that NSCs grew on various 3D nanofiber scaffolds and differentiated into many mature neurons with long axons (Fig. 6). The intercellular connections should have been established by these newly formed axons. The formation of neurites and possible connections by differentiated NSCs on PHBHHx nanofiber scaffold agreed with the results of SEM and CCK-8 assays (Figs. 6D and Fig. 8). Moreover, Western blotting revealed that NSCs grown on PHBHHx nanofiber scaffold expressed more β-tubulin III (Fig. 7), which is a specific neuronal marker [5], indicating that NSCs grown on PHBHHx nanofiber scaffold indeed differentiated favorably into neurons compared to other materials. When repairing CNS injury, it is very vital that cell matrix and niche enable the system to inhibit glial scar formation and promote axon elongation [4]. Compared with the other PHA materials, PHBHHx nanofiber scaffold as NSCs matrix may be suitable for neural tissue engineering to treat spinal cord injury.

5. Conclusion

This study investigated the effects of PHA films and PHA nanofiber scaffolds on NSCs growth and differentiation. Results showed that PHA nanofiber scaffolds, structurally similar to natural ECM, could support growth of NSCs. Compared with the PHA films, PHA nanofiber scaffolds showed stronger cellular adhesion, better connection and higher viability of NSCs. Nanofiber scaffolds made of PHBHHx were better than PHB and PHB4HB in promoting NSC differentiation into neuron-like cells. Combined with the biodegradability and strong mechanical properties as well as the compatability to NSCs, PHA nanofiber scaffolds, especially PHBHHx nanofiber scaffolds, could be promising biomaterials for repairing spinal cord injury.

Acknowledgements

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Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular Figs. 1, 2 and 6, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.01.132.

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