Biomaterials 31 (2010) 3967–3975

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The behaviour of neural stem cells on polyhydroxyalkanoate nanofiber scaffolds

Xian-Yi Xu [a], Xiao-Tao Li [b], Si-Wu Peng [a], Jian-Feng Xiao [c], Chao Liu [a], Guo Fang [a], Kevin C. Chen [a,**], Guo-Qiang Chen [d,*]

[a] Multidisciplinary Research Center, Shantou University, Shantou 515063, Guangdong, China
[b] Department of Anatomy, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China
[c] Department of Pharmacology, Shantou University Medical College, Shantou 515041, China
[d] Department of Biological Science and Biotechnology, School of Life Science, Tsinghua University, Beijing 100084, China

ARTICLE INFO

Article history:
Received 7 January 2010
Accepted 22 January 2010
Available online 12 February 2010

Keywords:
PHB
Polyhydroxyalkanoates
Nanofiber
Matrices
Scaffold
Neural stem cells

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

* Corresponding author. Tel.: +86 10 62783844; fax: +86 10 62794217.
** Corresponding author. Tel./fax: +86 754 82901180.
E-mail addresses: ckchen@stu.edu.cn (K.C. Chen), chengq@mail.tsinghua.edu.cn (G.-Q. Chen).

0142-9612/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2010.01.132

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