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PHBV microspheres as neural tissue engineering scaffold support neuronal cell growth and axon–dendrite polarization

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ABSTRACT

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) microspheres, with properties such as slower degradation and more efficient drug delivery properties, have important benefits for neural tissue engineering. Our previous studies have shown PHBV microspheres to improve cell growth and differentiation. This study aimed to investigate if PHBV microspheres would support neurons to extend these benefits to neural tissue engineering. PHBV microspheres' suitability as neural tissue engineering scaffolds was investigated using PC12 cells, cortical neurons (CNs), and neural progenitor cells (NPCs) to cover a variety of neuronal types for different applications. Microspheres were fabricated using an emulsion-solvent-evaporation technique. DNA quantification, cell viability assays, and immunofluorescent staining were carried out. PC12 cultures on PHBV microspheres showed growth trends comparable to two-dimensional controls. This was further verified by staining for cell spreading. Also, CNs expressed components of the signaling pathway on PHBV microspheres, and had greater axon–dendrite segregation (4.1 times for axon stains and 2.3 times for dendrite stains) than on coverslips. NPCs were also found to differentiate into neurons on the microspheres. Overall, the results indicate that PHBV microspheres, as scaffolds for neural tissue engineering, supported a variety of neuronal cell types and promoted greater axon–dendrite segregation.

1. Introduction

Scaffolds are important, if not essential, to the viability of cells used in regeneration therapies [1]. Physical and biochemical cues provided by scaffolds are crucial to keeping implanted cells viable and to improve therapeutic effects. Furthermore, scaffolds can localize cells at therapeutic sites. Cell-replacement, drug-delivery, and cell-delivery therapies are being developed for neurodegenerative diseases and traumatic injuries to the nervous systems. Some challenges for effective therapy include avoiding further inflammation, providing neuroprotection, stimulating tissue regeneration, overcoming inhibitory cues, and promoting functional recovery [2–5].

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) microspheres offer benefits as tissue engineering scaffold for neural therapy. Firstly, it has well-established drug delivery properties and controllable drug release profiles, providing sustained and controlled release of neurotrophins for neuroprotection, as well as various biocompounds for stimulating tissue regeneration, overcoming inhibitory cues and promoting functional recovery, which was demonstrated in our previous studies [6–12]. Secondly, its biodegradability enables it to disappear from implant sites, making space for regenerated tissue [13]. Thirdly, its slow degradation, as compared to PLGA [9], better matches the longer duration required for neural tissue treatment [14–17], therefore there is no accumulation of acidic degradation products in vivo which could be a problem [18]. Furthermore, PHBV could be added to slow down degradation of other polymers, as we have shown [9]. Fourthly, PHBV microspheres cause less inflammation and require less invasive surgery due to their biocompatibility, small sizes and spherical shape [19–23]. Finally, the three-dimensional (3-D) nature of PHBV microsphere scaffolds benefits cell growth and differentiation. Our previous studies showed PHBV microspheres to promote higher hepatocyte proliferation and function as compared to two-dimensional (2-D) cultures [9,12].

Despite all these, PHBV has not been widely studied for neural tissue engineering (TE), where recent literature reviews did not report on its use for neurons [24–26]. This work aims to investigate the suitability of PHBV microsphere in neural TE, so as to utilize its advantages as a suitable biomaterial. In neuronal TE research, different neuronal cell types have been used in different applications and models. For example, PC12 is a neuronal cell line that responds to nerve growth factor by differentiating to a neuronal phenotype, and thus has been used as a model for neuronal differentiation and biomolecule secretion [27–30]. It has also been used in the development of therapies for neural diseases as a highly potent cell

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source [31,32]. However, cell lines can only serve as robust and rapid models for neuronal studies, while they represent normal neurons to a limited extent [27,30]. Thus, primary neurons are also needed for studying normal in vivo and higher neuronal functions. Another example of primary cortical neurons (CNs) from mouse fetuses have functions and behaviors that are very close to in vivo neurons, supporting their use as a model for neuronal maturation [30]. While PC12 and CNs are useful models for studying neuronal proliferation and maturation, they have limited use in actual therapies compared to neuronal progenitor cells (NPCs) [19,21,30]. NPCs have both the advantages of being able to proliferate like PC12 cells to produce more neurons for therapy, and differentiate into highly matured neurons like CN [30]. However, differentiation of NPCs has to be complete and maintained to avoid tumorigenesis [19,30]. In this study, PC12, CN, and NPC were cultured on PHBV microspheres to investigate their capacity to support the growth of a variety of neuronal cell types and applications, in an attempt to generate functionally differentiated neurons with segregated axons and dendrites.

2. Materials and methods

2.1. Materials

Chloroform and poly(vinyl alcohol) (PVA, MW 6000) were acquired from Fluka and Polysciences respectively. PC12 cells were purchased from ATCC. C57/BL6 mice were obtained from the Centre for Animal Resources (CARE, National University of Singapore). Medium components for PC12, CNs and NPCs were purchased from Invitrogen. Antibodies to synaptophysin and NR1 were acquired from Santa Cruz Biotechnology Inc. Antibodies to GFAP and Map2 were purchased from Millipore and Cell Signalling Technology respectively. SMI312 was purchased from Abcam. Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories Inc. Polyhydroxy-3-butyrate-*co*-3-valerate (PHBV, 5% PHV), all other chemicals and antibodies were purchased from Sigma–Aldrich unless otherwise stated.

2.2. Microsphere fabrication

PHBV microspheres were fabricated using the water-in-oil-in-water (w/o/w) double emulsion solvent evaporation technique [33]. 0.6 g PHBV was dissolved in 12 ml chloroform at 60 °C, while 0.15 g PVA was dissolved in 300 ml phosphate buffered saline (PBS). The first water-in-oil emulsion was formed by sonicating (SONICS Vibra-Cell™, Boston, MA, USA) 1 ml of the PVA–PBS solution in the PHBV–chloroform solution. The first emulsion was added to the remaining PVA–PBS solution and stirred at 300 rpm with a mechanical stirrer (RW20, Ika Labortechnik, Staufen, Germany) for 4.5 h. This final w/o/w double emulsion mixture was maintained at 37 °C using a water bath on a magnetic hotplate stirrer (Cimarec2, Barnstead/Thermolyne, Iowa, USA). The microspheres that were formed were collected and washed five times with deionized water, then freeze-dried (Alpha 1-4, Martin Christ, GmbH, Germany) for 36 h.

2.3. Scanning electron microscope imaging of microspheres

Freeze-dried microspheres were mounted onto brass stubs using carbon tape, then sputter-coated with platinum (JFC-1300, JEOL, Tokyo, Japan) and viewed by scanning electron microscopy (SEM, JSM-5600VL, JEOL, Tokyo, Japan). Sample sizes of no less than 100 microspheres per fabricated batch were used. Representative images of the spheres' surface morphology were taken at various magnifications. The diameters of the microspheres were measured from the SEM images using the software Smile View.

2.4. PC12 cell culture

PC12 cells were cultured in F-12K medium, supplemented with fetal bovine serum (2.5%) and horse serum (15%), at 37 °C and 95% CO_2 according to the supplier's protocol. The medium was changed at 50% every 48 h.

2.5. Mouse fetal cortical neuron harvesting and culture

Cortical neurons were harvested from E16 embryos (mouse, C57/BL6) and cultured in neurobasal medium supplemented with B27 and L-glutamine. A caesarean section was performed on a pregnant C57/BL6 mouse. The uterine horns were extracted and the embryos removed. Following this, the embryos were decapitated, the brains removed, and the cortex was dissected from the brain. All steps from removing the embryos to isolating the cortex were carried out in a harvest medium consisting of Hank's buffered saline solution supplemented with 1% v/v HEPES and 1% v/v penicillin–streptomycin. The cortices were minced with microscissors, then further dissociated by incubation at 37 °C in 2.5% v/v trypsin and 1% v/v DNase for 15 min. The digestion was stopped by the addition of fetal bovine serum (FBS). The dissociation solution and FBS were subsequently removed, and the cells resuspended in plating medium consisting of MEM supplemented with 0.05% v/v $NaHCO_3$, 1 mM pyruvic acid, 20% w/v glucose, 10% v/v FBS, and 1% v/v penicillin–streptomycin. The cells were seeded accordingly onto coverslips and microspheres. After 24 h, the medium was switched to a maintaining medium consisting of neurobasal medium supplemented with 2% v/v B27, and 10 μM L-glutamine.

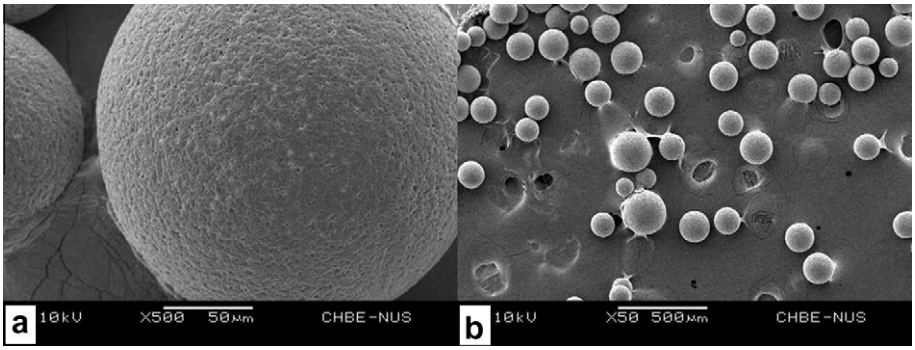

Fig. 1. Fabrication and characterization of PHBV microspheres. SEM images showing morphology of PHBV microspheres prepared by solvent evaporation technique (a, b). The average size of microspheres used was 205.2 ± 12.3 μm. The number of batches sampled was four, and a total of 574 microspheres were measured.

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