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# Differentiation of human bone marrow mesenchymal stem cells grown in terpolyesters of 3-hydroxyalkanoates scaffolds into nerve cells

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ABSTRACT

Polyhydroxyalkanoates, abbreviated as PHA, have been studied for medical applications due to their suitable mechanical properties, blood and tissue tolerance and in vivo biodegradability. As a new member of PHA family, terpolyester of 3-hydroxybutyrate, 3-hydroxyvalerate and 3-hydroxyhexanoate, abbreviated as PHBVHHx, was compared with polylactic acid (PLA), copolyester of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) for their respective functions leading to differentiation of human bone marrow mesenchymal stem cell (hBMSC) into nerve cells. Results indicated that 3D scaffolds promoted the differentiation of hBMSC into nerve cells more intensively compared with 2D films. Smaller pore sizes of scaffolds increased differentiation of hBMSC into nerve cells, whereas decreased cell proliferation. PHBVHHx scaffolds with pore sizes of 30–60 μm could be used in nerve tissue engineering for treatment of nerve injury. The above results were supported by scanning electron microscope (SEM) and confocal microscopy observation on attachment and growth of hBMSCs on PLA, PHBHHx and PHBVHHx, and by CCK-8 evaluation of cell proliferation. In addition, expressions of nerve markers nestin, GFAP and β-III tubulin of nerve cells differentiated from hBMSC grown in PHBVHHx scaffolds were confirmed by real-time PCR.



## 1. Introduction

The gold standard for treatment of nerve defects is to bridge the peripheral nerve gap with an autograft [1]. However, limitations still exist in these repairs of nerve injuries, partly because of the limited availability of donor tissues and partly due to the severity of the local pain suffered at the donor operative site [2]. Despite the good surgical advances, functional recovery was often poor [3,4]. The development of biomaterials for stem cell carriers has been regarded as a useful source of alternative tissue equivalents that provide a favorable microenvironment for tissue regeneration [5]. Tissue engineering techniques which enhance the beneficial endogenous responses to nerve injury could provide an alternative repair strategy [3].

Human bone marrow stromal cells (hBMSC) can continuously self-renew and differentiate into nerve cells in vitro under certain conditions [6]. There are also many evidences showing that hBMSC may be non-immunogenic or hypo-immunogenic [7]. hBMSC transplanted at sites of nerve injury are thought to promote functional recovery by producing trophic factors that induce survival and regeneration of host neurons [8]. Many researchers have attempted to regenerate nerve tissue by combining suitable biomaterials with hBMSC [9]. Artificial nerve scaffolds with cell and tissue compatibility have been used as carriers of cells to improve regeneration for nerve injury repair [10–15]. In the last few years, various biodegradable and non-biodegradable scaffolds have been tested using different experimental models of nerve injury [12,13]. In rat hemi-section spinal cord injury model, PLGA scaffolds with neural stem cell were implanted into the injured site, resulted in a functional improvement. Tissue loss and glial scarring were reduced by transplantation [14].

PHA, with their biocompatibility, biodegradability and strong mechanical properties, have been widely investigated for tissue engineering applications. Previous studies showed that PHB and PHBHHx could be used as potential candidate materials for peripheral nerve tissue engineering [15,16]. Novikova et al. demonstrated that a PHB scaffold promoted attachment, proliferation and survival of adult Schwann cells, and supported marked

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axonal regeneration within the graft [16]. An ideal scaffold should have a high affinity for cells to attach and proliferate, should be compatible to in vivo tissues and blood, should have durable strength. PHBHHx appears to be a material meeting these requirements [17]. Similar to PHBHHx, PHBVHHx is a new member of PHA family, our previous study showed that PHBVHHx had better biocompatibility compared with tissue cuture plates (TCP), PLA and PHBHHx [18]. Based on this finding, it had become interesting to investigate the possibility of PHBVHHx for hBMSC proliferation and differentiation.

Scaffold spatial structures have been shown to have effects on cell proliferation and differentiation in 3D directions [19]. In this study, porous PHBVHHx scaffolds intended for nerve tissue engineering were fabricated using thermally induced phase separation (TIPS), and these scaffolds with different pore sizes were studied for hBMSC proliferation and differentiation.

## 2. Materials and methods

### 2.1. Cell isolation and culture

Human bone marrow samples were acquired from donors (5–36 years old) without metabolic diseases at First Affiliated Hospital of Shantou University Medical College. The patients were informed and agreed on the sampling and the purposes, and local Ethical Committee approval was obtained for the use of the samples for this research. hBMSC cell isolation and culture were performed as previously described [20]. In brief, bone marrow samples were layered over a lymphoprep gradient and centrifuged at 2000 rpm for 15 min at room temperature. Mononuclear cells were washed twice with Hank's solution. Cells were resuspended in regular growth medium containing Dulbecco's modified Eagle's medium (low glucose content) (DMEM-LG, Invitrogen, California, USA) supplemented with 10 vol% fetal bovine serum (FBS, Hyclone, USA), penicillin/streptomycin, 2 mM glutamine, 5 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, California, USA) and $10^3$ U/ml leukemia inhibitory factor (LIF, Chemicon) to maintain their undifferentiated state. The cells were seeded in 6-well tissue culture plate (Corning Inc, Acton, Massachusetts, USA) at a density of $1 \times 10^6$ cells/cm$^2$. Cells were cultured in a humidified atmosphere with 5% $CO_2$ at 37 °C. After 72 h, non-adherent cells were removed, and the medium was changed every other day until cells became 90% confluent. The cells were digested with 0.25% trypsin solution (GIBCO, USA) and seeded with a density of $1 \times 10^4$ cells/cm$^2$ in regular growth medium for expansion purpose.

For differentiation studies, cells were cultured in nerve induction medium containing Dulbecco's modified Eagle's medium, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, California, USA), 500 mM IBMX (sigma, USA), 200 mM, INDO (sigma, USA), and 5 mg/ml insulin (sigma, USA) without FBS.

### 2.2. Analysis of cell phenotype by flow cytometry

Flow cytometry study was performed similarly as described previously [21]. Cell surface antigen phenotyping study was carried out using the hBMSC. The following cell surface epitopes were detected with anti-human antibodies: CD73-phycoerythrin (PE), CD105-PE, CD45-fluorescein isothiocyanate (FITC), CD34-FITC, HAL-DR-PerCP, CD90-FITC. $5 \times 10^5$ cells were analyzed using a FACScan flow cytometer (Epics XL, Beckman Coulter, Miami, FL, USA).

### 2.3. Preparation of 2D membranes and 3D porous scaffolds

Films of PLA, PHBHHx and PHBVHHx were prepared as previously described [22], Briefly, 1 g material was dissolved in 50 ml chloroform under vigorous agitation for 45 min at 60 °C. Subsequently, the chloroform solution was poured into Petri dishes. The dishes were maintained at room temperature for 24 h to allow the complete evaporation of chloroform. Vacuum drying was applied to completely remove any possible solvent remaining in the films.

Scaffolds of PHBVHHx were prepared by thermally induced phase separation (TIPS) [23,24]. Briefly, 1 g of PHBVHHx was dissolved in 25 ml of 1,4-dioxane under vigorous agitation for 60 min at 65 °C. The clear polymer solution was frozen for 2 h under varied temperature conditions of −20, −80 and −196 °C. Solvent was removed by a freeze-drying process lasting 24 h.

### 2.4. Scanning electron microscopy (SEM) examination

Materials were treated for SEM as previously described [25]. Cell-seeded films were washed three times using phosphate buffered saline (PBS), and immersed in PBS containing 5% glutaraldehyde (pH 7.4) for 1 h. They were then dehydrated in increasing concentrations of ethanol (from 30%, 50%, 70%, 90%, and 95%–100%), followed by lyophilization. No cell-seeded scaffold did not go through the above treatments. All samples were mounted on aluminum stumps coated with gold in a sputtering device (JFC-1600, Japan) for 10 min, followed by examination under a SEM (JSM-6360LA, Nikon, Japan).

### 2.5. Cell viability study

A cell count kit-8 (CCK-8, Beyotime, China) was employed in this experiment to quantitatively evaluate hBMSC viability [26]. Briefly, approximately $1 \times 10^4$ cells were seeded on each film placed in the 24-well plates for 72 h, the culture medium was removed and the cultures were washed with PBS twice. Approximately 900 μl serum-free DMEM medium and 100 μl CCK-8 solution were added to each sample, followed by incubation at 37 °C for 3 h. Supernatant was transferred to 96-well plate, the optical density (OD) at 450 nm was determined using a microplate reader (Multiskan MK33, Thermolabsystems, Finland). Six parallel experiments in each sample were used to assess the cell viability.

### 2.6. Cell seeding in PHBVHHx 3D scaffolds

PHBVHHx 3D scaffolds fabricated under different temperature gradients were evaluated for cell differentiation and proliferation [27]. Briefly, 3D scaffolds were pre-sterilized with 75% (v/v) ethanol followed by repeated washing with PBS to remove any residual alcohol. Scaffolds were first conditioned with DMEM for 2 h before cell seeding. Scaffolds were placed in 6-well culture plates (1 scaffold/well), each scaffold was seeded with $3 \times 10^5$ cells in 20 μl of the cell suspension. Seeded scaffolds were incubated for 2 h in a humidified atmosphere under 37 °C and 5% $CO_2$. Then, 3 ml DMEM was added to each well and scaffolds were incubated for 72 h. Half of the scaffolds were moved and transferred to nerve induction medium for differentiation study and then real-time PCR analysis, the other halfs were used to determine cell proliferation and distribution on scaffolds.

### 2.7. Study of cell proliferation and distribution in scaffolds using confocal microscopy

hBMSC proliferation and distribution on PHBVHHx scaffolds were studied using confocal microscopy. Briefly, scaffolds with cells were washed with phosphate buffered saline, fixed for 5 min in 3.7% formaldehyde in PBS. They were then immersed in 0.1% Triton X-100 for 3 min, followed by washing again in PBS. The so treated samples were taken for phalloidin-FITC (Sigma, USA) staining for 40 min under room temperature. Imaging experiments were conducted under laser scanning confocal microscope (LSM 510 Meta, Zeiss, Germany).

### 2.8. Real-time PCR analysis of gene expression

Total cellular RNA was extracted from cells grown on films of PLA, PHBHHx, PHBVHHx and in three independent PHBVHHx scaffolds immersed in nerve induction medium, hBMSC cultured in standard medium was used as a control. RNeasy mini kit (Qiagen, GmbH, Hilden, Germany) was employed in this process following manufacturer's instructions with DNase I (Qiagen) treatment. Complementary DNA synthesis was performed with 1 μg total RNA using PrimeScript RTase (TaKaRa, Japan). Real-time PCR study was conducted as described previously [28]. SybrGreen PCR MasterMix (Toyobo, Japan) was used in each reaction. PCR reaction was carried out under conditions of 95 °C, 15 s; 60 °C, 15 s; 72 °C, 30 s for 40 cycles using ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Expression level of GAPDH gene was used as an internal control. The PCR primers and probes were as synthesized with following sequences:

GAPDH forward primer: 5′-GCACCGTCAAGGCTGAGAAC-3′;
GAPDH reverse primer: 5′-TGGTGAAGACGCCAGTGGA-3′.
Nestin forward primer: 5′-CTCCAAGAATGGAGGCTGTAGGAA-3′;
Nestin reverse primer: 5′-CCTATGAGATGGAGCAGGCAAGA-3′.
GFAP forward primer: 5′-TGGCAGAGCTTGTTAGTGGTAAAGG-3′;
GFAP reverse primer: 5′-GTGAGACAGAGGCTGCTGCTTG-3′.
β-III tubulin forward primer: 5′-GAACCCGGAACCATGGACAG-3′;
β-III tubulin reverse primer: 5′-GACCCTTGGCCCAGTTGTTG-3′.

The expression level of GAPDH [GAPDH(ct) = 18] was used for normalization. A Ct of 38 was designated arbitrarily as 1 [29].

### 2.9. Statistical analysis

All data were calculated with the mean and standard deviation. The statistical significance of the data obtained was analyzed by the Student's *t*-test. Probability values of $p < 0.05$ were interpreted as denoting statistical significance.

## 3. Results

### 3.1. Characterization of hBMSC

Human bone marrow mononuclear cells were isolated using density gradient centrifugation. The resuspended cells were seeded in cell culture plates. Non-adherent hematopoietic cells were removed. When adherent cells grew to confluence, they were harvested and passaged. Flow cytometry study was performed to characterize surface markers on mesenchymal stem cells. The hBMSC expressed CD73, CD90 and CD105 without expression of CD34 (hematopoietic lineage marker), CD45 and HLA-DR (Fig. 1). These hBMSC showed spindle fibroblast-like morphology, a characteristic feature of hBMSC (Fig. 2). Most hBMSC displayed rapid changes in cellular morphology when exposed to nerve induction medium. The cytoplasm of hBMSC retracted towards the nucleus, forming contracted cell bodies with emitted cellular protrusions that were in contact with neighboring cells (Fig. 2). These results showed that hBMSC used for this study possessed the typical hBMSC phenotypes shown by other studies [20].

### 3.2. Morphology of PLA, PHBHHx and PHBVHHx films and cell attachment

PHBHHx and PHBVHHx film showed a rough surface compared with a smooth surface on a PLA film under SEM (Fig. 3). The distinct whorl-like surface morphology was observed only on the PHBVHHx film (Fig. 3C). When hBMSC were cultured for 3 days on different polymer films, more cells were observed on the PHBVHHx film than that on the PHBHHx and PLA ones (Fig. 3). This agreed well with results of the previous study [18].

### 3.3. Cell proliferation and viability

The CCK-8 assay was employed to compare hBMSC viability on different films. The poorest and the strongest cell viabilities were observed on PLA and PHBVHHx, respectively. At the end of the 72 h growth study, cell viability on the PHBVHHx was 33% and 31% higher than that on PHBHHx and on PLA, respectively (Fig. 4). The results demonstrated again that PHBVHHx significantly promoted cell proliferation compared with PLA and PHBHHx. The mechanism for better biocompatibility of PHBVHHx needs to be investigated further.

### 3.4. Morphology of PHBVHHx scaffolds

Since PHBVHHx demonstrated better potential for cell attachment and growth, it was used to prepare 3D scaffolds for following studies. To generate various pore sizes, the scaffolds were prepared at −20, −80 and −196 °C, respectively, using 4 wt% PHBVHHx 1,4-dioxane solution. After the 24 h freeze-drying process, the PHBVHHx scaffolds treated at −20 °C produced maximum pore sizes in the range of 110–170 μm (Fig. 5). Treatments under lower freezing temperatures such as at −80 and −196 °C led to smaller pore sizes (Fig. 5D). Temperatures appeared to be a good parameter for regulating PHBVHHx scaffold porosity.

### 3.5. Distribution of hBMSC grown in PHBVHHx scaffolds

Three-dimensional polymer scaffolds may enhance cell regeneration by creating and maintaining a space that facilitates cell adherence, migration and proliferation [30]. PHBVHHx has been proven to be suitable for cell attachment and proliferation, to

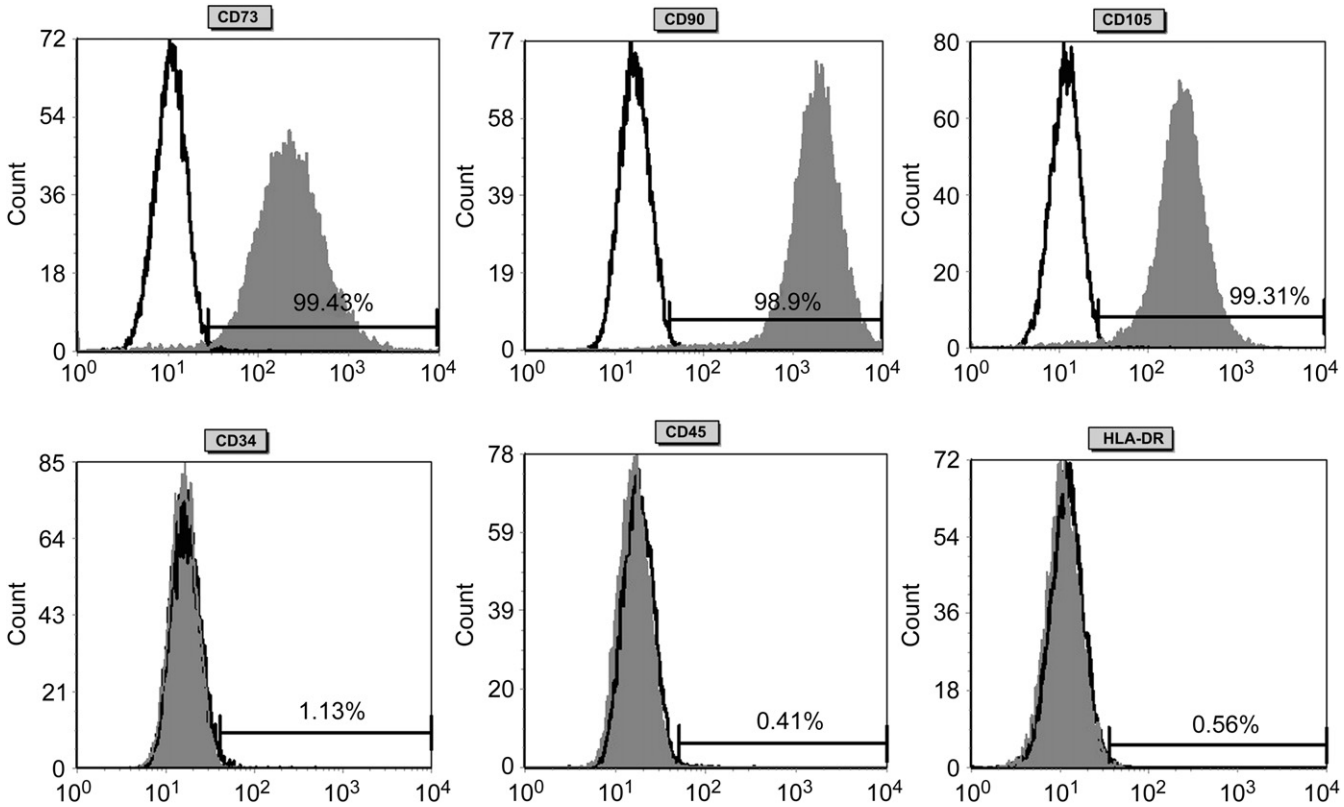


**Fig. 1.** Study of typical surface marker expression in hBMSC using flow cytometry. hBMSCs were confirmed by staining with CD73, CD90 and CD105 antibodies against the indicated human markers. CD34, CD45 and HLA-OR which are not expressed in hBMSC were not observed in our cells.

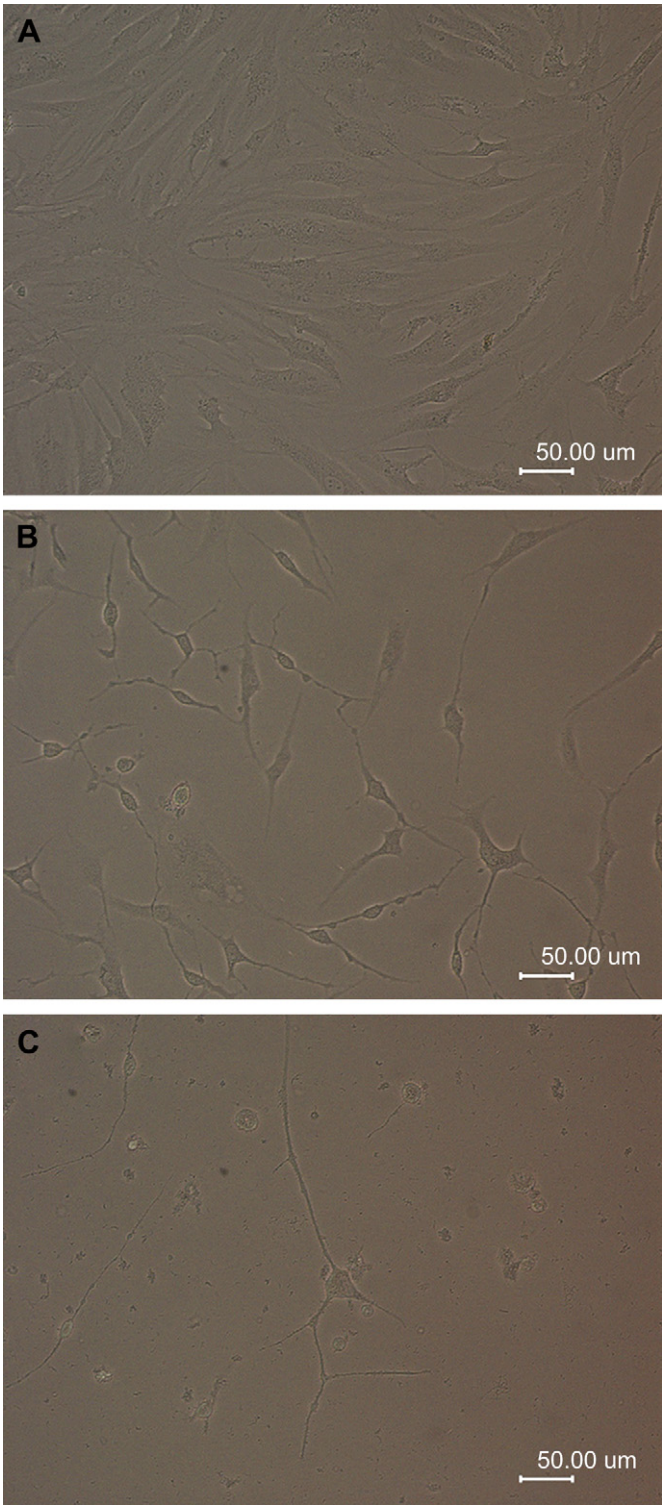


**Fig. 2.** Differentiation of hBMSC grown in a nerve induction medium. (A) Original hBMSC; (B) hBMSC grown for 36 h in the nerve induction medium; (C) hBMSC grown for 60 h in the nerve induction medium.

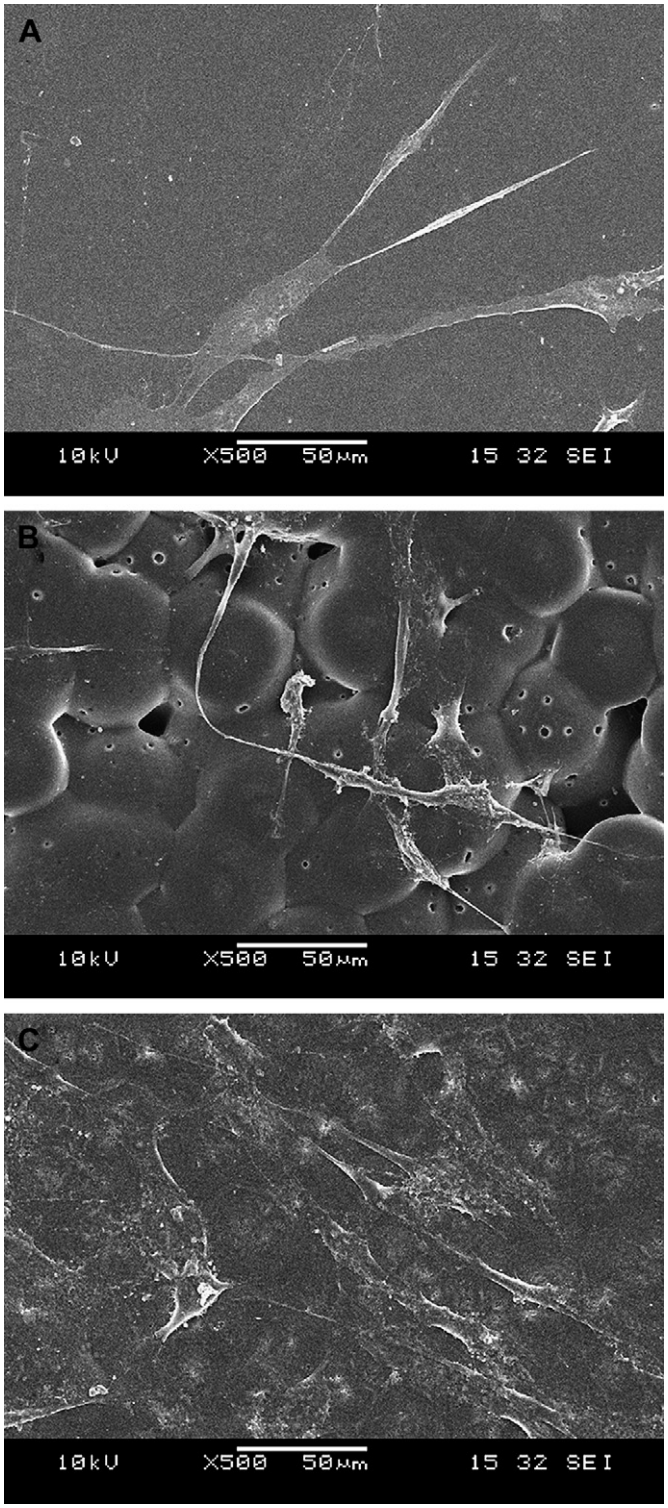


**Fig. 3.** Morphologies of (A) PLA, (B) PHBHHx, (C) PHBVHHx films and growth of hBMSC on respective films under SEM. (500×) hBMSC were cultured for 72 h in a non-differnetiation medium.

investigate the effect of porosity on cell growth in the 3D scaffolds, equal numbers of hBMSC were seeded on scaffolds having various pore sizes fabricated under different temperatures stated above. As shown in Fig. 6, cell proliferation was better in the scaffolds with bigger pore size, and cell number decreased with decreased pore size, which was confirmed by the decreased fluorescence intensities (Fig. 6D).

### *13.6. Gene expression study related to nerve cells differentiated from hBMSC*

Studies of gene expression by real-time PCR was performed to evaluate the differentiation of hBMSC grown in PHBVHHx 3D scaffolds of various pore sizes and films PLA, PHBHHx and

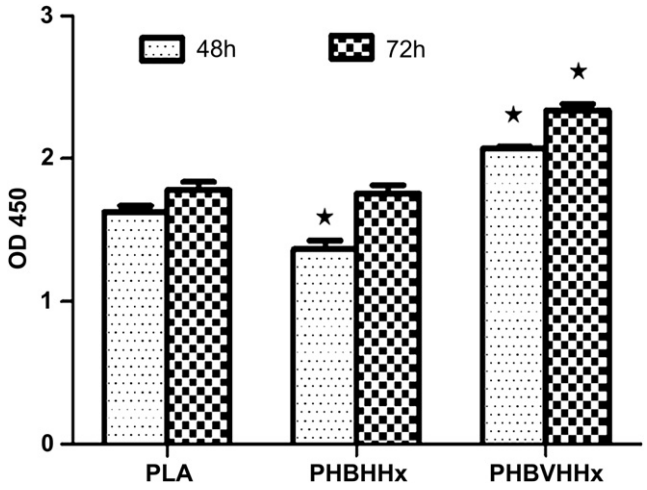


**Fig. 4.** Study of hBMSC proliferation using CCK-8 assays. hBMSC were cultured on films of PLA, PHBHHx or PHBVHHx for 48 h and 72 h, respectively ($n = 6$).

PHBVHHx, respectively. hBMSC were seeded on above samples and cultured in nerve induction medium for 3 days. Three key nerve cells related marker proteins, namely, nestin, an intermediate filament protein expressed by neural stem cells or progenitor cells; glial fibrillary acidic protein (GFAP), a structural element of fibrillary astrocytes; and β-III tubulin, a neuronal cytoskeletal dimmer [31], were studied. Nestin and GFAP exhibited a similar expression in hBMSC grown on PLA, PHBHHx and PHBVHHx films, β-III tubulin showed a higher expression for those grown on PHBVHHx films compared with that on PLA and PHBHHx films (Fig. 7). Stronger expression of three nerve marker genes was clearly observed on scaffolds compared with that on 2D films. Intensity of nestin gene expression increased with decreased pore size (Fig. 7A). β-III tubulin and nestin exhibited comparatively high expression when hBMSC were grown on PHBVHHx scaffolds prepared at −80 °C and −196 °C, however, undesirable factor GFAP also had high expression when hBMSC were grown on PHBVHHx scaffolds prepared at −196 °C. These results imply that microenvironment of PHBVHHx scaffolds with suitable pore sizes promote to generate more neuron and less astrocytes, which is useful to avoid gliar scar formation resulted from astrocytes from spinal cord injury. The above results suggest that −80 °C is a suitable preparation temperature for PHBVHHx scaffold for nerve differentiation.

## 4. Discussion

PHA have attracted increasing interest as tissue engineering materials due to their adjustable physical properties, biodegradability and good biocompatibility [32–34]. Copolyesters PHBHHx have been reported as a potential implant material [15]. In vitro studies demonstrated that PHBHHx has good compatibility for several cells including fibroblasts, chondrocytes and osteoblasts [22,35,36].

Recently terpolyester PHBVHHx was revealed to have stronger cell adhesion and proliferation for hBMSC compared with other materials including tissue culture plates (TCP), PLA and PHBHHx [18]. Among all the PHA available for biomaterial studies, PHBVHHx is so far the only material to show better cell proliferation compared with that on TCP.

In this study, PHBVHHx was confirmed to promote growth of hBMSC, the proliferative hBMSC grown on PHBVHHx scaffolds was then found to differentiate into nerve cells in the nerve induction medium.

PHBVHHx film showed a rougher surface with more hydrophobicity than that of PLA and PHBHHx [18]. Strong hydrophobicity

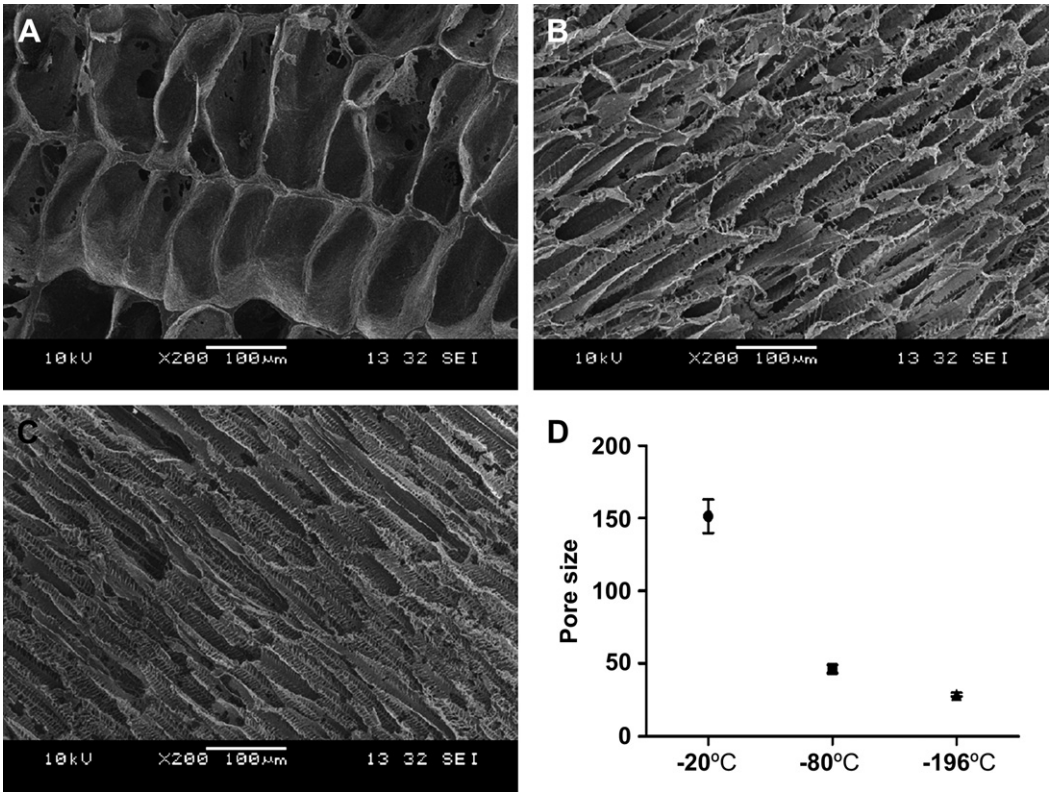


**Fig. 5.** SEM images of PHBVHHx scaffolds with various pore sizes. (A) Scaffolds prepared in 1,4-dioxane under −20 °C; (B) Scaffolds prepared in 1,4-dioxane under −80 °C; (C) Scaffolds prepared in 1,4-dioxane under −196 °C; (D) Effect of preparation temperature on scaffold pore size.

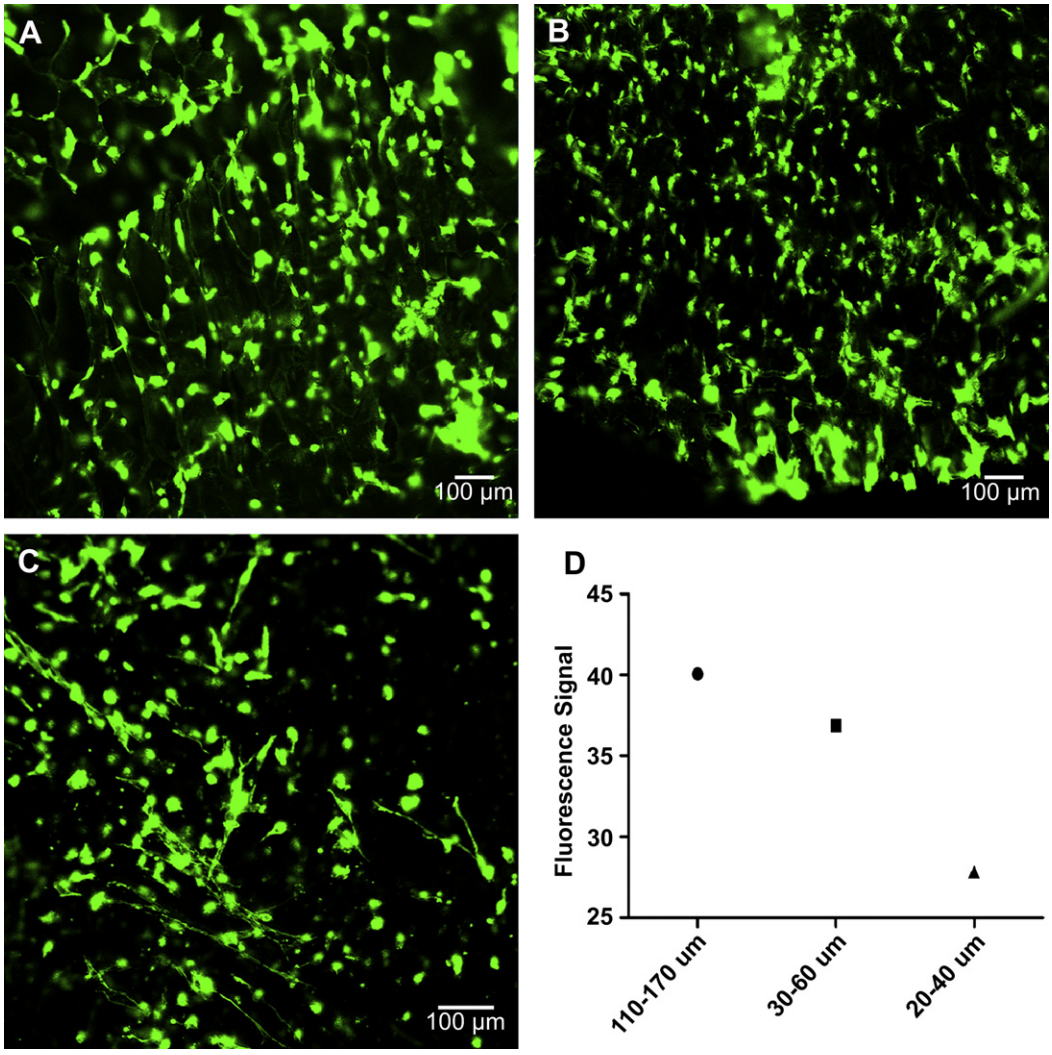


**Fig. 6.** Imaging of hBMSC grown for seven days in 3D PHBVHHx scaffolds under laser scanning confocal microscopy. (A) Phalloidin-FITC staining on cell F-actin in scaffold prepared under −20 °C; (B) Phalloidin-FITC staining on hBMSC in scaffold prepared under −80 °C; (C) Phalloidin-FITC staining on hBMSC in scaffold prepared under −196 °C; (D) The intensities of fluorescence signals.

helps to promote protein adsorption and hBMSC adhesion. Beside the hydrophilicity, surface morphology of a biomaterial can also influence cell attachment and growth behavior [37,38], different cells had different responses to different surface roughness and hydrophilicity [38,39].

Surface properties of materials seemed to be appropriate for cell adhesion and proliferation, they also affect cell differentiation [40]. In this study hBMSC grown in nerve induction medium exhibited a slight higher β-III tubulin expression when grown on PHBVHHx films compared with that on PLA and PHBHHx ones, yet no significant difference in nestin and GFAP expression was observed on three films (Fig. 7). However, much stronger gene expressions related to neural phenotypes were observed on hBMSC grown in 3D PHBVHHx scaffolds (Fig. 7). Improved cellular communication in a 3D culture grown in scaffolds may explain the enhanced differentiation compared with that in a 2D culture. Of course for functional cell formation, optimal 3D culture conditions should be further investigated [30].

Strong nerve differentiation from hBMSC grown in PHBVHHx 3D scaffolds immersed in nerve induction medium was clearly observed by real-time PCR results (Fig. 7). Obviously, porosity of the 3D scaffolds strongly affected intensity of nerve differentiation (Fig. 7), this agrees with previous studies suggesting that scaffold pore sizes were important for tissue engineering [41], larger pore sizes hindered differentiation, smaller pore sizes enhanced differentiation. This observation agreed with our results achieved in PHBVHHx scaffolds (Figs. 6 and 7).

Recent study showed that astrocytes may form glia scar, which is undesirable in therapies targeting spinal cord injuries [42]. Many studies emphasized the importance of minimizing astrocytic differentiation [43]. Cell differentiation has been demonstrated to be strongly affected by interactions with external physical and chemical stimuli [44]. An aligned nanofiber matrix could discourage the differentiation of ES cells into astrocytes [42], although other influences of a nanomaterial on human health and environments are not yet well understood [45]. To select an optimal scaffold pore size for limiting glia scar formation, we found that PHBVHHx scaffolds prepared under −80 °C with pore sizes of 30–60 μm induced a lower GFAP expression, a higher nestin and β-III tubulin expression to be the most beneficial ones for nerve injury repair.

Previous studies suggested that bigger pore size led to a better cell proliferation [46,47]. Our results also indicated that there were an intensity decrease of fluorescence signals for hBMSC grown in 3D PHBVHHx scaffolds with decreased pore size prepared at

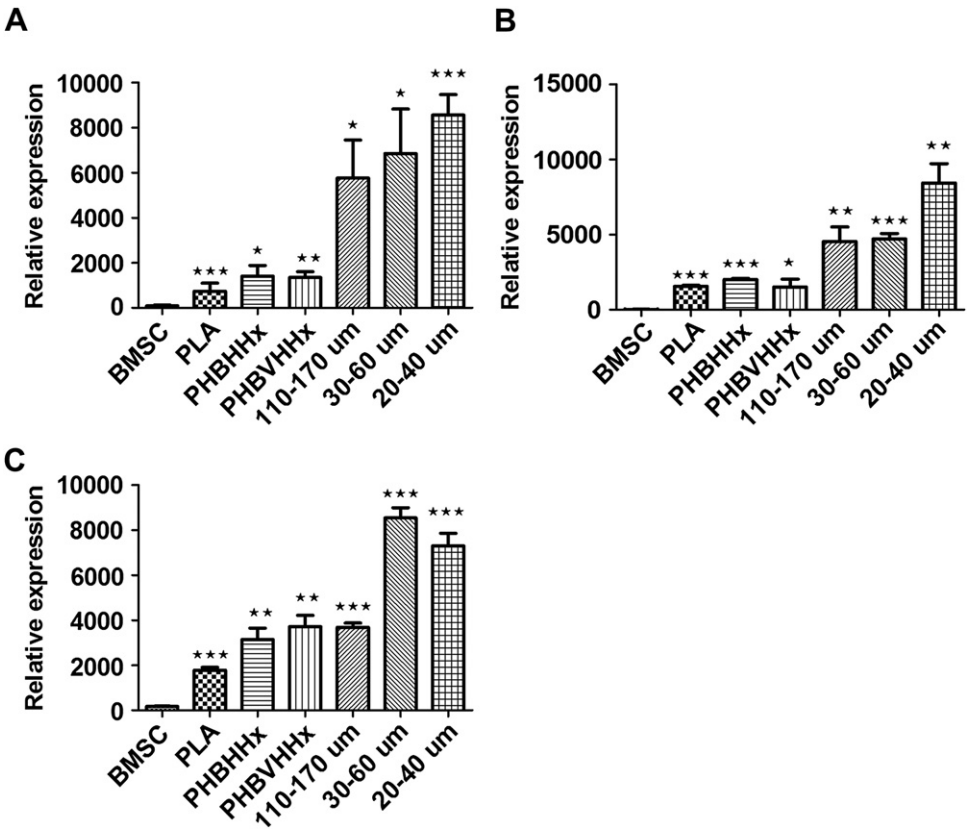


**Fig. 7.** Real-time PCR study of gene expressions related to nerve cells differentiated from hBMSC grown in nerve differentiation medium on films of PLA, PHBHHx or PHBVHHx, or on PHBVHHx scaffolds prepared under −20, −80 or −196 °C, leading to pore sizes of 110–170 μm, 30–60 μm and 20–40 μm, respectively. Relative expression of (A) Nestin; (B) GFAP; (C) β-III tubulin. ★: $p < 0.05$; ★★: $p < 0.01$; ★★★: $p < 0.001$ ($n = 3$).

−20 °C, −80 °C and −196 °C. The biggest pores in the range of 110–170 μm in scaffolds prepared at −20 °C supported the highest cell proliferation (Fig. 6), and there was only slight decrease of fluorescence intensity from −20 °C to −80 °C scaffolds. It is possible that suitable pore size and interconnectivity within the scaffolds facilitated cells migration, leading to lesser cell death in the initial growth phase due to reduced overcrowding situation. On the other hand, physical and chemical properties of PHBVHHx definitely played an important role in influencing microenvironment in the scaffolds.

Scaffolds with pore sizes of 30–60 μm prepared at −80 °C were showed to promote neuron differentiation (Figs. 6 and 7), decrease glial scar formation and enhance cell proliferation compared with scaffolds with larger and smaller pore sizes prepared at −20 °C and −196 °C, respectively. The results suggest that fabrication of PHBVHHx scaffolds with pore sizes of 30–60 μm is more suitable for nerve tissue engineering via hBMSC differentiation.

## 5. Conclusion

Terpolyester PHBVHHX films showed stronger cell adhesion, proliferation and differentiation for hBMSC compared with that of well studied PLA and PHBHHx. PHBVHHx scaffolds immersed in nerve differentiation medium were shown to promote hBMSC differentiation compared with their 2D cultures. PHBVHHx scaffolds prepared at −80 °C provided pore sizes of 30–60 μm, these revealed the most suitable environment for cell proliferation and nerve differentiation from hBMSC. PHBVHHx scaffolds with right pore sizes based on hBMSC differentiation can be used for nerve injury repair.

## Acknowledgement

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## Appendix

Figures with essential colour discrimination. Figs. 2 and 6 of this article may be difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2009.11.053.

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