CHAPTER V
ENHANCEMENT OF BIOCOMPATIBILITY ON ALIGNED ELECTROSPUN POLY(3-HYDROXYBUTYRATE) SCAFFOLDS IMMOBILIZED WITH LAMININ TOWARDS MURINE NEUROBLASTOMA CELL LINE AND RAT BRAIN-DERIVED NEURAL STEM CELLS

5.1 ABSTRACT

Electrospinning has been extensively used to construct tissue-engineered scaffolds because it is a simple fabrication process that can easily produce nano- and micro-size synthetic polymeric fibers. Electrospun fibers are structurally analogous to the naturally occurring protein fibrils/fibers in the extracellular matrix (ECM) of the body. Although electrospinning can produce sub-micron fibrous scaffolds, modification of electrospun scaffolds with bioactive molecules is beneficial as this can create an environment that consists of biochemical cues to further promote cell adhesion, proliferation and differentiation. In the present contribution, we investigated the feasibility to immobilized laminin onto the electrospun poly(3-hydroxybutyrate) (PHB) fibrous scaffold. Laminin was successfully added to scaffold using covalent binding combination with physical adsorption. The potential for use of the surface modified fibrous scaffolds for neural regeneration was evaluated *in vitro* towards murine neuroblastoma Neuro 2a cell line and, mouse brain-derived neural stem cells (NSCs). Both types of laminin coupled on the PHB fibrous scaffold supported the attachment and the proliferation of Neuro 2a very wells. Despite the good attachment and proliferation of Neuro 2a, NSCs were not able to proliferate on the neat PHB, hydrolyzed PHB and laminin coupled on hydrolyzed PHB fibrous scaffold.

(**Keywords**: Electrospinning; Nervous scaffold; Poly(3-hydroxybutyrate); Protein immobilization)

5.2 Introduction

Tissue engineering is an emerging technology in the contemporary human health care administration, in which the basic understanding of cellular biology and bioengineering are combined together for developing feasible substitutes to aid in the clinical treatment [1]. An attempt to repair the nerve injury has been explored for several hundred years with a major problem associated with the spinal cord treatment requires an additional care or new medical therapy, because axons do not regenerate appreciably in their native environment [2-4]. Clinically, recent advances in the neural tissue engineering provide to mimic the biological structure by creating the suitability environmental substitutes for neural regeneration [4–6]. The primary objectives of these substitutes are to restore, maintain and/or improve tissue functions [5]. In addition, a functional scaffold should mimic the structure and biological function of native extracellular matrix (ECM) proteins [7], so as to provide mechanical support and maintain the normal differentiated state of cells within the cellular compartment [8].

A wide variety of fabrication techniques have been used to generate 3D polymeric scaffolds for potential use in neural tissue engineering [9-14]. Among the various fabrication techniques, electrospinning has received more heavily study because of their attractive features such as high surface area, interconnecting pores, and good mechanical support [15-17]. In particular, electrospinning techniques have allowed the production of fibers with average diameters in the submicrometer to nanometer range which can mimic the architectural and topographic features of the extracellular matrix (ECM) [7,15]. These small fibers not only provide high surface area, but also present a means to control cellular and behaviors, such as adhesion and proliferation [14-16]. In addition, the fiber diameter and fiber alignment has been found to regulate cellular behaviors including contact guidance and differentiation [14,18].

Recently, a number of biological and synthetic biomaterials have been investigated for their suitability as matrixes for nerve regeneration [10-14, 18-25]. Polyhydroxyalkanoates (PHAs) are a class of naturally occurring biodegradable and biocompatible thermoplastic polyesters produced by various microorganisms,

making them attractive materials to be used as scaffolds for engineered tissues. Poly(3-hydroxybutyrate) (PHB) is the most thoroughly investigated member of the PHA family and has shown good biocompatibility for *in vitro* and *in vivo* studies [26-28]. PHB is an ideal biomaterial, since it completely degrades to release a normal component of blood and tissue, d,l-β-hydroxybutyrate (HB) [29]. These outstanding properties render PHB good candidates for tissue-engineered scaffolds. However, the sole use of scaffolding materials may not be sufficient for the treatment of an injured nerve, likely as a result of the lack of chemical cues that help to promote the neural regeneration [3-6]. In order to enhance the cell-scaffold interaction, polymer surface modification may potentially be used to create the suitability environment. These modifications generally involve enriching surfaces with extracelluar matrix (ECM) components such as the adhesive protein (e.g. fibronectin (FN) [30-31], and laminin [32-33]), or their functional domains biomolecules [34-41]. Laminin, a family of glycoproteins which is an integral part of the basement membrane is known to be important on cell differentiation, migration and adhesion, will be used as the contact guidance biochemical cues for axonal outgrowth [42-45]. Several studies have illustrated that even as Schwann cells could proliferate and migrate along axons, differentiation of myelinating phenotype was not observed without the presence of laminin [46,47]. Furthermore, *in vitro* experiments have shown that neurite outgrowth is enhanced on scaffolds that were covalently bound or physical adsorption with laminin [48-50]. Therefore, the incorporation of laminin onto nanofibers can potentially improve the ability of neural regeneration.

Despite the numerous reports on the *in vitro* responses of difference cell lineages on the various substrates coupled with laminin, a similar report that study and compare the *in vitro* responses and cellular behavior of any type of chemical surface modification is still lacking. In the present contribution, we report the *in vitro* biological evaluation of surface modified aligned electrospun PHB fibrous substrate to serve as scaffold for neural regeneration. The study was preliminary evaluated *in vitro* towards murine neuroblastoma Neuro 2a cell line (American Type Culture Collection : ATCC). However, for potential uses in specific application, all of the fibrous scaffolds were further evaluated with mouse brain-derived neural stem cells

(mNSCs). The neat electrospun PHB fibrous scaffold was used as the internal controls.

5.3 Experimental

5.3.1 Materials

Materials used in the fabrication of the fibrous scaffolds were poly(3-hydroxybutyrate) (PHB; Mw = 300,000 $g \cdot mol^{-1}$; Sigma-Aldrich, USA). Chloroform used as the solvent, was purchased from Lab-scan (Asia), Thailand. For the surface modification of the electrospun PHB fibrous scaffolds, natural mouse laminin and phosphate buffer saline (PBS; pH = 7.4), were purchased from Invitrogen Corporation, USA. 1,6-hexamethylenediamine (HMD), N-Hydroxysuccinimide (NHS), N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and (N-morpholino) ethanesulfonic acid (MES) were purchased from Sigma–Aldrich, USA. Sodium hydroxide and isopropanol (IPA) were purchased from UNIVAR-Ajax Finechem, Australia. All other chemicals were of analytical reagent grade and used without further purification.

5.3.2 Fabrication of the Aligned Electrospun PHB Fibrous Scaffolds

The aligned electrospun PHB fibrous scaffolds were prepared according to the conditions previously described [51]. Briefly, the spinning solutions were prepared by dissolving 14% (w/v) PHB in chloroform at 60 °C. Each of the spinning dopes was contained in a 50-ml glass syringe, the open end of which was connected to a gauge 20 stainless steel needle (OD= 0.91mm), used as the nozzle. A rotating drum (width and OD of the drum ≈ 15 cm; rotational speed = 1000 rpm) was used as a collector. The outer surface of the rotating drum was covered with an aluminum sheet and set about 20 cm from the tip of the needle. The temperature of the spinning solution was maintained at 60 °C via a home-made programmable heater band, wrapped around the glass syringe. A Gamma High Voltage Research DES30PN/M692 power supply was used to generate a fixed dc potential of 15 kV. The collection time was also fixed at about 8 h. Fiber alignment was quantified by measuring the mean fiber angle from 5 SEM images. These values were then

normalized to 90 degrees and plotted in a histogram. Closer to 90 degrees indicates more alignment.

5.3.3 Covalent Binding of Laminin onto Nanofibers

Figure 5.1 summarized the strategy used to immobilize laminin on the surface of the electrospun PHB fibrous scaffolds. The PHB fibrous scaffold was first immersed in an ethanolic aqueous solution (1:1 v/v) for 2 to 3 h to clean the fiber surface and then washed with a large quantity of deionized water. The surface aminolysis was performed according to the modified method reported by previous work [37]. To maintain enough mechanical properties for practical application, electrospun scaffold was aminolyzed for 15 min in 0.04 g/ml of HMD/IPA solution at room temperature. The aminolyzed scaffold was then rinsed successively with deionized water to remove unreacted HMD and dried *in vacuo* to rich a constant weight. For surface hydrolysis, the neat scaffold was performed according to the method reported by Cai *et al* [39]. After cleaned the fiber surface, the scaffold was subsequently immersed in an aqueous solution of 1.0 M NaOH for 15 min at room temperature. After which time, the scaffold was taken out, washed with 1.0 M HCl to neutralize the residue NaOH. Finally, the hydrolyzed scaffold was rinsed successively with deionized water to remove unreacted HCl and dried in vacuo to rich a constant weight.

Subsequently the aminolysis and hydrolysis treatment, the surface modified fibrous scaffold was immersed in (N-morpholino) ethanesulfonic acid (MES) buffered solution (0.10 M, pH 5.0) contained of 5 mg/mL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 5 mg/mL of N-hydroxysuccinimide (NHS) for 4 h at room temperature. The scaffold was then rinsed with MES buffer and immersed in laminin solution (1 μg/mL) for 24 h at 4 °C. The laminin was immobilized through a condensation reaction between the amino or carboxylic group on the fibrous surface and functional group of laminin. Then, covalently bounded laminin-PHB fibrous scaffold was rinsed successively with 0.10 M PBS to remove unreacted laminin that adsorbed on the fibrous surface and finally dried at room temperature prior to further investigation.

5.3.4 Characterization of Surface Modified PHB Fibrous Scaffolds

The morphological and fiber arrangement of electrospun fibrous scaffold was studied by a JEOL JSM-5200 scanning electron microscope (SEM) with an accelerating voltage of 15 kV. Before the observation, the scaffolds were coated with gold using a JEOL JFC-1100E sputtering device for 3 min prior to SEM observation. The diameter of the fiber was measured from the SEM photographs using image analysis software (SemAfore; JEOL, Insinööritoimisto J.Rimppi Oy, Finland).

The wettability of the neat and surface modified fibrous scaffold was assessed by water contact angle measurements. The static water contact angle was measured by a sessile drop method using a KrÜss contact angle measurement system. A distilled water droplet of about 8 μl was gently plated on the surface of each specimen. At least 10 readings on different parts of the specimen were averaged to obtain a data point. All samples were dried under vacuum at 30°C for 24 h and the measurements were processed at room temperature.

To determine the amount of amino ($-NH_2$) groups on the surfaces of aminolyzed fibrous membrane, ninhydrin assay was employed. First, the fibrous scaffold was immersed in 1.0 M ninhydrin/ethanol solution for 1 min in a glass tube, followed by heating at 80°C for 15 min to accelerate the reaction between ninhydrin and the NH_2 groups that might be present on the surfaces of the scaffolds. As an evidence for the actual presence of the NH_2 groups, the surfaces would turn blue. After complete evaporation of absorbed ethanol, chloroform was added in the tube to dissolve the scaffolds. IPA was then added to stabilize the blue compound. The amount of NH_2 groups was finally quantified by observing the absorbance of the obtained mixture at 538 nm using a Shimadzu UV-2550 UV-Vis spectrophotometer against a predetermined calibration curve that was obtained from HMD solutions in chloroform/IPA (1:5 v/v). For the amount of carboxyl groups on the surfaces of hydrolyzed fibrous membrane, the reaction with TBO was determined. The hydrolyzed fibrous scaffold was immersed into a 0.5 mM TBO aqueous solution with a pH value of 10. The formation of ionic complex between the COOH groups and the cationic dye was allowed to proceed for 12 h at room temperature. After the immersion, the fibrous scaffold was rinsed with 0.1 mM NaOH solution to remove

the unbound TBO molecules. Finally, the bonded TBO on the fibrous surface was desorbed by incubation in 4 mL 50% acetic acid solution for 10 min. The absorbance at 633 nm was recorded against a predetermined calibration curve that was obtained from TBO in 50% acetic acid solution. The calculation is based on the assumption that 1 mol TBO has complexed exactly with 1mol carboxyl groups [52].

To quantify the surface atomic composition, X-ray photoelectron spectroscopy (XPS) was also investigated. XPS was carried out using a Thermo Fisher Scientific Theta Probe XPS instrument equipped with a monochromatic Al Kα X-ray. The analysis area was 400 μm x 400 μm on the polymer surfaces. The maximum analysis depth lay in the range of ~4-8 nm. The atomic ratio of carbon, oxygen and nitrogen on the surface was used as a marker to analyze the relative amount of functional groups and laminin-immobilized on the modified PHB surface.

Since XPS only examined the surface of the functionalized fibrous scaffold, quantification of laminin coupled onto the nanofibers was achieved using BCA protein assay. The protein assay (The Thermo Scientific Pierce® BCA Protein Assay Kit, USA) was used according to manufacturer's test tube protocol for analysis of the amount of laminin immobilized onto the surface fibrous scaffold. Laminin concentration was calculated from laminin standard curve.

5.3.5 Cell Culture and Cell Seeding

To evaluate the potential for use of the modified electrospun PHB fibrous membrane as neural scaffolding materials, their biocompatibility in terms of cytotoxicity, cell adhesion, cell proliferation and neurite outgrowth toward murine neuroblastoma Neuro 2a cell line (American Type Culture Collection : ATCC) and mouse brain-derived neural stem cells (mNSCs) was evaluated in comparison with that of unmodified fibrous scaffold.

Neuro 2a were first cultured as a monolayer in MEM/EBSS medium (HyClone), supplemented by 10% fetal bovine serum (HyClone), 2 mM L-glutamine (Gibco) and 1X Pen/Strep (Gibco). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2, and the culture medium was replaced once every 2 d. Each of the fibrous scaffolds was cut into circular disks (~15 mm in diameter), and the disk specimens were placed in the wells of a 24-well TCPS (Biokom System, Poland), which were later sterilized in 70% ethanol for 10 min. The specimens were

then washed with autoclaved deionized water and subsequently immersed in MEM/EBSS overnight. To ensure a complete contact between the specimens and the wells, each specimen was pressed with a stainless steel ring (~15 mm in diameter). The reference cells from the cultures were trypsinized [0.25% Trypsin-EDTA (Gibco)], and seeded on the fibrous scaffold specimens.

Primary culture of NSCs was prepared from neonatal one-day mice. Briefly, rat cerebral cortices were dissected, cut into small pieces and mechanically triturated in PBS. The dissociated cells were collected by centrifugation and were resuspended in neuorobasal medium containing 20 ng/mL bFGF, 20 ng/mL EGF, 2 mM L-glutamine, 1X Pen/Strep and 1X B27 supplement. Cells were cultured in an incubator which was maintained at 37°C in 5% CO_2. After 1 day of culture, suspended cells underwent cell division. Cell division continued for an additional 2 ~ 3 days, after which proliferating cells formed neurospheres. Subsequently, adherent cells were discarded and the suspended neurospheres were collected by centrifugation, mechanically dissociated and replated as single cells in the fresh culture medium at a density of 1×10^5 cells/ml.

5.3.6 Cytotoxicity Evaluation

The indirect cytotoxicity evaluation of the modified PHB fibrous scaffolds was conducted in adaptation from the ISO10993-5 standard test method. First, the extraction media were prepared by immersing the specimens, cut from both the fibrous and the film scaffolds (~7 mm in diameter), in wells of a 96-well TCPS in a serum-free medium (SFM; containing MEM-EBSS, 1% L-glutamine, and 1% Pencilinstreptomycin) and incubated for 24 h. In the preparation of the reference cells, Neuro-2a cells were seeded in the wells of a 96-well TCPS at a density of 1.0 x 10^4 cells/well and incubated in 5% SFM to allow cell attachment on the plate. After 24 h, the culture medium was removed and the as-prepared extraction media were added to the wells. The cells were incubated further for 24 h, after which time the number of viable cells was quantified with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The viability of the cells that were cultured with fresh SFM was used as the control.

5.3.7 Cell Attachment and Cell Proliferation

For the cell attachment study, the reference cells were seeded on both the modified and unmodified fibrous scaffold specimens (~15 mm in diameter) in the wells of a 24-well TCPS at a cell density of 4.0×10^4 cells/well and allowed to attach to the scaffold specimens for 4 h. The viability of the cells on the unmodified scaffold was used as the control. At specified seeding time, the viability of the attached cells was quantified by the MTT assay. Each specimen was rinsed with phosphate-buffered saline (PBS;Sigma-Aldrich) to remove unattached cells prior to MTT assay.

For the cell proliferation study, the cells were first seeded on both the modified and unmodified fibrous scaffold specimens (~15 mm in diameter) in the wells of a 24-well TCPS at a cell density of 4.0×10^4 cells/well and allowed to attach to the scaffold specimens for 24 h. After the attachment period of 24 h, the cells were starved with SFM twice (i.e., the medium was changed with SFM once after the 24 h attachment period and again after 2 d). The proliferation of cells on the specimens was quantified by MTT assay after days 1 and 3 of cell culture. Each experiment was carried out in triplicate.

5.3.8 Quantification of Viable Cells (MTT Assay)

The MTT assay is the method used to quantify the viability of cells on the basis of the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals relates to the number of the viable cells in a linear manner. First, the culture medium of each cultured specimen was removed and replaced with 500 μl/well of MTT solution (Sigma–Aldrich, USA) and then the plate was incubated for 3 h. After incubation, the MTT solution was removed. Then, 500 μl well of dimethyl sulfoxide (DMSO; Riedel-de Haën, Germany) was added to dissolve the formazan crystals and the plate was left at room temperature in darkness for 2 h on a rotary shaker. Finally, the absorbance at 570 nm, representing the proportion of the viable cells, was recorded by a Tecan infinite M200 instrument using microplates (Tecan, Germany).

5.3.9 Statistical Analysis

The data are presented as means ± standard errors of the means (n = 3). A one-way ANOVA was used to compare the means of different data sets and a statistical significance was accepted at a 0.05 confidence level.

5.3.10 Morphological Observation of Cultured Cells

After the culture medium had been removed, the cell cultured fibrous membranes were rinsed with PBS twice and the cells were fixed with 3% glutaraldehyde solution (diluted from 50% glutaraldehyde solution with PBS) at 500 μl/well. After 30 min, they were rinsed again with PBS. After cell fixation, the specimens were dehydrated in an ethanol solution of varying concentration (i.e., 30%, 50%, 70% and 90%) and, finally, with pure ethanol for about 2 min each. The specimens were then dried in 100% hexamethyldisilazane (HMDS; Sigma–Aldrich, USA) for 5 min and later dried in air after the removal of HMDS. After being completely dried, the specimens were mounted on copper stubs, coated with gold using a JEOL JFC-1100E sputtering device for 3 min and observed by a JEOL JSM-5200 scanning electron microscope.

5.3.11 Immunocytochemistry

Immunostaining on the various fibrous scaffolds were performed after day 2 of culturing. 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-b-tubulin III : 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 dihydrochloride (Invitrogen) for 10 min at room temperature. Images were taken under a fluorescence microscope equipped with a charge-coupled device camera (Carl Zeiss Axio Observer Z1).

5.4 Results and Discussions

The neural regeneration is a complex biological phenomenon. A major problem associated with the treatment of nerve injury requires an additional care or new medical therapy, because nervous do not regenerate appreciably in their native environment. Recent advances in the artificial nervous scaffolds can be designed to create a favorable environment for neural regeneration, have become an alternative strategy for the restoration of nerve injuries. Many strategies have been developed to fabricate the neural scaffolding materials. Among the various techniques, fibrous scaffold derived from electrospinning exhibits an excellence candidate due to the possibility of generating the fiber diameter analogous to the extracellular matrix (ECM) of native tissue. A few studies demonstrated that surface topography introduced by electrospun fibers of affects cell morphology and cell proliferation [9,14-16]. In addition, electrospun fibers can be readily aligned in to uniaxial array. The resulting anisotropic material properties have been shown to be effective cue to direct and enhance the neural regeneration process [9,14-16]. Recently, a number of biological and synthetic biomaterials have been investigated for their suitability as matrixes for nerve regeneration [10-14,18-25]. PHB is a promising scaffold material due to its biocompatibility and biodegradability. Studies related to the *in vitro* biocompatibility of electrospun PHB with different type of cells have usually been studied [25-29]. Despite the inherent biocompatibility and biodegradability of PHB, actual utilization of this material as artificial scaffolding material is limited by its hydrophobicity. Many chemical strategies were used to improve biocompatibility such adsorption or immobilization of certain cell recognition molecules on the surface of a substrate to obtain controlled interaction between cells and the synthetic substrate [30-41].

Here, electrospinning technique was used to fabricate aligned PHB fibrous membrane, to be used as artificial nervous scaffold. A selected SEM image of the as spun PHB fibrous scaffold is shown in Figure 5.2a. Clearly, fibers with smooth and bead-free structure were obtained and the diameter of these fibers was 2.15 ± 0.22 μm with fiber alignment between -25° and +40° degrees normalized to 90 degrees (see Figure 5.2b). More than 35%, 25% and 15& of fibrous scaffold was found in the

orientation of 0°, +5° and -5°, respectively. After consecutive spinning for 8 h, the thickness of the fibrous scaffold was 364 ± 10 μm. As mention, the sole use of scaffolding materials may not be sufficient for regeneration process due to its hydrophobicity surface, likely as a result of the lack of chemical cues that help to promote the cellular ingrowth [3-6]. One approach to solve this problem, polymer surface modification may potentially be used to enhance the cell-scaffold interaction. In the present work, the potential use of surface modified electrospun PHB fibrous scaffold in neural tissue engineering was preliminary evaluated *in vitro* towards murine neuroblastoma Neuro 2a cell line (American Type Culture Collection : ATCC). However, the potential use in specific application such cell replacement in central nerve injury was evaluated *in vitro* towards mouse brain-derived neural stem cells (mNSCs). The evaluation was carried out in terms of the ability of the scaffold to support the attachment of cells (i.e., the cell attachment study) and to promote the increase in the number of the attached cells (i.e., the proliferation study). The morphology and neurite outgrowth of the cultured cells on the membranes was also evaluated. The neat electrospun PHB fibrous scaffold was used as the internal controls.

5.4.1 Morphology and Physio-chemical Properties of Surface Modified Electrospun PHB Fibrous Scaffolds

The major motivating factors supporting the use of electrospun fibrous scaffolds in neural tissue engineering is the ability to mimic the scale and 3D-dimensional arrangement of collagen fibrils in the ECM and ease of control the fiber direction in order to provide the contact mediation for neural regeneration process. However, such a hydrophobicity of various synthetic polyester polymers has been known to be unfavorable for cell adhesion and growth. In the present work, electrospinning was employed to produce the aligned electrospun PHB fibrous scaffold, and further subject to surface modification via surface-specific lysis reaction. For aminolysis, one amino group ($-NH_2$) of HMD reacts with the ester group (–COO–) of PHB to form the amide linkage (–CONH–), leaving another free amino group. However, for hydrolysis, the ester groups of polymer chain react with hydroxide anion (HO^-), leading to breakage of the ester linkage and formation of

carboxyl (-COOH) groups on the chain termini. The introduction of $-NH_2$ or -COOH groups onto the fiber surface not only improve the hydrophobicity, but also provide the necessary active sites use for covalent binding with cell-adhesive protein. Laminin is a well-known cell-adhesive protein that helps to promote the cellular adhesion and spreading [42-45]. The morphological structures of aligned electrospun PHB fibers before and after subject to the surface treatment reaction are shown in Figure 2. The topographies of fibrous scaffold could possibly change as a result of chemical modifications. SEM analysis showed that, while the original PHB fibers had smooth texture (Figure 5.3a), the topographies changed to a somewhat small wedge structure on the surface (Figure 5.2b and 5.3c) as they are subjected to chemical modification. However, after protein immobilization, the fibers have uniform textures with roughness suggesting that the thin layer of laminin is coverage of the surface (Figure 5.3d and 5.3e).

To assess the degree of surface aminolysis and hydrolysis, the quantity of free amino and carboxyl groups existing on the surface of the A-PHB and H-PHB fibrous scaffold was employed. Without the presence of chemical treatment, amino and carboxylic groups could not be detected. In the presence of HMD, the average areal density of the amino groups was 2.42×10^{-7} mol $\cdot$ cm^{-2}, whereas the average areal density of the carboxylic groups was 4.33×10^{-7} mol $\cdot$ cm^{-2} in the presence of NaOH. The wettability of the surface modified PHB fibrous scaffold with respect to double-distilled water was shown in Table 5.1. After the introduction of hydrophilic group such amino and carboxylic onto the fiber surface, water contact angles decrease from 135.3 ± 0.47 for original PHB fibrous scaffold to 97.6 ± 0.96 and 89.5 ± 0.79 for surface aminolysis and hydrolysis, respectively. The difference in hydrophilicity of aminolysis surface comparison with that of hydrolysis surface should be a result of the surface roughness and the amount of surface functional groups that presence on the fibrous surface. In addition, the water contact angles decreased again after laminin was immobilized on the modified scaffold. These results demonstrate that the surface wettability is greater enhanced by protein immobilization.

The composition of the outermost layer of PHB fibrous scaffold before and after surface modification was further determined by X-ray photoelectron

spectroscopy (XPS), and the results are shown in Table 5.2 and Table 5.3. The presence of nitrogen atom indicates the introduction of amino groups on the PHB fibrous scaffold. In addition, the increase in the N/C ratio from 0.0133 for A-PHB to 0.0271 for LA-PHB also reveals the success of laminin binding on the fibrous surface. For surface hydrolysis, there is a little difference between the O/C ratio of PHB from 0.4245 to 0.4326 of H-PHB surface. This result indicated that the NaOH treatment could not significantly alter the atomic composition of the top most layer of polymers. The surface of LH-PHB scaffold contained nitrogen as was expected after laminin immobilization. However, the N1s peaks of LH-PHB scaffold were resolved into two peaks with binding energies of 401.9 and 399.7 eV, which were attributed to nitrogen in C-N bond and $-NH_3^+$, respectively (see supporting information). The presence of $-NH_3^+$ also reveals the success of laminin binding on the fibrous surface by complexation.

Since XPS only examined the presence of functionalized surface of modified PHB fibrous scaffold, the quantification of laminin coupled on the surface was achieved using BCA protein assay. The amount of laminin coupled on A-PHB and H-PHB fibrous surface is shown in Table 5.4. The amount of laminin immobilized on the H-PHB fibrous scaffold was significantly greater than that of laminin immobilized on the A-PHB fibrous scaffold. This result correlated to the number of functional groups on the fibrous surface that provided the active site to bind with laminin molecule.

5.4.2 Effect of Laminin-PHB Scaffolds on Neuro 2a and NSCs Cellular Behavior

The incorporation of bioactive molecules on the tissue scaffolds has been shown to enhance neural regeneration, since interaction between materials and neural cells is tuned by materials' interface. In this study, PHB fibrous scaffold was modified with specific-surface lysis reaction and then immobilized with laminin through covalent binding and physical adsorption. Aiming at promotion of neural regeneration, we thus focused the cellular behavior of Neuro 2a cell line and NSCs on the modified scaffolds by using an *in vitro* cell culture method. The ability to support the attachment and to promote the proliferation of cells is one of the foremost important aspects of tissue scaffold materials. To evaluate such

characteristics, the reference cells were cultured on these scaffolds for various culturing time intervals. The results were compared with that obtained for the corresponding electrospun PHB fibrous scaffold (i.e.,control).

5.4.2.1 Cytotoxicity

Cytotoxicity is a basic property of scaffolding materials. Figure 5.4 shows the viability of the cells obtained from MTT assay after the cells had been cultured with extraction media from neat electrospun PHB and surface modified (A-PHB, H-PHB, LA-PHB and LH-PHB) fibrous scaffolds as compared with that obtained after the cells had been cultured with the fresh SFM. The viability of the cells was reported as the percentage with respect to that of the control. Evidently, the viability of Neuro 2a and NSCs cultured with the extraction media from all of fibrous scaffolds were equivalent to that of the cells cultured with fresh SFM. This indicated that the fibrous scaffolds do not release some cytotoxic substance to the culture media, implying the biocompatibility of these materials toward Neuro2a and NSCs. Previous reports showed that PHB had been repeatedly demonstrated with a good biodegradability and without cytotoxicity *in vitro* and *in vivo* [25-29,53-57] Results from this work confirmed that the surface modification PHB fibrous scaffolds obtained from the studied materials can be used as scaffolds for Neuro 2a and NSCs culture.

5.4.2.2 Cell Attachment and Cell Proliferation

The biocompatibility of the neat PHB and the surface modified fibrous scaffolds in terms of cell attachment and cell proliferation was further investigated by observing the viability of Neuro 2a and NSCs that were cultured on the surface modify fibrous scaffolds as well as the neat PHB fibrous scaffold (i.e., control) for 4 h, 1, and 3 d (see Figure 5.5). The viability of the attached cells at 4 h after cell seeding was taken as that of the attachment 4 h, whereas the viability of the attached cells at day 1 and day 3 after cell seeding was taken as that of the proliferated cells. The viability of the cells that were cultured on neat PHB fibrous scaffold at 4 h was used as the reference value to obtain the relative viability of the viability cells shown in the figure. Figure 5.5 shows the viability of Neuro 2a on various fibrous surface modify scaffolds in comparison with that on neat PHB fibrous scaffold (i.e., control) after the cells were seeded on these surfaces for 4

h, day 1 and day 3. The viability of cells on the neat PHB at 4 h after cell seeding was used as the reference value to arrive at the relative viability of the cells shown in the figure. For any given substrate, the viability of the attached cells increased with increasing cell seeding time. At 4 h of cell seeding, the viability of the cells on any given type of the fibrous scaffolds showed equivalent values. However, the viability of cells that had been seeded on the surface modified scaffold for day 1 and day 3 was inferior to that of the cells on the neat PHB scaffold. At day 1 after cell seeding, the viability of the cells on the LH-PHB fibrous scaffold was greater than A-PHB, LA-PHB, H-PHB and neat PHB. At day 3 after cell seeding, the viability of the cells on the LA-PHB and LH-PHB fibrous scaffold was the greatest and appeared to be quite comparable to one another.

The ability of the different substrates in promoting the attachment and proliferation of the cells could be evaluated further by observing the viability of the cells attached on a given type of substrates whether it was either increased or decreased between two adjacent seeding time points. Between 4 h and day 1 after cell seeding, the largest increase in the viability of the cells was observed on the LH-PHB fibrous scaffold. Between day 1 and day 3 after cell seeding, the largest increase in the viability of the cells was observed on the LA-PHB fibrous scaffold. Among the various fibrous scaffolds, the LH-PHB and the LA-PHB fibrous scaffolds showed better support for the attachment and proliferation of Neuro 2a cell line than the other fibrous scaffolds. This result indicated that the LA-PHB and LH-PHB fibrous scaffold was a better support for Neuro 2a at any given cell seeding time. Among the fibrous scaffolds, the viability of cells on the LA-PHB and LH-PHB was the greatest, followed by that on the A-PHB, H-PHB, and neat PHB fibrous scaffolds, respectively.

Tables 5.5 show selected SEM images of Nero2a that were seeded on the surfaces of the neat PHB fibrous (i.e., control) and the surface modified PHB fibrous scaffolds at 4 h, day 1 and 3 after cell seeding at magnifications of 3500x (scale bar 5 μm). The difference in the cellular behavior was observed when Neuro 2a were seeded on the fibrous scaffolds of different surface topographies due to the variation in the surface roughness and hydrophilicity. At 4 h after cell seeding time, the Neuro 2a cells attached rather well over the neat PHB

fibrous scaffold and were still in a round shape, suggesting that the cells might not be fully attached on the surfaces. For the cells that were seeded on the surface modify fibrous substrates, at the same given cell seeding time, the cells attached well over the surfaces and started to extend their cytoplasm. With increasing the cell seeding time to day 1 and day 3, the cells on all other surfaces became more stretched and elongated their cytoplasm along the fibrous surfaces, except for these on the surface of the neat PHB fibrous scaffold, which were still in a round shape and slightly less elongation. However, the sizes of most pores were much larger than those of the cells, thus allowing the cells to penetrate to the inner side of the scaffolds.

For potential for uses as neural scaffold in specific application such CNS disorders, all of the fibrous scaffolds were further evaluated with NSCs. Figure 5.6 shows the viability of the attached and proliferated NSCs on various surface fibrous scaffolds in comparison with that on the neat PHB fibrous scaffold (i.e., control) after the cells were seeded on these surfaces for 4 h, day 1, and 3. The viability of the cells on the neat PHB at 4 h after cell seeding was used as the reference value to arrive at the relative viability of the cells shown in the figure. For any given cell type, the viability of the cells on these substrates generally increased with an increase in the cell seeding time. At any given cell seeding time point, the viability of the cells on any given type of the A-PHB and LA-PHB was superior to that of the cells on the neat PHB, H-PHB and LH-PHB fibrous scaffold. Among the various fibrous scaffolds, the viability of the cells on the LA-PHB fibrous scaffold, at 4 h after cell seeding, was the greatest. However, the viability of the cells on the neat PHB, A-PHB, H-PHB and LH-PHB appeared to be quite comparable at this interval time. At 8 h after cell seeding, the viability of the cells on the LH-PHB fibrous scaffolds was the greatest, followed by that of the cells on the A-PHB fibrous scaffolds, respectively. At 24 h after cell seeding, the viability of the cells on the LA-PHB fibrous scaffold was still greater than that of the cells on the other fibrous scaffolds.

In order to investigate the ability of the different substrates in promoting the attachment and proliferation of the cells, the viability of the between two adjacent seeding time points was evaluated. Comparatively, between 4 h and day 1 after cell seeding, the largest increase in the viability of the cells was observed on

the A-PHB and LA-PHB fibrous scaffold. Between day 1 and day 3 after cell seeding, the largest increase in the viability of the cells was observed on the LA-PHB fibrous scaffold. At any given cell seeding time, the viability of the cells on any given type of the LA-PHB fibrous scaffolds was superior to that of the cells on the other scaffold. Between the neat and the surface modified fibrous scaffolds, the A-PHB and LA-PHB fibrous scaffolds showed better support for the attachment and proliferation of NSCs than H-PHB, LH-PHB and neat PHB fibrous scaffolds. This result indicated that the A-PHB and LA-PHB fibrous scaffold was a better support for NSCs than neat PHB fibrous scaffold at any given cell seeding time.

Tables 5.6 show selected SEM images of NSCs that were seeded on the surfaces of the neat PHB fibrous (i.e., control) and the surface modified PHB fibrous scaffolds at 4 h, day 1 and 3 after cell seeding at magnifications of 3500x (scale bar 5 μm). The difference in the cellular behavior was observed when NSCs were seeded on the fibrous scaffolds of different surface topographies. At 4 h after cell seeding time, the NSCs attached rather well over the neat PHB, A-PHB and H-PHB fibrous scaffold and were still in a round shape, suggesting that the cells might not be fully attached on the surfaces. For the cells that were seeded on the LA-PHB and LH-PHB fibrous scaffold, at the same given cell seeding time, the cells attached well over the surfaces and started to extend their cytoplasm. With increasing the cell seeding time to day 1 and day 3, the cells on LA-PHB and LH-PHB fibrous scaffold became more stretched and elongated their cytoplasm along the fibrous surfaces, except for these on the A-PHB, H-PHB and neat PHB fibrous scaffold, which were slightly less elongation.

The successful nerve regeneration relies on the extensive growth of axonal processes. To observe the orientation and outgrowth of neurites as well as the relationship between the surface chemistry and cell recognition proteins of the scaffolds, neurofilament immunocytochemistry staining and LSCM observation was performed on the cell cultured scaffolds at day 2. Figure 5.7 shows the LSCM micrographs of NSCs cultured on the various fibrous scaffolds. It can be seen that the NSCs cultured on the LA-PHB and LH-PHB fibrous scaffolds exhibited classical contact guidance by growing parallel to the fibers. However, some exceptional cases were also found occasionally (Fig. 5.7b, arrows). It might be due to

the neurites outgrowth was not affected by the fiber alignment at the initial period; they all turned through large angles in order to grow parallel to the fiber alignment [14]. This suggests that the favorite growing direction of NSC neurites is parallel to the direction of fibers and the process is dynamically directed over time [14]. Interestingly, it can be seen that the amount of NSCs cultured on the A-PHB and LA-PHB fibrous scaffolds greater than that of the cells on the H-PHB and the LH-PHB fibrous scaffolds, which supports and cooperates with the results of the viability of cells from MTT assay. Smetana *et al.* [58] provides the information on the behavior of macrophages on the surface of the implanted hydrogel strips. The fusion of macrophages into multinucleate cells was inhibited on the surface of materials containing acidic groups such carboxylic group. However, the cellular mechanism behind the surface chemistry of substrate on the behavior of NSCs needs to be studied further.

The differences in the cellular behavior of Neuro 2a and NSCs on the various fibrous scaffolds can be hypothetically explained by electrostatic interaction between cell membrane and substrates as well as the cellular characteristic of individual reference cell. The extracellular matrix (ECM) proteins have the capacity to regulate cell behaviors such as adhesion, spreading, growth, and migration [59]. In a cell culture experiment, the factors influencing the adsorption of proteins are surface wettability and surface charge [60, 61]. It is a known fact that cells carry a negative surface charge at physiological pH and the magnitude of the charge depends mainly on the composition of the cell membranes [62, 63]. According to the cellular characteristic, Neuro 2a is a cell line whereas NSCs is a stem cell, so the sensitivity of the cells toward the surface chemistry and surface topology were different. As a result of Neuro 2a, the difference in the viability of cell on the scaffolds could be contributions from the enhancement of the adsorption of specific proteins on the surface of a substrate. Thus scaffold coupled with laminin (LA-PHB and LH-PHB) was postulated as the reason for the ability of the materials to enhance the attachment and proliferation of Neuro 2a. However, NSCs seem to be sensitive to various factors such contact guidance and chemical guidance [6]. In addition, it is well known that laminin contains bioactive neurite binding sites for neural cell attachment and differentiation (namely YIGSR and IKVAV), and studies

had shown enhanced neurite extensions on substrates that were modified with laminin peptides [33]. Thus, the LA-PHB and LH-PHB fibrous scaffold was better in supporting the neurite extension seen on the fluorescence microscope. However, the neat PHB, H-PHB and LH-PHB exhibited cytostatic property (i.e., cytostatic property: this is not cytotoxic but inhibits cell proliferation [64]) towards NSCs, as suggested by the relative constancy in the MTT viabilities and the relatively low number of cells seen on the fluorescence microscope.

5.5 Conclusions

Immobilization of laminin on the surface of the electrospun PHB fibrous scaffolds was successfully accomplished by using the amino and carboxylic groups introduced on the scaffold surface via surface-specific lysis reaction and further covalent immobilization of the biomolecules. The potential for use of the surface modified fibrous scaffolds for neural regeneration was evaluated *in vitro* towards murine neuroblastoma Neuro 2a cell line (ATCC, CCL-131). However, for potential uses in specific application, all of the fibrous scaffolds were further evaluated with rat brain-derived neural stem cells (NSCs). Both types of laminin coupled on the PHB fibrous scaffold supported the attachment and the proliferation of Neuro 2a very wells. Despite the good attachment and proliferation of Neuro 2a, NSCs were not able to proliferate on the neat PHB, H-PHB and LH-PHB fibrous scaffold.

5.6 Acknowledgements

This work was supported in part by (1) the Ratchadaphisek Somphot Endowment Fund for Research and Research Unit, Chulalongkorn University and (2) the Center for Petroleum, Petrochemicals and Advanced Materials (CPPAM). P. Sangsanoh thanks a doctoral scholarship (PHD/0191/2550) received from the Royal Golden Jubilee Ph.D. Program, the Thailand Research Fund (TRF).

5.7 References

[1] Langer, R.; Vacanti, J.P. Tissue engineering. *Science* **1993**, 260, 920–926.

[2] Fine, E.G.; Valentini, R.E.; Aebischer, P. Nerve regeneration. In: Lanza, R.P.; Langer, R.; Vacanti, J.P. editors. Principles of tissue engineering. *San Diego Academic Press*, **2000**, 785–798.

[3] Tresco, P.A. Tissue engineering strategies for nervous system repair. In: Seil, F.J. editor. Progress in brain research. New York: *Elsevier Science B.V., 2000*, 349–63.

[4] Schmidt, C.E.; Leach, J.B. Neural tissue engineering: strategies for repair and regeneration, *Ann Biomed Eng* **2003**, 5, 293–347.

[5] Willerth, S.M.; Sakiyama-Elbert, S.E. Approaches to neural tissue engineering using scaffolds for drug delivery, *Adv Drug Deliver Rev* **2007**, 59, 325–38.

[6] Yu, L.M.Y.; Leipzig, N.D. Shoichet, M.S. Promoting neuron adhesion and growth, *Mater Today* **2008**, 11, 36-43.

[7] Teo, W.E.; He, W.; Ramakrishna, S. Electrospun scaffold tailored for tissue-specific extracellular matrix, *Biotechnol J* **2006**, 1, 918–29.

[8] Rho, K.S.; Jeong, L.; Lee, G.; Seo, B.M.; Park, Y.J.; Hong, S.D.; Roh, S.; Cho, J.J.; Park, W.H.; Min, B.M. Electrospinning of collagen nanofibers: effects on the behavior of normal human keratinocytes and early-stage wound healing, *Biomaterials* **2006**, 27, 1452–61.

[9] Venugopal, J.; Low, S.; Choon, A.T.; Ramakrishna, S. Interaction of Cells and Nanofiber Scaffolds in Tissue Engineering, *J Biomed Mater Res B* **2008**, 84, 34-48.

[10] Hadlock, T.; Elisseeff, J.; Langer, R.; Vacanti, J.P.; Cheney, M. A tissue-engineered conduit for peripheral nerve repair, *Arch Otolaryngol Head Neck Surg* **1998**, 124, 1081–1086.

[11] Ceballos, D.; Navarro, X.; Dubey, N. Wendelschafer-Crabb, G.; Kennedy, W.R.; Tranquillo, R.T. Magnetically aligned collagen gel filling a collagen nerve guide improves peripheral nerve regeneration, *Exp Neurol* **1999**, 158, 290–300.

[12] Dubey, N.; Letourneau, P.C.; Tranquillo, R.T. Neuronal contact guidance in magnetically aligned fibrin gels: effect of variation in gel mechano-structural properties, *Biomaterials* **2001**, 22, 1065–75.

[13] Yang, F.; Murugan, R.; Ramakrishna, S.; Wang, X.; Ma, Y.X.; Wang, S. Fabrication of nano-structured porous PLLA scaffold intended for nerve tissue engineering, *Biomaterials* **2004**, 25, 1891–1900.

[14] Yang, F.; Murugan, R.; Wang, S.; Ramakrishna, S. Electrospinning of nano/micro scale poly(L-lactic acid) aligned fibers and their potential in neural tissue engineering, *Biomaterials* **2005**, 26, 2603–2610.

[15] Pham, Q.P.; Sharma, U.; Mikos, A.G. Electrospinning of polymeric nanofibers for tissue engineering applications, *Tissue Eng* **2006**, 12, 1197–1211.

[16] Lim, S.H.; Mao, H.Q. Electrospun scaffolds for stem cell engineering, *Adv Drug Deliver Rev* **2009**, 61, 1084–1096.

[17] Murugan, R.; Ramakrishna, S. Nano-featured scaffolds for tissue engineering: A review of spinning methodologies, *Tissue Eng* **2006**, 12, 435–447.

[18] Shin, M.; Ishii, O.; Sueda, T.; Vacanti, J.P. Contractile cardiac grafts using a novel nanofibrous mesh, *Biomaterials* **2004**, 25, 3717–3723.

[19] Yuan, Y.; Zhang, P.; Yang, Y.; Wang, X.; Gu, X. The interaction of Schwann cells with chitosan membranes and fibers in vitro, *Biomaterials* **2004**, 25, 4273-4278

[20] Jansen, K.; van der Werff, J.F.A.; van Wachem, P.B.; Nicolai, J.P.A.; de Leij, L.F.M.H.; van Luyn, M.J.A. A hyaluronan-based nerve guide: in vitro cytotoxicity, subcutaneous tissue reactions, and degradation in the rat, *Biomaterials* **2004**, 25, 483-489.

[21] Stang, F.; Fansa, H.; Wolf, G.; Reppin, M.; Keilhoff, G. Structural parameters of collagen nerve grafts influence peripheral nerve regeneration, *Biomaterials* **2005**, 26, 3083-3091.

[22] Chen, Y.S.; Chang, J.Y.; Cheng, C.Y.; Tsai, F.J.; Yao, C.H.; Liu, B.S. An in vivo evaluation of a biodegradable genipin-cross-linked gelatin peripheral nerve guide conduit material, *Biomaterials* **2005**, 26, 3911-3918.

[23] Schlosshauer, B.; Müller, E.; Schröder, B.; Planck, H.; Müller, H.W. Rat Schwann cells in bioresorbable nerve guides to promote and accelerate axonal regeneration, *Brain Res* **2003**, 963, 321-326.

[24] Bryan, D.J.; Tang, J.B.; Doherty, S.A.; Hile, D.D.; Trantolo, D.J.; Wise, D.L.; Summerhayes, I.C. Enhanced peripheral nerve regeneration through a poled bioresorbable poly(lactic-co-glycolic acid) guidance channel, *Neural Eng* **2004**, 1, 91-8.

[25] Sangsanoh, P.; Waleetorncheepsawat, S.; Suwantong, O.; Wutticharoenmongkol, P.; Weeranantanapan, O.; Chuenjitbuntaworn, B.; Cheepsunthorn, P.;. Pavasant, P.; Supaphol, P. In vitro biocompatibility of Schwann cells on surfaces of biocompatible polymeric electrospun fibrous and solution-cast film scaffolds, *Biomacromolecules* **2007**, 8, 1587-1594.

[26] Wang, Y.W.; Yang, F.; Wu, Q.; Cheng, Y.C.; Yu, P.H.F.; Chen, J.; Chen, G.Q. Effect of composition of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) on growth of fibroblast and osteoblast, *Biomaterials* **2005**, 26, 755-761.

[27] Williams, S.F.; Martin, D.P.; Horowitz, D.M.; Peoples, O.P. PHA applications: addressing the price performance issue, I. Tissue engineering, *Int J Biol Macromol* **1999**, 25, 111–121.

[28] Volova, T.; Shishatskaya, E.; Sevastianov, V.; Efremov, S.; Mogilnaya, O. Results of biomedical investigations of PHB and PHB/PHV fibers, *Biochem Eng J* **2003**, 16, 125-133.

[29] Cheng, S.; Chen, G.Q.; Leski, M.; Zou, B.; Wang, Y.; Wu, Q. The effect of D,L-β-hydroxybutyric acid on cell death and proliferation in L929 cells, *Biomaterials* **2006**, 27, 3758-3765.

[30] Pierschbacher, M.D.; Ruoslahti, E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule, *Nature* **1984**, 309, 30–33.

[31] Nagai, M.; Hayakawa, T.; Makimura, M.; Yoshinari, M. Fibronectin Immobilization using Water-soluble Carbodiimide on Poly-L-lactic Acid for Enhancing Initial Fibroblast Attachment, *J Biomater Appl* **2006**, 21, 33-47.

[32] Koh, H.S.; Yong, T.; Chan, C.K.; Ramakrishna, S. Enhancement of neurite outgrowth using nano-structured scaffolds coupled with laminin, *Biomaterials* **2008**, 29, 3574-3582

[33] Yu, T.T.; Shoichet, M.S. Guided cell adhesion and outgrowth in peptide-modified channels for neural tissue engineering, *Biomaterials* **2005**, 26, 1507–1514.

[34] Zhu, Y.; Gao, C.; Liu, X.; He, T.; Shen, J. Immobilization of biomacro-molecules onto aminolyzed poly(L-lactic acid) toward acceleration of endothelium regeneration, *Tissue Eng* **2004**, 10, 53-61.

[35] Ma, Z.; Gao, C.; Ji, J.; Shen, J. Protein immobilization on the surface of poly-L-lactic acid films for improvement of cellular interactions, *Eur Polym J* **2002**, 38, 2279-2284.

[36] Cui, Y.L.; Qi, A.D.; Liu, W.G.; Wang, X.H.; Wang, H.; Ma, D.M.; Yao, K.D. Biomimetic surface modification of poly(L-lactic acid) with chitosan and its effects on articular chondrocytes in vitro, *Biomaterials* **2003**, 24, 3859-3868.

[37] Mattanavee, W.; Suwantong, O.; Puthong, S.; Bunaprasert, T.; Hoven, V.P.; Supaphol, P. Immobilization of Biomolecules on the Surface of Electrospun Polycaprolactone Fibrous Scaffolds for Tissue Engineering, *Appl. Mater. Interfaces* **2009**, 1, 1076-1085.

[38] Ho, M.H.; Wang, D.M.; Hsieh, H.J.; Liu, H.C.; Hsien, T.Y.; Lai, J.Y.; Hou, L.T. Preparation and characterization of RGD-immobilized chitosan scaffolds, *Biomaterials* **2005**, 26, 3197-3206.

[39] Cai, K.; Yao, K.; Lin, S.; Yang, Z.; Li, X.; Xie, H.; Quing T. Poly(d,l-lactic acid) surfaces modified by silk fibroin: effects on the culture of osteoblast in vitro, *Biomaterials* **2002**, 23, 1153-1160.

[40] Bhang, S.H.; Lee, T.J.; Yang, H.S.; La, W.G.; Han, A.M.; Kwon, Y.H.; Kim, B.S. Enhanced nerve growth factor efficiency in neural cell culture by immobilization on the culture substrate, *Biochem Biophys Res Commun* **2009**, 382, 315-320.

[41] Edlund, U.; Dänmark, S.; Albertsson, A.C. A strategy for the covalent functionalization of resorbable polymers with heparin and osteoinductive growth factor, *Biomacromolecules* **2008**, 9, 901-905.

[42] Timpl, R.; Rohde, H.; Robey, P.G.; Rennard, S.I.; Foidart, J.M.; Martin, G.R. Laminin-a glycoprotein from basement membranes, *J Biol Chem* **1979**, 254, 9933-9937.

[43] Rutka, J.T.; Apodaca, G.; Stern, R.; Rosenblum, M. The extracellular-matrix of the central and peripheral nervous systems-structure and function, *J Neurosurg* **1988**, 69, 155-170.

[44] McDonald, D.; Cheng, C.; Chen, Y.Y.; Zochodne, D. Early events of peripheral nerve regeneration, *Neuron Glia Biol* **2006**, 2, 139-147.

[45] Luckenbill-Edds, L. Laminin and the mechanism of neuronal outgrowth, *Brain Res Rev* **1997**, 23, 1-27

[46] Milner, R.; Wilby, M.; Nishimura, S.; Boylen, K.; Edwards, G.; Fawcett, J.; Streuli, C.; Pytela, R.; Ffrench-Constant, C. Division of labor of Schwann cell integrins during migration on peripheral nerve extracellular matrix ligands, *Developmental Biol* **1997**, 185, 215-228.

[47] Chen, Z.L.; Strickland, S. Laminin gamma 1 is critical for Schwann cell differentiation, axon myelination, and regeneration in the peripheral nerve, *J Cell Biol* **2003**, 163, 889-899.

[48] Yu, X.J.; Dillon, G.P.; Bellamkonda, R.V. A laminin and nerve growth factor-laden three-dimensional scaffold for enhanced neurite extension, *Tissue Eng* **1999**, 5, 291-304.

[49] Miller, C.; Jeftinija, S.; Mallapragada, S. Synergistic effects of physical and chemical guidance cues on neurite alignment and outgrowth on biodegradable polymer substrates, *Tissue Eng* **2002**, 8, 367-378.

[50] Labrador, R.O.; Buti, M.; Navarro, X. Influence of collagen and laminin gels concentration on nerve regeneration after resection and tube repair, *Exp Neurol* **1998**, 149, 243–252.

[51] Sombatmankhong, K.; Sanchavanakit, N.; Pavasant, P.; Supaphol, P. Bone Scaffolds from Electrospun Fiber Mats of Poly(3-hydroxybutyrate), Poly(3-hydroxybutyrate-co-3-hydroxyvalerate), and Their Blend, *Polymer* **2007**, 48, 1419-1427.

[52] Uchida, E.; Uyama, Y.; Ikada, Y. Sorption of low-molecular-weight anions into thin polycation layers grafted onto a film, *Langmuir* **1993**, 9, 1121-1124.

[53] Suwantong, O.; Waleetorncheepsawat, S.; Sanchavanakit, N.; Pavasant, P.; Cheepsunthorn, P.; Bunaprasert, T.; Supaphol P. In Vitro Biocompatibility of Electrospun Poly(3-hydroxybutyrate) and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) Fiber Mats, *Int J Biol Macromol* **2007**, 40, 217-223.

[54] Chen, G.Q.; Wu, Q. Polyhydroxyalkanoates as tissue engineering materials, *Biomaterials* **2005**, 26, 6565–6578.

[55] Rivard, C.H.; Chaput, C.; Rhalmi, S.; Selmani, A. Bio-absorbable synthetic polyesters and tissue regeneration: A study of three-dimensional proliferation of ovine chondrocytes and osteoblasts, *Ann Chir* **1996**, 50, 651-658.

[56] Teixeira, A.I.; Duckworth, J.K.; Hermanson, O. Getting the right stuff: controlling neural stem cell state and fate in vivo and in vitro with biomaterials, *Cell Res* **2007**, 17, 56–61.

[57] Xu, X.Y.; Li, X.T.; Peng, S.W.; Xiao, J.F.; Liu, C.; Fang, G.; Chen, K.C.; Chen, G.Q. The behaviour of neural stem cells on polyhydroxyalkanoate nanofiber scaffolds, *Biomaterials* **2010**, 3967-3975.

[58] Smetana, K.; Vacik, J.; Souckova, D.; Krcova, Z.; Sulc, J. The influence of hydrogel functional groups on cell behavior, *J Biomed Mater Res B* **1990**, 24, 463-470.

[59] Wenling, C.; Duohui, J.; Jiamou, L.; Yandao, G.; Nanming, Z.; Zhang, X. Effects of the degree of deacetylation on the physicochemical properties and Schwann cell affinity of chitosan films, *J Biomater Appl* **2005**, 20, 157-177.

[60] Burns, N.L.; Holmberg, K.; Brink, C. Influence of surface charge on protein adsorption at an amphoteric surface: effects of varying acid to base ratio, *J Colloid Interf Sci* **1996**, 178, 116–122.

[61] Webb, K.; Hlady, V.; Tresco, P.A. Relative importance of surface wettability and charged functional groups on NIH3T3 fibroblast attachment, spreading, and cytoskeletal organization, *J Biomed Mater Res* **1998**, 41, 422–430.

[62] Prasitsilp, M.; Jenwithisuk, R.; Kongsuwan, K.; Damrongchai, N.; Watts, P. Cellular responses to chitosan in vitro: the importance of deacetylation, *J Mater Sci –Mater Med* **2000**, 11, 773–778.

[63] Knopf, B.; Wollina, U. Electrophoretic mobilities of keratinocytes from normal skin and psoriatic lesions, *Arch Dermatol Res* **1992**, 284, 117–118.

[64] Chatelet, C.; Damour, O.; Domard, A. Influence of the degree of acetylation on some biological properties of chitosan films, *Biomaterials* **2001**, 22, 261–268.

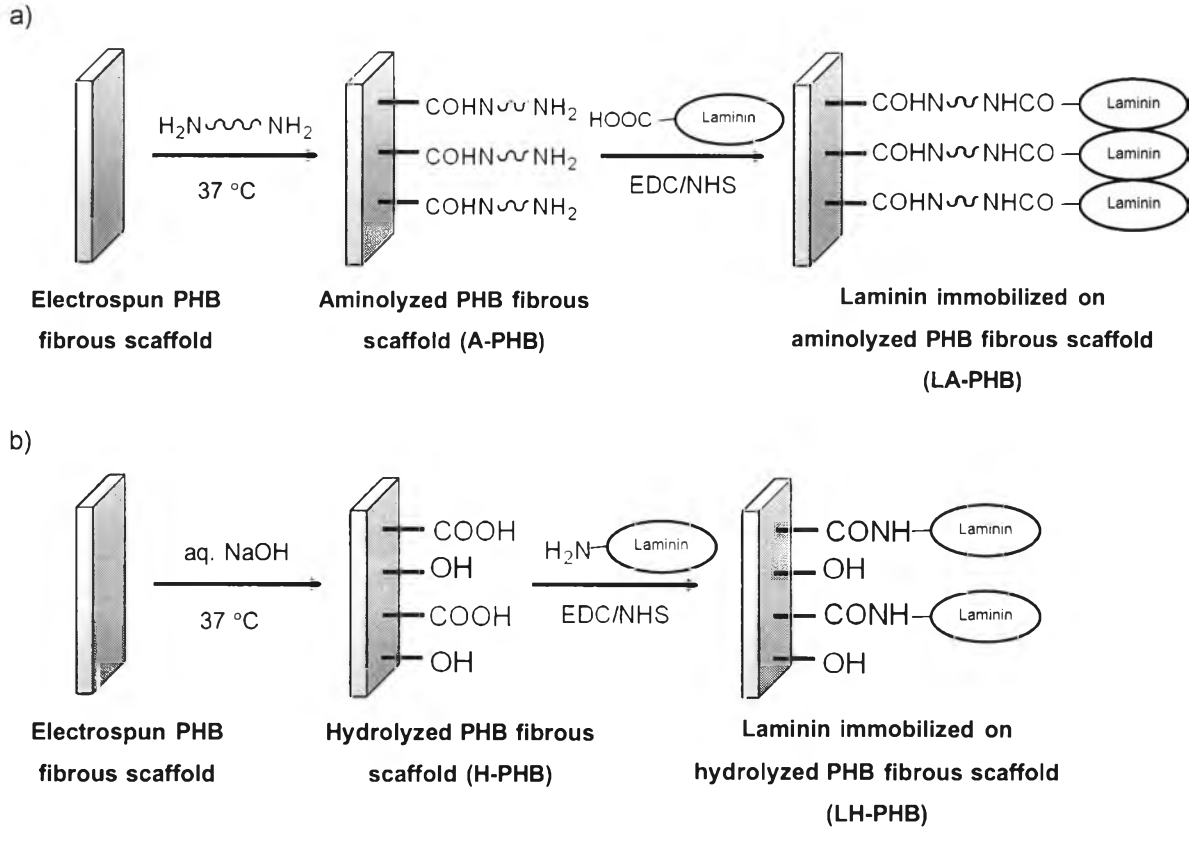


Figure 5.1 Summarizes the chemical pathway for the immobilization of laminin on the surface of the electrospun PHB fibrous scaffolds; a) aminolysis reaction and b) hydrolysis reaction.

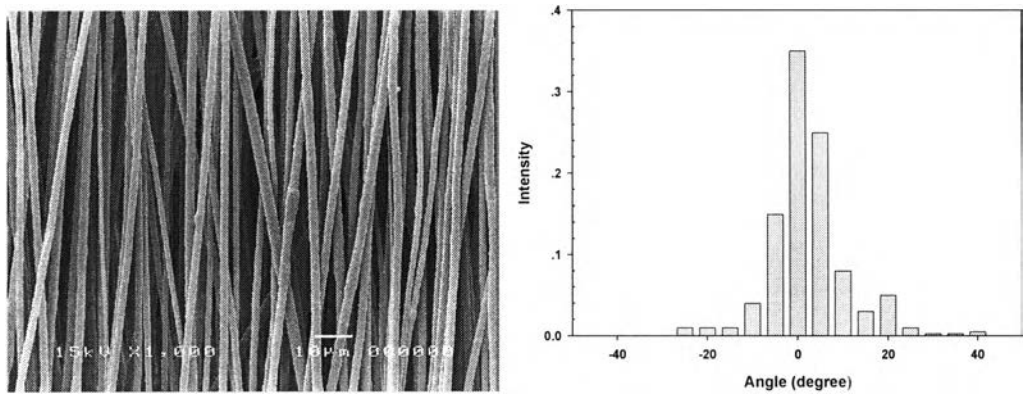


Figure 5.2 Selected SEM image of neat electrospun PHB fibrous scaffold (a) and histogram represent angular distributions of electrospun fibers (b).

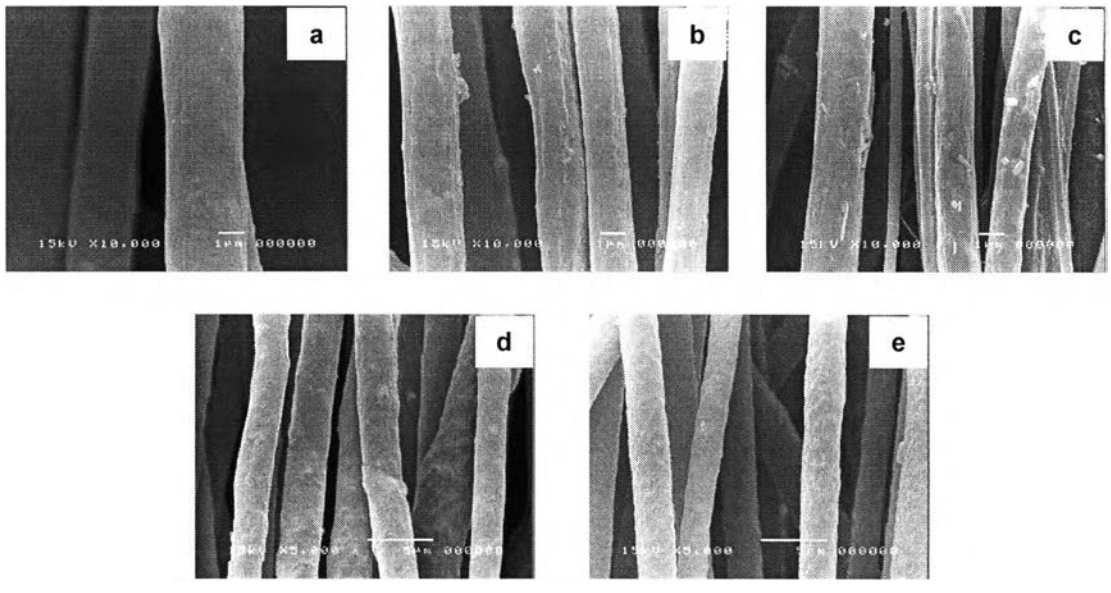


Figure 5.3 Selected SEM images of neat electrospun PHB fibrous membrane (a), Aminolysis PHB fibrous scaffold (A-PHB) (0.04 g/ml of HMD/IPA solution for 15 min at room temperature) (b), hydrolysis PHB fibrous scaffold (H-PHB) (1.0 M NaOH solution for 15 min at room temperature) (c), laminin immobilized on aminolyzed PHB fibrous scaffold (LA-PHB) (d), and laminin immobilized on hydrolyzed PHB fibrous scaffold (LH-PHB) (e).

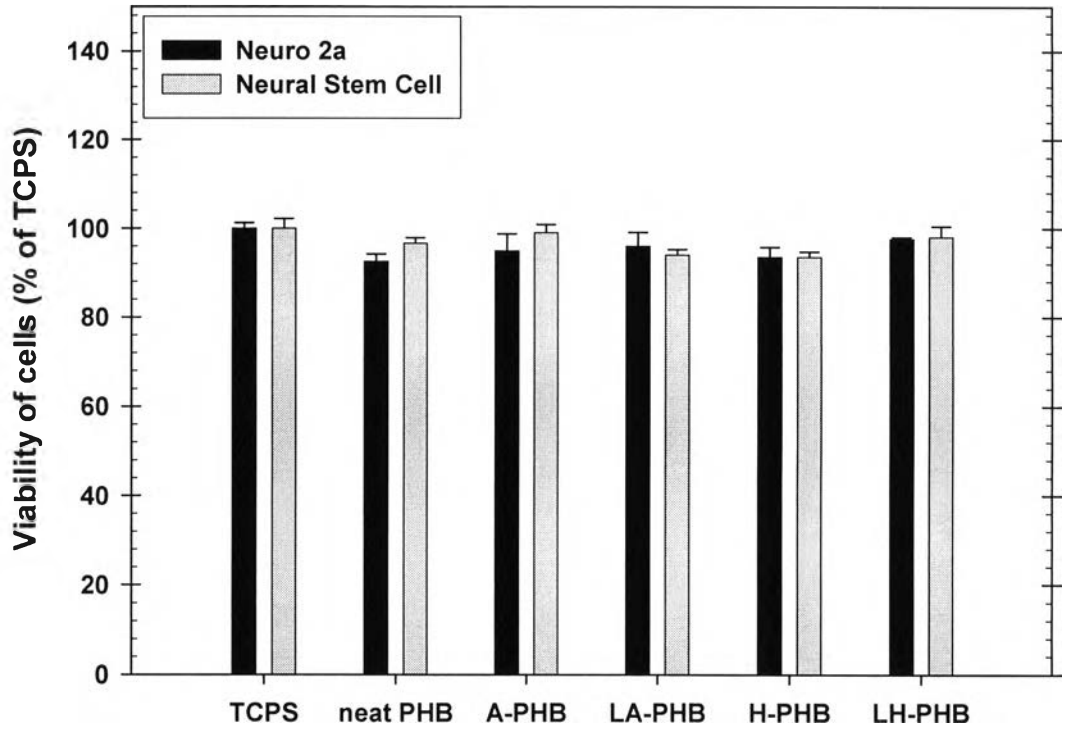


Figure 5.4 Indirect cytotoxicity evaluation of various electrospun fibrous scaffolds based on the viability of Neuro 2a and mNSCs that were cultured with the extraction media from these materials for 24 h. The viability of the cells that were cultured with fresh culture medium (SFM) (i.e., control) was used as the reference to arrive at the viability of the attached cells shown in the figure.

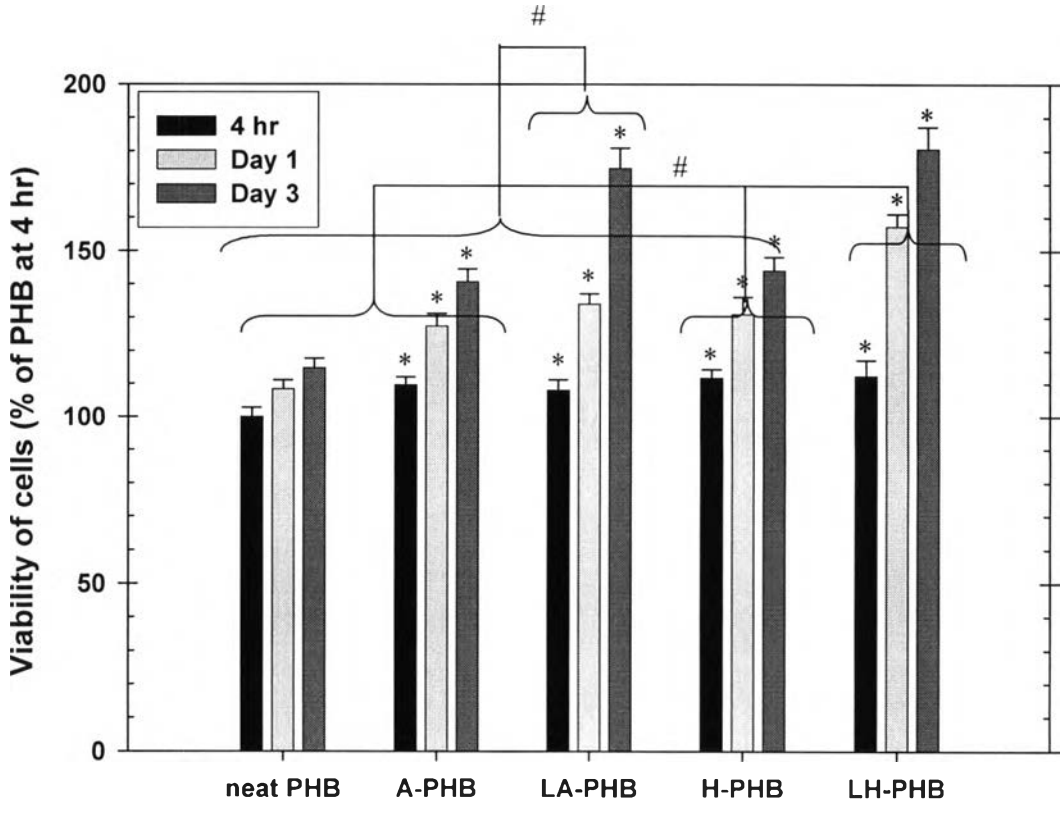


Figure 5.5 Attachment and proliferation of Neuro2a cells that were seeded on various electrospun fibrous for 4 h, days 1, and 3. The viability of the cultured cells that were seeded on the neat PHB fibrous scaffold for 4 h was used as the reference to arrive at the viability of the cultured cells shown in the figure. $^{*,\#}$ Significantly different at $p < 0.05$.

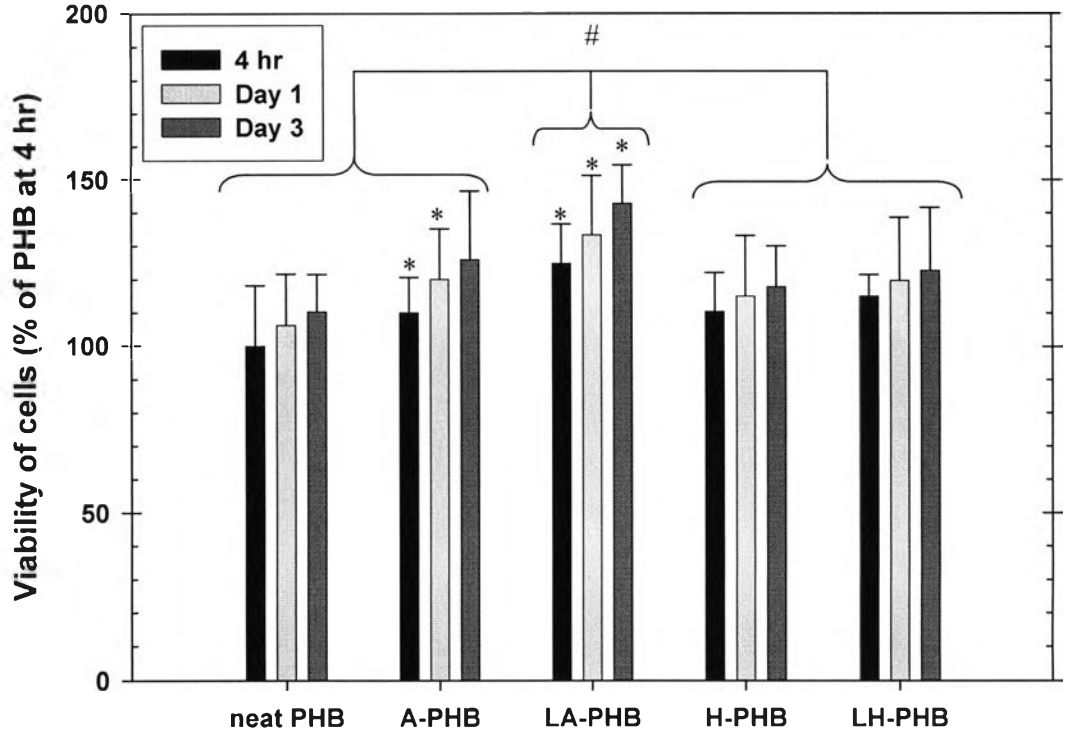


Figure 5.6 Attachment and proliferation of mNSCs that were seeded on various electrospun fibrous for 4 h, days 1, and 3. The viability of the cultured cells that were seeded on the neat PHB fibrous scaffold for 4 h was used as the reference to arrive at the viability of the cultured cells shown in the figure. *,# Significantly different at $p < 0.05$.

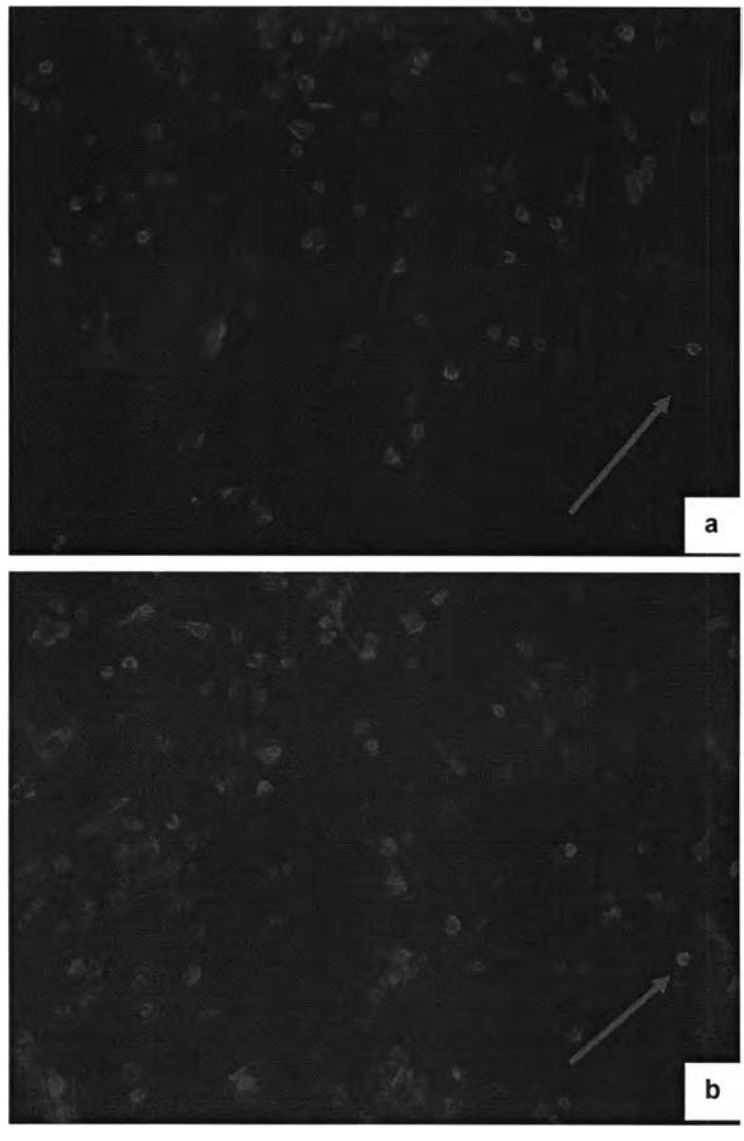


Figure 5.7 Representative fluorescence microscope of mNSCs that were seeded on neat PHB (a), A-PHB (b), LA-PHB (c), H-PHB (d) and LH-PHB) (e) at 10x Magnification. The observation was performed on the cell cultured scaffolds at day 2.

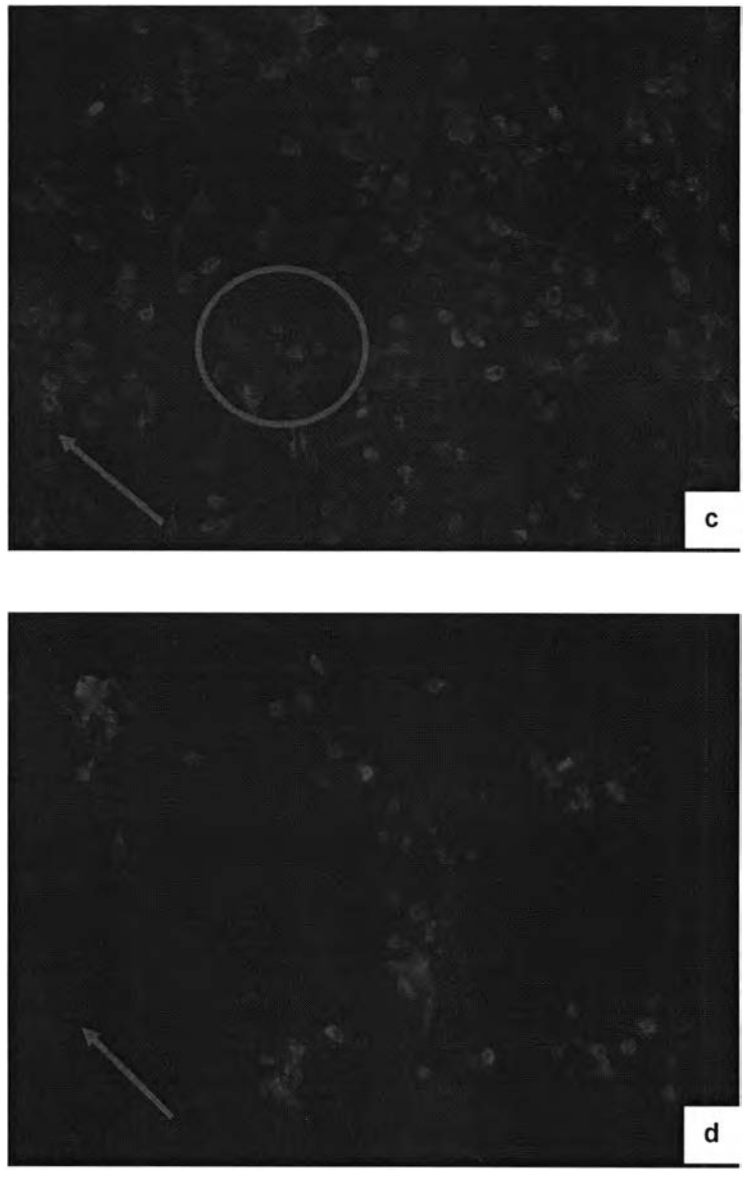


Figure 5.7 (cont.)

Figure 5.7 (cont.)

Table 5.1 The resulting static water contact angles of the original and modified PHB fibrous scaffolds.

Type of substrates	Static contact angle (degree)
neat PHB	135.3 ± 0.47
A-PHB	97.6 ± 0.96
LA-PHB	81.2 ± 0.52
H-PHB	89.5 ± 0.79
LH-PHB	80.5 ± 0.41

Table 5.2 Element composition of carbon, oxygen, and nitrogen on the surface of original PHB and surface modified PHB fibrous scaffolds as determined by X-ray photoelectron spectrometry.

Type of substrates	C atomic concentration (%)	O atomic concentration (%)	N atomic concentration (%)
neat PHB	70.2	29.8	0
A-PHB	67.8	31.3	0.9
LA-PHB	66.3	31.9	1.8
H-PHB	69.8	30.2	0
LH-PHB	66.8	31.1	2.1

Table 5.3 Atomic ratios of N1s/C1s and O1s/C1s on the surface of neat PHB and surface modified PHB fibrous scaffolds.

Type of substrates	N1s/C1s ratio	O1s/C1s ratio
neat PHB	0	0.4245
A-PHB	0.0133	0.4617
LA-PHB	0.0271	0.4811
H-PHB	0	0.4326
LH-PHB	0.0314	0.4656

Table 5.4 The amount of laminin coupled on the surface modified PHB fibrous scaffolds.

Electrospun fibrous membranes	Quantity of laminin coupled per surface area of polymer (mg/cm^2)
neat PHB	0
LA-PHB	144.4
LH-PHB	162.5

Table 5.5 Representative SEM images of murine neuroblastoma Neuro2a cell line (ATCC, CCL-131) that had been seeded or cultured on surface modified electrospun PHB fibrous scaffold for 4 h, 1 d and 3 d.

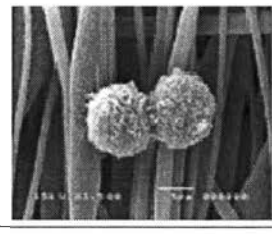
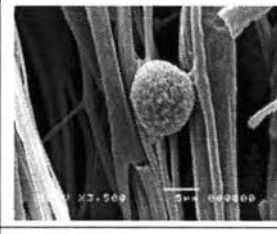
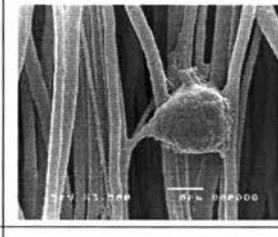
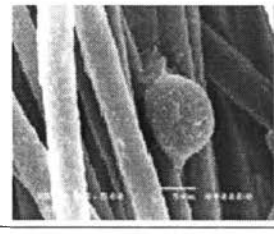
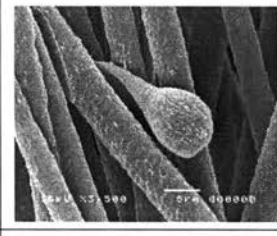
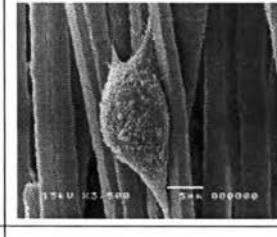
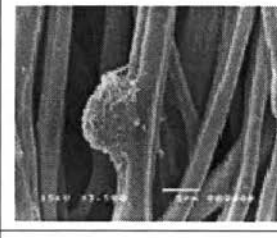
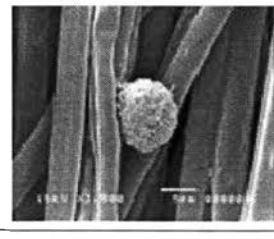
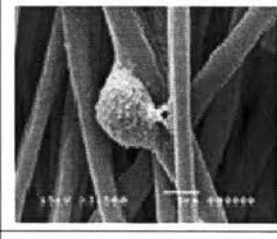
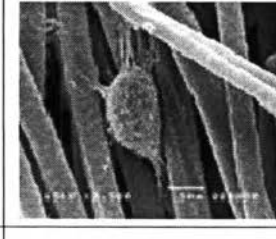
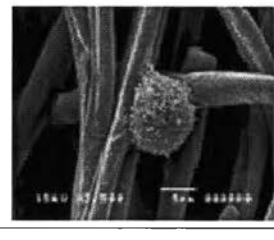
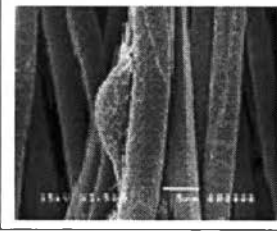
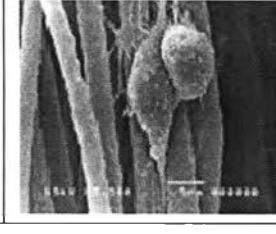
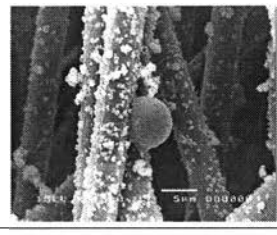
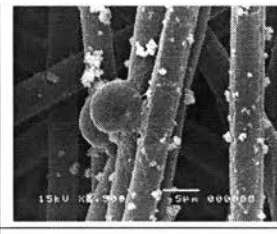
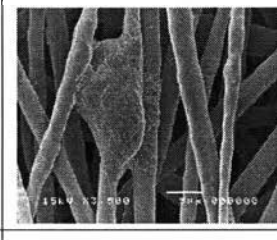
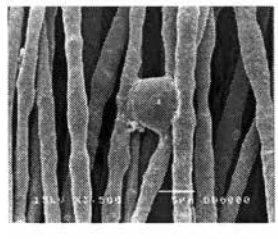
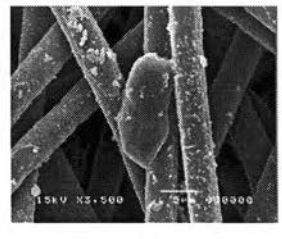
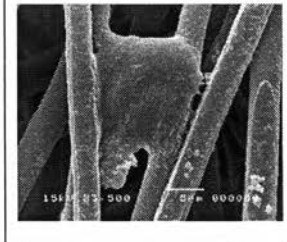
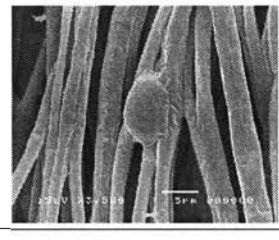
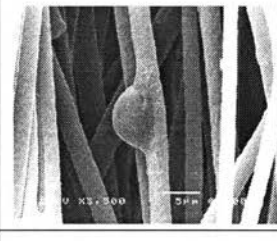
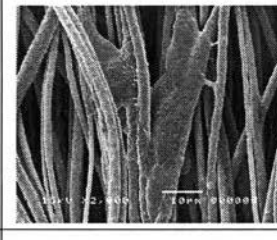
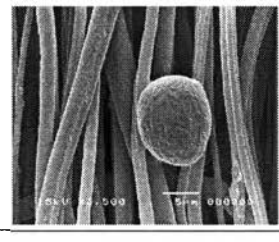
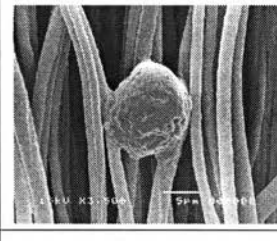
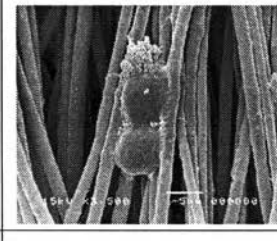
Type of substrate	Cell seeding/culturing time point		
	4 h	1 d	3 d
Neat PHB			
Aminolyzed PHB (A-PHB)			
Laminin coupled on aminolyzed PHB (LA-PHB)			
Hydrolysis PHB (H-PHB)			
Laminin coupled on hydrolyzed PHB (LH-PHB)			

Table 5.6 Representative SEM images of neural stem cells (NSCs) that had been seeded or cultured on surface modified electrospun PHB fibrous scaffold for 4 h, 1 d and 3 d.

Type of substrate	Cell seeding/culturing time point		
	4 h	1 d	3 d
Neat PHB			
Aminolyzed PHB (A-PHB)			
Laminin coupled on aminolyzed PHB (LA-PHB)			
Hydrolysis PHB (H-PHB)			
Laminin coupled on hydrolyzed PHB (LH-PHB)