CHAPTER IV
EFECT OF SURFACE TOPOGRAPHY AND CHEMISTRY OF POLY(3-HYDROXYBUTYRATE) SUBSTRATES ON THE CELLULAR BEHAVIOR TOWARDS MURINE NEUROBLASTOMA NEURO 2A CELL LINEAGE

4.1 ABSTRACT

Cell interactions with substrates play an importance role for tissue development in the process of regeneration of damaged tissue. Substrates that mimic extracellular matrix (ECM) surface topography and chemical composition will be enhanced cellular interactions. Electrospinning can easily produce the alignment fibrous substrates with architecture that structurally resembles the ECM of tissue that can provide the contact guidance during tissue regeneration. However, the sole use of substrate materials may not be sufficient for the treatment of damage tissue due to the lack of biochemical guidance that helps to promote cell adhesion and proliferation. In the present contribution, we evaluated the effect of surface properties of various surface modified electrospun fibrous and solution-cast film PHB substrates *in vitro* towards murine neuroblastoma Neuro 2a cell line. The neat electrospun fibrous and solution-cast PHB scaffold was used as the internal controls. The results from cell studies suggested that the surface topography and chemistry has a significant impact on the particular cell line of Neuro2a. The introduction of contact guidance such fiber diameter and alignment and biochemical guidance such immobilization of adhesive protein enhanced the attachment and proliferation of the cells. All of these results emphasized the importance of the surface properties on the cellular behavior.

(**Keywords**: Electrospinning; Fibrous membranes; Solution-cast film membranes; Surface modification; Protein immobilization)

4.2 Introduction

Recent advances in the tissue engineering, the most important effort is to regulate the growth and behavior of cells on artificial biodegradable scaffolds in order to provide functional tissue which creating the permissive environmental substitutes for tissue regeneration [1–3]. The primary objectives of these substitutes are to restore, maintain and improve tissue functions by mimicking the structure and biological function of native extracellular matrix (ECM) [2, 4]. Therefore, the selection of polymer, the manufacturing process and the modification of substitute materials play an important role in scaffold fabrication. Over the last three decades, electrospinning has been a popular technique to produce 3D fibrous scaffold because its possibility to produce nano- and micro-fibers which structurally analogous to the naturally occurring protein fibers in the ECM. Fibrous scaffolds with different topographical feature can be readily obtained by adjusting fiber diameter, alignment and fiber surface [5]. Many studies have shown that electrospun scaffolds are able to support the growth and promote the proliferation of the cultured cells [6]. In neural tissue engineering, some researchers have shown that fiber direction and surface topography may be directed the cell orientation and promoted the cell differentiation [7]. A few studies have investigated that aligned electrospun fibrous scaffolds are able to provide contact guidance to cultured cells, resulting in an elongation and alignment of cells along the axes of the fibers [8-10].

A wide variety of biocompatible and biodegradable synthetic polymers have been studied for their potential use in tissue engineering application, because of their suitable mechanical strength, processability and their controllable degradation rates in biological environment [6]. Among these, poly(3-hydroxybutyrate) (PHB) is the most thoroughly investigated member of the polyhydroxyalkanoates (PHAs) family, has shown good biocompatibility for *in vitro* and *in vivo* studies [11-13] due to its biocompatibility and completely biodegrades to release a normal component of blood and tissue, d,l-β-hydroxybutyrate (HB) [14]. Despite its inherent biocompatibility and biodegradability of PHB, actual utilization of this material as artificial scaffolding material is limited by its hydrophobicity which diminishes the initial response of cells to materials. A variety of surface modification techniques have been

used for improving cell affinity of scaffolding materials such as plasma treatment [15], surface alkaline hydrolysis treatment [16] and surface aminolysis treatment [17]. Most of those methods need to combine the immobilization of some bioactive molecules to achieve cell affinity on polymer surface such as fibronectin [16], laminin [18] or their functional domains biomolecules [17, 19-21] for enhancing the discrete biological information which is transmitted to the cell through cell surface receptors. 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 [22, 23]. Furthermore, *in vitro* experiments have shown that neurite outgrowth is enhanced on scaffolds that were covalently bound or physical adsorption with laminin [24-26].

It is well known that, the initial response of cells to the biomaterial mostly depends on surface properties [27]. ECM proteins and positively charged functional groups play the dominant role in neuronal adhesion [6, 27, 28]. Despite the numerous reports on the *in vitro* responses of neuronal cell lineages on the various types of substrates, a similar report that examined the influence of surface topography compared with ECM proteins is still lacking. In the present contribution, we report the *in vitro* responses and cellular behavior on the modified electrospun fibrous and corresponding solution-cast film PHB substrates with positively charge surface compared with those obtained from immobilizing ECM proteins. The study was preliminary evaluated *in vitro* towards murine neuroblastoma Neuro 2a cell line (American Type Culture Collection: ATCC). The neat electrospun fibrous and solution-cast PHB scaffold was used as the internal controls.

4.3 Experimental Details

4.3.1 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 surface modification process, 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. All other chemicals were analytical reagent grade and used without further purification.

4.3.2 Preparation of Aligned Electrospun Fibrous and Solution-cast Film PHB Scaffolds

The aligned electrospun PHB fibrous scaffolds were prepared according to the conditions previously described [29]. Briefly, the spinning solutions were prepared by dissolving 14% (w/v) PHB in chloroform at 60 °C. The spinning solution 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. For comparison purpose, PHB was also fabricated into films by the solution-casting technique. The casting solution was prepared by dissolving the polymer in chloroform at 60 °C. After being stirred until clear solutions were obtained, the solutions were cast on glass Petri dishes and dried in vacuo at room temperature prior to further investigation.

4.3.3 Surface Modification and Laminin Immobilization on Aligned Electrospun Fibrous and Solution-cast Film PHB Scaffolds

Figure 4.1 summarizes the chemical pathway of surface aminolysis and protein immobilization on the surface substrate. Before the aminolysis reaction, both PHB fibrous and film substrates were firstly immersed in an ethanolic aqueous solution (1:1 v/v) for 2 to 3 h to clean the surface and then washed with a large

quantity of deionized water. The surface aminolysis treatment was performed according to the modified method reported by previous work [17]. To maintain enough mechanical properties for practical application, PHB scaffolds were aminolysed for 15 min in 0.04 g/ml of HMD/IPA solution at room temperature. The aminolysed scaffolds were then rinsed successively with deionized water to remove unreacted HMD and dried *in vacuo* to rich a constant weight. Subsequently, the aminolysed PHB substrates were 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 substrates were 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 groups on modified scaffolds and carboxylic groups of laminin. Then, covalently bounded laminin-PHB substrates were 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.

4.3.4 Characterization of Surface Modified Aligned Electrospun Fibrous and Solution-cast Film PHB Substrates

The morphological of electrospun fibrous and solution-cast film PHB substrates were 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 morphological appearance was measured from the SEM photographs using image analysis software (SemAfore; JEOL, Insinööritoimisto J. Rimppi Oy, Finland).

The wettability of the unmodified and modified substrates 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 for 24 h and the measurements were processed at room temperature.

To examine the functionalized surface, 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 success of functionalized and immobilized on the modified PHB substrates.

4.3.5 Cell Culture and Cell Seeding

To evaluate the biological response of modified electrospun fibrous and solution-cast film PHB substrates as neural scaffolding materials, their biocompatibility in terms of cytotoxicity, cell adhesion and cell proliferation toward murine neuroblastoma Neuro 2a cell lineage (American Type Culture Collection : ATCC) was studied in comparison with that of unmodified substrates.

Neuro 2a cells 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 substrates 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 substrates and the wells, each substrate 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 substrate specimens.

4.3.6 Biological Evaluation

The indirect cytotoxicity evaluation of the modified and unmodified electrospun fibrous comparison with solution-cast film PHB substrates 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 substrates (~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.

For the cell attachment study, the Neuro 2a cells were seeded on both the fibrous and film substrates (~15 mm in diameter) in the wells of a 24-well TCPS at a cell density of 4.0 x 10^4 cells/well and allowed to attach to the specimens for 4 h. The viability of the cells on the unmodified film substrates 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 fibrous and film substrate (~15 mm in diameter) in the wells of a 24-well TCPS at a cell density of 4.0 x 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. The viability of the cells on the unmodified film substrates was used as the control. Each experiment was carried out in triplicate.

4.3.7 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 Haün, 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).

4.3.8 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.

4.3.9 Morphological Observation of Cultured Cells

After the culture medium had been removed, the cell cultured substrates 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.

4.4 Results and Discussion

The initial response of cells to the biomaterial mostly depends on surface properties of the substrates [6, 27, 28]. Positive charge is considered to facilitate cellular adhesion [28]. The most important aspect in the field of tissue engineering is to control the growth and behavior of cells on artificial biodegradable scaffolds in order to create the permissive environmental substitutes for tissue regeneration. A lot

of biocompatible and biodegradable synthetic materials have been studied in tissue engineering, because of their suitable mechanical strength, processability and their controllable degradation rates in biological environments. Many strategies have been developed to fabricate the 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 [27, 30-32]. 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.

Recently, a number of biocompatibility and biodegradability synthetic polymeric material have been investigated for potential use as matrixes for tissue regeneration [33-40]. PHB is a promising polymeric material due to its biocompatibility and biodegradability. Despite the inherent biocompatibility and biodegradability of PHB, actual utilization of this material as artificial scaffolding material is limited by its hydrophobicity. Many surface modification techniques have been developed for improving cell affinity, especially the surface immobilization of some ECM protein molecules [16-21]. Milner R. *et al.* [22] 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. And also, *in vitro* experiments have shown that neurite outgrowth is enhanced on scaffolds that were covalently bound or physical adsorption with laminin [22-24].

In the present contribution, the alignment electrospun fibrous and solution-cast film of PHB was carried out in a manner similar to a previous report [40]. In order to enhance the cell-substrate interaction, the aminolysis treatment and immobilization of ECM protein were carried out. The *in vitro* responses and cellular behavior on the modified electrospun fibrous and corresponding solution-cast film PHB substrates were investigated. The study was preliminary evaluated *in vitro* towards murine neuroblastoma Neuro 2a cell line (American Type Culture Collection: ATCC). The unmodified electrospun fibrous and solution-cast PHB scaffold was used as the internal controls.

4.4.1 Morphology and Physio-chemical Characterization of Electrospun Fibrous and Film PHB Substrates

As previous mention, the surface topography and surface chemistry have a strong effect on cell-surface interactions. The selected SEM images of unmodified electrospun fibrous and film PHB substrates are shown in Figure 4.2. The surface of the PHB film substrate was generally smooth with lots of island-like domains on the membrane surface as compared to the electrospun fibrous substrate. For electrospun fibrous substrate, smooth fibers without the presence of beads were obtained and the diameters of these fibers were 1.95 ± 0.19 μm. The fiber arrangement was oriented between -25° to +40° normalized to 90° (small Figure). More than 35%, 25% and 15% of fibrous scaffold was found in the orientation of 0°, +5° and -5°, respectively. The morphological appearances of electrospun fibrous and film substrates after subjected to the aminolysis treatment are shown in Figure 4.3a and 4.3b. The morphology of film substrate was changed to more roughness while the fibrous substrate appeared to have some wedge on the surface. However, the fibrous and film substrates were smooth again, which indicated that the thin layer of laminin is coverage of the surface after protein immobilization (Figure 4.3c and 4.3d).

The wettability of the surface plays an important role in the cell culture [27, 38]. For enhancing the wettability of the substrate surface, aminolysis treatment on the as prepared surface substrates were performed. In the aminolysis reaction, the mechanism proceeds via nucleophilic attack of one amino group of HMD on the carbonyl carbon of polymer to form a positively charged tetrahedral intermediate, resulting in the chain scission of polymer, the formation of an amide linkage and a free alcoholic group and the another free amino group of HMD. This treatment not only improves the hydrophilicity on the surface substrates, but also provides the necessary active sites for interacting with cell-adhesive molecules. In addition, the presence of functionality relevant biochemical cues shown to influence on the cellular response. To evaluate the effect of the aminolysis treatment on the surface, the wettability of unmodified and modified substrates were studied with static contact angle measurement. Figure 4.4 illustrated the change of the static water

contact angle after the modification. Evidently, all of the fibrous substrates exhibited the water contact angles in the range of about 81.2° – 135.3°, while all of the film counterparts showed the value in the range of about 77.6° – 98.4°. The observed hydrophobicity of the fibrous substrates in comparison with that of the films was probably a result of the surface roughness that introduced multiple contacting points on the surface of the water droplet such that the interface between the water droplet and the fibrous surface was not exactly solid/liquid. After the aminolysis treatment, the aminolysed substrates show a great change on hydrophilicity compare to the unmodified substrates. The difference in hydrophilicity should be a result of the presence of functional groups on the surface. The greater change in hydrophilic was observed on the fibrous substrates due to their highly surface area for providing the aminolysis reaction. The water contact angle dropped down again after laminin was immobilized on the scaffolds. This result indicated that the surface wettability is greater enhanced by protein immobilization.

The success of the modifications was also confirmed by the result from XPS analysis. Table 4.1 indicated the intensities (normalized to 100% total intensity) of the elements on the surfaces. There was no nitrogen signal appeared on the unmodified PHB substrates. The nitrogen signal appeared after aminolysis treatment, indicated the presence some of amino groups on the PHB substrates. After subsequent reaction with laminin protein, the intensity of nitrogen signal increased with N/C ratio from 0.0074 for aminolysed to 0.0164 for laminin immobilized on film substrate and 0.0133 for aminolysed to 0.0271 for laminin immobilized on fibrous substrate, respectively (see Table 4.2). These results revealed the success of laminin binding on the substrates, which was in accord with the increasing in wettability of the substrates.

4.4.2 Effect of Physio-chemical Characteristic of Electrospun Fibrous and Film PHB Substrates on Neuro 2a Cellular Behavior

4.4.2.1 Cytotoxicity

Cytotoxicity is a basic property of scaffolding materials. The potential for use of the modified and unmodified PHB substrates was assessed by indirect cytotoxicity assay using Neuro2a as the reference cell line. Figure 4.5 shows

the viability of the cells obtained from MTT assay after the cells had been cultured with extraction media from modified and unmodified PHB substrates 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 cultured with the extraction media from all of substrates were equivalent to that of the cells cultured with fresh SFM. This indicated that the substrates do not release some cytotoxic substance to the culture media, implying the biocompatibility of these materials toward Neuro2a. Previous reports showed that PHB had been repeatedly demonstrated with a good biodegradability and without cytotoxicity *in vitro* and *in vivo* [11-14, 40] Results from this work confirmed that the surface modified PHB substrates obtained from the studied materials can be used as scaffolds for Neuro 2a culture.

4.4.2.2 Cell Attachment and Cell Proliferation

The ability to support the attachment and to promote the proliferation of cells is one of the most important aspects of a functional scaffold. To evaluate such characteristics, Neuro 2a cells were either seeded or cultured on these scaffolds for 4 h, 1, and 3 d (see Figure 4.6). 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 unmodified PHB film substrates at 4 h was used as the control value to obtain the relative viability of the viability cells shown in the figure. For any given substrate, the viability of the cells increased with increasing cell seeding time. Comparatively, between the fibrous and the film substrates, the viability of Neuro 2a cells that had been seeded on the fibrous substrates, in most cases, was greater than that of the cells on the film counterparts, with an exception to Neuro 2a that had been seeded for 4 h which showed a comparable value between aminolysed and laminin-PHB substrates. At any given cell seeding time point, the viability of the cells cultured on laminin-PHB substrates were generally greater than that of the cells cultured on both the unmodified and the aminolysed substrates, with an exception to Neuro 2a on the aminolysed substrates which showed an equivalent values in their viability at 4 h of cell seeding. At day 1 after cell seeding, the viability of the cells on the Laminin-

PHB fibrous substrate was greater than laminin-PHB film, aminolysed fibrous, aminolysed film and unmodified substrates. At day 3 after cell seeding, the viability of the cells on the Laminin-PHB fibrous substrate was the greatest.

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 Laminin-PHB fibrous substrate. Between day 1 and day 3 after cell seeding, the largest increase in the viability of the cells was also observed on the Laminin-PHB fibrous substrate. Among the various substrates, the Laminin-PHB fibrous substrate showed better support for the attachment and proliferation of Neuro 2a cells than the other substrates. Among the fibrous scaffolds, the viability of cells on the Laminin-PHB fibrous substrate was the greatest, followed by that on the Laminin-PHB film, Aminolysed fibrous and unmodified substrates, respectively. This result indicated that the Laminin-PHB fibrous substrate was a better support for Neuro 2a at any given cell seeding time.

Tables 4.3 show selected SEM images of Nero 2a that were seeded on the surfaces of the unmodified and the surface modified PHB fibrous and film substrates at 4 h, day 1 and 3 after cell seeding at magnifications of 3500x (scale bar 5 μm), 2000x (scale bar 10 μm) and 1500x (scale bar 10 μm), respectively. 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 unmodified PHB substrates 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 modified 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, most of the cells on the flat surfaces of all the film substrates became more stretched and elongated their cytoplasm along the surfaces. For the fibrous substrates, between day 1 and day 3 after cell culture, the cells on the surfaces of the macrofibrous substrates also

elongated their cytoplasm along the fiber direction, with the cells being able to penetrate to the inner side of the scaffolds even more. Additionally, some of the cells wrapped around the individual fibers, while others anchored to multiple fibers.

The differences in the cellular behavior of Neuro 2a on the various substrates 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 [41]. In a cell culture experiment, the factors influencing the adsorption of proteins are surface wettability and surface charge [42, 43]. 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 [44, 45]. Thus, the difference in the viability of cell on the substrates could be contributions from the enhancement of the fiber direction that provided contact mediation and the adsorption of specific proteins on the surface of a substrate that provided chemical mediation, respectively. Thus the fibrous substrate coupled with laminin (Laminin-PHB fibrous) was postulated as the reason for the ability of the materials to enhance the attachment and proliferation of Neuro 2a. However, the unmodified substrates exhibited cytostatic property (i.e., cytostatic property: this is not cytotoxic but inhibits cell proliferation [46]) towards Neuro 2a, as suggested by the relative constancy in the MTT viabilities. All of the results on both the attachment and the proliferation of Neuro 2a on the surfaces of these various types of PHB substrates with different surface characteristics emphasized the importance of the surface topography of the substrates on the behavior of the particular cell line of Neuro2a, in which the cells showed a greater preference toward fibrous surfaces over flat ones. The fibrous surfaces could help the cells to elongate along the fiber direction and form cell-to-cell contacts with adjacent cells much easier, thus rendering the cells to perform their role during nerve regeneration much more conveniently.

4.5 Conclusions

In the present paper we report the *in vitro* responses of murine neuroblastoma Neuro 2a cell line on various types of surface modified electrospun fibrous and solution-cast film PHB substrates. The results were compared with those obtained on unmodified corresponding substrates that used as the internal control. The results from *in vitro* cell studies suggested that the alteration of surface topography and chemistry has a significant impact on adhesion and proliferation of cells. The introduction of contact guidance such fiber alignment and biochemical mediation such immobilization of adhesive protein enhanced the attachment and proliferation of the cells. On laminin immobilized fibrous substrate, Neuro 2a aligned and elongated unidirectionally along the fiber axes. Some of cells wrapped around the individual fibers, while others anchored to multiple fibers. This result showed a greater preference toward fibrous surfaces over flat ones. However, the unmodified substrates of both film and fibrous substrates exhibited cytostatic property in which inhibited the cellular proliferation. All of these results emphasized the importance of the surface topography and chemistry of the substrates on the behavior of the particular cell line of Neuro2a.

4.6 Acknowledgments

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).

4.7 References

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

[2] Willerth, S.M.; Sakiyama-Elbert, S.E. Approaches to neural tissue engineering using scaffolds for drug delivery, *Adv Drug Deliver Rev* **2007**, 59, 325–338.

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

[4] Teo, W.E.; He, W.; Ramakrishna, S. Electrospun scaffold tailored for tissue-specific extracellular matrix, *Biotechnol J* **2006**, 1, 918–929.

[5] He, L.; Liao, S.; Quan, D,; Ma, K.; Chan, C.;, Ramakrishna, S.; Lu, J. Synergistic effects of electrospun PLLA fiber dimension and pattern on neonatal mouse cerebellum C17.2 stem cells, *Acta Biomaterialia* **2010**, 6, 2960-2969.

[6] Supaphol, P.; Suwantong, O.; Sansanoh, P.; Srinivasan, S.; Jayakumar, R.; Nair, S.V. Electrospinning of biocompatible polymers and their potentials in biomedical applications, *Adv Polym Sci* **2012**, 246, 213-240.

[7] Wang, B.; Cai, Q.; Zhang, S.; Yang, X.; Deng, X. The effect of poly (l-lactic acid) nanofibers orientation on osteogenic responses of human osteoblast-like MG63 cells, *J Mech Behav Biomed* **2011**, 4, 600-609.

[8] Lee, C.H.; Shin, H.J.; Cho, I.H.; Kang, Y.M.; Kim, I.A.; Park, K.D.; Shin, J.W. Nanofiber alignment and direction of mechanical strain affect the ECM production of human ACL fibroblast, *Biomaterials* **2005**, 26, 1261-1270.

[9] Zong, X.; Bien, H.; Chung, C.Y.; Yin, L.; Fang, D.; Hsiao, BS.; Chu, B.; Entcheva, E. Electrospun fine-textured scaffold for heart tissue constructs, *Biomaterials* **2005**, 26, 5330-5338.

[10] Schnell, E.; Klinkhammer, K.; Blazer, S.; Brook, G.; Klee, D.; Dalton, P.; Mey, J. Guidance of glial cell migration and axonal growth on electrospun nanofibers of poly-epsilon-caprolactone and a collagen/poly-epilon-caprolactone blend, *Biomaterials* **2007**, 28, 3012-3025.

[11] 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.

[12] 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.

[13] 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.

[14] 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.

[15] Vert, M.; Li, S.M.; Spenlehaver, G.; Guerin, P. Bioresorbability and biocompatibility of apliphatic polyesters, *J Mater Sci-Mater M* **1992**, 3, 432-446.

[16] 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.

[17] 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.

[18] 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

[19] 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.

[20] 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.

[21] 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.

[22] 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.

[23] 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.

[24] 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.

[25] 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.

[26] 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.

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

[28] Liu, B.F.; Ma, J.; Xu, Q.Y.; Cui, F.Z. Regulation of charged groups and laminin patterns for selective neuronal adhesion, *Colloid Surface B* **2006**, 53, 175-178.

[29] 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.

[30] 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.

[31] 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.

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

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

[34] 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

[35] 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.

[36] 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.

[37] 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.

[38] 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.

[39] 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, 91-98.

[40] 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.

[41] 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.

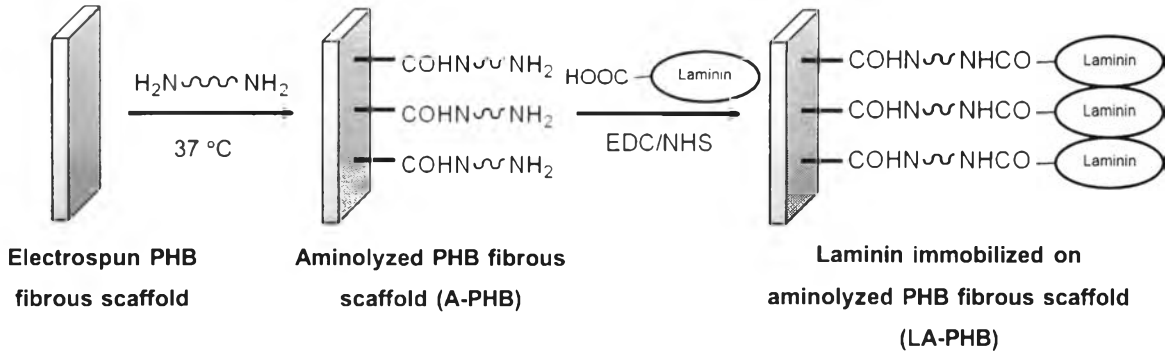
[42] Burns, N.L.; Holmberg, K.; Brink, C. Influence of surface charge on protein adsorption at an amphoteric surface: effects of varying acid to base ratio, *J Colloid Interf Sci* **1996**, 178, 116–122.

[43] 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.

[44] 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.

[45] Knopf, B.; Wollina, U. Electrophoretic mobilities of keratinocytes from normal skin and psoriatic lesions, *Arch Dermatol Res* **1992**, 284, 117–8.

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



Electrospun PHB fibrous scaffold | **Aminolyzed PHB fibrous scaffold (A-PHB)** | **Laminin immobilized on aminolyzed PHB fibrous scaffold (LA-PHB)**

Figure 4.1 Summarizes the chemical pathway for aminolysis treatment and laminin immobilization on the surface of the electrospun PHB fibrous scaffolds.

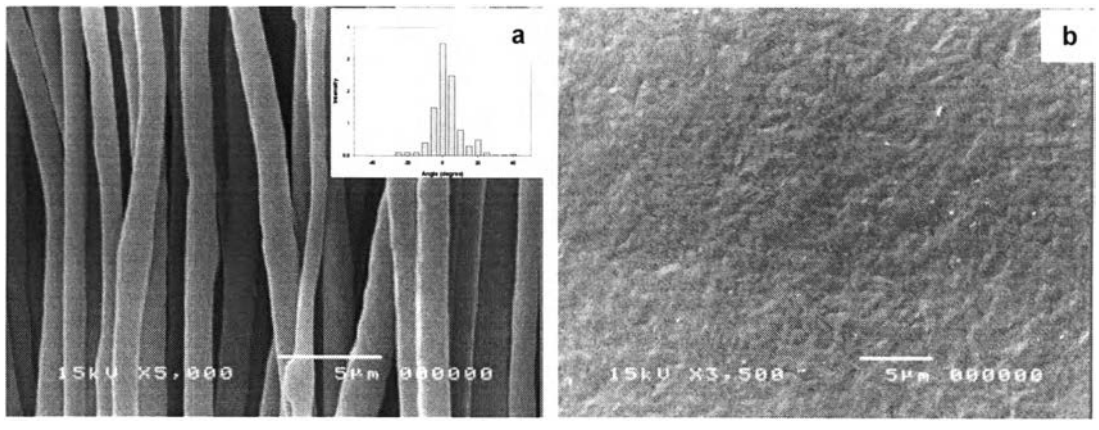


Figure 4.2 Selected SEM images of (a) unmodified electrospun fibrous and (b) film PHB substrate.

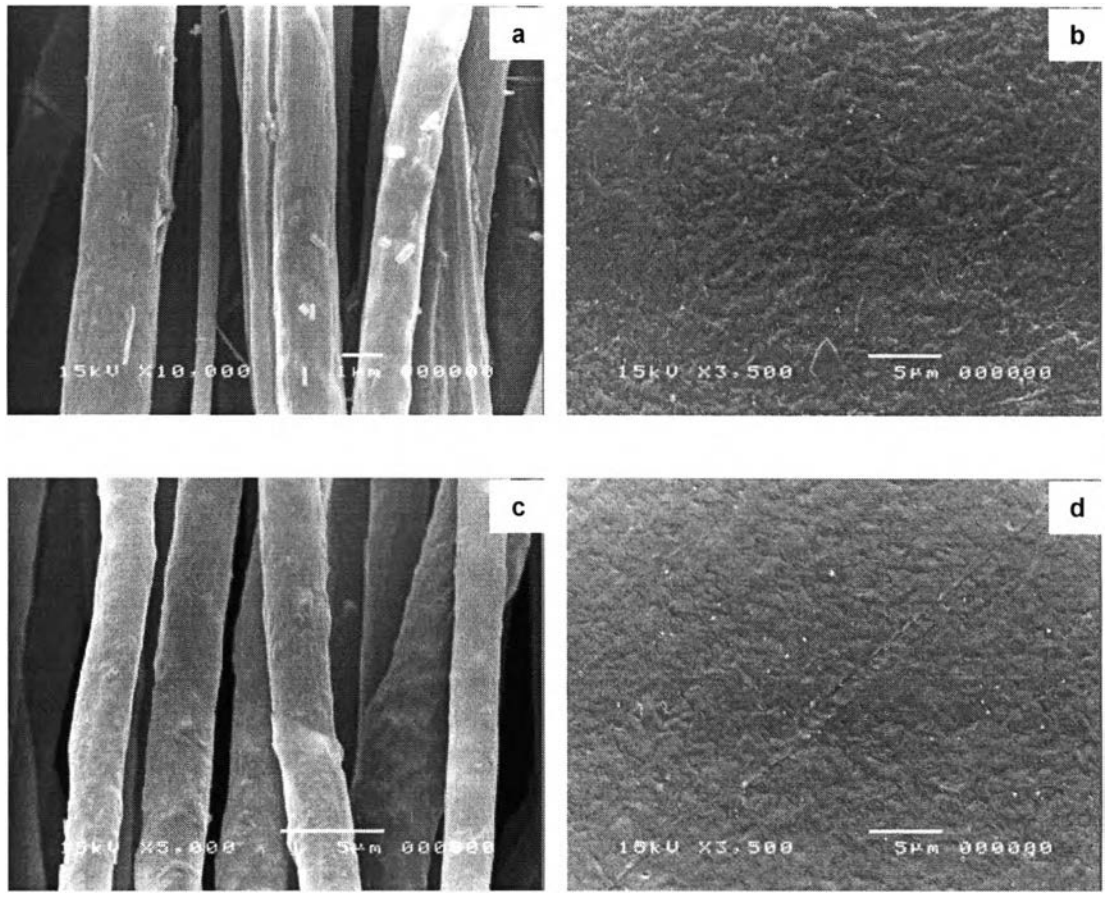


Figure 4.3 Selected SEM images of (a) aminolysed PHB fibrous, (b) film substrates using (0.04 g/ml of HMD/IPA solution for 15 min at room temperature), (c) laminin immobilized on aminolysed PHB fibrous and (d) film substrates.

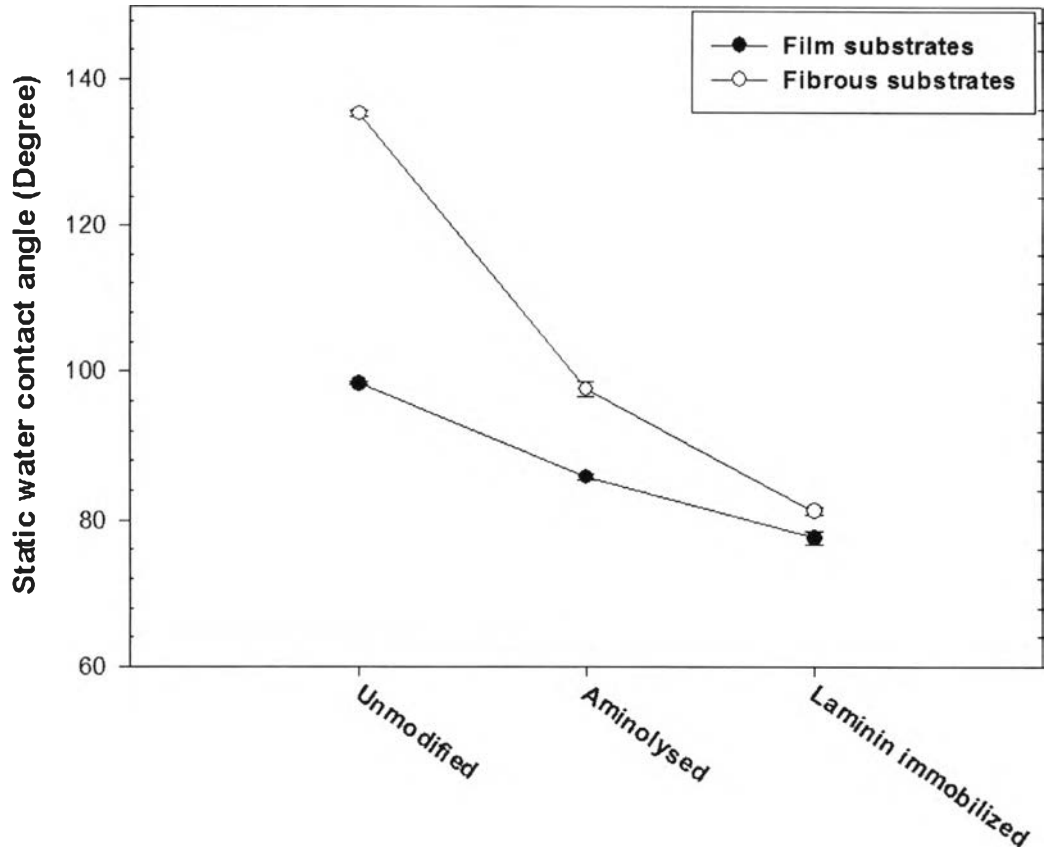


Figure 4.4 The resulting static water contact angles electrospun fibrous and film PHB substrates.

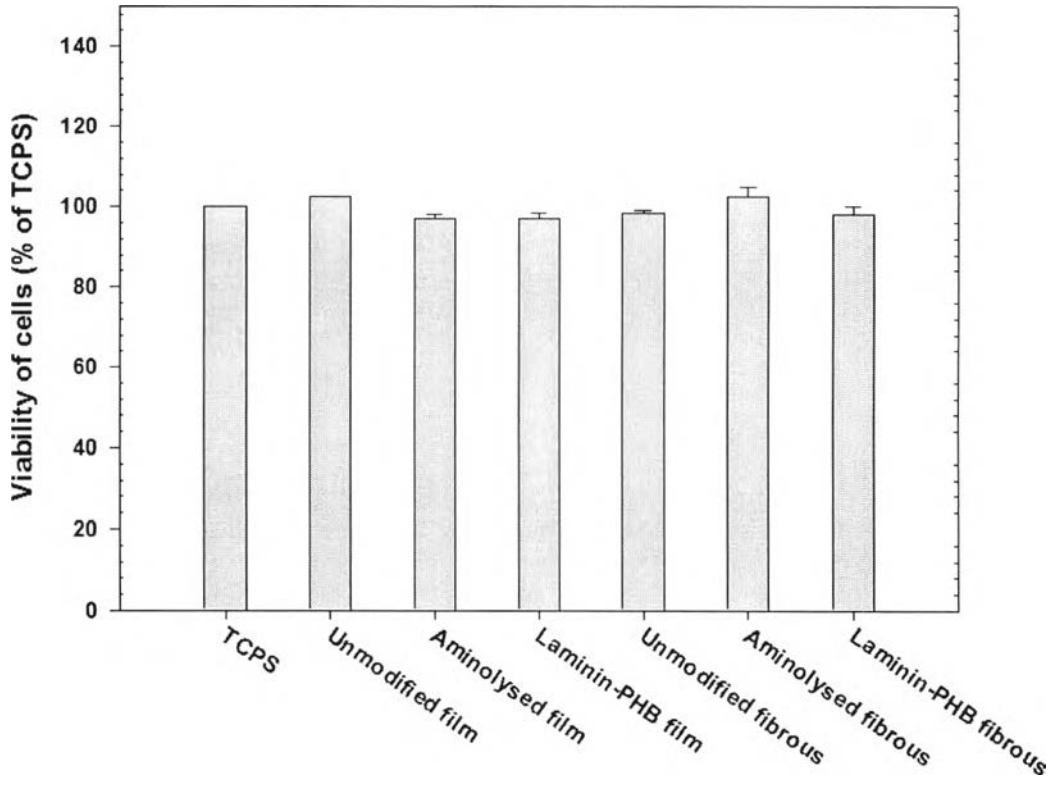


Figure 4.5 Indirect cytotoxicity evaluation of (a) unmodified PHB film, (b) unmodified PHB fibrous, (c) aminolysed PHB film, (d) aminolysed PHB fibrous, (e) laminin immobilized on aminolysed PHB film and (d) laminin immobilized on aminolysed PHB fibrous substrates based on the viability of Neuro 2a that 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.

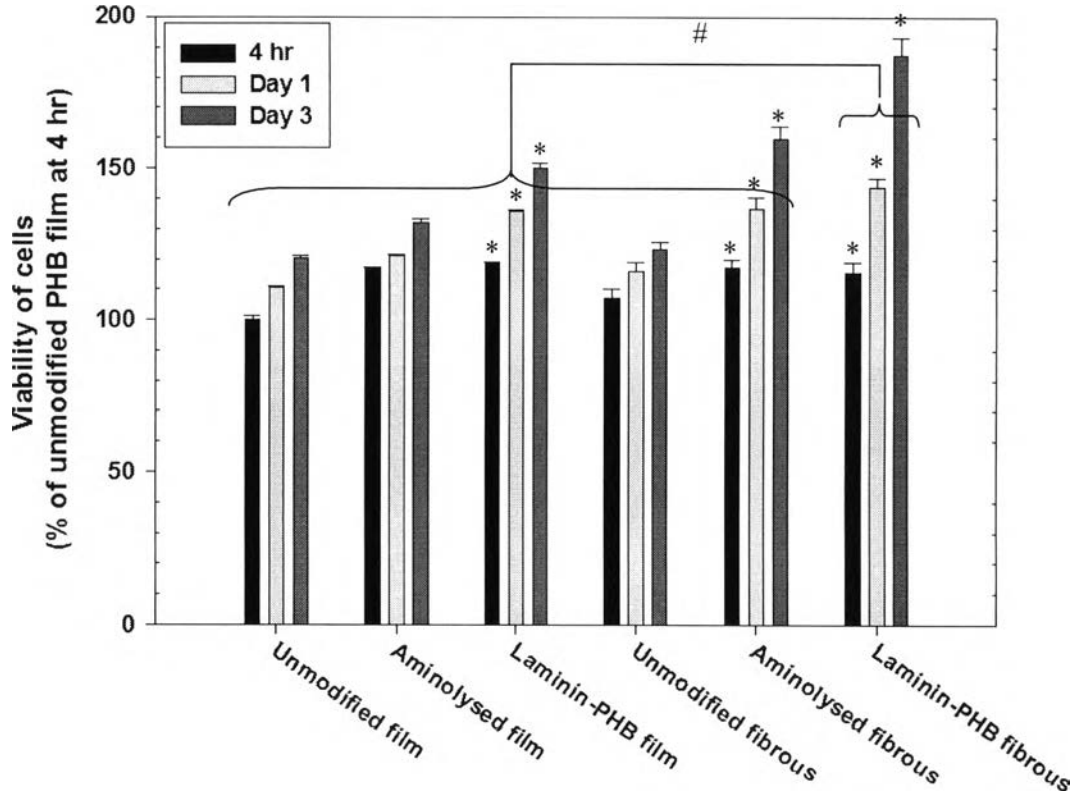


Figure 4.6 Attachment and proliferation of Neuro2a cells that were seeded on various PHB substrates for 4 h, days 1, and 3. The viability of the cultured cells that were seeded on the unmodified PHB film for 4 h was used as the reference to arrive at the viability of the cultured cells shown in the figure. $^{*,\#}$ Significantly different at $p < 0.05$.

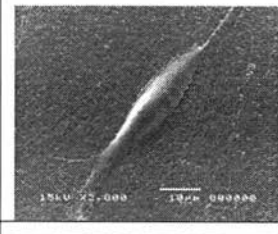
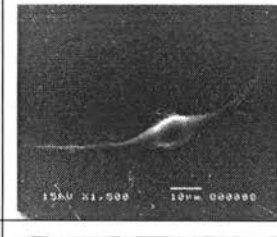
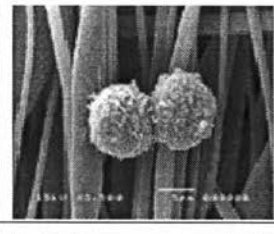
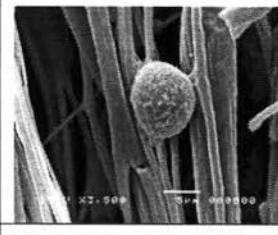
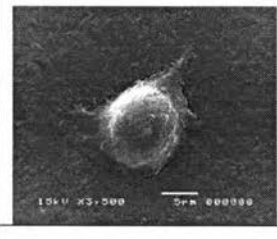
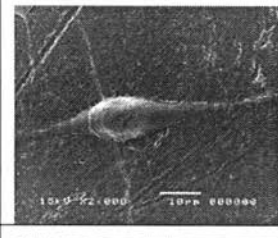
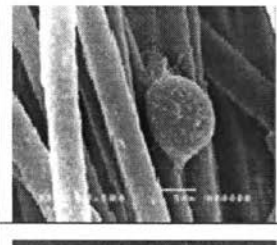
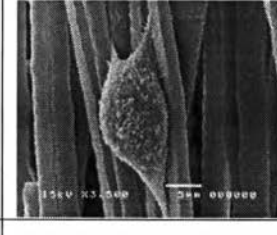
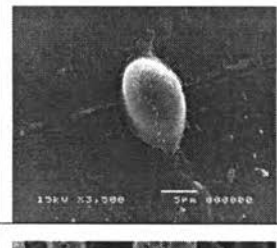
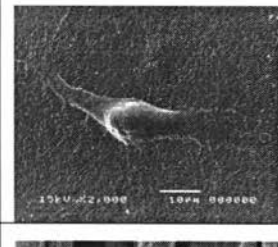
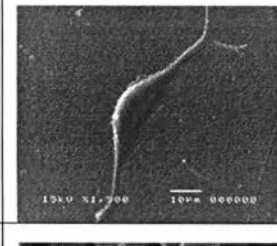
Table 4.1 Element composition of carbon, oxygen, and nitrogen on the various surfaces of PHB substrates as determined by X-ray photoelectron spectrometry.

Type of substrates	C atomic concentration (%)	O atomic concentration (%)	N atomic concentration (%)
Unmodified PHB film	69.9	30.1	0
Aminolysed PHB film	68.0	31.5	0.5
Laminin immobilized PHB film	66.9	32.0	1.1
unmodified PHB fiber	70.2	29.8	0
Aminolysed PHB fiber	67.8	31.3	0.9
Laminin immobilized PHB fiber	66.3	31.9	1.8

Table 4.2 Atomic ratios of N1s/C1s and O1s/C1s on the various surfaces of PHB substrates as determined by X-ray photoelectron spectrometry.

Type of substrates	N1s/C1s ratio	O1s/C1s ratio
Unmodified PHB film	0	0.4306
Aminolysed PHB film	0.0074	0.4632
Laminin immobilized PHB film	0.0164	0.4783
Unmodified PHB fiber	0	0.4245
Aminolysed PHB fiber	0.0133	0.4617
Laminin immobilized PHB fiber	0.0271	0.4811

Table 4.3 Representative SEM images of murine neuroblastoma Neuro2a cell line (ATCC, CCL-131) that had been seeded or cultured on various types of PHB substrates for 4 h, 1 d and 3 d.

Type of substrate	Cell seeding/culturing time point		
	4 h	1 d	3 d
Unmodified PHB film substrate			
Unmodified PHB fibrous substrate			
Aminolysed PHB film substrate			
Aminolysed PHB fibrous substrate			
Laminin immobilized on aminolysed PHB film substrate			
Laminin immobilized on aminolysed PHB fibrous substrate