CHAPTER IV
RESULTS AND DISCUSSION

4.1 Preparation and Characterization of As-Spun Chitosan Nanofibers Scaffold

Electrospinning has been recognized as an efficient method for the fabrication of ultrafine polymer filaments by introducing a high electrical charge into a polymer solution or melt. When an electric field is applied between a needle capillary end and a collector, the surface charge is induced on a polymer fluid deforming a spherical pendant droplet to a conical shape. As the electric field surpasses a threshold value where electrostatic repulsion force of surface charges overcome surface tension, the charged fluid jet is ejected from the tip of the Taylor cone [Ramakrishna et al., 2003]. The charge density on the jet interacts with the external field to produce instability and stretched polymeric fiber with simultaneous rapid evaporation of the solvent. Nanofibers have amazing characteristics such as high specific surface area and high porosity with very small pore size. Therefore, nanofibers can be promising materials for many biomedical applications such as tissue templates, medical prostheses, artificial organ, wound dressing, drug delivery, and pharmaceutical composition. Several biocompatible materials have been electrospun into nanofibers, such as DNA [Fang X. et al., 1997], silk [Min BM. et al., 2004], collagen [Matthews JA. et al., 2002], fibrinogen [Wnek GE. et al., 2003], gelatin [Huang ZM. et al., 2004], hyaluronic acid [Um IC. et al., 2004], and oxidized cellulose [Son WK. et al., 2004].

Recently, much attention has been paid to chitosan; a natural polysaccharide obtained from N-deacetylation of chitin and it is a copolymer of D-glucosamine and N-acetyl-D-glucosamine. Chitosan is an interesting biopolymer because of it has several distinctive biological properties including good biocompatibility, biodegradability, antimicrobial activity, antitumor effect and wound healing properties. Moreover, it has a similar molecular structure to the glycosaminoglycans in the basal membrane and extracellular matrix; therefore, it can provide the interaction between the extracellular adhesive molecules such as laminin, fibronectin and collagen [Pangburn SH. et al., 1982]. These suitable properties provide it into

many applications such as wound dressing, wound healing, drug delivery system and various tissues engineering application [Vasudev SC. et al., 1997].

The major complication in electrospinning chitosan is the poor solubility of chitosan and the high viscosity of its aqueous solution. At low polymer concentrations, the solutions do not contain sufficient material to produce stable solid fibers. With increasing polymer concentration, the number of direct interchain associations of chitosan molecules in the solution increases rapidly causes a highly viscous gel, rendering the solution unspinnable.

From the literature reviews, Yamamoto et. al. [Ohkawa K. et al., 2004] studied the effect of electrospinning solvent on the morphology of nonwoven fabric. They found that trifluoroacetic acid/dichloromethane co-solvent system has the ability to form homogeneity fibrous structure with a mean diameter of 330 nm. There are two possible reasons why the electrospinning of chitosan is successful when using TFA: (i) TFA forms salts with the amino groups of chitosan [Hasegawa et al., 2004] and this salt formation destroys the rigid interaction between the chitosan molecules, making them ready to be electrospun; (ii) the high volatility of TFA is advantageous for the rapid solidification of the electrified jet of the chitosan-TFA solution. In a more recent report, concentrated acetic acid solution was used as electrospinning solvent to fabricate chitosan nanofibers, which showed that the uniform nanofibrous structures with an average fiber diameters of 130 nm was successfully electrospun in 90% aqueous acetic acid solution [Geng X. et al., 2005].

In this work, a number of parameters that affect on the fiber morphology were study by using TFA as solvent.

4.1.1 Effect of Polymer Concentration on as-Spun Fibers

The effects of polymer concentration on morphological appearance of the as-spun chitosan fibers at a given apply voltage (25 kV) and collecting distance (15 cm.) was evaluated. When the chitosan concentration was 5 wt.-%, a combination between beads and fibers was observed (Figure 4.1-a). Solution with low concentrations did not have enough chain entanglement to withstand the force acting on polymer jet. Once the charged jet was broken up, surface tension resulted in the formation of discrete droplets. Increased the polymer concentration to 6 wt.-%,

fibers were predominantly deposited on the collector because the chain entanglement high enough to prevent the breaking of charge jet (Figure 4.1-b). When the chitosan concentration further increased to 7 wt.-%, the bead fraction remarkably decreased, resulting in more uniform fiber with interconnection. (Figure 4.1-c; average diameter, 142±15 nm). Although, when the chitosan concentration was 8 wt.-%, the bead-liked fibrous structure appeared again. Additionally, the sized of beads in this case were larger than 5 and 6 wt.-% concentration. The possible reason to explain this evidence due to the increasing in viscosity of polymer solution decreased the continuous flow through the needle (Figure 4.1-d).

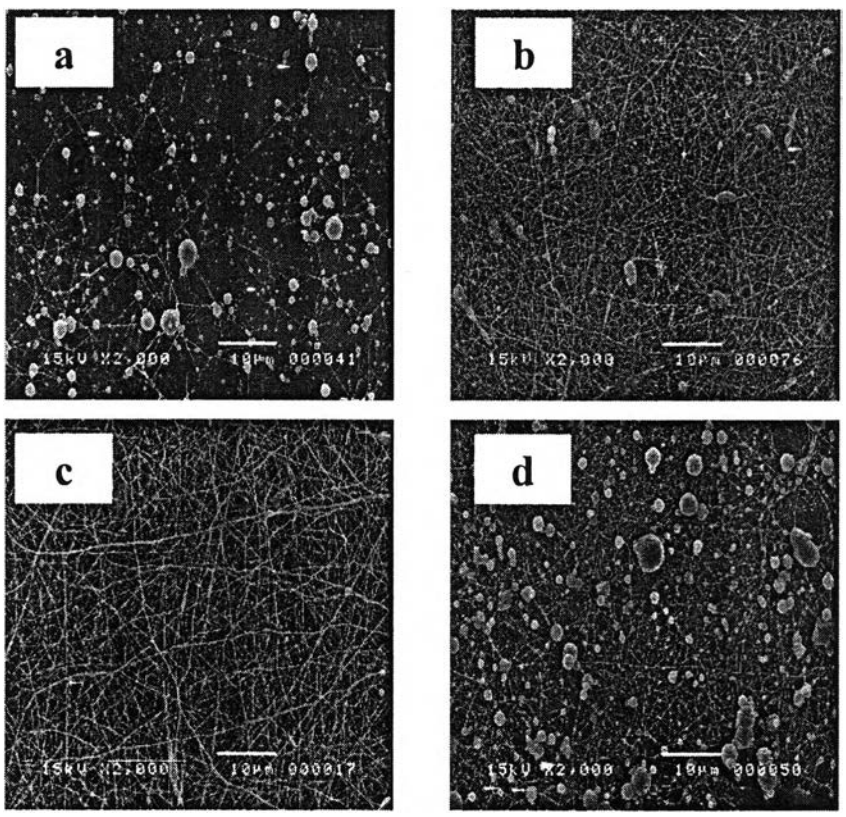


Figure 4.1 Morphological changes in the electrospun chitosan nanofibers dissolved in triffluoroacetic acid (TFA) at various concentrations. Chitosan concentration were 5 wt.-%(panel a; magnification, x 2000), 6 wt.-%(panel b; magnification, x 2000), 7 wt.-%(panel c; magnification, x 2000), 8 wt.-%(panel d; magnification, x 2000).

However, in the case of 7 wt.-% chitosan-TFA solution, small beads and interconnected fibers (Figure 4.2) were still found because the fibers have not enough time to dry completely before deposited on the collector. To optimize the condition for preparing the as-spun scaffold, co-solvents were used in this experiment.

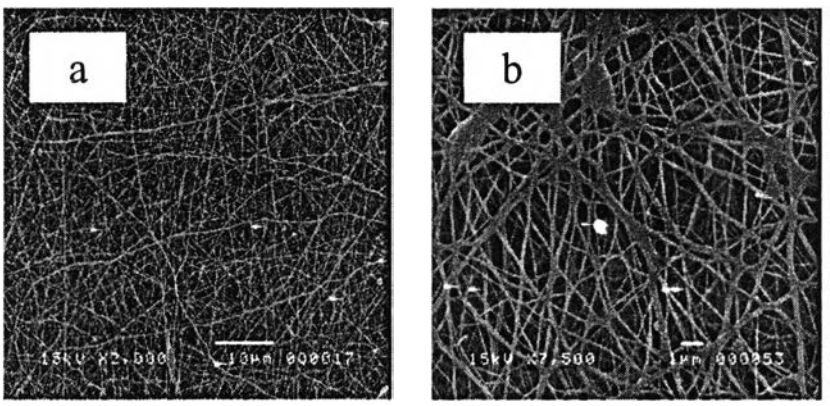


Figure 4.2 SEM images of chitosan fibers at 7 wt.-% chitosan-TFAsolution; a) magnification, x 2000, b) magnification, x 7500.

4.1.2 Effect of Trifluoroacetic Acid (TFA)/dichloromethane (MC) Ratios on Morphology of as-Spun Fibers

In the application, the optimization of the electrospinning conditions would be necessary for the preparation of a homogenous (not interconnected) fiber network of chitosan. One possible approach to this optimization was to mix a volatile organic solvent with TFA. In this work, dichloromethane (MC) was chosen as a mixed solvent. The experimental performed under the applied voltage of 25 kV, and 15 cm of collecting distance.

When the mixing ratio was lower than 80:20, the bead-like fiber structure was obtained. This evidence can be explained by the increased in non-solvent part (dichloromethane) caused the polymer chain contracted together and reduced the chain entanglement, resulting in the charged jet being broken up to form the discrete droplet. However, the average fiber diameter increased with the amount of dichloromethane in co-solvent ratio (Figure 4.3-a, b, and c: average diameter, 145±6 nm, 147±10 nm, 150±8 nm, respectively). These could be explained by the

reduction in the polymer viscosity caused by the polymer chain contracted together and reduce the chain entanglement. The network morphology prepared from the TFA:MC = 70:30 solvent became more homogenous due to it might balanced the ratio between trifluoroacetic acid and dichloromethane to form enough the chain entanglement for resisting the force acting on the polymer jet. The small amount of beads and interconnected fibers also be seen, indicating that a homogenous fiber network could be prepared under this condition. However, by increasing the MC ratio above 70:30, the chitosan partly separated from the solution.

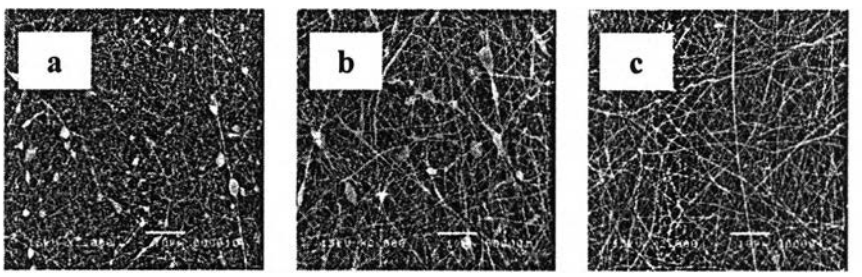


Figure 4.3 SEM images of chitosan fibers at 7 wt.-% chitosan in TFA:DC at various ratio; a) 90:10 (magnification, x 2000, b) 80:20 (magnification, x 2000), c) 70:30 (magnification, x 2000).

4.1.3 Effect of Collecting Distance and Applied Voltage on The Fibers Morphology of as-Spun Fibers

The effect of collecting distance and applied voltage on morphological appearance of as-spun chitosan fiber was shown in figure 4. The chitosan concentration and co-solvent ratio was fixed at 7 wt.-% chitosan in 70:30, TFA:CM ratio. Interestingly, when the collecting distances was 15 cm (Figure 4.4-a, b and c), a combination of beads and fibers was observed in every applied voltage. This could be explained by the shorter path length for traveling to the target causing the fiber having not enough time to elongate, resulting in the bead-liked fibers depositing on the target. Likely at 15 cm. collecting distance, the bead-liked fibers were observed again due to the chain entanglement might not prevent the breaking-up of charge jet when the collecting distance was increased more than 20 cm (Figure 4.4-g, h, and i). However, when the collecting distance was 20 cm (Figure 4.4-d, e,

and f), the small amount of beads still could be observed, expected at 25 kV of applied voltage. The mean diameter of the chitosan fiber that obtained from this condition was 130±10 nm.

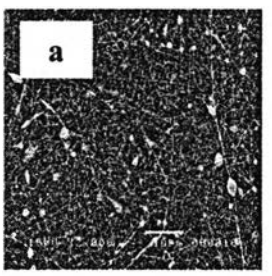
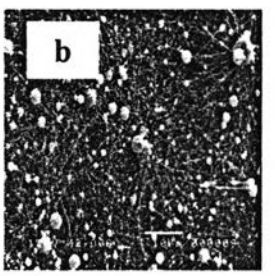
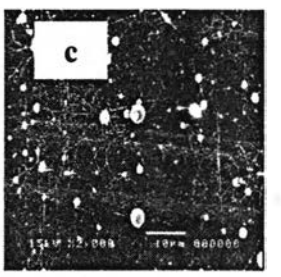
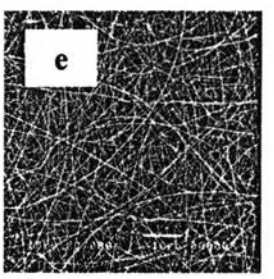
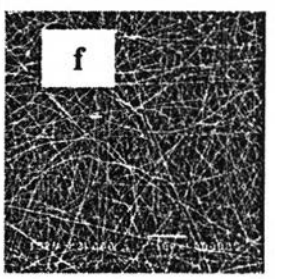
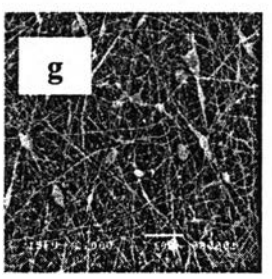
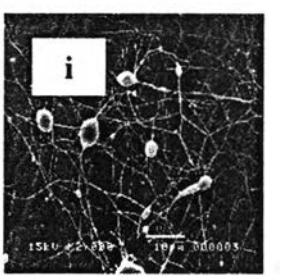
Applied Voltage (kV) / Collecting Distance (cm)	20 kV.	25 kV.	30 kV.
15 cm.			
20 cm.			
25 cm.			

Figure 4.4 SEM images of chitosan fibers at 7 wt.-% chitosan in TFA:DC = 70:30 at various applied voltage (kV) and collecting distance (cm).

Thus, the optimum condition used for this experiment was 7 wt.-% chitosan dissolved in 70:30, TFA:CM ratio, an applied voltage was 25 kV, and 20 cm of collecting distance. The mean diameter of the chitosan fiber that obtained from

this optimum condition was 130±10 nm. To further evaluate the physical and mechanical properties, and the feasibility to use as scaffolds, the as-spun chitosan mats were collected onto a rotating drum until 20±5 μm were obtained (around 24 h).

4.2 Physical Characterization of as-Spun Scaffold

4.2.1 Weight Loss and Swelling Behaviors of Fibrous Scaffold

The ability to maintain the fibrous structure in an aqueous environment, particularly in vivo applications, was the major concern. Thus, the weight loss and the swelling behavior of scaffold were evaluated by using chitosan film as references. Without treating with the aqueous alkaline solution, as-spun chitosan mats dissolved completely in water in a few minute. After the neutralization treatment, the loss in the weight of the fibrous memebrane samples increased very rapidly during the first three weeks and gradually increased until it leveled off after about 6 weeks. For comparison, the loss in the weight of the cast film samples also increased very rapidly during the first three weeks in submersion, but, after three weeks, no significant change was observed. After 12 weeks, the loss in the weight of the film samples was about half that of the fibrous samples (i.e. 9 and 16%, respectively), a result of the very much greater surface area of the fibrous structure in comparison with that of the film (Figure 4.5).

In analogy to the weight loss results, the degree of swelling was found to increase with time (Figure 5.6). Interestingly, the degree of swelling of as-spun chitosan at the initial state was rapidly increased when compared with chitosan film; its possible reason might be explained because it had three dimensional structure which made the fibrous scaffold could absorb more water into its matrix. However, for a long immersion time, the swelling degree of chitosan film was still higher than the as-spun fibers. The reason on the increasing in the degree of swelling in chitosan film might be caused by the lower degree of crystallinity when compared with chitosan fibers. From these results suggested that the neutralization treatment provided the close packing in the chitosan structures which resisted the water molecule to penetrate and swell the fibers matrixes.

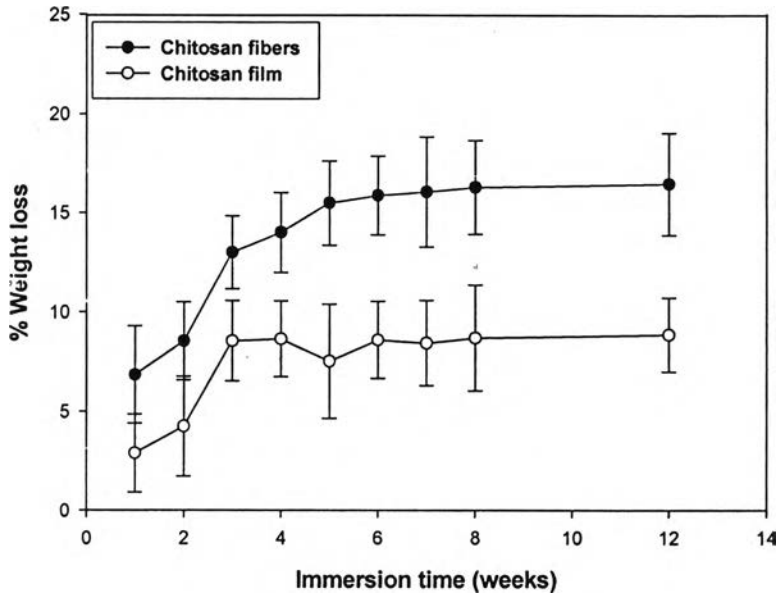


Figure 4.5 Percentage of weight loss of (-•-) chitosan fibers and (-O-) chitosan film after the neutralization with 5M of Na_2CO_3.

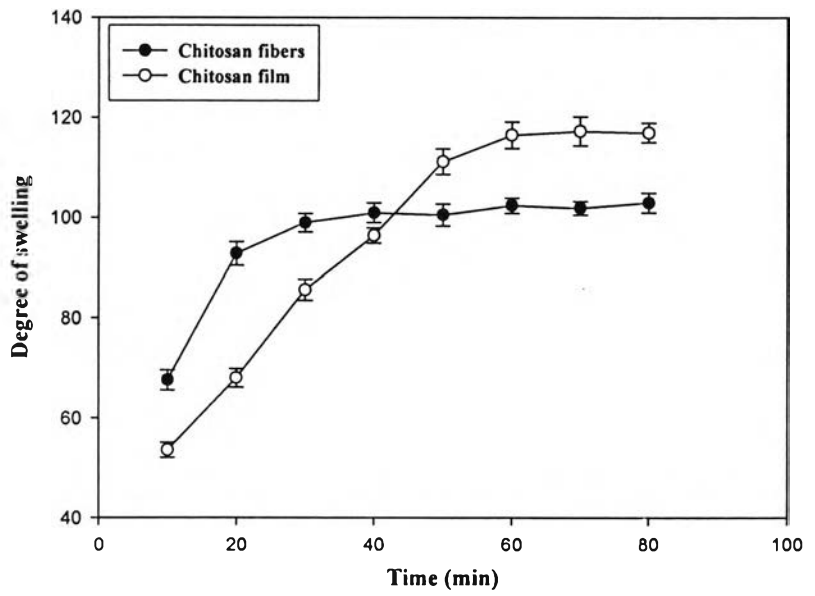


Figure 4.6 Degree of swelling of (-•-) chitosan fibers and (-O-) chitosan film after the neutralization with 5M of Na_2CO_3.

4.3 In vitro Nerve Regeneration

A major problem related to the treatment of peripheral nerve injury is the bridging of large gaps between the cut ends of the transected nerve. Three dimensional materials have recently been used as scaffolds for tissue regeneration to take the place of traditional direct suture, autograft, and allograft techniques, which have some disadvantages, such as the limited availability of donor tissue and morbidity related to its sacrifice. The use of artificial nerve materials that create a favorable environment for nerve regeneration has become a strategy for repairing major nerve defects.

In recent years the possibility of using artificial materials such as fibronectin mats through which axons could regenerate has been explored [Brown RA. et al., 1994 and Ejim OS. et al., 1993]. Another possibility would be filaments composed of poly-glycolic acid [3–5]. Yet despite these advances and contributions in the field of tissue engineering, current results with nerve conduits have failed to equal nerve regeneration equivalent to autogenous grafts for large distances. The failure is proposed to be the result of a combination factors [Lundborg G. et al., 1982 and Gu X., et al., 1995], such as the inability of neurotrophic factors produced by distal stumps to reach the proximal stump, the lack of a matrix bridge as a supporting structure, and the absence of Schwann Cells (SCs) [Williams LR. et al., 1987].

It is well known that SCs play a crucial role during nerve regeneration through the production of growth factors and the excretion of extracellular matrix [Bunge RP. et al., 1994], such as nerve growth factor, brain-derived neurotrophic factor, laminin, fibronectin and collagen IV, among others. Recently, there have been interests to introduce cultured SCs into nerve regeneration as a persistent source of neurotrophic factors. A promising alternative for the repair of peripheral nerve injuries is the bioartificial nerve graft, comprising biomaterials pre-seeded with SCs, which is an effective substrate for enhancing nerve regeneration. Therefore, there is a need for a new material that can take the place of autografts and naturally degrade in the body [Rutkowski GE. et al., 2002]. This material could repair certain distant nerve injury and provoke very little or no immune rejection by the body.

Among various biomaterials, chitosan becomes one of the most widely studied polymers since it has several distinctive biological properties including good biocompatibility, biodegradability, antimicrobial activity, antitumor effect and wound healing properties. Moreover, it has a similar molecular structure to the glycosaminoglycans in the basal membrane and extracellular matrix; therefore, it can provide the interaction between the extracellular adhesive molecules such as laminin, fibronectin and collagen [Pangburn SH. et al., 1982]. These suitable properties render its many applications such as wound dressing, wound healing, drug delivery system and various tissues engineering application [Vasudev SC. et al., 1997].

There have been no reports comparing chitosan membranes with as-spun chitosan scaffold. Moreover, the effectiveness of chitosan scaffolds were compared with most widely used biocompatible materials, likely PLLA film. The possibility for use of the as-spun chitosan nanofiber mats as a tissue scaffolding material was conducted by using the schwann cell line RT4-D6P2T and the mouse fibroblast cell line L929 as reference cells. For such a purpose, as-spun chitosan nanofiber mats, chitosan films and PLLA films with 20±5 μm in thickness were used in this experiment and bare culture plates were used as control.

This study uses several tests to investigate the possibility for use chitosan as scaffolding material in terms of biocompatibility, cell adhesion, and cell proliferation. The quantitative measure of cell viability by MTT assay was used in this studied.

4.3.1 Biocompatibility Test

Biocompatibility is a basic property that provides the material to use for the tissue scaffolding. In this present work, the biocompatibility of chitosan films, chitosan fibers and PLLA films were evaluated based on indirect cytotoxicity method. This experiment was conducted by using the Schwann cell line RT4-D6P2T and the mouse fibroblast cell line L929 as reference cells. Figure 4.7 shows the numbers of living cells after the mouse fibroblast and Schwann cells were cultured in the extraction media solution from chitosan fibers, chitosan films, and PLLA films for 24 h. The experimental results showed that no significantly difference in the cell

viability of mouse fibroblast between the groups of material. Similar to mouse fibroblasts, there are no significant difference in the viability of Schwann cells.

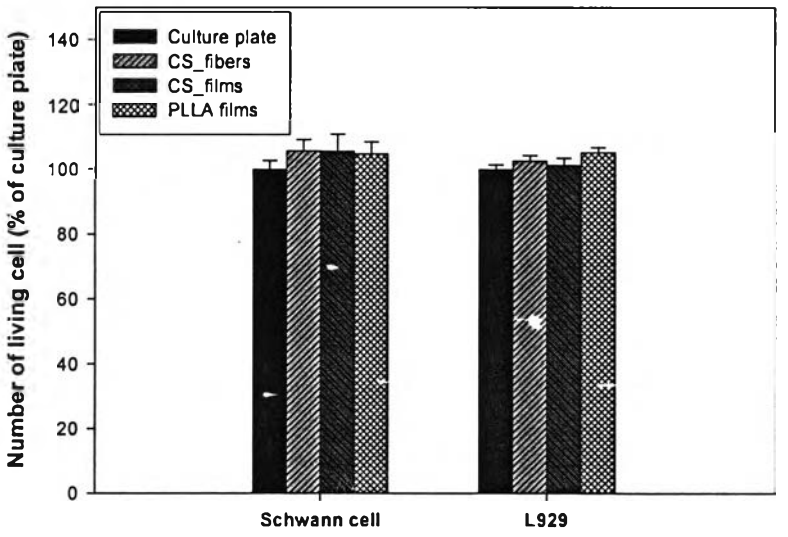


Figure 4.7 MTT-tetrazolium (MTT) assay. The mitochondrial metabolic activity of mouse fibroblast L929 and Schwann cells line RT4-D6P2T cultured for 24 h in the extracted media of chitosan fiber, chitosan film and PLLA film.

Chitosan biocompatibility had already been proved in vitro towards myocardial endothelial and epithelial cells [Popowicz P. et al., 1985], fibroblasts [Izume M. et al., 1989], hepatocytes [Elcin Ym. Et al., 1999], chondrocytes and keratinocytes [Denuziere A., 1998]. It was also noticed that chitosan is biocompatible for mammals and humans in vivo [Muzzarelli RAA et al., 1995]. All these results implied that chitosan fibers, chitosan films, and PLLA films were cytocompatibility and did not release cytotoxic substances in the culture medium towards mouse fibroblasts and schwann cells.

4.3.2 Cell Attachment and Cell Proliferation Tests

Attachment and proliferation of cells on the scaffold is one of the most important aspects of scaffolding materials. The number of cells attached and proliferation on a substrate could be determined by the UV absorbance from the MTT assay. In this work, two types of scaffolding materials, i.e. as-spun chitosan fibers, and chitosan films, were evaluated in comparison with PLLA films. Figure

4.8 shows attachments of Schwann cells on chitosan fibers, chitosan film, and PLLA film. The experimental results showed that the adhesion of Schwann cells on chitosan and PLLA films was significantly lower than that on chitosan fibers. In case of the attachment on a given substrate, the attachment on chitosan fiber continuously increased with time, whereas the amount of cell adhesion on chitosan and PLLA films at a given time was nearly constant. This behavior could be related to the increasing in surface area that provides more available surface for cell attachment in comparison with film scaffold, thus promoting the adhesion.

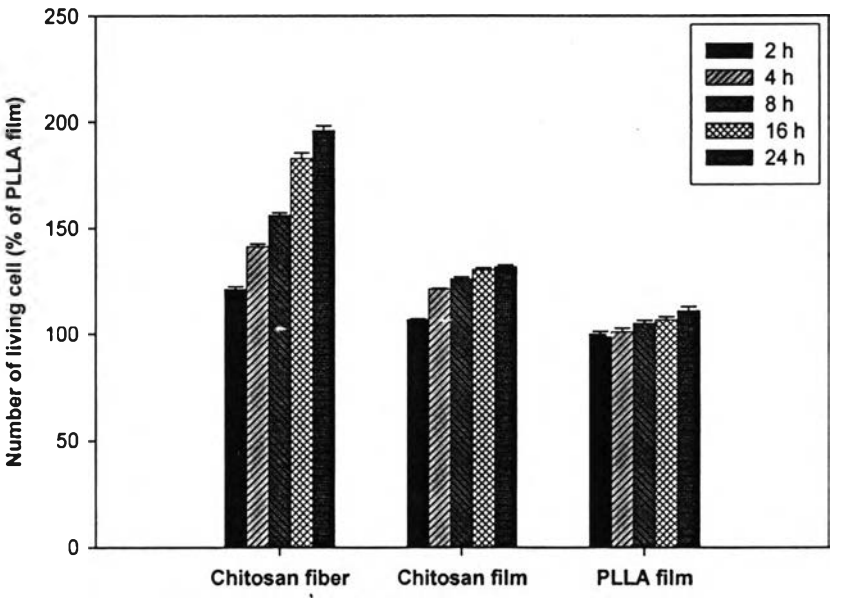
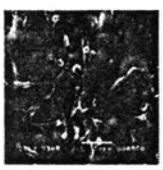
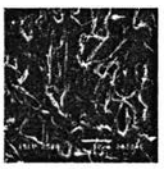
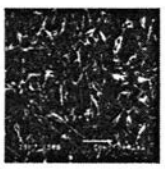
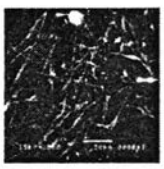
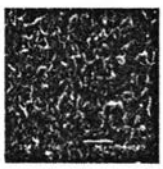
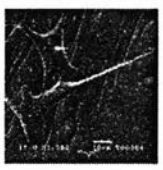
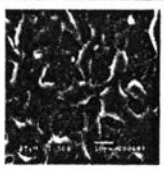
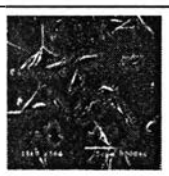
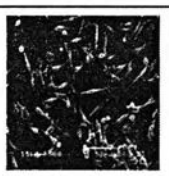
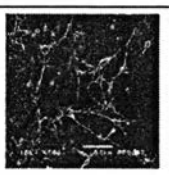
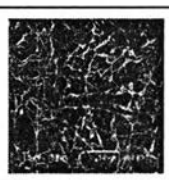
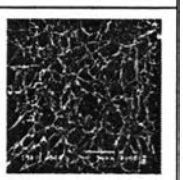
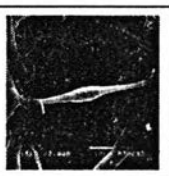
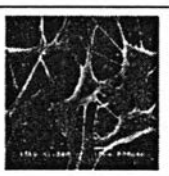
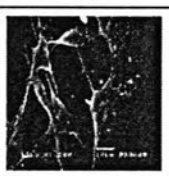
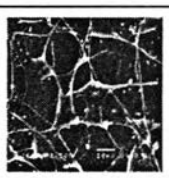
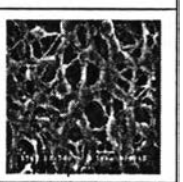
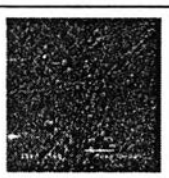
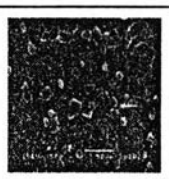
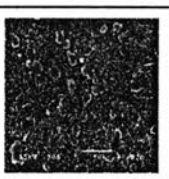
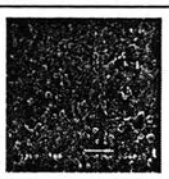
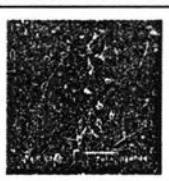
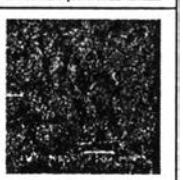
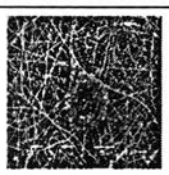
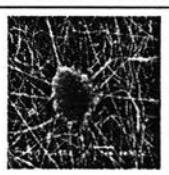
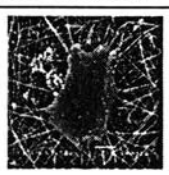
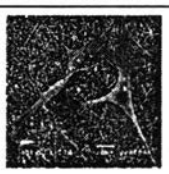
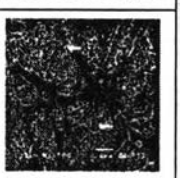


Figure 4.8 Attachment of Schwann cell on bare culture plate, chitosan fiber, chitosan film, and PLLA film as a function of time in culture. The number of living cells was determined by MTT-tetrazolium (MTT) assay.

For confirming the MTT assay results, with regards to cell interaction and spreading, on various scaffolds were observed by SEM images as shown in Table 4.1. At 2 h after seeding, most Schwann cells on film scaffolds adhered and stretched out, although there were also a number of cells still in round shape, indicating that the cells were not yet fully attached. After 4 h, cells completely changed from their original round shape to elongated and spindle-like shape, expect chitosan film. After 8 h of culturing, most of cells started to expand and elongated

along film surface. This cell morphology was corresponded to the previous work in which Schwann cells were cultured onto a flat surface like film and glass substrates [Chatelet C. et al., 2000].

Table 4.1 SEM images of Schwann cells culture on various scaffolding materials at different times in culture

	2h	4h	8h	1 day	3 day	5day
PLLA film						
						
Chitosan film						
						
Chitosan fiber						
						

In a more recent reported [Yuan Y. et al., 2003], Schwann cells were cultured on chitosan fibers (15 μm in diameter) which could remodel the anatomical of the nerve shape. The results showed that the cells migrated spiraling along the fibers end to end and the long olivary cells encircled the fibers in three-dimensional fashion. However, the difference in cell morphology was observed when Schwann cells were cultured on the as-spun fibrous scaffold which exhibited the fiber diameter in sub-micron range. SEM images indicated the influence of the size and fiber directional on the significant changing in phenotypes of Schwann cells as compared to film scaffold and glass substrate. At 2 h of seeding, the cells that were cultured on the fibrous scaffold were still in round shape and demonstrated small branches on their surfaces. When the culturing time increased to 4 h, the cells became expanded along the fibers axis and start to stretch their branches to help attach them on the fiber surface, and cells already expanded after reach 8 h of culturing. It is interesting to see that branches of cells tended to attach to and grow along the polymer nanofibers whose diameter is similar to that of anchoring ligand.

For further evaluation the possibility to use as-spun chitosan as scaffolding material, the cell proliferation was investigated. The effect of biomaterials on the proliferation of Schwann cells at day 1, 3, and 5 after being allowed for cell attachment for 24 hr (i.e. the attachment at 24 hr was taken as the proliferation day 1) is illustrated in Figure 4.9. The results from MTT assay showed the similar trends of proliferation between the culturing scaffolds were observed. Based on the results measuring from the metabolic activity of living cells indicated that there were no different in an ability to promote the cell proliferation in terms of the types of the scaffolding biomaterials. However, these results were not corresponded to the cell morphology and the number of living cells obtained from SEM images and by counting under optical microscopy (Table 4.2), respectively. The possible reasons used to explain this evidence was the increasing in metabolic activity for surviving when the reference cells were culture on the impractical environment, result in more the reduction of the yellow Tetrazolium salt MTT to purple crystals whereas the number of living cells did not increase.

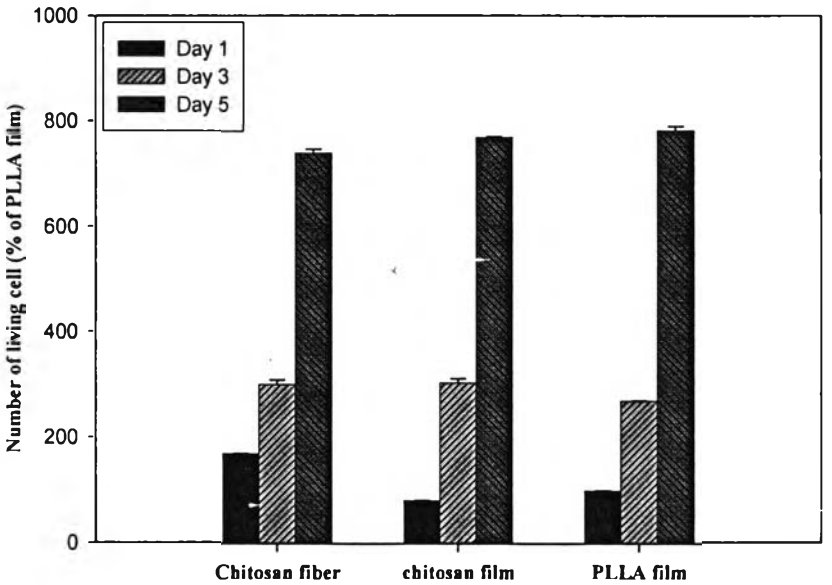


Figure 4.9 Proliferation of Schwann cell on bare culture plate, chitosan fiber, chitosan film, and PLLA film as a function of time in culture. The number of living cells was determined by MTT-tetrazolium (MTT) assay.

Table 4.2 The number of living cells obtained by counting under the optical microscopy

	Types of scaffold (Cellsx10^4)		
Days	**Chitosan fibers**	**Chitosan film**	**PLLA film**
1	59,000±930	40,240±550	42,000±670
3	77,400±670	155,420±780	168,000±980
5	84,000±551	196,000±1,050	214,000±1,120

In the Schwann cells morphology for long time of cell culturing on film scaffold (Table 4.1), the cells took on radiating vortex end to end arrangement and exhibited a number of cells that still in round shape. These cells might be the new generation of Schwann cell that proliferated. This SEM images corresponded to the number of cell viability determined from MTT assay that increased with time. On the contrary, the cells that were cultured on fibrous scaffold during day 1 to 3 exhibited a few number of new proliferated cells. However after day 5 of culturing, most of cells elongated and assumed spindle-like shape.

This result implied that chitosan fibers only promoted cell attachment, but they did not promote the proliferation of cells very well. This could be related to the fact that the cell proliferation was favored by the smoothness of the surface which allows an easier mobility of the cells that is necessary for their proliferation [Chatelet C. et al., 2000]. The obtained results emphasized the important role played by the surface morphology of the substrates on the adhesion and proliferation towards to Schwann cells. Although as-spun chitosan fibers not only have an ability to promote cell proliferation, but also its cytocompatibility and the ability to promote the cell adhesion towards Schwann cells allows its use in combination with other material, such as collagen.