CHAPTER V
IN VITRO BIOCOMPATIBILITY OF ELECTROSPUN HEXANOYL CHITOSAN FIBROUS SCAFFOLDS TOWARDS HUMAN KERATINOCYTES AND FIBROBLASTS

5.1 Abstract

With the ability to form a submicron-sized fibrous structure with interconnected pores mimicking the extracellular matrix (ECM) for tissue formation, electrospinning was used to fabricate ultra-fine fiber mats of hexanoyl chitosan (H-chitosan) for potential use as skin tissue scaffolds. In the present communication, the *in vitro* biocompatibility of the electrospun fiber mats was evaluated. Indirect cytotoxicity evaluation of the fiber mats with mouse fibroblasts (L929) revealed that the materials were non-toxic and did not release substances harmful to living cells. The potential for use of the fiber mats as skin tissue scaffolds was further assessed in terms of the attachment and the proliferation of human keratinocytes (HaCaT) and human foreskin fibroblasts (HFF) that were seeded or cultured on the scaffolds at different times. The results showed that the electrospun fibrous scaffolds could support the attachment and the proliferation of both types of cells, especially for HaCaT. In addition, the cells cultured on the fibrous scaffolds exhibited normal cell shapes and integrated well with surrounding fibers. The obtained results confirmed the potential for use of the electrospun H-chitosan fiber mats as scaffolds for skin tissue engineering.

(**Key-words:** fibrous scaffold; electrospinning; chitosan; hexanoyl chitosan; keratinocytes; fibroblasts)

5.2 Introduction

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences into the development of biological substitutes that restore, maintain, or improve tissue function [1]. Such tissue regeneration is achieved

with the aid of polymeric scaffolds which serve as matrices for cellular in-growth, proliferation, and new tissue formation in three-dimensions [2,3]. Various polymeric scaffolds with interconnected pores of a few tenths to several hundreds of micrometer scale were produced via several techniques which include foaming [3,4], salt leaching [5,6], and freeze-drying [2,7]. However, the scaffolds in foam or sponge form, manufactured from these techniques, exhibit poor mechanical properties and an unacceptable physical structure for many specific tissues regeneration [8]. This is due to the great differences between the scaffolds and the native extracellular matrix (ECM), in which the collagen structure is organized in a three-dimensional network of nano-scale diameter fibers [9]. Thus, the design and the fabrication of submicron to nano-scale fibrous scaffolds has received much attention in recent years [10-12], as scaffolds that are fabricated in this fashion may be an alternative to some of the practical limitations offered by the other types of scaffolds.

Electrospinning is a fiber spinning technique, capable of producing fibers with diameters in the sub-micron to nanometer range from materials of diverse origins [13]. This process involves the application of a strong electric field over a conductive capillary attaching to a reservoir containing a polymer solution or melt and a conductive collective screen. Upon increasing the electric field strength to a critical value, the charge species accumulating on the surface of a pendant drop destabilizes the hemispherical shape into a conical shape (commonly known as Taylor's cone). Beyond the critical value, a charged polymer jet is ejected from the apex of the cone (as a way to relieve the charge built-up on the surface of the pendant drop). The ejected charged jet is then carried to the collective screen via the electrostatic force. Both the electrostatic and the Coulombic repulsion forces are responsible for the thinning of the charged jet during its trajectory to the collective screen. Meanwhile, the solvent evaporates or the melt solidifies, leaving behind polymer fibers on the collective screen.

In an attempt to create biomimetic tissue *in vitro* in the form of fibrous structures that exhibit fiber diameters in the range close to the collagen fibers found in the natural ECM, i.e., ~30 to 130 nm [14], a number of natural and synthetic biodegradable polymers have been electrospun: they are, for examples, native collagens from calfskin (type I) and human placenta (types I and III) [14], bovine

fibrinogen fraction I [15], *Bombyx mori* and *Samia cynthia ricini* silk fibroins [16], dextran and methacrylated dextran [17], chitin and chitosan [18,19], hyaluronic acid (HA) and HA/gelatin blends [20], and polylactide (PLA), polyglycolide (PGA), polycaprolactone (PCL) and their copolymers [12,21,22]. In previous reports, hexanoyl chitosan (H-chitosan; an ideal structure shown in Figure 5.1), an acyl derivative of chitosan, was successfully synthesized and electrospun into sub-micron fiber mats [23,24]. In another report, H-chitosan film was found to be non-toxic and biocompatible due to its ability in supporting the attachment and the proliferation of fibroblasts *in vitro* [25].

In the present communication, biocompatible dressing or scaffolding materials for tissue engineering were developed from the electrospun fiber mats of H-chitosan. The electrospun fibrous scaffolds were evaluated for their cytotoxicity using L929 cells. The potential for use of the electrospun fiber mats as skin tissue scaffolds was assessed by *in vitro* cell culture with human keratinocytes (HaCaT) and human foreskin fibroblasts (HFF). A comparison was made against the tissue-culture polystyrene plates (i.e., positive control) and the solvent-cast H-chitosan films (i.e., internal control).

5.3 Experimental

5.3.1 Materials

Hexanoyl chitosan [H-chitosan; degree of substitution (DS) of the hexanoyl groups ≈ 3.0] was prepared via heterogeneous acylation reaction between chitosan and hexanoyl chloride as previously described [23]. The viscosity-average molecular weight and the degree of deacetylation (DD) of the base chitosan was 5.76 $\times$ 10^5 g·mol^{-1} and 88%, respectively [23]. All chemical reagents were of analytical grade and were used as received.

5.3.2 Electrospinning

H-chitosan solution (8% w/v) was prepared by dissolving H-chitosan in chloroform. To improve the electrospinability of the solution, 7.5% w/v of pyridinium formate (PF), an organic salt, was added. In the electrospinning process,

an electrical potential of 12 kV from a Gamma High Voltage Reserch ES30P-5W power supply was applied to the solution by attaching the emitting, positive electrode to a blunt hypodermic stainless needle (inner diameter = 0.9 mm), which was attached to a syringe within which the solution was contained. The electrospun fibers were collected on an aluminum sheet wrapped around a rotating cylinder (outer diameter and width ≈ 15 cm; rotational speed ≈ 40 rpm), which was placed 12 cm from the tip of the needle. The collection time was 8 h.

5.3.3 Cells and Cell Culture

Mouse connective tissue, fibroblast-like cells (L929) were cultured as a monolayer in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, BIOCHROM, Germany), 1% L-glutamine (Invitrogen, USA), together with 100 units/mL penicillin (Invitrogen, USA) and 100 μg/mL streptomycin (Invitrogen, USA). The medium was replaced once in every 3 d and the cultures were maintained at 37°C in a wet atmosphere containing 5% CO_2. When the cells reached 80% confluence, they were trypsinized with 0.25% trypsin containing 1 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen, USA) and counted by a hemacytometer (Hausser Scientific, USA) prior to being used in the experiments. Human keratinocytes (HaCaT) and human foreskin fibroblasts (HFF) were also cultured based on the same procedure.

5.3.3.1 Indirect Cytotoxicity Evaluation

The indirect cytotoxicity evaluation of the H-chitosan fiber mats was conducted in adaptation from the ISO10993-5 standard test method. That of the H-chitosan films was also conducted for comparison purpose. The H-chitosan films were cast from H-chitosan solution (1% w/v in chloroform) on a polytetrafluoroethylene plate, dried at room condition, and further dried *in vacuo* for another 24 h. The thickness of the films was 59 ± 10 μm [25]. Both the fiber mat and the film specimens (circular discs; 14 mm in diameter) were pre-washed with 70% ethanol for 30 min, washed with autoclaved phosphate buffer saline (PBS; Sigma–Aldrich, USA) two times, and finally washed once with the culture medium. They were then incubated at 37°C in fresh culture medium for 24 h to prepare the extraction media. The extraction ratio was 10 $mg \cdot mL^{-1}$. L929 were seeded in empty

wells of a 24-well tissue-culture polystyrene plate (TCPS; Corning, USA) at 4×10^4 cells/well. After the attachment period of 24 h, the culture medium was removed and replaced with a serum-free medium (SFM; DMEM containing 100 units·ml^{-1} penicillin and 100 μg·ml^{-1} streptomycin, but without FBS) and incubated for another 24 h. The medium was then replaced with the extraction medium for each type of the specimens and the cells were further incubated. After 24 h, the extraction medium was removed. The viability of the cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The viability of the cells cultured with fresh SFM was used as control.

5.3.3.2 Viability of Attached Cells and Cell Proliferation

Both the fiber mat and the film specimens (circular discs; 14 mm in diameter) were put in empty wells of TCPS. The specimens were sterilized by 70% ethanol for 5 min, washed two times with PBS, and then washed once with the culture medium. Prior to cell seeding, each scaffold specimen was pressed with a metal ring (diameter = 12 mm) and 500 μL of the culture medium was pipetted into each well. For the attachment study, 2×10^4 cells/well of HaCaT were seeded and allowed to attach on TCPS (i.e., control) and both the fiber mat and the film scaffolds for 2, 6, or 24 h. At each specified seeding time point, the viability of the attached cells was quantified by MTT assay. Each specimen was rinsed with PBS 2 times to remove unattached cells prior to MTT assay. Since no studies related to the expression of attachment proteins or the strength of the attached cells were carried out, this evaluation only served as the qualitative measure of the cell attachment study. For the proliferation study, 2×10^4 cells/well of the cells were cultured and incubated for 1, 2, or 3 d. At each culturing time point, the viability of the cells was determined by MTT assay. Such studies for HFF cells were carried in the same procedure.

5.3.4 Quantification of Viable Cells (MTT Assay)

The MTT assay is based on the fact that metabolically active cells interact with a tetrasolium salt in an MTT reagent to produce a soluble formazan dye, which absorbs light at the wavelength of 570 nm. The intensity of the absorbance is proportional to the number of viable cells. First, each specimen was incubated with

0.5 $mg \cdot mL^{-1}$ MTT solution in DMEM without phenol red at 250 μL/well. The incubation was 10 and 25 min for HaCaT and HFF, respectively. After the incubation, the MTT solution was removed. A buffer solution containing dimethylsulfoxide (DMSO; Carlo Erba, Italy; at 900 μL/well) and glycine buffer (pH = 10; at 125 μL/well) was added to each well to dissolve the dye. The solution was then transferred to a cuvette and placed in a Thermospectronic Genesis10 UV–vis spectrophotometer, from which the absorbance at 570 nm was measured.

5.3.5 Morphology of Fibrous Scaffolds and Cultured Cells

Morphology of H-chitosan fibrous scaffolds was investigated by a JEOL JSM-5410LV scanning electron microscope (SEM). Each specimen was coated with a thin layer of gold using a JEOL JFC-1100E ion sputtering device prior to SEM observation. Diameters of the electrospun fibers were measured directly from SEM images ($n \geq 50$), while the thickness of the fiber mats was measured with a digital micrometer ($n \geq 10$). The SEM was also used to observe morphology of the cells that had either been seeded (for 2-24 h) or cultured (for 24 h-7 d) on both the fiber mat and the film substrates. Briefly, the cellular constructs were harvested, washed with PBS, and then fixed with 3% glutaraldehyde solution [diluted from a 50% glutaraldehyde solution (Electron Microscopy Science, USA) with PBS] at 500 μl/well for 30 min. After rinsing twice with PBS, the construct specimens were dehydrated through a series of graded DMSO and later dried in air. The specimens were coated with a thin layer of gold and then subjected to observation by SEM.

5.3.6 Statistical Analysis

All of the quantitative data were expressed as means ± standard deviations. Statistical comparisons were performed using one-way ANOVA with SPSS 13.0 for Windows software (SPSS, USA). P values of less than 0.05 were considered statistically significant ($n = 4$ for indirect cytotoxicity evaluation and $n = 3$ for the attachment and the proliferation studies).

5.4 Results and Discussion

5.4.1 Electrospun H-chitosan Fiber Mats

To mimic a three-dimensional structure resembling that of a natural ECM, i.e., nanoscale fibrous network of collagens and proteoglycans, that promote the attachment, the proliferation, and the differentiation of the cells, electrospinning has been proven as a novel and effective method to produce such fibrous structures [12,21,22]. In the present communication, electrospun H-chitosan fiber mats were fabricated from 8% w/v H-chitosan solution in chloroform with an addition of 7.5% w/v PF. Figure 5.2 shows a selected SEM image of an electrospun H-chitosan fiber mat. Obviously, the individual fibers of the fiber mats were randomly oriented with large interconnected pores. Diameters of the individual fibers ranged from 0.4 to 3.2 μm, with the average value being ~1.5 μm, while the thickness of the fiber mats, after continuous electrospinning for 8 h, was 83 ± 9 μm. The large variation in the fiber diameters was postulated to be as result of the flow instabilities that are responsible for the observed whipping action of the charged jet.

5.4.2 Indirect Cytotoxicity Evaluation

Even though it was proven that H-chitosan in the form of solvent-cast films poses no threat to L929 [25], the assessment with regards to cytotoxicity of H-chitosan in the form of electrospun fiber mats is necessary as chloroform and PF, known toxic organic substances, were used during the fabrication of the materials. L929 were again used in the assessment.

Figure 5.3 shows the viability of L929 after being cultured with the extraction medium, prepared by immersing both the fiber mat and the film scaffolding materials in SFM for 24 h, as compared with that of the cells that were cultured in fresh SFM (i.e., control). The viability of the control condition was used as the base value to arrive at the relative viability reported in the figure. For the solvent-cast films (i.e., internal control), the viability of the cells (~102%) cultured in the extraction medium from the films (10 $mg \cdot mL^{-1}$) was equivalent to that of the cells cultured in fresh SFM. In our previous report [25], these values were found to be ~85%, 88%, and 85% for the extraction media with the extraction ratios of 0.25, 0.5,

and 1 $mg \cdot mL^{-1}$, respectively. Despite the greater extraction ratio of the extraction medium used, the H-chitosan films investigated here were less toxic than those investigated in the previous report. The discrepancy could be a result of the difference in the size of the TCPS used (i.e., 24 versus 96 wells) and the difference in the number of cells seeded (i.e., 4×10^4 versus 10^3 cells/well). With regards to the electrospun fiber mats, the viability of the cells cultured in the extraction medium from the fiber mats was ~130%, which was significantly greater than that of the cells cultured in the extraction medium from the film counterparts. All of the obtained results suggested that the extraction media from both the fiber mat and the film specimens were free from any substance that is harmful to the cells and these materials could be use as scaffolds for further investigation.

5.4.3 Cell Attachment and Cell Proliferation

Since both the topography and the porosity of a scaffold play a significant role in the attachment and the proliferation of mammalian cells, biocompatibility of the electrospun H-chitosan fibrous scaffolds was evaluated against the solvent-cast film scaffolds and TCPS. To further evaluate the potential for use of these materials in wound dressing and skin tissue engineering applications, human keratinocytes (HaCaT) and human foreskin fibroblasts (HFF) were used as referenced cell lines. Both the attachment (at 2-24 h after cell seeding) and the proliferation (at 24-72 h after cell culturing) of HaCaT that were either seeded or cultured on the fibrous scaffolds, the film scaffolds, and TCPS (i.e., control) are graphically shown in Figure 5.4a. The viability of the cells seeded or cultured on TCPS was used as the base value to arrive at the relative absorbance shown in the figure. A similar plot for HFF that were either seeded or cultured on the fibrous scaffolds, the film scaffolds, and TCPS (i.e., control) are graphically shown in Figure 5.4b.

At 2 h after cell seeding or at 48 and 72 h after cell culturing, the viability of HaCaT attached or proliferated on both the fibrous and the film scaffolds was less than that on TCPS. At 6 h after cell seeding however, the viability of the cells attached on both types of H-chitosan substrates was greater than that on TCPS, while, at 24 h after cell seeding or cell culturing, only the viability of the cells

attached or proliferated on the fibrous scaffolds was greater than that on TCPS. At all of the time points investigated, the viability of the cells attached or proliferated on the H-chitosan fibrous scaffolds was greater than that on the film counterparts. The greater amount of cells that were able to attach or proliferate on the fibrous scaffolds than that of the film counterparts could be due to the larger surface area of the fibrous scaffolds that is available for the cells to attach. For HFF, the viability of the cells attached or proliferated on both the fibrous and the film scaffolds, at any given time point, was less than that on TCPS, while a similar tread in terms of the ability of the fibrous scaffolds to provide better support for the attachment and the proliferation of the cells was observed. The general better support for both the attachment and the proliferation of both types of cells on TCPS over those on both types of the scaffolds is possibly due to the greater hydrophilicity of TCPS over that of the scaffolds [25-27], as hydrophilicity is one factor influencing the cell response to a substrate [28,29].

Based on the actual absorbance values (results not shown), TCPS supported both the attachment and the proliferation of HFF much better than HaCaT at most of the time points investigated; however, the opposite trend was observed at 72 h after cell culturing. On the other hand, both the H-chitosan fibrous and the film scaffolds showed much better support for the attachment and the proliferation of HaCaT than those of HFF at all of the time points investigated. Based on this as well as on the facts that the relative viability values for both the attachment and the proliferation of HaCaT on both types of the H-chitosan scaffolds ranged between 77 and 140% (relative to those on TCPS) and that of HFF ranged between 35 and 62% (relative to those on TCPS), it could be postulated that HaCaT prefer a more hydrophobic surface than HFF do. With regards to the proliferation rate of the cells (considering only during 24-72 h of cell culturing),

5.4.4 Morphology of Seeded or Cultured Cells

Table 5.1 shows selected SEM images of HaCaT that had either been seeded or cultured on the surfaces of the film and the fibrous scaffolds at different times of cell seeding or cell culturing. At 2 h after cell seeding, HaCaT attached on both types of the H-chitosan substrates were still in round shape, indicating that the

cells were not yet fully attached on the surfaces. At 6 h after cell seeding, the cells on the film scaffold started to extend their cytoplasm in the form of filopodia to help their anchorage to the film surface, while those on the fibrous scaffold were still round. During the proliferation period (i.e., 24 h-7 d), the cells on both types of the H-chitosan substrates were well expanded, with strong evidence of filopodia. At 24 h after cell culturing however, the cells appeared to aggregate into groups and, at longer culturing time points, propagation of the cells to form a connected tissue-like structure on the scaffold surface was evident. This is the nature of keratinocytes, both in native tissue and in culture conditions [30,31]. Interestingly, the cells on the film scaffolds reached the confluence since day 3 after cell culturing, while those on the fibrous scaffolds were still propagating between days 3 and 7. Despite this, the viability of the cells on the fibrous scaffolds was found to always be greater than that on the film counterparts, as previously mentioned. This should be a result of the much greater surface area of the fibrous scaffolds in comparison with that of the film counterparts.

SEM images of HFF that had either been seeded or cultured on the surfaces of the film and the fibrous scaffolds at different times of cell seeding or cell culturing are shown in Table 5.2. The extension of the cytoplasm in the form of filopodia of HFF was observed even at 2 h after cell seeding. Notwithstanding, the majority of the cells on the fibrous scaffolds was still round, while those on the film counterparts exhibited the characteristic spindle-like morphology. At 6 h after cell seeding, the cells on the fibrous scaffolds appeared in their characteristic spindle-like shape as well. At 24 h after cell culturing, conjoined cellular boundary between adjacent cells was evident. This could be a result of either the inter-cellular contacts or an evidence of the on-going cellular mitosis during the proliferation of the cells. With further increasing the culturing time, the viability of the cells increased further from that observed at 24 h after cell culturing to cover more empty space on both the fibrous and the film scaffolds. Evidently, on day 7 after cell culturing, the cells on the film scaffolds reached the confluence to the hypothetical monolayer construct on the surface of the films, while those on the fibrous scaffolds almost filled up the empty space, with an evidence of the cells being able to penetrate into and propagate within the inner layer of the fibrous scaffolds to form a three-dimensional cell-

scaffold construct (see also SEM image of the HFF-cultured H-chitosan fibrous scaffold on day 3).

5.5 Conclusions

In the present communication, electrospun hexanoyl chitosan [H-chitosan; degree of substitution (DS) of the hexanoyl groups ≈ 3.0] fiber mats were prepared from 8% w/v H-chitosan solution in chloroform with the addition of 7.5% w/v pyridinium formate. These fiber mats (thickness ≈ 83 ± 9 μm after continuous electrospinning for 8 h; diameters of the individual fibers ≈ 0.4-3.2 μm) were evaluated for their potential use as biocompatible dressing or scaffolding materials for skin tissue engineering, using human keratinocytes (HaCaT) and human foreskin fibroblasts (HFF). A comparison was made against the tissue-culture polystyrene plates (i.e., positive control) and the solvent-cast H-chitosan films (i.e., internal control). Indirect cytotoxicity evaluation using mouse fibroblasts (L929) as referenced cells indicated that the H-chitosan fiber mats were non-toxic and could be used as biocompatible dressing or scaffolding materials. *In vitro* biocompatibility of the H-chitosan fibrous scaffolds towards HaCaT and HFF was assessed at various time points after cell seeding (2-24 h) or cell culturing (24 h-7d). It was found that the H-chitosan fibrous scaffolds showed much greater viability of both the seeded and the proliferated cells than the film counterparts did and both the fibrous and the film scaffolds showed much better support for the attachment and the proliferation of HaCaT than those of HFF. Visual observation based on scanning electron microscopy revealed that both types of cells integrated well with surrounding fibers to form a three-dimensional cellular network and that the cells maintained their characteristic morphology during the cell culture. Since HaCaT and HFF are anchorage-dependent cells, the high surface area-to-volume ratio and the overall porous structure of the electrospun fiber mats are favorable parameters for promoting the attachment and the proliferation of the cells. As a result, cellular responses of both types of cells on the fibrous scaffolds were superior to those on the film counterparts.

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Table 5.1 Selected SEM images of HaCaT that were either seeded (during 2-24 h) or cultured (during 24 h-7 d) on H-chitosan fibrous and film scaffolds at different times of cell seeding or cell culturing

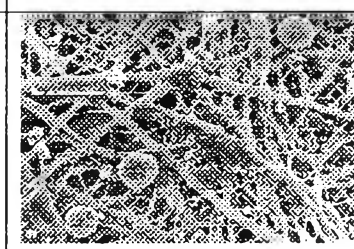
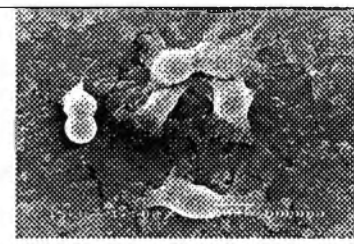
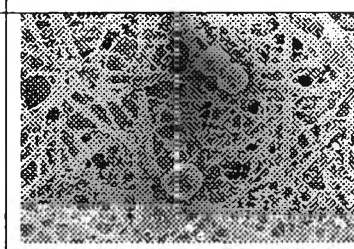
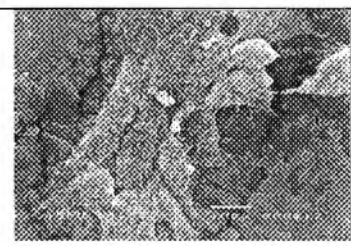
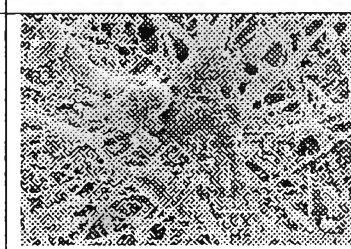
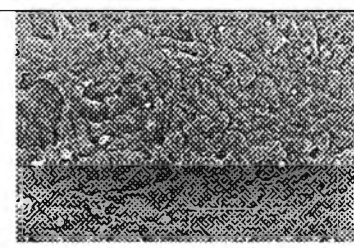
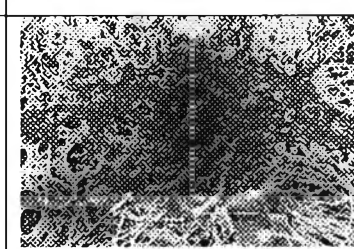
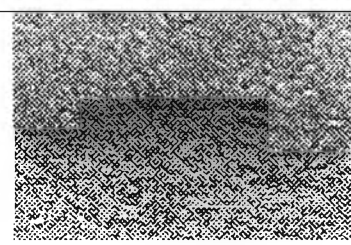
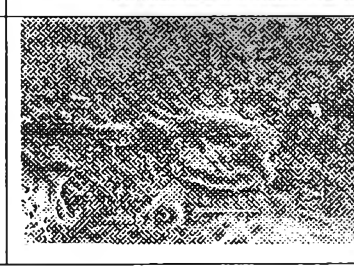
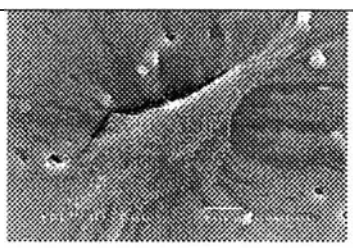
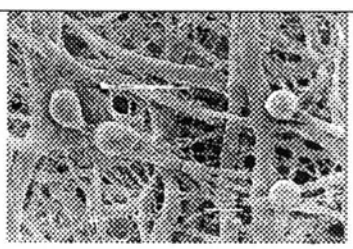
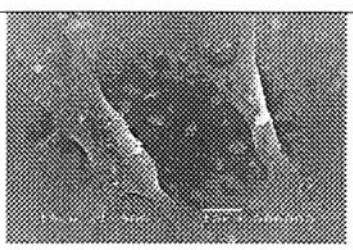
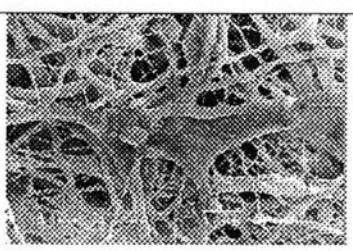
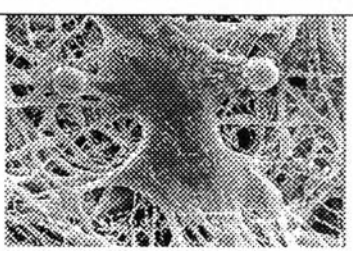
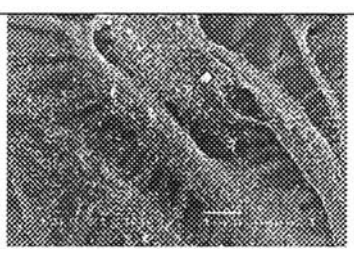
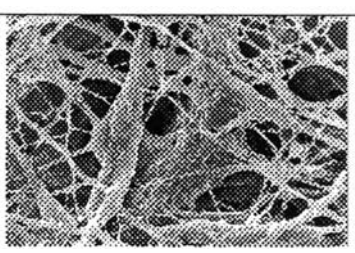
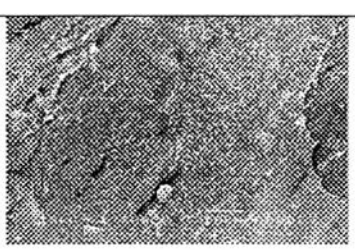
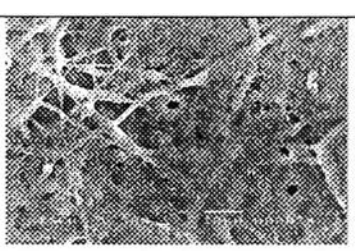
Time point after cell seeding (2-24 h) or cell culturing (24 h-7d)	HaCaT-cultured H-chitosan film scaffold	HaCaT-cultured H-chitosan fibrous scaffold
2 h		
6 h		
24 h		
3 d		
7 d		

Table 5.2 Selected SEM images of HFF that were either seeded (during 2-24 h) or cultured (during 24 h-7 d) on H-chitosan fibrous and film scaffolds at different times of cell seeding or cell culturing

Time point after cell seeding (2-24 h) or cell culturing (24 h-7d)	HFF-cultured H-chitosan film scaffold	HFF-cultured H-chitosan fibrous scaffold
2 h		
6 h		
24 h		
3 d		
7 d		

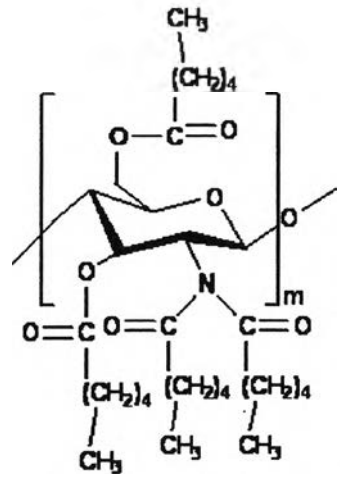


Figure 5.1 Chemical structure of fully-substituted hexanoyl chitosan (H-chitosan).

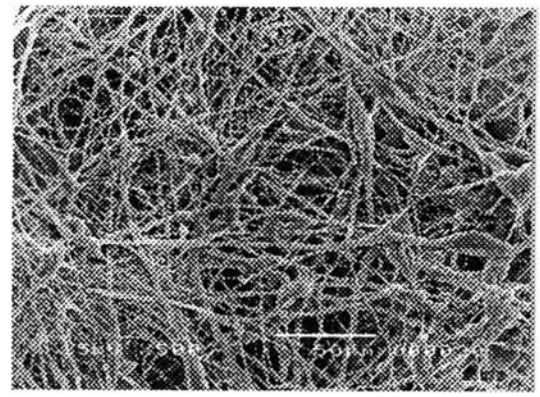


Figure 5.2 Selected SEM images of electrospun H-chitosan fiber mat from 8% w/v H-chitosan solution in chloroform with 7.5% w/v pyridinium formate.

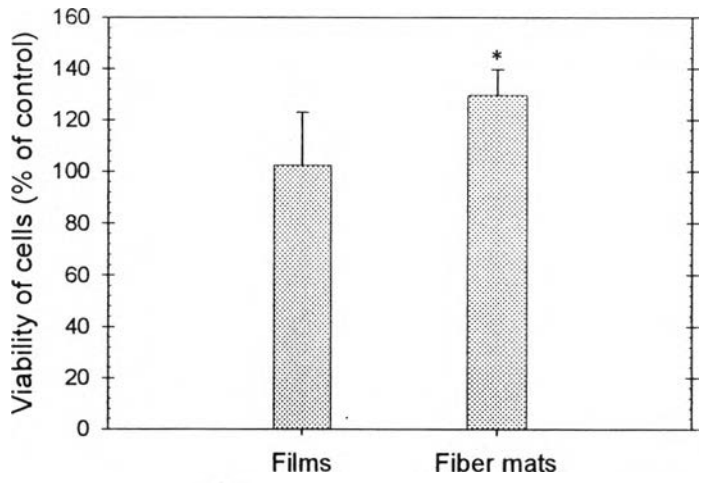


Figure 5.3 Indirect cytotoxicity evaluation of H-chitosan fiber mats and films based on the viability of mouse fibroblasts (L929) that were cultured in the extraction media (10 $mg \cdot mL^{-1}$) from these materials. The viability of the cells cultured in fresh media was used as control. (*) $p < 0.05$ compared with control.

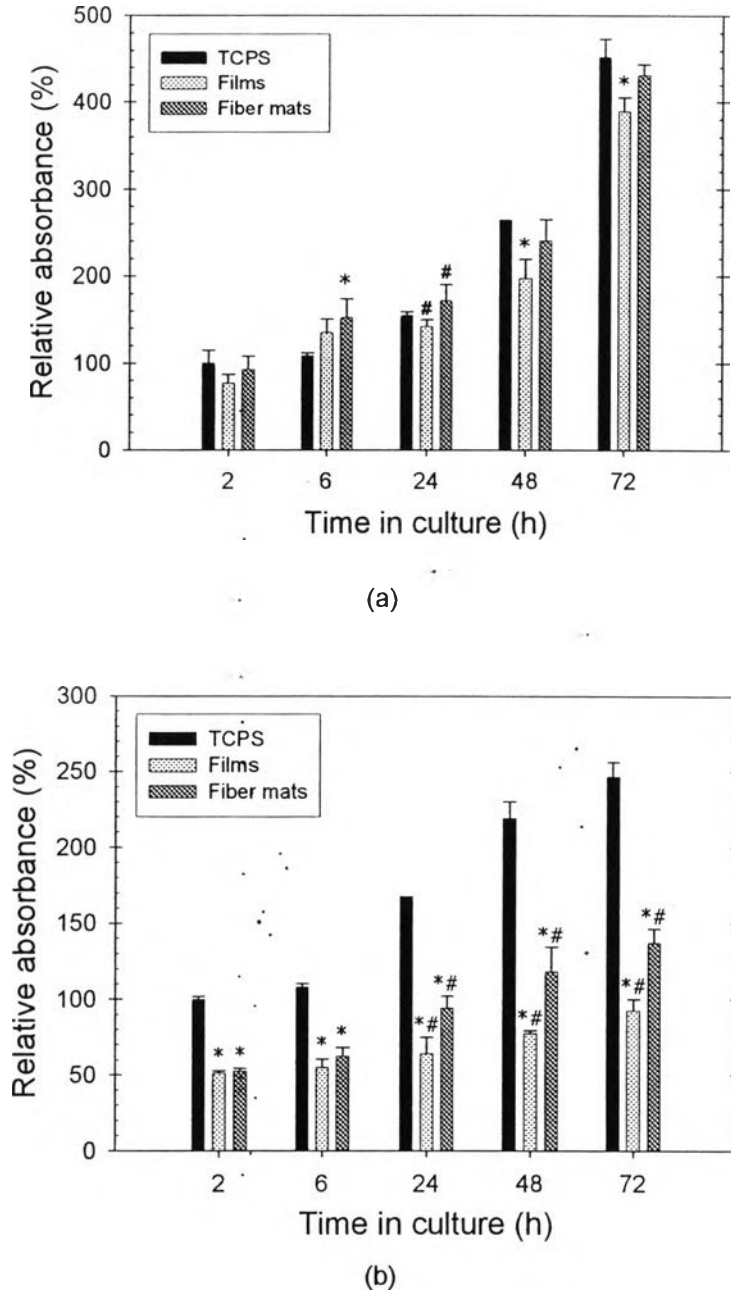


Figure 5.4 Attachment and proliferation of (a) HaCaT or (b) HFF that were either seeded or cultured on tissue-culture polystyrene plate (TCPS; positive control) and H-chitosan fibrous and film scaffolds at various time points after cell seeding or cell culturing. 2-24 h represents the attachment period and 24-72 h represents the proliferation period. (*) $p < 0.05$ compared with TCPS and (#) $p < 0.05$ compared between the fibrous and the film scaffolds at a given time point.