CHAPTER VI
IN VITRO BIOLOGICAL EVALUATION OF ELECTROSPUN CELLULOSE ACETATE FIBER MATS CONTAINING CURCUMIN OR ASIATICOSIDE

6.1 Abstract

In the present contribution, ultra-fine cellulose acetate (CA; $M_w \approx 30{,}000$ Da; degree of acetyl substitution ≈ 2.4) fiber mats containing either curcumin (CM; from the plant *Curcuma longa* L.) or asiaticoside [from the plant *Centella asiatica* L.; either in the form of a crude extract (CACE) or pure substance (PAC)] were aimed at being used as topical/transdermal patches or wound dressings. Here, the potential for use of these herb-loaded CA fiber mats as wound dressings was evaluated in terms of the stability and the antioxidant activity of the as-loaded herbal substances, the ability to support both the attachment and the proliferation of fibroblasts and the ability of the cultured fibroblasts to synthesize collagen. Normal human dermal fibroblasts (NHDF) were used as the reference fibroblastic cells in this study. The results showed that the as-loaded herbal substances were stable even after the herb-loaded CA fiber mats had been aged either at room temperature or at 40 °C for a period of up to 4 months. While the inclusion of asiaticoside (either 2 wt.% CACE or 40 wt.% PAC) in the CA fiber mats was responsible for the superiority of the resulting CA fiber mats in supporting the attachment, promoting the proliferation, and to up-regulating the production of collagen of the seeded and/or the cultured NHDF to the corresponding solvent-cast films as well as the neat CA fiber mats, the inclusion of CM in the CA fiber mats helped impart the antioxidant activity to the resulting materials better than did the asiaticoside-containing substances.

(**Key-words**: Topical/transdermal drug delivery; Wound dressing; Normal human dermal fibroblast cells; Electrospinning; Cellulose acetate)

6.2 Introduction

Over the past decade, electrospinning (e-spinning) has been heavily explored, due mainly to its simplicity and capability of producing ultra-fine fibers that possess a high surface area, rendering their vast possibilities for surface functionalization (Reneker, 2008). The highly porous nature of the electrospun (e-spun) fiber matrices provides a greater surface area to volume or mass ratio, when compared with the corresponding solvent-cast films. This characteristic is particularly inductive for applications in life science, such as cell/tissue culture and drug delivery (Khil, 2003), as the e-spun fiber matrices would allow the nutrients to diffuse into and, at the same time, the wastes to diffuse out from the cellular construct and allow drug molecules to diffuse out from the matrix much more conveniently. They also allow for high oxygen permeation and easy profusion of exudates, as well as provide a good protection of wounds from infection and dehydration, which are important characteristics of functional wound dressings (Khil, 2003). Due to the aforementioned characteristics of the e-spun fiber matrices, the proposed use for these materials in life science are particularly as scaffolds for cell/tissue culture (Li, 2002; Yoshimoto, 2003; Suwantong, 2007; Wutticharoenmongkol, 2007), carriers for delivery of drugs (Kenawy, 2002; Taepaiboon, 2007; Tungprapa, 2007; Suwantong, 2007; Suwantong 2008), and wound dressings (Noh, 2006; Han, 2007; Zhou, 2008).

Among various pharmacological agents, extracts from some plants that are known to promote the healing of wounds and, at the same time, to suppress the inflammation of the wounds have been heavily explored (Pierce, 1995). *Centella asiatica* (L.) Urban, commonly known as Asiatic Pennywort, has long been used as the traditional medicine in many Asian countries to heal wounds, burns and ulcerous abnormalities of the skin, cure stomach and duodenal ulcers and are effective in the treatment of leprosy, lupus, sceleroderma and diseases of the veins (Kartnig; 1998). It has been shown to increase collagen synthesis *in vitro* and extracellular matrix (ECM) accumulation *in vivo* as well as to enhance tensile strength of wound tissues (Suguna, 1996; Maquart, 1990; Maquart, 1999).Among the four major triterpenoid components of the plant extracts (i.e., asiatic acid, asiaticoside, madecassic acid and

madecassoside), asiatic acid and asiaticoside are both essential for biological activities. Asiaticoside (see chemical structure in Figure 1a), a trisaccharide triterpene, is supposedly the most active compound associated with the healing of wounds, as evidenced by the observed increase in antioxidant levels at an initial stage of the healing of excision-type cutaneous wounds in rats (Shukla, 1999), the observed increase in the proliferation and the production of types I and III pro-collagen mRNAs and the levels of the corresponding proteins of human dermal fibroblasts (Maquart, 1990; Shim, 1996), and the stimulation of ECM accumulation in murine experimental wounds (Suguna, 1996; Maquart, 1999), in response to the presence of this substance. Curcumin (see chemical structure in Figure 1b), widely known for its antitumor, antioxidant and anti-inflammatory properties (Sharma, 2005; Jayaprakasha, 2005; Jayaprakasha, 2006; Maheshwari, 2006), is another interesting herbal substance from the plant *Curcuma longa L.* It was reported to enhance cutaneous wound healing in rats and guinea pigs (Sidhu, 1999). Wounds of the animals treated with curcumin showed early re-epithelialization, improved neovascularization, increased migratory activity of various cells including dermal myofibroblasts, fibroblasts and macrophages into the wound bed and a higher collagen content (Sidhu, 1999). Moreover, incorporation of curcumin in collagen films was shown to help increase wound reduction, enhance cell proliferation and provide efficient free radical scavenging activity (Gopinath, 2004).

Various e-spun polymeric fiber matrices have been developed as carriers for delivery of drugs (Kenawy, 2002; Taepaiboon, 2007; Tungprapa, 2007; Suwantong, 2007; Suwantong, 2008). Recently, we reported the successful preparation of ultra-fine cellulose acetate fiber mats that contained various amounts of curcumin (CM; 5-20 wt.% based on the weight of CA) by e-spinning (Suwantong, 2007). The average diameters of the CM-loaded CA fibers ranged between 314 and 340 nm. The release characteristic of CM from the CM-loaded CA fiber mats and the corresponding solvent-cast films was carried out by the total immersion and the transdermal diffusion through a pig skin method in the B/T/M medium at 37 °C. In the total immersion method, about 90-95% of CM was released from the CM-loaded CA fiber mat specimens, while only about 3-9% of the substance did so from the CM-loaded CA film counterparts (Suwantong, 2007). Moreover, we successfully prepared the

ultra-fine CA fiber mats containing either a *Centella asiatica* crude extract (CACE) or pure asiaticoside (PAC) by e-spinning (Suwantong, 2008). Incorporation of either CACE or PAC (40 wt.% based on the weight of CA) in the neat CA solution did not affect the morphology of the obtained fibers, as both the neat and the herb-loaded CA fibers were smooth. The average diameters of these fibers ranged between 301 and 545 nm. The release characteristic of asiatisocide from the CACE- and the PAC-loaded CA fiber mats and the corresponding solvent-cast films was tested by the total immersion and the transdermal diffusion through a pig skin method in the A/B/M or the P/B/M medium at the skin or the physiological temperature of 32 or 37 °C, respectively. In the total immersion method, the maximum amounts of asiaticoside released from the CACE- and the PAC-loaded CA fiber mats into the A/B/M medium were about 77 and 98%, while those released into the P/B/M medium were about 92 and 99%, respectively (Suwantong, 2008).

The aim of the present contribution is to investigate further the potential for use of the e-spun CA fiber matrices that contained either CM or asiaticoside (in the form of either CACE or PAC) as topical/transdermal patches or wound dressings. These e-spun fiber mats were evaluated *in vitro* with normal human fibroblast cells (NHDF), in terms of the indirect cytotoxicity, antioxidant activity of the as-loaded herbal substances, the attachment and the proliferation of the seeded/cultured cells and the production of collagen of the cultured cells. Morphological observation of the cultured cells was also investigated by scanning electron microscopy (SEM). Comparisons were made against the corresponding solvent-cast films. Moreover, the stability of the as-loaded substances in the e-spun CA fiber mats was also investigated at room temperature and at 40 °C as a function of the aging period (ranging from 1 to 4 months).

6.3 Experimental

6.3.1 Materials

Cellulose acetate (CA; white powder; $M_w \approx 30{,}000$ Da; acetyl content = 39.7 wt.%; degree of acetyl substitution ≈ 2.4) was purchased from Sigma-Aldrich

(Switzerland). Curcumin (CM; ≥95.0% purity) was purchased from Fluka BioChemika (USA). *Centella asiatica* crude extract [CACE; triterpene content = 95%; asiaticoside = 37.5% (HPLC) and madecassic and asiatic acids = 56.2% (HPLC)] and pure asiaticoside (PAC; 90% purity) were purchased from Shanghai Angoal Chemical Co., Ltd. (China). Acetone (Carlo Erba, Italy), *N,N*-dimethylacetamide [DMAc, Labscan (Asia), Thailand], sodium chloride, anhydrous disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate (Ajax Chemicals, Australia) were of analytical reagent grade and used without further purification.

6.3.2 Preparation of Neat and Herb-Loaded CA Fiber Mats and Films

An amount of CA powder was dissolved in 2:1 v/v acetone/DMAc to prepare the base CA solution (at 17% w/v). CM-containing CA solutions were prepared by dissolving the same amount of CA and CM powder at 5, 10, 15 or 20 wt.% (based on the weight of CA) in the acetone/DMAc mixture. Similarly, CACE- and PAC-containing CA solutions were prepared by dissolving the same amount of CA and either CACE or PAC powder at 40 wt.% (based on the weight of CA) in the acetone/DMAc mixture. However, according to a previous study (Suwantong, 2008), the 40 wt.% CACE-loaded CA fiber mats were toxic towards normal human dermal fibroblasts (NHDF) at the extraction ratios of ≥5 $mg \cdot mL^{-1}$. Consequently, the CACE-loaded CA fiber mats were also prepared from the CA solution that contained 2 wt.% (based on the weight of CA) of CACE. Each of the as-prepared solutions was then subjected to the e-spinning (ES-30P 5W; Gamma High Voltage Research, USA). The electric field applied to the solution was fixed at 17.5 kV/15 cm and the feeding rate of the solution was fixed at 1 $mL \cdot h^{-1}$ (by means of a Kd Scientific syringe pump). The solution was e-spun continuously for 18 h, resulting in the fiber mats of 90 ± 10 μm in thickness. For comparison purposes, the neat and the herb-loaded CA films were also prepared by solvent-casting from 4% w/v CA solution in 2:1 v/v acetone/DMAc and the CA solutions that contained the same amounts of the herbal substances used to prepare the herb-loaded e-spun CA fiber mats, respectively. The thicknesses of the as-cast films were 80 ± 10 μm.

6.3.3 Stability of Herb Substances in Herb-Loaded CA Fiber Mats

The stability of CM, CACE and PAC in the respective herb-loaded e-spun CA fiber mats was evaluated after the materials had been aged for different time intervals (i.e., 1-4 months) at room temperature and at 40 °C. The herb-loaded materials, sealed in plastic bags, were either stored in a desiccator (for the samples that had been aged at room temperature) or an oven (for the ones that had been aged at 40 °C). At a given time point, each specimen (circular disc of 2.8 cm in diameter) was dissolved in 4 mL of 2:1 v/v acetone/DMAc. Then, 0.5 mL of the solution was mixed with 8 mL of phosphate buffer saline (PBS, pH 7.4; Sigma-Aldrich, USA) and the amount of the respective herbal substance in each specimen was determined either by UV-vis spectrophotometry (to quantify the amount of CM in the CM-loaded CA fiber mats; see detail of the analytical procedure in ref. (Suwantong, 2007)) or HPLC (to quantify the amount of asiaticoside in either the CACE- or the PAC-loaded CA fiber mats; see detail of the analytical procedure in ref. (Suwantong, 2008)) against the predetermined calibration curve for each respective herbal substance.

6.3.4 Cell Culture

NHDF (sixth passage) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., USA), supplemented by 10% fetal bovine serum (FBS; Invitrogen Corp., USA), 1% L-glutamine (Invitrogen Corp., USA) and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate and amphotericin B (Invitrogen Corp., USA)]. The medium was replaced once in every 2 d and the cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

6.3.5 Indirect Cytotoxicity Evaluation

The indirect cytotoxicity evaluation of the 2 wt.% CACE-loaded e-spun CA fiber mats and the corresponding as-cast CA films was conducted in adaptation from the ISO 10993-5 standard test method in a 96-well tissue-culture polystyrene plate (TCPS; Nunclon™, Denmark) using NHDF (seventh passage). The specimens, cut from the herb-loaded fiber mat and film samples, were sterilized by UV radiation for 1 h and then were immersed in serum-free medium (SFM;

containing DMEM, 1% L-glutamine, 1% lactalbumin and 1% antibiotic and antimycotic formulation) for 24 h in incubation to produce the extraction media of varying concentrations (i.e., 10, 5 and 0.5 $mg \cdot mL^{-1}$). NHDF were separately cultured in wells of TCPS at 8,000 cells/well in serum-containing DMEM for 24 h to allow cell attachment. The cells were then starved with SFM for 24 h. After that, the medium was replaced with an extraction medium and cells were re-incubated for 24 h. Finally, the viability of the cells that had been cultured with each of the extraction media was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (see later), with the viability of the cells that have been cultured by the fresh SFM being used as control.

6.3.6 Antioxidant Activity

NHDF were plated in 90 (μL of DMEM at a density of 8,000 cells/well in 96-well TCPS. After the cultures reached confluence (typically, 48 h after plating), the test solutions containing a certain amount of the herbal substance that had been released from the herb-loaded fiber mat and film specimens (circular discs of 2.8 cm in diameter) at 6, 12, 24 and 48 h after submersion in PBS were added at 10 μL/well. At 16 h after further incubation, a culture medium supplemented with 12 μM H_2O_2 solution was added into each well at 10 μL/well. After 3 h of further incubation, the viability of the cells following the treatment with H_2O_2 was quantified by the MTT assay (see later). The viability of the cells that had been cultured with the fresh and the H_2O_2-supplemented DMEM was used as controls.

6.3.7 Viability of Attached Cell and Cell Proliferation

The specimens, cut from the neat and the herb-loaded e-spun CA fiber mats and the corresponding as-cast films into circular discs of 15 mm in diameter, had been sterilized by UV radiation for 1 h prior to being immersed in DMEM overnight in wells of a 24-well TCPS. To ensure a complete contact between the specimens and the wells, each specimen was pressed with a metal ring. NHDF from the cultures were trypsinized [0.25% trypsin containing 1 mM EDTA (Invitrogen Crop., USA)], counted by a hemacytometer (NEUBAUER improved bright-line, HBG, Germany) and seeded at 30,000 cells/well on the specimens and empty wells of TCPS (i.e., control). The cultures were maintained in an incubator. In the

attachment study, NHDF were allowed to attach on the specimens and TCPS for 2, 6 and 24 h, respectively. At each cell seeding time point, the viability of the attached cells was quantified by the MTT assay (see later). Each specimen was rinsed with PBS to remove unattached cells prior to the quantification. Since no studies related to the expression of the 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. In the proliferation study, the cells at 30,000 cells/well were cultured on the specimens and empty wells of TCPS (i.e., control) and incubated for 1, 3, 5 and 7 d. At each cell culturing time point, the viability of the proliferated cells was quantified by the MTT assay (see later).

6.3.8 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 550 nm. The intensity of the absorbance is proportional to the number of viable cells. First, each sample was incubated for 3 (1) h at 37 °C with 300 (100) μL/well of MTT solution at 0.5 (5) $mg \cdot mL^{-1}$ without phenol red for a 24-well (or 96-well) TCPS. After further incubation for about 4 h, MTT solution was removed. A buffer solution containing DMSO at 500 (100) μL/well was then added into each well to dissolve the dye. The solution was then transferred to a cuvette and placed in a microplate reader (SpectraMax M2; Molecular Devices, USA), from which the absorbance at 550 nm was measured.

6.3.9 Morphological Observation of Cultured Cells

After the culture medium had been removed, the cell-cultured specimens were rinsed with PBS twice and the cells were then fixed with 3% glutaraldehyde solution [diluted from 50% glutaraldehyde solution (Electron Microscopy Science, USA) with PBS] at 500 μL/well. After 30 min, they were rinsed with PBS twice. After cell fixation, the specimens were dehydrated in an ethanol solution of varying concentrations (i.e., 30, 50, 70 and 90%, respectively) and pure ethanol, for about 2 min each. The specimens were then dried in 100% hexamethyldisilazane (HMDS; Sigma, USA) for 5 min and then in air after the removal of HMDS. After completely dried, the specimens were mounted on SEM stubs, coated with gold and observed by SEM.

6.3.10 Quantification of Synthesized Collagen

The amount of collagen synthesized by the cultured cells was quantified on day 7 after cell culturing using a Sircol^{TM} collagen assay (Biocolor, UK). Briefly, NHDF were first cultured on each specimen, cut from the neat and the herb-loaded e-spun CA fiber mat and the corresponding as-cast film samples into circular discs of 15 mm in diameter, at 30,000 cells/well. On day 7 after cell culturing, the supernatant from each cell-cultured specimen was pipetted out at 50 μL. It was later mixed with 50 μL of 0.5 M acetic acid and then shaken for 2 h at room temperature. After that, 1 mL of the dye reagent was added and gently mixed at room temperature for 30 min. Dyed collagen was precipitated out by centrifugation for 10 min and the bound dye was then recovered with 1 mL of the alkali dye-release reagent. Optical density of the recovered dye was then measured at 540 nm using the microplate reader. The actual amount of the synthesized collagen was finally quantified against a standard curve of the manufacturer-provided acid-soluble collagen standard to cover the amount of collagen in the range of 0-100 μg.

The amount of DNA signifying the number of proliferated cells that had been cultured on each fiber mat or the film specimen for 7 d was quantified by a DNA Quantification Kit (Sigma-Aldrich, USA). Briefly, after the supernatant from each cell-cultured specimen was taken out for collagen quantification, the cultured cells were thoroughly washed twice with 400 μL of PBS. The cells were then lysed with 300 μL of a cell lysis buffer. The obtained suspension was centrifuged for 10 min to precipitate the cell debris. The supernatant was pipetted out at 20 μL, which was later mixed with 2 mL of 0.1 $\mu g \cdot mL^{-1}$ Bisbenzimide H 33258 solution in 10X Fluorescent Assay Buffer. The fluorescent emission intensity of the obtained solution was then measured at 460 nm, after it had been excited at 360 nm, using the microplate reader. The actual amount of DNA was finally quantified against a standard curve of the manufacturer-provided DNA standard to cover the amount of DNA in the range of 20-1000 ng.

6.3.11 Statistical Analysis

Data were presented as means ± standard errors of means. Statistical analysis was carried out by the one-way analysis of variance (one-way ANOVA) and

Scheffe's post hoc test in SPSS (SPSS). The statistical significance was accepted when $p < 0.05$.

6.4 Results and discussion

6.4.1 Stability of Herbal Substances in Herb-Loaded CA Fiber Mats

The stability of curcumin (CM) or asiaticoside [AC; either in the form of *Centella asiatica* crude extract (CACE) or pure asiaticoside (PAC)] in the CA fiber mats that had been aged at room temperature or 40 °C for various time intervals, ranging from 0 to 4 months, was investigated. Basically, the stability of the herbal substances was assessed based on the detectable amounts of the substances in comparison with those of the substances initially contained in the spinning solutions. While the initial amounts of the herbal substances in the CM- and the PAC-loaded CA fiber mats were calculated based on the weight of the herb powder that had been dissolved in the spinning solutions, that of AC in the CACE-loaded CA fiber mats was calculated based on the weight of the incorporated CACE powder multiplied by 0.507 (according to the actual content of AC in CACE as determined by HPLC which was 50.7%). Figure 2 shows the results of such analyses. Immediately after fabrication (i.e., at 0 month of aging), the relative amounts of the herbal substances in the herb-loaded CA fiber mats that had been aged at both conditions ranged between 87.4-97.6%. An increase in both the aging period and the aging temperature generally caused the relative, detectable amounts of the herbal substances to decrease. Among the various herbal substances, the relative, detectable amounts of AC in the CACE-loaded CA fiber mats were the lowest, with the values ranging between 81.3-91.6% and 74.6-90.0% for the specimens that had been aged at room temperature and 40 °C, respectively.

6.4.2 Indirect Cytotoxicity Evaluation of 2 wt.% CACE-Loaded CA Fiber Mats and Films

The cytotoxicity of the CM-, CACE- and PAC-loaded CA fiber mats and the corresponding films had already been evaluated with NHDF in previous reports (Suwantong, 2007; Suwantong, 2008), based on the indirect cytotoxicity

evaluation. While most of the herb-loaded materials released no substances in the levels that were harmful to the cells, the 40 wt.% CACE-loaded ones, at the extraction ratios greater than or equal to 5 $mg \cdot mL^{-1}$, released certain substances in the levels that appeared to be toxic to the tested cells (Suwantong, 2008). As a result, the content of CACE initially loaded in the CA solutions used to prepare the e-spun fiber mats and the solvent-cast films was decreased to 2 wt.% (based on the weight of CA). For the potential for use of the 2 wt.% CACE-loaded CA fiber mats and the corresponding films as topical/transdermal patches or wound dressings, it is necessary to evaluate these materials for their toxicity against NHDF as well. Figure 3 shows the viability of NHDF that had been cultured with the extraction media from these materials in comparison with that of the cells that had been cultured with the fresh culture medium (i.e., control). Evidently, the cells that had been cultured with the extraction media from both types of 2 wt.% CACE-loaded materials, at any given extraction ratio, exhibited the viability that was greater than those of the cells that had been cultured with the fresh culture medium. The obtained results indicated that these materials released no substances in the levels that were harmful to the cells.

6.4.3 Antioxidant Activity of Herbal Substances in Herb-Loaded CA Fiber Mats and Films

Hydrogen peroxide, H_2O_2, is produced naturally in a very small quantity as a by-product of oxygen metabolism, which can be catalytically decomposed into water and oxygen by enzymes known as peroxidases. It is one of the most powerful oxidizers, which, under certain circumstances, can be converted to hydroxyl radicals (•OH), of which reactivity second only to fluorine. When being present in a large enough quantity, H_2O_2 is toxic to cells and living tissues, as it involves in the oxidation of various cellular components, such as DNA as well as a number of proteins and lipids, leading to mutagenesis and apoptosis (Hampton, 1997; Tada-Oikawa, 1999, Kowaltowski, 2000). The cytotoxicity of H_2O_2 has also been reported to be a result of the diffusion of it into the mitochondrial matrix, causing a loss to the integrity of certain mitochondrial components that finally leads to cell death (Mronga, 2004). Based on the fact that the adverse effect of H_2O_2 has also been suggested to occur within the mitochondria (Mronga, 2004), MTT should be an ideal

method for the assessment of the antioxidant activity of the studied herbal substances against H_2O_2 upon its exposure to NHDF (viz. since the reduction of MTT to formazan in viable cells takes place via reactions catalyzed by mitochondrial dehydrogenases, coupled with oxidative phosphorylation (Ekmekcioglu, 1999)).

Figure 4 shows the viability of NHDF that were treated with H_2O_2-supplemented culture medium after they had been pretreated with PBS solutions, each containing a certain amount of the herbal substance that was released from the CM-, CACE- and PAC-loaded CA fiber mats and the corresponding films. The viability of the cells that had been cultured with the fresh and the H_2O_2-supplemented culture media was used as controls, while that of the cells that had been cultured with the fresh culture medium was used as the base value to obtain the relative viability values reported in the figure. Evidently, for the cells that had been cultured with the H_2O_2-supplemented culture medium, their viability was only about 63-65% with respect to that of the cells that had been cultured with the fresh medium. This confirms the toxicity of H_2O_2 towards the cells. When the cells were pre-incubated with the PBS solutions containing CM that had been released from the majority of the CM-loaded CA fiber mats and films, their viability was significantly increased upon their exposure to H_2O_2. Specifically, the viability of NHDF pretreated with the PBS solutions containing CM that had been released from the CM-loaded CA films ranged between about 81 and 131%, while that of the cells pretreated with the solutions from the CM-loaded CA fiber mat counterparts ranged between 79 and 110%.

On the other hand, the viability of NHDF that had been pre-incubated with the PBS solutions containing CACE or PAC that had been released from the 40 wt.% CACE- or the 40 wt.% PAC-loaded CA fiber mats and films was relatively low, with the values ranging from 56 to 85%. Specifically, only the cells pretreated with the PBS solutions containing PAC that had been released from the 40 wt.% PAC-loaded CA fiber mats for 12 and 24 h showed the greatest increase in the viability from that of the cells that had been cultured with the H_2O_2-supplemented culture medium (82 and 85%, respectively). Evidently, a significant increase in the viability of the cells was observed with the cells that had been pretreated with the PBS solutions containing CACE that had been released from the 2 wt.% CACE-

loaded CA fiber mats (i.e., 112-114%). Such an improvement was not seen in the cells that had been pretreated with the PBS solutions containing CACE that had been released from the film counterparts. The much greater amount of CACE that was able to diffuse out from the fiber mats than from the film counterparts is the probable cause for such an observation.

The obtained results indicated clearly that CM was a better antioxidant than either CACE or PAC. Though not shown, the release characteristics of these herbal substances from the corresponding fiber mat and film specimens in PBS at the specified time points were also investigated. The results showed that the herbal substances were able to diffuse out from the fiber mats more readily than from the films and that the cumulative amounts of CACE or PAC released from both types of specimens was far greater than those of CM. Based on this, it is certain that the observed superiority in the antioxidant activity of CM in comparison with that of CACE and PAC was obviously not a result of the content, but should be a result of its chemistry. Considering the chemical structures of CM (in its enol form) and asiaticoside shown in Figure 1, it is quickly recognized that, due to the presence of the phenyl rings and the double bonds in the vicinity of the hydroxyl groups of each CM molecule, its electronic property is more stabilized. Upon its donation of a radical, the ability of the remaining electron to delocalize around the π-orbitals of the phenyl rings and the double bonds renders CM a better antioxidant than asiaticoside. Notwithstanding, in the case of the PBS solutions containing CACE that had been released from the 2 wt.% CACE-loaded CA fiber mats which showed the greatest antioxidant ability towards H_2O_2, its effective antioxidant activity could be due to the synergistic effect of the combination of the various triterpenoid components in addition to asiaticoside.

6.4.4 Attachment and Proliferation of Fibroblast Seeded/Cultured on CACE- and PAC-Loaded Fiber Mats and Films

To investigate the potential for use of the herb-loaded CA fiber mats and the corresponding films as wound dressings, NHDF were seeded or cultured on these matrices for various time intervals. The viability of the cells at various cell seeding/culturing time points was evaluated by MTT assay in comparison of that of the cells that had been seeded or cultured on TCPS (positive control) as well as the

neat CA fiber mat and film specimens (internal controls). It should be noted that, due to the interference from the yellow color of CM, MTT assay could not be used to evaluate the viability of the cells seeded or cultured on the CM-loaded materials.

Figure 5 shows the viability of the attached cells on the different substrates, reported as the relative values to the viability of the cells that had been seeded on TCPS for 2 h. For any given type of the substrates, the viability of the attached cells increased with an increase in the cell seeding time, except for that of the cells seeded on the 40 wt.% CACE-loaded CA fiber mats that showed a decrease in the value at 24 h after cell seeding, a result that suggested the toxicity of the loaded substances towards the cells (Suwantong, 2008). Generally, the fibrous substrates showed better support for the attachment of the cells than the film counterparts did. Interestingly, while the inclusion of either CACE or PAC in the CA films did not cause a significant increase in the viability of the seeded cells from that observed on the neat materials, a significant increase in such values was observed for cells that had been seeded on the fiber mats that contained either 2 wt.% CACE or 40 wt.% PAC for 24 h, with the latter showing the greatest value. Quite a similar result was obtained in the proliferation studies, shown in Figure 6. The toxicity of both the fiber mats and the films that contained 40 wt.% CACE became more obvious (Suwantong, 2008), as the viability of the cultured cells being found to decrease with increasing the cell culturing time. While the CA films that contained 2 wt.% CACE showed a marginal increase in the viability of the cultured cells, a significant increase was observed for the fiber mats that contained either 2 wt.% CACE or 40 wt.% PAC, with the latter showing the greatest values at all cell culturing time points.

Despite the inability to observe the viability of NHDF that had been either seeded or cultured on the CM-loaded CA fiber mat and film specimens based on the MTT assay because of the interference from the color of CM, the behavior of the cultured cells could be evaluated by observing their morphology using SEM instead (see Supporting Information). Representative SEM images of the cell-cultured specimens are shown, as examples, in Figure 7. The use of the glass substrate as control instead of TCPS was due to the ease of taking the cell-cultured specimens to the SEM observation. On the glass substrate, the cells proliferated to

cover most of the surface only after 3 d of cell culturing. On the other hand, much less coverage of the cultured cells was observed on both the neat CA fiber mat and film substrates at any given cell culturing time point, despite the observed increase in the number of proliferated cells with an increase in the cell culturing time. Clearly, most of the cells cultured on the fiber mat surface assumed their characteristic spindle-like morphology even after they had been cultured on the surface only for 1 d. On the film counterpart, even though most of the cells were in their characteristic spindle-like morphology, some of the cells that had been cultured for 1 and 3 d were still round.

With regards to the CM-loaded materials, the cells, after having been cultured on the film surfaces for 1 d, appeared in their typical, spreading morphology. Evidently, further increase in the cell culturing time resulted in an observed decrease in the number of the cells that had been cultured on these surfaces. In addition, increasing the loaded CM content in the films also resulted in an observed decrease in the number of the cells. These results suggest that CM, in the levels that were loaded and released into the culture medium, pose an adverse effect to the behavior of the cells. On the contrary, even though the number of the cells that had been cultured on the fiber mat surfaces, for any given CM loading, did not appear to decrease appreciably with an increase in the cell culturing time, as in the case of the film counterparts, the majority of the cells were still round, even after they had been cultured on the surfaces for more than 1 d. This is in contrast to the cells that had been cultured on the neat CA fiber mat surfaces, which appeared to spread rather well over the surfaces. The results clearly confirm that CM poses an adverse effect to the behavior of the cultured cells.

Even though the viability of NHDF that had been either seeded or cultured on the CACE- and the PAC-loaded CA fiber mat and film specimens could be observed by the MTT assay, the behavior of the cells that had been cultured on these materials was also evaluated by observing their morphology using SEM (see Supporting Information). Representative SEM images of the cell-cultured specimens are shown, as examples, in Figure 8. Only the cells that had been cultured on the surfaces for 1 and 7 d were observed. Consistent with the observation by the MTT assay, the number of the cells that had been cultured on the 2 wt.% CACE-loaded

CA films, at any given cell culturing time point, was greater than that on the 40 wt.% PAC-loaded CA films. Evidently, all of the cells appeared in their characteristic spindle-like morphology. In addition, the number of the cells was found to increase with an increase in the cell culturing time. On the other hand, a reverse result was observed between the 2 wt.% CACE-loaded and the 40 wt.% PAC-loaded CA fiber mats, in that the number of cells on the 40 wt.% PAC-loaded materials was greater than that observed on the 2 wt.% CACE-loaded ones. The number of the cells also increased with an increase in the cell culturing time. Comparing between the cells that had been cultured on the CM-loaded and the asiaticoside-loaded CA fiber mats, it is evident that the cells that had been cultured on both the CACE- and the PAC-loaded CA fiber mats expanded more readily on the surfaces than they did on the CM-loaded ones, the results that suggested the better biocompatibility of the asiaticoside-loaded materials than the CM-loaded counterparts.

6.4.5 Quantification of Synthesized Collagen

To investigate whether the loaded herbal substances influence the differentiation of the cultured fibroblasts, the amount of collagen synthesized by NHDF that had been cultured on the CM-, CACE- and PAC-loaded CA fiber mat and the corresponding film specimens, in comparison with those that had been cultured on TCPS, for 7 d was then quantified by the Sircol^{TM} collagen assay. To suitably compare the amount of collagen synthesized by the cells that had been cultured on these different substrates, the amount of the synthesized collagen, for a given cell-cultured specimen, needed to be normalized by the number of the cells. As previously mentioned, quantification for the number of the cells that had been cultured on the CM-loaded materials could not be completed with the MTT assay, due to the interference from the color of the loaded CM. Consequently, the number of the cells that had been cultured on the different substrates was then quantified based on the amount of DNA materials, using the DNA quantification assay.

Figure 9 shows the normalized amount of collagen synthesized by NHDF that had been cultured on the different substrates for 7 d. Evidently, the normalized amount of the collagen synthesized by the cells that had been cultured on the film substrates was significantly lower than that by the cells that had been cultured on both the fiber mat specimens and TCPS. The results are in agreement

with the fact that the number of the cells that had been cultured on the fiber mat specimens were generally greater than that of the cells that had been cultured on the film counterparts, even though the cells on the fiber mat surfaces appeared to be more round. The significantly greater number of cells on the fiber mat specimens than on the film counterparts could result in the cells on the fiber mat specimens exhibiting a cell-to-cell mediating interactions that up-regulated the production of collagen and other ECM materials faster than those on the film counterparts, hence the observed greater normalized amount of the synthesized collagen on day 7 after cell culturing. Apparently, the cells that had been cultured on the CM-loaded CA fiber mat specimens synthesized collageneous matters in the levels that were either equivalent or lower than the cells that had been cultured on TCPS. A significant increase in the normalized amount of the synthesized collagen was observed for the cells that had been cultured on the CA fiber mats that contained either 2 wt.% CACE or 40 wt.% PAC, with the cells grown on the latter exhibiting the greatest value. Based on the results shown here, asiaticoside, either in the form of CACE or PAC, was more effective than CM in the ability to upregulate the production of collageneous matters *in vitro*. However, whether or not asiaticoside was solely responsible for such an observation requires further systematic investigation.

6.5 Conclusions

Due to the implication of asiaticoside and curcumin (CM) as wound-healing mediators, asiaticoside [in the form of either crude extract (CACE) or pure substance (PAC)] and CM were individually incorporated with cellulose acetate (CA) in the form of electrospun (e-spun) fiber mats. These herb-loaded fiber mats were evaluated for the stability of the as-loaded herbal substances, the indirect cytotoxicity, antioxidant activity of the as-loaded herbal substances, the ability to support the attachment and the proliferation of the seeded/cultured fibroblasts and the ability of the cultured fibroblasts to synthesize collagen. In many cases, comparisons were made against the corresponding solvent-cast films. The results showed that the as-loaded herbal substances in the fiber mats, measuring either at room temperature or 40 °C, were stable up to 4 months of storage (i.e., ≥74.6% with respect to the initial

contents loaded in the spinning solutions). The neat CA fiber mats provided the better support for both the attachment and the proliferation of the fibroblasts. Similar results were obtained for all of the herb-loaded CA fiber mats. However, while the cells that had been cultured on the 2 wt.% CACE- and the 40 wt.% PAC-loaded CA fiber mats appeared in their phenotypic spindle-like shape, those that had been cultured on all of the CM-loaded CA fiber mats were round. Among these various herb-loaded CA fiber mat substrates, the 40 wt.% PAC-loaded ones exhibited the greatest ability to support the attachment and the proliferation of the fibroblasts, followed by the 2 wt.% CACE-loaded ones. The large numbers of the cells that had been proliferated on these substrates on day 7 agreed particularly well with the great amounts of collagen synthesized by these cells. Despite its ability to mediate the collagen production in the cultured fibroblasts, asiaticoside was inferior to CM in terms of the antioxidant activity.

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Supplementary data

Supplementary data associated with this research work can be found in the online version, at http://pubs.acs.org.

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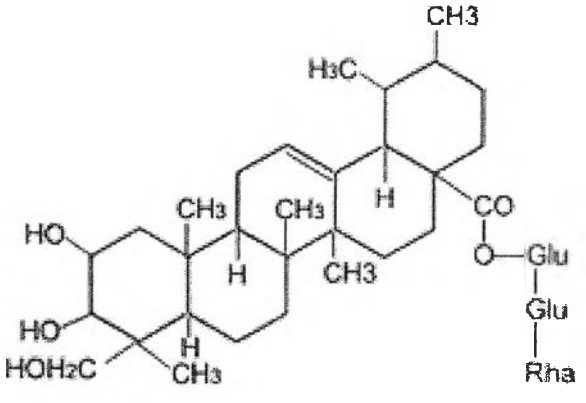

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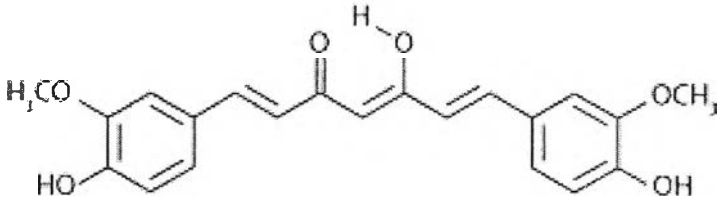
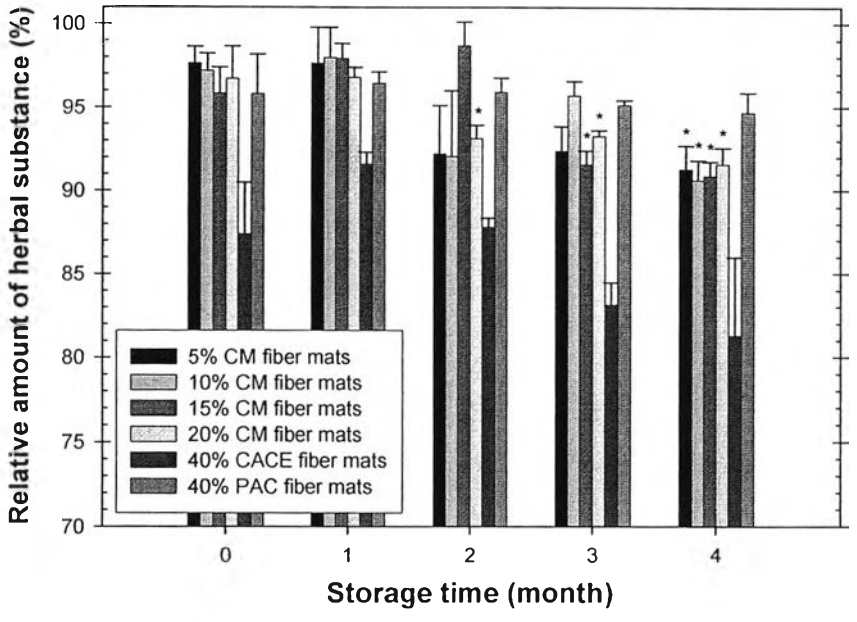
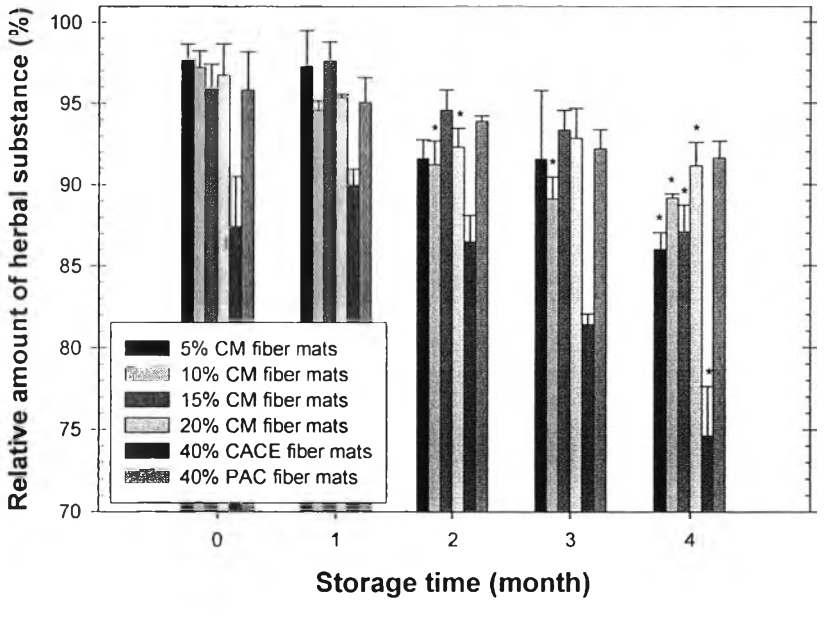


(b)

Figure 6.1 Chemical structures of (a) asiaticoside and (b) curcumin (enol form).





(b)

Figure 6.2 Stability of herbal substances in the herb-loaded CA fiber mats that had been aged for various time intervals ranging from 0 to 4 months at (a) room temperature and (b) 40 °C ($n = 3$). *$p < 0.05$ compared with the stability of the herbal substances in the herb-loaded CA fiber mats that had been investigated at 0 month.

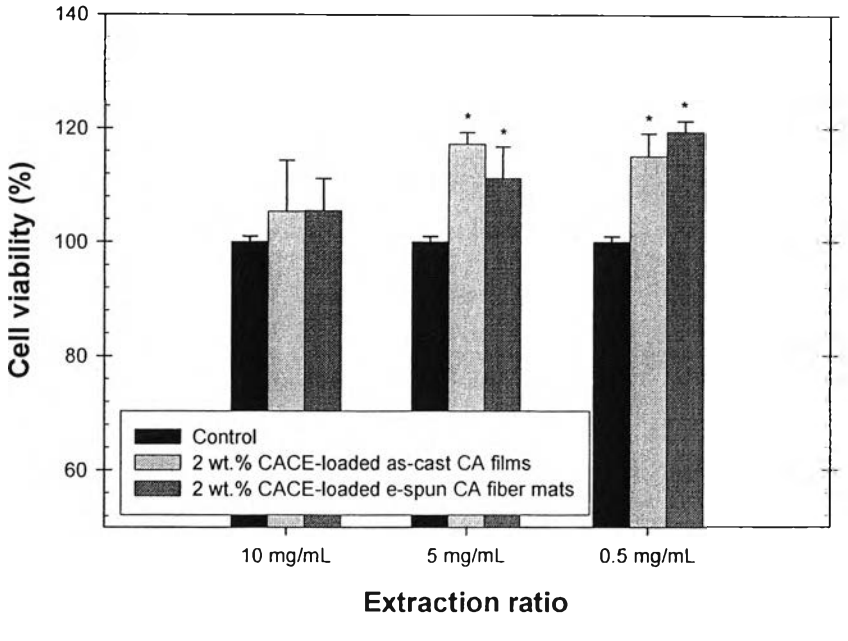
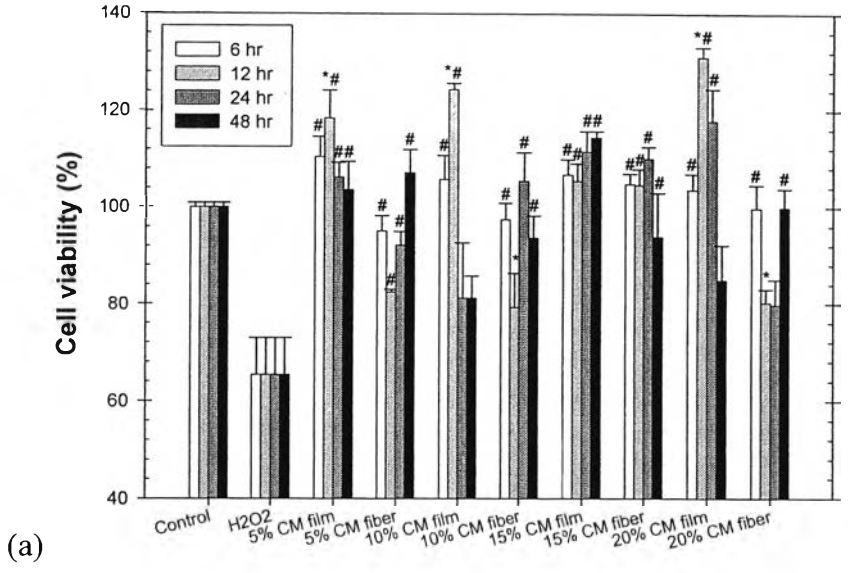


Figure 6.3 Indirect cytotoxicity evaluation of 2 wt.% CACE-loaded CA fiber mats and corresponding films, expressed in terms of the viability of normal human dermal fibroblast (NHDF) that had been cultured with the extraction media from these materials in comparison with that of the cells that had been cultured with fresh culture medium (i.e., control) ($n = 3$). *$p < 0.05$ compared with control at a given extraction ratio.



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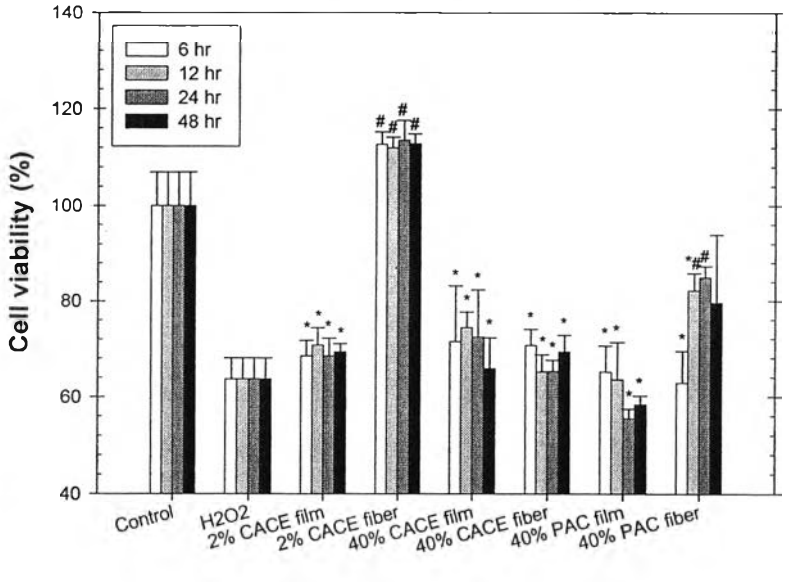


(b)

Figure 6.4 Viability of NHDF that were treated with H_2O_2-supplemented DMEM after they had been pretreated with PBS solution containing a certain amount of the herbal substance that was released from the (a) CM-, (b) CACE- and PAC-loaded CA fiber mats and corresponding films at different times after submersion in PBS (n = 3). The viability of the cells that had been cultured with the fresh and the H_2O_2-supplemented culture media was used as controls. $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared with H_2O_2.

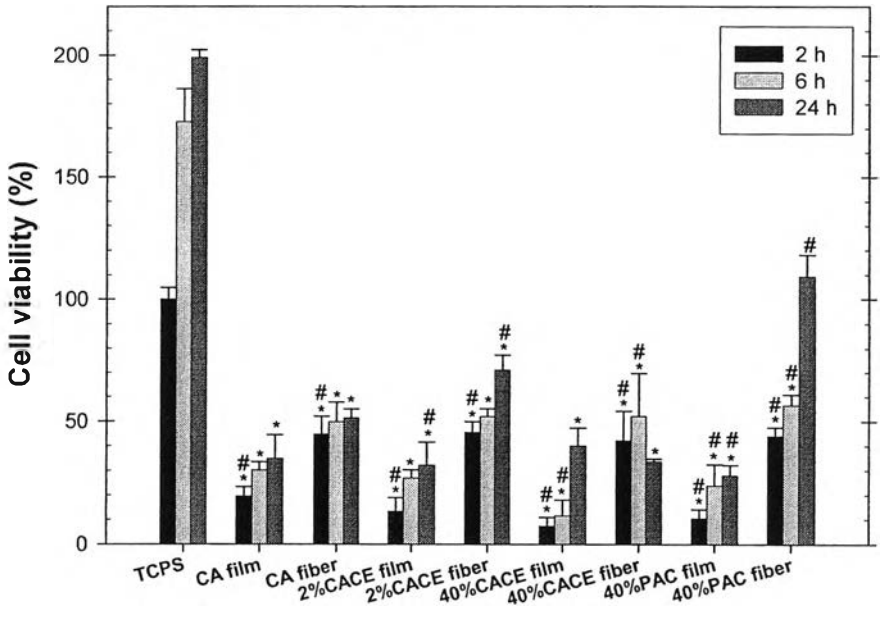


Figure 6.5 Attachment of NHDF that had been seeded on neat CA fiber mats and the CA fiber mats that contained CACE (at 2 or 40 wt.%) or PAC (at 40 wt.%) as well as their corresponding films for 2, 6 and 24 h ($n = 3$). The viability of the cells that had been seeded on TCPS was used as the positive control and the viability of the attached cells that had been seeded on TCPS for 2 h was used as the reference to obtain the relative viability values shown in the figure. $^{*}p < 0.05$ compared with TCPS at 2 h and $^{\#}p < 0.05$ compared between the fiber mats and the films at any given time point.

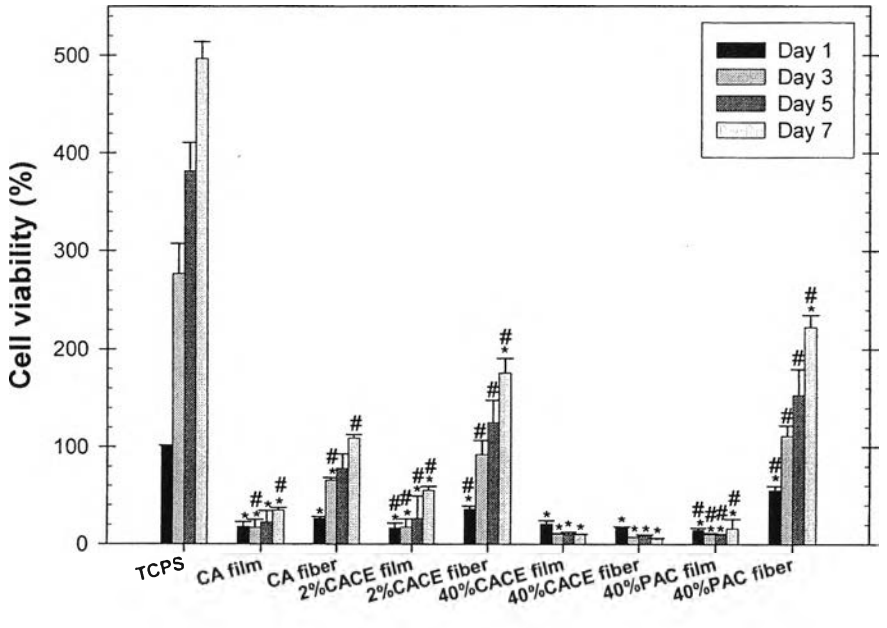
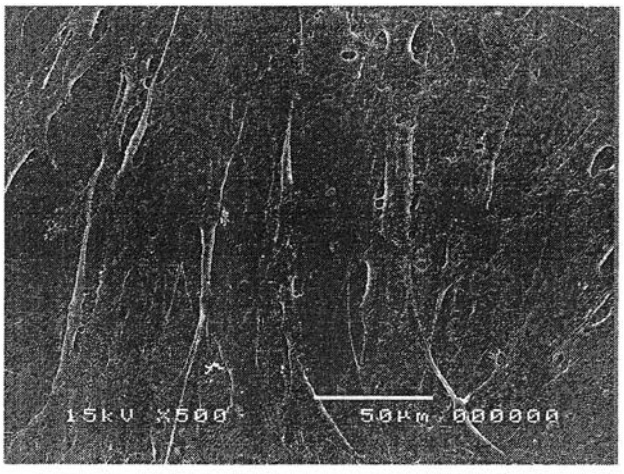


Figure 6.6 Proliferation of NHDF that had been cultured on neat CA fiber mats and the CA fiber mats that contained CACE (at 2 or 40 wt.%) or PAC (at 40 wt.%) as well as their corresponding films for 1, 3, 5 and 7 d ($n = 3$). The viability of the cells that had been seeded on TCPS was used as the positive control and the viability of the attached cells that had been seeded on TCPS for 1 d was used as the reference to obtain the relative viability values shown in the figure. $^{*}p < 0.05$ compared with TCPS on day 1 and $^{\#}p < 0.05$ compared between the fiber mats and the films at any given time point.



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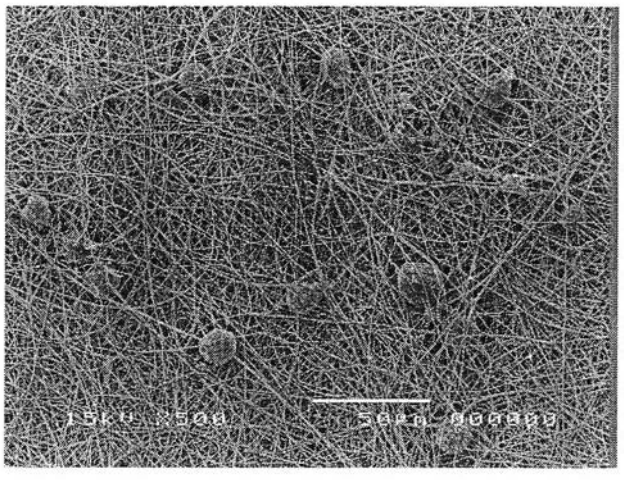
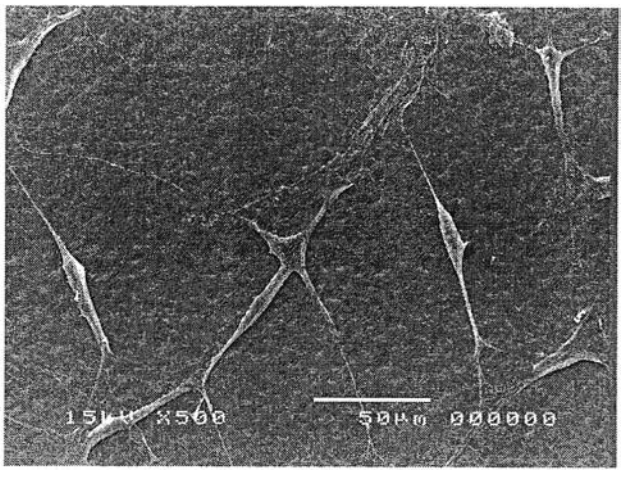


(b)

Figure 6.7 Representative scanning electron micrographs illustrating the morphology of NHDF that had been cultured on the surfaces of (a) CA film and (b) CA fiber mat that contained 20 wt.% CM for 1 d.



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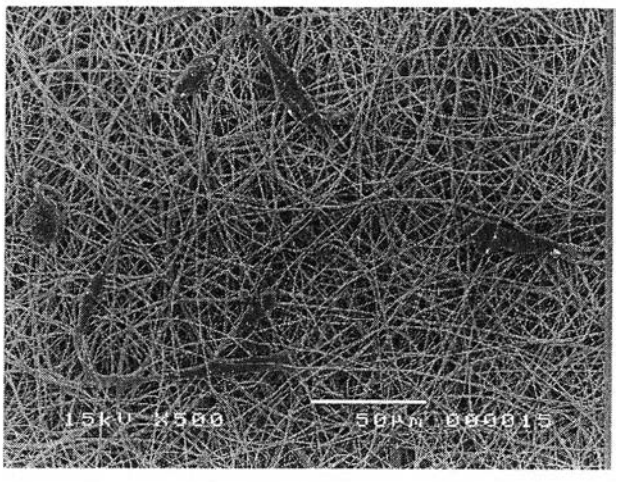

(b)

Figure 6.8 Representative scanning electron micrographs illustrating the morphology of NHDF that had been cultured on the surfaces of (a) CA film and (b) CA fiber mat that contained 40 wt.% PAC for 1 d.

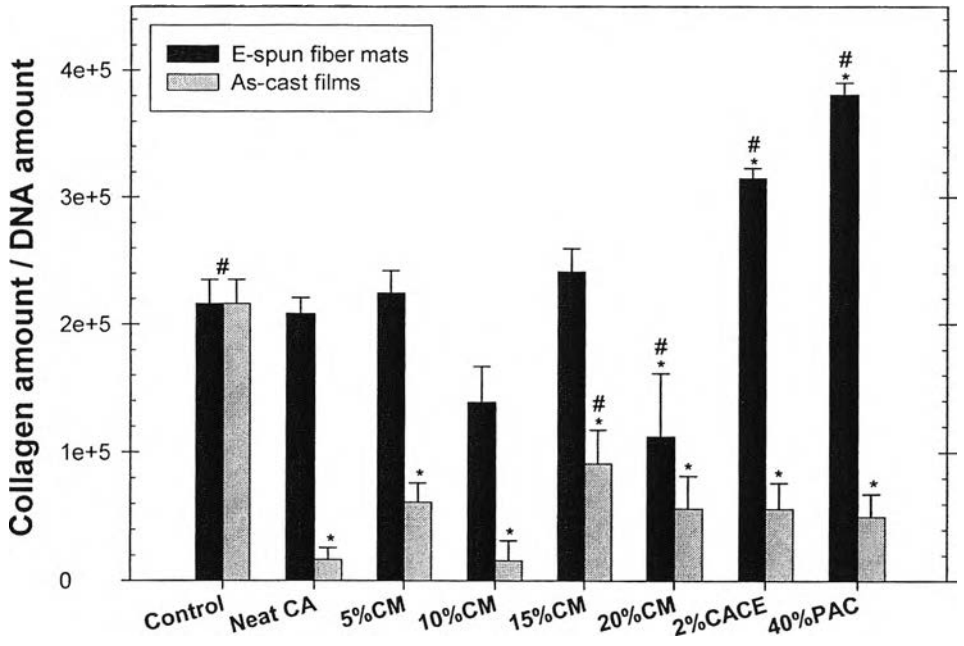

Figure 6.9 Normalized amount of collagen synthesized by NHDF that had been cultured on TCPS (i.e., control), neat CA fiber mat and film specimens, CM-loaded CA fiber mat and film specimens, CACE-loaded CA fiber mat and film specimens and PAC-loaded CA fiber mat and film specimens for 7 d ($n = 3$). $^{*}p < 0.05$ compared with TCPS and $^{\#}p < 0.05$ compared with the neat CA fiber mat or the film specimens.