CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials for Fabrication of Herb-Loaded Electrospun Fiber Mats and Films

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 (Switzerland). Pure asiaticoside (PAC; 90% purity) and *Centella asiatica* crude extract (CACE; triterpene content = 95%; asiaticoside = 37.5% (HPLC) and madecassic and asiatic acids = 56.2% (HPLC)) were purchased from Shanghai Angoal Chemical Co., Ltd. (China). Acetone and *N,N*-dimethylacetamide (DMAc) were purchased from Carlo Erba (Italy) and Labscan (Asia, Thailand), respectively.

3.1.2 Materials for Preparation of Buffer Solution

Methanol and Acetic acid were purchased from Labscan (Asia, Thailand). Tween 80 [Polyoxyethylene (20) sorbitan mono oleate] was purchased from BDH Ltd., UK. Sodium acetate, sodium chloride, anhydrous disodium hydrogen orthophosphate, and sodium dihydrogen orthophosphate (Ajax Chemicals, Australia), and glacial acetic acid (Carlo Erba, Italy) were of analytical reagent grade and used without further purification.

3.1.3 Materials for Cell Culture

Normal human dermal fibroblasts (NHDF) were used as reference cells. 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)].

3.2 Equipment

3.2.1 Equipment for Electrospinning Process

- High voltage power supply from Gamma High Voltage Research Inc. (Ormond Beach, Florida), model D-ES30PN/M692 DC is used to generate positive DC voltage
- Syringe with volume size 10 ml is served as a container for polymer solutions.
- Stainless steel needle with gauge number 20 (or the inner diameter of 0.91 mm) is used as the electrode to conduct the electrical from power supply to the solutions.
- The rotating drum which covered with aluminum sheet is used as a collector. Width and OD of the rotating drum were 14 and 15 cm, respectively.
- A Kd Scientific syringe pump was used to control the feed rate of the polymer solution at about 1 ml/h.

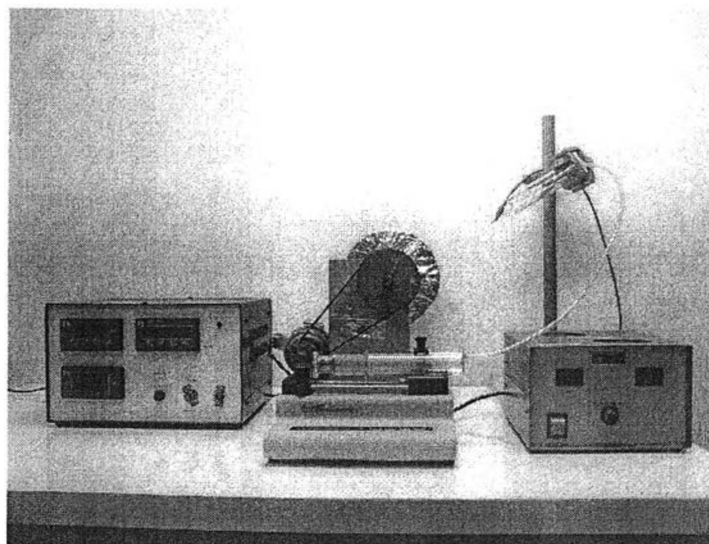

Figure 3.1 A schematic photograph of electrospinning apparatus.

3.2.2 Equipment for Characterization of Materials

- A Brookfield DV-III programmable viscometer was used to examine the viscosity of polymer spinning solutions.
- A SUNTEX conductivity meter was used to examine the conductivity of polymer spinning solutions.
- A JEOL JSM-6400 scanning electron microscope (SEM) was used to observe the morphology of the materials.
- A JEOL JFC-1100E sputtering device was used to coat the materials with a thin layer of gold prior to observation under SEM.
- A Bruker DRX400 ^{1}H nuclear magnetic resonance spectrometer (^{1}H NMR) was used to investigate the chemical integrity of herbal substance in the herb-loaded electrospun fiber mat samples.
- A Lloyd LRX universal testing machine was used to examine the mechanical integrity of the materials.
- A Shimadzu UV-2550 UV-vis spectrophotometer was used to measure the amount of CM in the sample solutions at wavelength of 426 nm.
- A microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601; Packard BioScience, USA) was used to measure the absorbance of solutions from antioxidant activity (DPPH assay) at wavelength of 550 nm.
- A Shimadzu LC-10 AD HPLC was used to quantify the amount of AC in the sample solutions.
- A SpectraMax M2 Microplate Reader was used to measure the absorbance of solution from MTT assay at wavelength of 570 nm.

3.3 Methodology

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

3.3.1.1 Preparation of Neat and CM-loaded CA Fiber Mats and Films

A weighed amount of CA powder was dissolved in 2:1 v/v acetone/dimethylacetamide (DMAc) to prepare the base CA solution at a fixed concentration of 17% w/v. CM-loaded CA solutions were prepared by dissolving curcumin powder (CM) in the amounts of 5, 10, 15, and 20 wt.% based on the weight of CA powder in the base CA solution. Prior to electrospinning, the as-prepared solutions were characterized for their viscosity and conductivity using a Brookfield DV-III programmable viscometer and a SUNTEX conductivity meter, respectively. All experiments were carried out at 25 °C. These mixtures were then electrospun under a fixed electric field of 17.5 kV/15 cm. The feeding rate of the solutions was controlled at ~1 ml h^{-1} by means of a Kd Scientific syringe pump. Unless otherwise noted, the collection time was ~18 h (resulting in the fiber mats of 90 ± 10 mm in thickness). For comparison purposes, both the neat and the CM-loaded CA films were also prepared by solvent-casting technique from 4% w/v CA solution in 2:1 v/v acetone/DMAc and the base CA solution that contained varying amounts of curcumin (5, 10, 15 and 20 wt.%). Unless otherwise noted, the thickness of the as-cast films was 90 ± 10 mm.

3.3.1.2 Preparation of Neat and PAC- or CACE-Loaded CA Fiber Mats and Films

A weighed amount of CA powder was dissolved in 2:1 v/v acetone/dimethylacetamide (DMAc) to prepare the base CA solution at a fixed concentration of 17% w/v. PAC- or CACE-containing CA solutions were prepared by dissolving the same amount of CA powder and either pure asiaticoside (PAC) or *Centella asiatica* crude extract (CACE) in the amount of 40 wt.% based on the weight of CA powder in the acetone/DMAc mixture. Prior to electrospinning, the as-prepared solutions were characterized for their viscosity and conductivity using a Brookfield DV-III programmable viscometer and a SUNTEX conductivity meter, respectively. The measurements were carried out at 25 ± 1 °C. The as-prepared

solutions were then electrospun under a fixed electric field of 17.5 kV/15 cm at a controlled feeding rate of ~1 $mL \cdot h^{-1}$ (by means of a Kd Scientific syringe pump). Unless otherwise noted, the collection time was ~18 h (resulting in the fiber mats of 90 ± 10 μm in thickness). For comparison purposes, both the neat and the herb-loaded CA films were also prepared by solvent casting technique from 4% w/v CA solution in 2:1 v/v acetone/DMAc and the same solution that contained either 40 wt.% of PAC or CACE. Unless otherwise noted, the thickness of the as-cast films was 80 ± 10 μm.

3.3.2 Characterization of Neat and Herb-Loaded CA Fiber Mats and Films

Morphological appearance of both the neat and the herb-loaded electrospun CA fiber mats and as-cast CA films was observed by a JEOL JSM-6400 scanning electron microscope (SEM). Each specimen was coated with a thin layer of gold using a JEOL JFC-1100E sputtering device prior to observation under SEM. Diameters of the electrospun fibers were measured directly from SEM images using a SemAphore 4.0 software.

A Bruker DRX400 ^{1}H nuclear magnetic resonance spectrometer (^{1}H NMR) was used to investigate the chemical integrity of herbal substances in the herb-loaded electrospun fiber mat samples (2-3 mg), using deuterated dimethylsulfoxide (DMSO-d_6) as solvent.

Mechanical properties in terms of stress at maximum load, strain at maximum load, tensile strength, and elongation at break of both the neat and the CM-load electrospun CA fiber mats were tested on a Lloyd LRX universal testing machine (gauge length = 50 mm and crosshead speed = 20 mm min^{-1}). The specimens of ~100 ± 10 mm in thickness (i.e., the collection time was ~24 h) were cut into a rectangular shape (10 mm × 100 mm).

The swelling and the weight loss behavior of both the neat and the CM-loaded electrospun CA fiber mats and as-cast films were measured in an acetate buffer solution containing 0.5% v/v polysorbate 80 (hereafter, Tween 80) and 3% v/v methanol (hereafter, the B/T/M medium) at the physiological temperature of 37 °C for 48 h. On the other hand, the swelling and the weight loss behavior of both the neat and PAC- or CACE-loaded electrospun CA fiber mats and the corresponding as-

cast films were measured in an acetate or a phosphate buffer solution containing 10% v/v methanol (hereafter, the A/B/M and the P/B/M medium, respectively; see below for the preparation of the media) at the skin and the physiological temperatures of 32 and 37 °C, respectively, for 24 h according to the following equations:

$$\text{Degree of swelling } (\%) = \frac{M - M_d}{M_d} \times 100, \tag{3.1}$$

and

$$\text{Weight loss } (\%) = \frac{M_i - M_d - M_r}{M_i - M_r} \times 100, \tag{3.2}$$

where M is the weight of each sample after submersion in the buffer solution for 24 h, M_d is the weight of the sample after submersion in the buffer solution for 24 h in its dry state, M_i is the initial weight of the sample in its dry state, and M_r is the weight of herbal substance that was released from the sample.

3.3.3 Release of Herb Substances from Herb-Loaded CA Fiber Mats and Films

3.3.3.1 Preparation of Releasing Medium

To prepare 1,000 mL of the acetate buffer solution, 150 g of sodium acetate was dissolved in 250 mL of distilled water. Exactly 15 mL of glacial acetic acid was then added very slowly into the aqueous sodium acetate solution. Finally, distilled water was added to the solution to fill the required volume. The pH of the as-prepared acetate buffer solution was 5.5. On the other hand, to prepare 1,000 mL of the phosphate buffer solution, 6.177 g of anhydrous disodium hydrogen orthophosphate and 1.014 g of sodium dihydrogen orthophosphate were dissolved in 100 mL of distilled water. Exactly 8.7 g of sodium chloride was then added into 20 mL of the solution. Finally, distilled water was added to the solution to fill the required volume. The pH of the as-prepared phosphate buffer solution was 7.4.

Due to the solubility limitation of CM in the acetate buffer solution, the B/T/M releasing medium (96.5% v/v acetate buffer with 0.5% v/v Tween 80 and 3% v/v methanol) was used. For intended use of both the PAC- and the CACE-loaded electrospun CA fiber mats and the corresponding as-cast films as

tropical/transdermal patches, the acetate buffer solution (pH = 5.5) containing 10% v/v of methanol (hereafter, the A/B/M medium) was used as the releasing medium. On the other hand, for intended use of both the PAC- and the CACE-loaded materials as wound dressings, the phosphate buffer solution (pH = 7.4) containing 10% v/v methanol (hereafter, the P/B/M medium) was instead used.

3.3.3.2 Actual Herb Content

The actual amount of CM in the CM-loaded electrospun CA fiber mats and as-cast CA films was determined. Each specimen (circular disc; ~2.8 cm in diameter) was dissolved in 4 ml of 2:1 v/v acetone/dimethylacetamide (DMAc). After that, 0.5 ml of the solution was added into 8 ml of the acetate buffer solution and the actual amount of CM was measured by a Shimadzu UV-2550 UV-vis spectrophotometer at the wavelength of 426 nm. The actual amount of CM in the CM-loaded electrospun CA fiber mat and film samples was back-calculated from the obtained data against a predetermined calibration curve for CM.

To determine the actual amount of AC in the PAC- or CACE-loaded electrospun CA fiber mats and the corresponding as-cast films, each specimen (circular disc; ~2.8 cm in diameter) was first dissolved in 4 mL of 2:1 v/v acetone/DMAc. After that, 0.5 mL of the solution was added into 8 mL of either A/B/M or P/B/M medium and the actual amount of AC was measured by a high-performance liquid chromatography (HPLC) (see later). The actual amount of AC in the PAC- or CACE-loaded electrospun CA fiber mats and the corresponding as-cast films was then back-calculated from the obtained data against a predetermined calibration curve for AC.

3.3.3.3 Herb-Release Assay

The release characteristic of herbal substances from the herb-loaded electrospun CA fiber mats and as-cast CA films was investigated by two types of the release assay, i.e., total immersion and transdermal diffusion through a pig skin method.

Due to the solubility limitation of CM in the acetate buffer solution, the B/T/M releasing medium (96.5% v/v acetate buffer with 0.5% v/v Tween 80 and 3% v/v methanol) was used. Each specimen (circular disc; ~2.8 cm in diameter) was immersed in 30 ml of the medium at the physiological temperature of

37 °C. At a specified immersion or diffusion period ranging between 0 and 48 h (2880 min), either 1 ml (for the total immersion method) or 0.3 ml (for the transdermal diffusion through a pig skin method) of a sample solution was withdrawn and an equal amount of the fresh medium was refilled. For the transdermal diffusion through a pig skin method, each CM-loaded fiber mat and film specimen was placed on a fresh piece of pig skin (abdomen; epidermal hair, subcutaneous fat, and underlying tissues removed; final thickness = 1-1.5 mm), which, in turn, was placed on top of the medium on a modified Franz diffusion cell. The amount of CM in the sample solutions was determined using the UV-vis spectrophotometer at the wavelength of 426 nm. The obtained data were calculated to determine the cumulative amount of CM released from the specimens at each immersion or diffusion time point. The experiments were carried out in triplicate and the results were reported as average values.

For the release characteristics of AC from the PAC- or CACE-loaded electrospun CA fiber mats and the corresponding as-cast films, two types of the releasing medium, i.e., A/B/M (pH 5.5) or P/B/M (pH 7.4), were used. Each specimen (circular disc; ~2.8 cm in diameter) was immersed in 20 mL of a medium at the skin and the physiological temperatures of 32 °C (for A/B/M medium) or 37 °C (for P/B/M medium). At a specific immersion or diffusion time point ranging between 0 and 24 h (1440 min), either 0.5 mL (for the total immersion method) or 0.3 mL (for the transdermal diffusion through a pig skin method) of a sample solution was withdrawn and an equal amount of the fresh medium was refilled. The amount of AC in the sample solutions was determined by HPLC (see later). The obtained data were carefully calculated to determine the cumulative amount of AC released from the specimens at each immersion or diffusion time point. The experiments were carried out in triplicate.

3.3.3.4 HPLC Analysis

A Shimadzu LC-10 AD HPLC was used to quantify the amount of AC in the sample solutions. Chromatographic separation of the herbal substances was achieved by the use of an Inertsil ODS-3 C18 column (particle size = 5 μm; column dimension = 4.6 x 250 mm) with an Inertsil ODS-3 guard column (particle size = 5 μm; column dimension = 4.0 x 10 mm) operating at 1 $mL \cdot min^{-1}$.

The mobile phase for AC separation was 26:24:50 v/v/v acetonitrile/methanol/distilled water. The injection volume was 50 μL. A UV detector for AC was set at (λ_{max}) 204 nm. All of the sample solutions were filtered through a nylon filter (average pore size = 0.45 μm) prior to injection. AC was separated out over a range of elution periods of 7.5-7.7 min. Due to the difference in the AC content in PAC (i.e., 90%) and CACE (i.e., 37%), the calibration curves for AC were carried out over the concentration ranges of PAC of 0.14-2.95 $mg \cdot mL^{-1}$ and CACE of 1.84-4.60 $mg \cdot mL^{-1}$, respectively.

3.3.4 Antioxidant Activity (DPPH assay)

The antioxidant activity of CM loaded in both the CM-loaded electrospun CA fiber mats and as-cast CA films was assessed with 1,1'-diphenyl-2-picrylhydrazyl (DPPH) radicals, following the method of Blois (1958). Each specimen (circular disc; ~2.8 cm in diameter) was first dissolved in 4 ml of 2:1 v/v acetone/dimethylacetamide (DMAc) and treated with a methanolic solution of DPPH (100 mM) for 30 min at the physiological temperature of 37 °C. The free radical scavenging activity was determined photometrically in a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601; Packard BioScience, USA) and the absorbance was measured at the wavelength of 550 nm. The antioxidant activity (%AA) of the as-loaded CM was expressed as the percentage of DPPH that was decreased in comparison with that of the control condition (i.e., the testing solution without the presence of the as-loaded CM), according to the following equation:

$$\%AA = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100, \tag{3.3}$$

where $A_{control}$ and A_{sample} are the absorbance values of the testing solution without and with the presence of the as-loaded CM, respectively.

3.3.5 Stability of 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

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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.

3.3.6 The Cell Study of Herb-Loaded CA Fiber Mats and Films

3.3.6.1 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.

3.3.6.2 Indirect Cytotoxicity Evaluation

The indirect cytoxicity evaluation of both the neat and the herb-loaded e-spun CA fiber mats and 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; Biokom Systems, Poland) using normal human dermal fibroblasts as reference. First, extraction media were prepared by immersing specimens in serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1% lactabumin, and 1% antibiotic and antimycotic formulation) for 24 h. NHDF cells were separately cultured in 96-well TCPS in serum-containing DMEM for 24 h to allow cell attachment on the plate. Then, the cells were starved with SFM for 24 h, after which

time the medium was replaced with an extraction medium. After 24 h of cell culturing in the extraction medium, the viability of the cells cultured by each of the extraction medium was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with the viability of the cells cultured by fresh SFM being used as control.

3.3.6.3 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.

3.3.6.4 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.

3.3.6.5 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.

3.3.6.6 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.

3.3.6.7 Quantification of Synthesized Collagen

The amount of collagen synthesized by the cultured cells was quantified on day 7 after cell culturing using a Sircol™ 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.