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Asiaticoside/2-Hydroxypropyl-β-cyclodextrin 포접화합물 함유 셀룰로오스 아세테이트 섬유 매트의 전기방사: 창상피복제로서 사용가능성과 방출특성

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Electrospinning of Asiaticoside/2-Hydroxypropyl-β-cyclodextrin Inclusion Complex-loaded Cellulose Acetate Fiber Mats: Release Characteristics and Potential for Use as Wound Dressing

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Abstract: Cellulose acetate (CA) fiber mats containing inclusion complexes of asiaticoside (AC) in 2-hydroxypropyl-β-cyclodextrin (HPβCD) for potential usage as wound dressings were developed. The AC/HPβCD complex-loaded CA fibers at various HPβCD to AC molar ratios of 0.5, 1, and 2 were prepared in 90:10 v/v mixture of 80% (v/v) acetic acid and *N,N*-dimethylacetamide (DMAc) via electrospinning. The maximum released amounts of AC depended on the HPβCD content and were much greater than those released from the AC-loaded CA fiber mat. In the *in vitro* study, indirect cytotoxic evaluation with human dermal fibroblasts (HDFa) showed that these materials released no substances in the levels that were harmful to the cells and the cells appeared to attach and proliferate well on these substrates. However, only the CA fiber mats containing AC/HPβCD complexes at the HPβCD to AC molar ratio of 0.5 was effective in up-regulating the production of collagen of the cultured cells.

Keywords: asiaticoside, 2-hydroxypropyl-β-cyclodextrin, cellulose acetate, wound dressings, electrospinning.

Introduction

Wound healing is a tissue regeneration process, involving the restoration of cellular structures and injured tissue layers. Upon an injury to the skin, a set of four overlapping sequential events occurs, starting with the hemostasis phase, followed by inflammation, proliferation, and finally maturation.[1,2] For several years, effective wound dressings have been heavily explored to obtain accelerated healing, based mainly on production techniques, materials and active substance-loaded matrices.[3-7] Such dressings should regulate the release of active substances, adsorb exudates, maintain a moist environment, allow gas and nutrient exchange, and prevent wound infections as well.[8,9] Moreover, it should be non-toxic, non-allergenic, able to accelerate the healing process and readily removed without secondary trauma.[10]

Among various techniques, electrospinning is one promising technique for producing non-woven-based dressings for wound healing. This owes to its simplicity, cost-effectiveness and capability to produce ultra-fine fibers with diameters ranging from micro- down to nanometers.[11,12] Electrospun fibers provide a range of useful characteristics: for instances, the high surface area to volume mass ratio which provides a better result in the release of substances that promote the healing of a wound and helps facilitate adsorption of excess exudates, and

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the ability of maintaining a moist environment during healing.[7,13] Lastly, the highly porous nature of the fibrous structure allows for the exchange of nutrient and waste from cellular activity and allows gaseous diffusion in/out of the wound area.[14] In the electrospinning process, ultra-fine fibers are electrically ejected from a conical apex of polymer liquid droplet at the tip of a spinneret, provided that a sufficiently high electric field is applied, resulting in the electrical forces overriding that of the surface tension. The ultra-fine fibers depositing on a collector screen are usually in the form of a non-woven fabric. The morphology of the fibers resembles the natural structure of an extracellular matrix (ECM).[15,16]

Among various pharmacological substances exhibiting an ability to heal wounds, asiaticoside (AC; see chemical structure in Figure 1(a)), an active substance extracted from *Centella asiatica* (L.) Urban or Buabok (in Thai), has been traditionally known for its ability to heal wounds, burns, and ulcerous abnormalities of the skin.[17] The presence of this substance improves the proliferation of human dermal fibroblasts, as well as the expression of types I and III pro-collagen mRNA and the proteins within the treated cells.[18,19] The up-regulation of collagen and the increase of tensile strength of guinea pig wounds with topical treatment of varying dosage were observed.[20] Moreover, Suwantong *et al*.[21] observed an increase in synthesized collagen by fibroblasts which had been cultured on a cellulose acetate (CA) fiber mat containing pure AC at a high loading amount (i.e., 40% (wt/wt), based on the weight of CA) in comparison with those cultured on a neat CA fiber.

Even though AC is capable of accelerating the wound healing process, the use of AC as a pharmacological agent is quite limited due to the low aqueous solubility of AC. 2-Hydroxypropyl-β-cyclodextrin (HPβCD), a cyclodextrin derivative, is a well-known complexing agent, capable of improving aqueous solubility and increasing bioavailability of many therapeutic agents.[22-24] Such complexation within the cavity of a cyclodextrin has also been reported to decrease toxicity, increase stability and is able to control the release of certain therapeutic agents.[25,26] Very recently, we reported the preparation of AC inclusion complex with HPβCD in the solution and solid state. We found that the cyclohexene ring moiety of AC is captivated well within the HPβCD cavity (Figure 1(b)).[27]

According to Suwantong *et al*.,[21] the efficacy of AC loaded within the electrospun CA fibers in promoting the production of collagen of cultured fibroblasts was only observed at a high concentration of 40% (wt/wt). Here, we aimed to use HPβCD to improve the efficacy of AC at a much lower concentration of AC (i.e., at 10% (wt/wt)). This was thought to be possible because HPβCD, in an inclusion complex with AC, would help increase the solubility of AC in an aqueous medium. Here, CA fiber matrices incorporating AC/HPβCD inclusion complexes at various AC to HPβCD molar ratios (i.e., 1:0.5, 1:1, and 1:2) were fabricated by electrospinning. These AC/HPβCD complex-loaded CA fiber mats (FINC fibers) were hereafter denoted as FINC 50, FINC 100, and FINC 200, respectively. Various characteristics and properties of the fiber

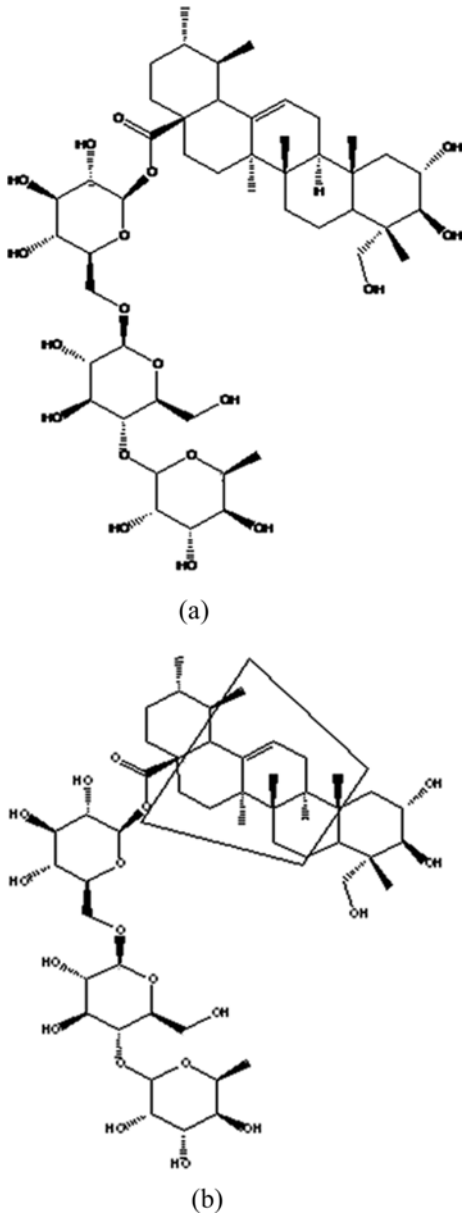


Figure 1. (a) Chemical structure of asiaticoside (AC); (b) schematic arrangement of AC with the cavity of HPβCD.

mat specimens such as morphology, thermal property and weight loss behavior were characterized. The presence of HPβCD on the release characteristics of AC from the fiber specimens was studied in a phosphate buffer saline solution (PBS). The potential for use of the FINC fibers as a wound dressing was evaluated *in vitro* with human dermal fibroblasts (HDFa). This was done in terms of indirect cytotoxicity, cell adhesion and cell proliferation, the ability of the cells to synthesize collagen, and the cell morphology.

Experimental

Materials. AC (90% purity) was purchased from Shanghai Angoal Chemical Co., Ltd. (China). HPβCD (average M_w≈ 1380 Da; 0.6 molar substitution per glucopyranose repeat unit) and CA (average M_w≈30000 Da by GPC; 39.7% (wt/wt) acetyl; degree of acetyl substitution≈2.4) were purchased from Sigma-Aldrich, Switzerland. Anhydrous disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, and sodium chloride (Ajax Chemicals, Australia), *N,N*-dimethylacetamide (DMAc, Lab-Scan Asia, Thailand), glacial acetic acid (Carlo Erba, Italy) were also used. All of the chemicals were used without further purification.

Preparation of the AC-, HPβCD- and AC/HPβCD Complex-loaded CA Fiber Mats. CA fiber mats containing an AC/HPβCD inclusion complex were prepared by the electrospinning technique. The inclusion complexes were prepared at various molar ratios between AC and HPβCD (i.e., 0.5, 1.0, and 2.0) in a 90:10 v/v mixture of 80% (vol/vol) acetic acid and DMAc (see Table 1). Each mixture of AC and HPβCD, according to Table 1, was stirred at room temperature (25±1°C) for 24 h to allow for the formation of an inclusion complex.[27] An exact amount of CA powder at the final concentration of 25.5% (w/v) was subsequently added and stirred at room temperature (25±1 °C) for 3 h until of a clear solution. Each of the sample solutions, loaded in a 10 mL glass syringe, equipped with a needle (inner diameter = 0.8 mm), was electrospun under a fixed electric field of 25 kV/18 cm and a controlled feed rate of 1 mL h^{-1} (by means of a Kd Scientific syringe pump) to obtain electrospun fiber mats with thicknesses of 90±10 μm. These fiber mats were kept in a desiccator *in vacuo* prior to further use.

Table 1. Composition of AC/HPβCD Complex-loaded CA Fiber Mats at Various HPβCD to AC Molar Ratios and Those Electrospun Fiber Mats

Electrospun fiber mats	CA (%w/v)[a]	AC (%w/w)[b]	HPβCD (%w/w)[b]	AC/HPβCD (molar ratio)
Neat CA	25.5	-	-	-
AC 10	25.5	10	-	1:0
HPβCD 50	25.5	-	7.19	0:0.5
HPβCD 100	25.5	-	14.39	0:1
HPβCD 200	25.5	-	28.78	0:2
FINC 50	25.5	10	7.19	1:0.5
FINC 100	25.5	10	14.39	1:1
FINC 200	25.5	10	28.78	1:2

[a]With respect to the solvent (90:10 v/v mixture of 80 vol% acetic acid and DMAc). [b]With respect to the weight of CA powder.

In addition, the neat CA fiber mat, the CA fiber mats containing AC at 10% (wt/wt) (hereafter, AC 10) and CA fiber mats containing an equivalent amount of HPβCD to FINC 50, FINC 100, and FINC 200 fiber mats (hereafter, HPβCD 50, HPβCD 100, and HPβCD 200, respectively) were also prepared as control fiber mat samples (Table 1).

Measurement and Characterization of AC-, HPβCD- and AC/HPβCD Complex-loaded CA Fiber Mats. The morphologies of the electrospun fiber mats were studied by using a JEOL JSM-6400 scanning electron microscope (SEM). All of the fiber specimens were vacuum-coated with a thin layer of gold using a JEOL JFC-1100E sputtering device. The average diameters of the fiber specimens were determined from at least 100 different data points collected from 2 or 3 SEM images at 2000x magnification, using SemAfore4.0 software.

To confirm the successful preparation of the FINC fiber mats, the thermal property of the fiber specimens was investigated by using a differential scanning calorimeter (DSC) (Netzsch, 204 F1 Phoenix), operating at a temperature range of 30 to 270 °C under nitrogen gas.

The degree of weight loss behavior of the fiber mats was investigated in a phosphate-buffered saline (PBS, pH 7.4) medium. The materials were immersed for 24 h at 37 °C. After immersion, the weight loss behavior was calculated as follows:

$$\text{Weight loss}(\%) = \frac{M_i - M_d}{M_d} \times 100 \quad (1)$$

where M_d is the weights of dried fiber mats, after submersion in PBS at different time intervals. M_i is the initial, dry weights of the specimens. All measurements were carried out in triplicate.

Release of AC from AC- and AC/HPβCD Complex-Loaded CA Fiber Mats. The actual amounts of AC in the

specimens were first quantified according to a published method.[28] Briefly, the 1.5 cm circular disc samples were dissolved in 4 mL of 2:1 v/v acetone/DMAc. Then, 0.5 mL of solution was withdrawn and diluted in 8 mL of the phosphate buffer saline solution (PBS, pH 7.4) containing 10% (vol/vol) methanol. Next, the diluted solution was filtered through a nylon filter (average pore size = 0.45 μm) and was separated by HPLC (Shimadzu LC-10 AD) in an Intersil ODS-3 C18 column (particle size = 5 μm; column dimension = 4.6 mm× 250 mm), accompanied by an Inertsil ODS-3 guard column (particle size = 5 μm; column dimension = 4.0 mm×10 mm). A flow rate for separation was fixed at 1 mL·min^{-1} and an UV-Visible detector was set at λ_{max} = 204 nm.

In the release assay, the FINC fiber mats and the AC 10 fiber mats (1.5 cm circular disc) were immersed in 25 mL of the PBS (pH 7.4) at a temperature of 37 ºC. At given time intervals, ranging between 0 and 24 h (1440 min), 1 mL of the releasing medium was taken out and an equal volume of fresh buffer solution was refilled. The withdrawn solution was then quantified for AC content by high performance liquid chromatography (HPLC). The amount of AC released from the specimens was calculated using a calibration line.

Cell Culture. For the *in vitro* studies in this experiment, primary human dermal fibroblasts, isolated from adult skin (HDFa; Invitrogen, USA), were used as a reference cell line. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA), 1% L-glutamine (Invitrogen, USA), penicillin (100000 U/L) and streptomycin (100 mg/L). The culture medium was changed every 2 days and maintained in 5% CO_2 at 37 ºC in a 95% relative humidity atmosphere.

Indirect Cytotoxic Evaluation. The biocompatibility of the fiber mat specimens, based on a procedure adopted from the ISO 10993-5 standard test method in a 24-well tissue-culture polystyrene plate (TCPS), was evaluated by indirect cytotoxic evalution. The fiber mat specimens sterilized by UV radiation for 1 h, were immersed in serum-free medium (SFM) containing DMEM, 1% L-glutamine, 1% L-lactabumin and penicillin and streptomycin at varying extracting ratios of 10, 5, 0.5 mg/mL, respectively, for 24 h in an incubator. Thirty thousand cells of HDFa per well were separately cultured in serum-containing DMEM for 24 h to allow cell attachment, and then were cultured in serum-free medium (SFM) for 24 h to starve the cells. After that, the SFM medium was replaced by the extraction medium and the cells were incubated for 24 h. Finally, the viability of the cells was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Viability of Cell Attachment and Cell Proliferation. The sterilized fiber mat specimens were placed in 24 TCPS well plates. Sterilized Al-metal rings were subsequently placed on top of the specimens to prevent them from floating. Next, 500 μL of DMEM medium was added into each well and incubated for 30 min. After removing the medium, the fiber specimens and empty wells of TCPS, (i.e., positive control) were seeded with HDFa in 500 μL of culture medium at a density of 30000 cells/well. In the attachment studies, the seeded cells were allowed to attach on the surface of the fiber specimens and TCPS for 2 and 5 h, respectively. The unattached cells on those specimens were gently removed by rinsing with PBS and the viability of the attached cells was quantified by MTT assay. To study the cell proliferation, the HDFa cells were cultured on the fiber mat specimens and TCPS (i.e., positive control) for either 1 or 7 days. Subsequently, the fiber mat specimens were gently washed with PBS and viability of the cells was quantified by MTT.

Quantification of Viable Cells (MTT Assay). The MTT assay, involving the reduction of yellow tetrazolium salt to purple formazan crystals by mitochondrial activity in the living cells,[29] was used to quantify the amounts of viable cells on the surface of the fiber specimens. Firstly, the specimens were washed with PBS and then 300 μL of 0.5 mg/mL MTT solution, without phenol red, was added into each well, followed by incubation for 1 h. Next, the MTT solution was removed and replaced with 500 μL of a buffer solution containing dimethyl sulfoxide (DMSO; Carlo Erba, Italy) to dissolve the formazan crystals. Lastly, the formazan solution, proportional to the viable cells, was measured by a UV-visible spectrophotometer (Thermospectinic Genesis 10) at a wavelength of 570 nm.

Morphological Observation of Cultured Cells. The cultured cells on the fiber mat specimens were rinsed with PBS twice and then fixed with 3% glutaraldehyde/PBS solution for 30 min. After the fixation, the fiber mat specimens were later dehydrated in various ethanol solutions of 30, 50, 70, 90 and 100% for about 2 min for each concentration and dried in hexamethyldisilazane (HDMS; Sigma, USA) for 5 min, and subsequently left in air until completely dried. Finally, the dried fiber mat specimens were mounted on an SEM stub.

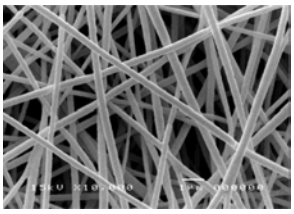
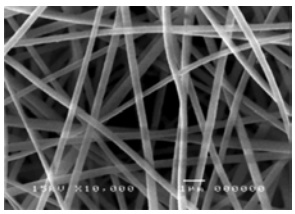
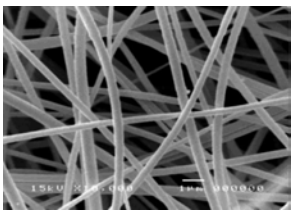
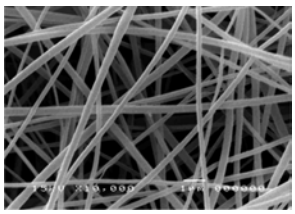
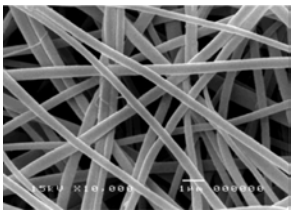
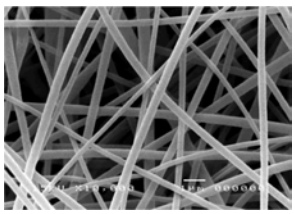
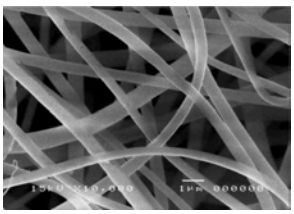
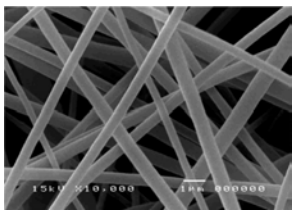
Quantification of Synthesized Collagen. The collagen synthesis of the HDFa cells on day 7 of cell culturing was quantified by using a SIRCOL® assay kit. Briefly, HDFa cells

(30000 cells/well) were seeded and cultured on the fiber mat specimens and TCPS (i.e., positive control) for 7 days. The supernatant from each well was collected for each 50 μL sample in an eppendorf tube and then 50 μL of 0.5 M acetic acid was added and gently mixed at room temperature for 2 h. To precipitate the collagen synthesized by the cells, 1 mL of dye reagent was pipetted in, followed by gently shaking at room temperature for 30 min. During this step, a collagen-dye complex was formed and later collected by centrifugation at 12000 rpm for 10 min. The slurry of collagen-dye complex was redissolved in 1 mL of alkaline dye-releasing reagent. Finally, 200 μL of each diluted solution was spectrophotometrically measured at 540 nm. To determine the actual amount of synthesized collagen, the obtained data was measured against a calibration curve of the manufacture-provided acid soluble collagen standard in the range of 0-100 μg in cultured medium was back-calculated.

The actual amounts of DNA on the fiber specimens and the corresponding TCPS well were determined by using a DNA quantification kit (Sigma-Aldrich, USA). The cell-cultured specimens on day 7, after collecting the supernatant for collagen quantification, were gently washed with 500 μL of PBS twice. After washing, the cells cultured on the fiber mat specimens were lysed with 300 μL of cell lysis buffer solution and then the suspension was centrifuged at 12000 rpm for 10 min. After that, 20 μL of supernatant was pipetted into 2 mL of 0.1 μg/mL Bisbenzimide H 33258 solution in 10x fluorescent assay buffer, followed by gently mixing. Next, the obtained solution was spectrophotometrically measured at 460 nm, after having been excited at 360 nm. The emission intensity of the samples, representing the actual amount of DNA, was finally determined against a standard DNA calibration curve that was provided with the kit.

Statistical Analysis. The means of different data sets were analyzed by using One-way ANOVA. The significance for all of the tests was accepted at a 0.05 confidence level.

Table 2. Representative SEM Images at ×10000 Magnification and Fiber Diameters of the AC-loaded CA Fiber Mats (AC 10 Fiber Mats), the HPβCD Fiber Mats (HPβCD 50, HPβCD 100, and HPβCD 200 Fiber Mats) and the FINC Fiber Mats (FINC 50, FINC 100, and FINC 200 Fiber Mats)

Type of materials	Electrospun fiber mats	Type of materials	Electrospun fiber mats
CA *d*=356±41 nm		AC 10 *d*=353±50 nm	
FINC 50 *d*=408±115 nm		HPβCD 50 *d*=357±90 nm	
FINC 100 *d*=483±134 nm		HPβCD 100 *d*=427±102 nm	
FINC 200 *d*=515±99 nm		HPβCD 200 *d*=507±92 nm	

Results and Discussion

Measurement and Characterization of AC-, HPβCD- and AC/HPβCD Complex-loaded CA Fiber Mats. The morphologies of all of the electrospun fiber mats are represented in Table 2. Smooth surfaces with no aggregation on the cross-sectionally round fibers are observed on all of the fiber mat samples. This is in contrast to the aggregates of AC/HPβCD inclusion complexes that were clearly observed on the surfaces of AC/HPβCD complex-loaded CA films.[27] The non-existence of the aggregates on the surfaces of the AC/HPβCD complex-loaded CA fiber mats as compared with those of the film counterparts should be due to the difference in the time used during the fabrication of the materials. While 24 h was required to fabricate the films, only a fraction of a second was needed in case of the electrospun fiber mats. Such a long period of time would allow adjacent AC/HPβCD inclusion complexes to interact to form into large aggregates. In the same analogy, even though cyclodextrins and cyclodextrin derivatives are well known for forming self-assembling aggregates,[30-32] such a short processing time would lessen the possibility for HPβCD to form into large aggregates in case of the HPβCD fibers. As for the AC 10 fibers, the non-existence of any aggregate agreed well the work of Suwantong *et al.*,[28] which showed that the CA fiber mats containing 40% (wt/wt) AC were smooth without any aggregate on the fiber surfaces.

The diameters of CA and AC 10 fibers were 356±41 and 353±50 nm, respectively. Comparatively, Suwantong *et al.*[28] reported that the diameter of AC-loaded CA fibers at 40% (wt/wt) of AC loading was 485±91 nm. The larger diameters of the AC 40 fibers (as in the Suwantong *et al.*[28]'s work) as opposed to those of the AC 10 fibers should be due to the greater amount of AC used, which could affect to the viscosity of the spinning solution in an increasing manner. The diameters of the HPβCD fibers ranged between 357±90 and 507±92 nm, while those for the FINC fibers were between 408±115 and 515±99 nm. Detailed values of the diameters can be obtained from Table 2. Generally, one of the major factors contributing to an observed increase in the diameters of electrospun fibers is an increase in the viscosity of the spinning solution.[28,33] The presence of foreign substances (i.e., AC, HPβCD, and AC/HPβCD, in this case) would cause the viscosities of the AC-, HPβCD-, and AC/HPβCD-containing CA solutions to increase from that of the neat CA solution. As a result, larger diameters from those of the neat CA fibers were observed.

DSC is a useful technique for investigating the inclusion complex of HPβCD with various organic compounds in the solid state.[34-36] The absence or the presence of the melting point of organic compounds in their free or complex form could be used to confirm the formation of the inclusion complex within fiber mats.[33] Figure 2 shows that pure AC powder exhibited a broad endothermic peak over a temperature range of 40 to 160 °C, associating with the loss of moisture during the heating process, and a melting peak at about 248 °C, corresponding to the melting point of pure AC. The incorporation of AC within the CA fibers (i.e., AC 10 fibers) shows the peak of moisture loss between 40 and 130 °C and two additional endothermic peaks at 218 and 224 °C. While the latter is clearly correspondent to the melting of the CA matrix (cf. the thermogram of the neat CA fibers), the former is assumed to associate with the thermal dissociation of AC aggregates. The very short time during fiber formation would only allow adjacent AC molecules to aggregate, hence requiring much less thermal energy

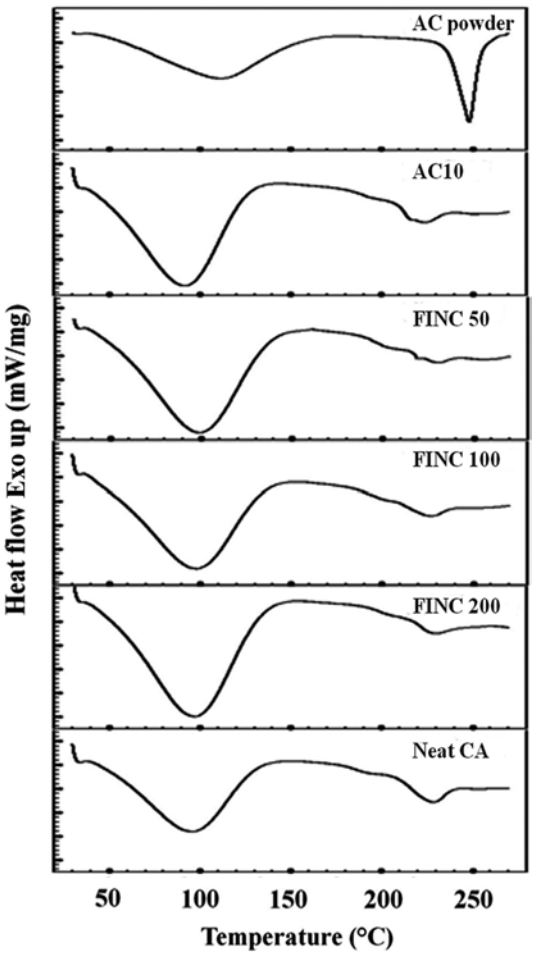


Figure 2. DSC thermograms of AC powder and AC 10, FINC 50, FINC 100, FINC 200 and neat CA fiber mats.

to dissociate them during a heating scan in the DSC. Similarly, all of the FINC fibers also exhibited a moisture loss peak over similar temperature ranges to those of the CA powder and the AC 10 fibers. However, only the FINC 50 fibers showed a small endothermic peak that was supposed to be that of the AC aggregates (at about 218 °C), while other FINC fibers did not. This implies that there were some free AC molecules being present in the FINC 50 fibers (in a similar manner with the AC 10 fibers). The inexistence of this small endothermic peak in both the FINC 100 and the FINC 200 fibers suggests that most, if not all, of the AC molecules were in complexation with HPβCD, hence no free AC molecules, in a large enough quantity, were available to form aggregates that would result in the presence of the small endothermic peak.

The loss in the weights of the fiber specimens after they had been submerged in PBS for 24 h was investigated (Figure 3). The CA fiber mats showed an average weight loss at about 2%, which could be associated with the loss of retained moisture prior to the submersion in the medium. For the AC 10 fiber mats, about 3.9% of their initial weight was lost in the medium, suggesting that only about 1.9% of AC was released into the medium. As for the HPβCD fiber mats, the loss in the weights of the HPβCD 50 and the HPβCD 100 fiber mats was about the same at about 3.0 and 3.2%, while it was about 17.2% for the HPβCD 200 fiber mat specimens. This suggests that only about 1 and 1.2% of the incorporated HPβCD was released from the HPβCD 50 and the HPβCD 100 fibers, while about 15.2% did so from the HPβCD 200 counterparts. Despite the fact that electrospinning process would lessen the possibility for HPβCD to form into large aggregates in case of the HPβCD fibers as previously mentioned, the much larger amount of HPβCD in the HPβCD 200 fiber mats, as compared with those in the HPβCD 50 and the HPβCD 100 fiber mats may lead to the formation of small aggregates, which cannot be visualized by SEM. Such aggregates would allow for an easy access to water molecules, hence water solubility was significantly enhanced. Compared with the AC 10 fibers, the greater values of weight loss that were observed on the FINC fiber mats, with the values being 4.3, 8.6, and 22.8% for the FINC 50, FINC 100, and FINC 200 fiber mats, respectively. This suggests that about 2.3, 6.6, and 20.8% of AC, HPβCD, and/or the AC/HPβCD inclusion complexes were likely to be released from these fiber mats, respectively.

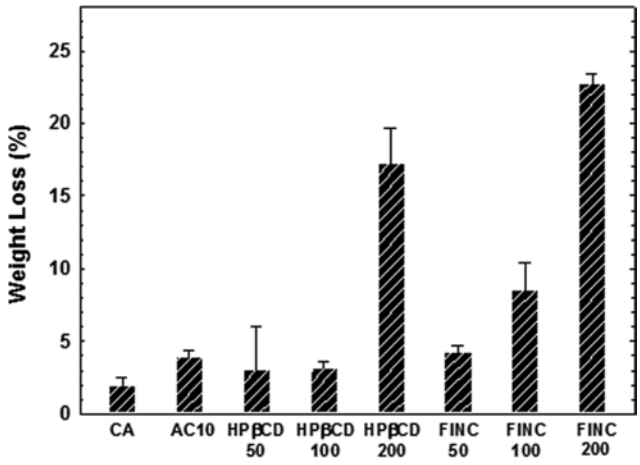


Figure 3. Weight loss behavior of neat CA and all fiber mats after immersion in PBS medium at 37 °C for 24 h (n=3).

Release of AC- and AC/HPβCD Complex-loaded CA Fiber Mats. Prior to the release studies, the actual amounts of AC contained within the AC 10 and the FINC fiber mats had to be determined (based on HPLC technique). It should be noted that a mixture of acetone/DMAc was used, instead of that of acetic acid/DMAc, to dissolve all the fiber mats is to prevent the damage to the HPLC column. In case of the AC 10 fiber mats, the actual loading of AC was determined to be 62.4% (based on the initial weight of AC loaded into the spinning solution), while the values were 54.8, 58.7, and 62.6% for the FINC 50, the FINC 100, and the FINC 200 fiber mat specimens, respectively.

Figure 4 shows the release profiles of AC from all of the AC-loaded CA fiber mat specimens. Two types of graphs are shown. In the first, the cumulative released amounts of AC were calculated based on the actual weights of the as-loaded AC (Figure 4(a)), while, in the second, such values were calculated based on the actual weights of the fiber mat specimens (Figure 4(b)). All of the releasing profiles exhibited an initial burst release of AC, within the first 300 min after submersion in the medium. After that, gradual increases to reach plateau values at a long submersion time were observed in all sample types. Interestingly, the AC 10 fiber mats exhibited the slowest releasing rate of AC in the initial burst release region (cf. the slope), while the releasing rate of AC was found to increase with an increase in the amount of HPβCD incorporated within the fibers. Based on the actual weights of the as-loaded AC, the maximal cumulative amounts of AC released from the AC 10, FINC 50, FINC 100, and FINC 200 fiber mats were about 28, 53, 84, and 93%, respectively. Based on the actual weights of the fiber mat specimens, the maximal cumulative amounts of AC released from the AC 10, FINC 50, FINC 100, and FINC 200 fiber mats were about 1.4, 2.0, 4.8, and 4.9%,

respectively.

The obtained results clearly suggest that the presence of HPβCD improves both the releasing rate and the maximal cumulative released amount of AC from the fiber mat specimens. As previously described by us,[27] the inclusion complex of AC within the cavity of HPβCD disfavored the crystallization of AC. The prevention of AC to form crystals was also observed in this work (see the thermal analysis results). In the weight loss studies, it was shown that AC could be released into the PBS medium, especially for the fiber mats containing the highest amounts of HPβCD (i.e., the HPβCD 200 fiber mats). Both the presence of HPβCD in the medium (upon its release) and the prevention of AC from crystallizing (upon the formation of the inclusion complex with HPβCD) clearly facilitate the release of AC out of the fibers.

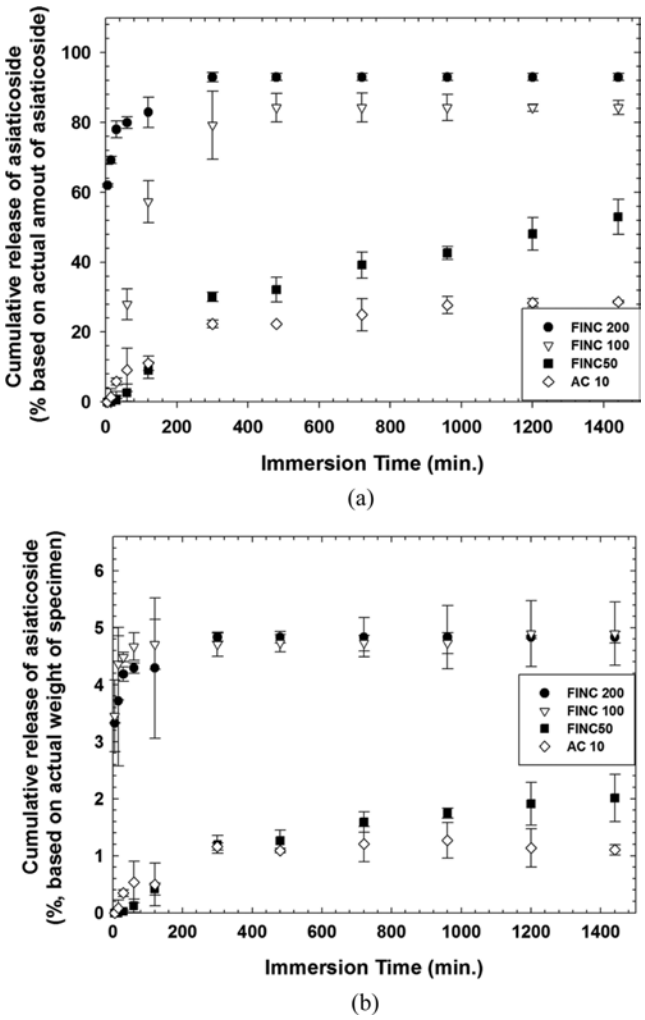


Figure 4. Release characteristics of AC from AC 10, FINC 50, FINC 100 and FINC 200 fiber mats upon immersion in the PBS medium at 37 °C reported as a percentage of the as-released AC to (a) the amounts of AC actually loaded within the fiber mats; (b) the actual weights of the fiber mats.

Cell Culture. Indirect Cytotoxic Evaluation: To investigate the potential for use of the AC/HPβCD complex-loaded CA fiber mats as wound dressing materials, the FINC 50, FINC 100, and FINC 200 fiber mats were evaluated in terms of indirect cytotoxicity with HDFa cells. The viabilities of HDFa cells that had been cultured with different amounts of extraction media (0.5, 5, or 10 mg/mL) from these specimens, in comparison with that of cells that had been cultured with the fresh culture medium (i.e., control) are shown in Figure 5. The relative viabilities of HDFa that had been cultured with the extraction media from the CA fiber mats ranged between about 83 and 95%. Those of the cells that had been cultured with the extraction media from the AC 10 fiber mats, on the other hand, ranged between 89 and 101%. These values suggested that these materials released no substances in the levels that were harmful to the cells. Suwantong *et al.*[28] showed that CA fiber mats containing AC at 40% (wt/wt) were non-toxic to the tested fibroblasts, while Sikareepaisan *et al.*[37] showed that alginate films containing AC at 20% (wt/wt) also posed no threat to the cells. As for the HPβCD fiber mats, the viabilities of the cells after having been submerged in the extraction media were found to range between 92 and 99%, while the values were in the range of 82 and 99% for the AC/HPβCD fiber mats. The viability values greater than 80% for all of the HPβCD and the AC/HPβCD fiber mats clearly indicated non-toxicity of these materials towards the tested cells.

Cell Adhesion and Cell Proliferation: The ability of the AC/HPβCD complex-loaded CA fiber mats in supporting the

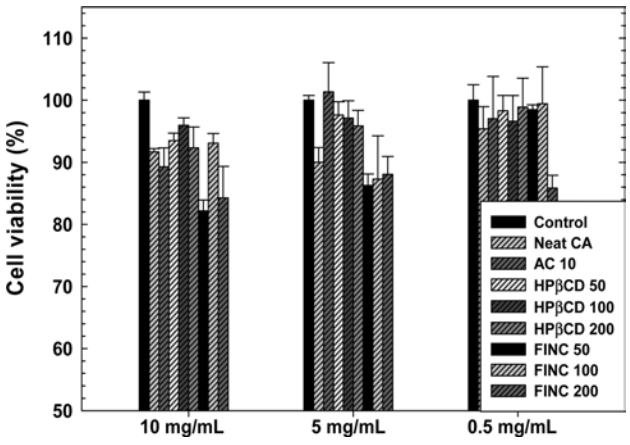


Figure 5. Indirect cytotoxic evaluation of the neat CA, AC 10, HPβCD and FINC fiber mats at different extraction media (i.e., 0.5, 5, 10 mg/mL) based on the viability of HDFa cells.

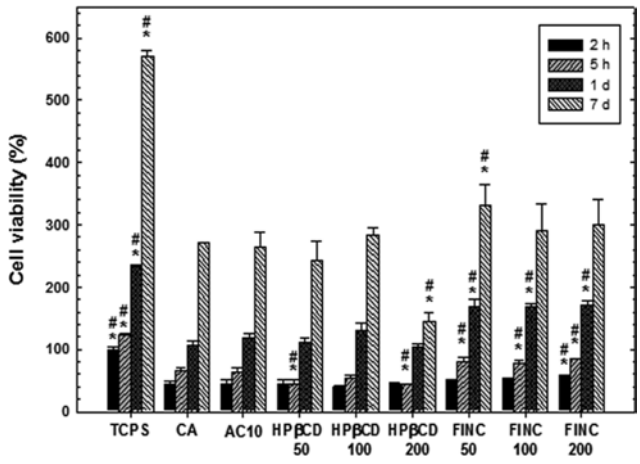


Figure 6. Attachment (2 h and 5 h) and proliferation (1 d and 7 d) of HDFa cells cultured on the surfaces of the neat CA, AC 10, HPβCD (HPβCD 50, 100 and 200 fiber mats) and FINC fiber mats (FINC 50, 100 and 200 fiber mats) were reported as the viability of the cells on those specimens relative to the cells that had been seeded/cultured on TCPS for 2 h. *$p<0.05$ and $^{\#}p<0.05$ as compared to the neat CA and AC 10 fiber mats, respectively.

adhesion and proliferation of HDFa was further investigated, as shown in Figure 6. While TCPS was used as a positive control, the CA, the AC 10, and the HPβCD fiber mats were used as internal controls. Clearly, all of the fibrous substrates were inferior to TCPS in supporting the adhesion and the proliferation of the cells after having been cultured for 2 h, 5 h, 1 d, and 7 d, respectively. Between the CA and the AC 10 fiber mats, the viabilities of the cells, at any time point, were not significantly different. In the release studies, only about 1.4% of AC (based on the weight of the specimens) was able to release from the AC 10 fiber mats into PBS. In the cell culturing medium, such a value might be a little different. Such a small amount of AC released into the medium may not be enough to regulate any cellular activity.

As for the effect of incorporated HPβCD, both the HPβCD 50 and the HPβCD 100 fiber mats, at most time points investigated, showed no significant difference in the cell viabilities when compared with those of the CA and the AC 10 fiber mats. Only the viabilities of the cells grown on the HPβCD 200 fiber mats at 5 h and 7 d showed significantly lower values. This could imply that a high amount of HPβCD that had been released into the medium (cf. about 15.2%, based on the weight loss studies) may present an adverse effect to the cells. As for the AC/HPβCD complex-loaded CA fiber mats, they all seemed to promote both the adhesion and the proliferation at time points shorter than about 1 d. Only the FINC 50 fiber mats provided a better support for the proliferation of the cultured cells on day 7. In the release studies, the maximal cumulative amounts of AC released from the FINC 50, FINC 100, and FINC 200 fiber mats were about 2.0, 4.8, and 4.9%, respectively. Despite the fact that greater amounts of AC were released from FINC 100 and FINC 200 fiber mats, it should be also noted that, AC that was released from these specimens would likely be present in its complexation with HPβCD.[27] For the FINC 50 fiber mats, despite the lower amount of AC being released into the medium, the amount of HPβCD present in the fiber mats was not enough to encapsulate all of AC molecules,[27] hence there would be more free AC molecules available to regulate the proliferation of the cells.

To support such claim, the loss in the weights of the FINC fibers after 72 h of submersion in PBS was additionally studied. Such values for the FINC 50, FINC 100, and FINC 200 fiber mats were about 11.0, 11.3, and 24.1%, respectively. This suggests that about 9, 9.3, and 22.1% of AC, HPβCD, and/or the AC/HPβCD inclusion complexes were likely to be released from these fiber mats, respectively. After 24 h of submersion, however, these values were about 2.3, 6.6, and 20.8%, respectively. This suggests that about 6.7, 2.7, and 1.3% of AC, HPβCD, and/or the AC/HPβCD inclusion complexes were additionally released from the FINC 50, FINC 100, and FINC 200 fiber mats into the medium, after additional 2 days of submersion. A sharp increase in the amount of AC, HPβCD, and/or the AC/HPβCD inclusion complexes released from the FINC 50 fiber mats, along with the lowest ratio of HPβCD to form the AC/HPβCD inclusion complexes, should result in a larger amount of free AC to up-regulate the cell proliferation.

Table 3 represents the SEM images of HDFa cells that had been cultured on TCPS and on the various fiber mat specimens at time intervals of 2 h, 5 h, 1 d, and 7 d. During the cell adhesion periods of 2 and 5 h, HDFa cells seeded on all types of the fiber specimens could expand on the surface of these specimens and exhibited spindle-like morphology at 2 h of cell seeding, while the cells on TCPS were still round at 2 h, and most of the cells was later in a more spreading form at 5 h of cell seeding. The rapid spreading of the seeded cells on all types of the fiber mat specimens in comparison with those on TCPS may be due to the nature of a nanofibrous matrix that can mimic the structure and functions of a natural extracellular matrix (ECM)[15,16] and provide a large surface area that would be indirectly interacting with the cells. During the proliferation periods of 1 and 7 d, the numbers of cells on all types of the fibrous matrices were found to significantly increase with an

Table 3. SEM Images of HDFa Cells Seeded/Cultured on the Surfaces of TCPS, AC 10, HPβCD and FINC Fiber Mats for 2 h, 5 h, 1 d and 7 d

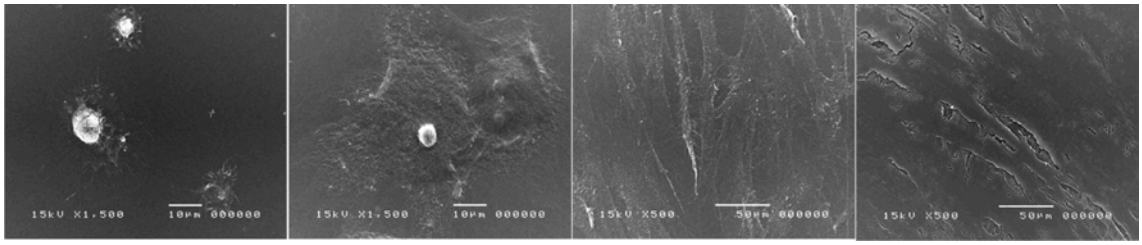
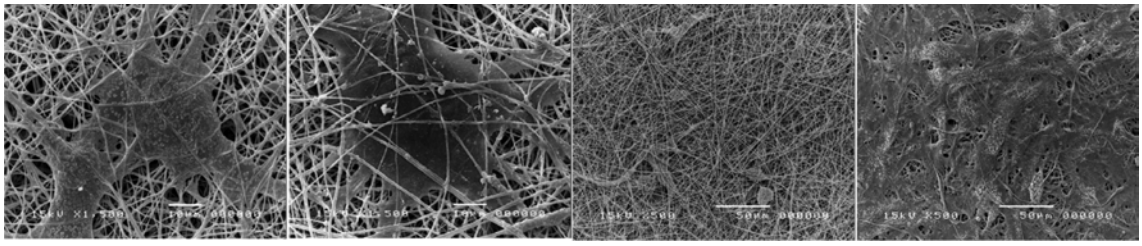
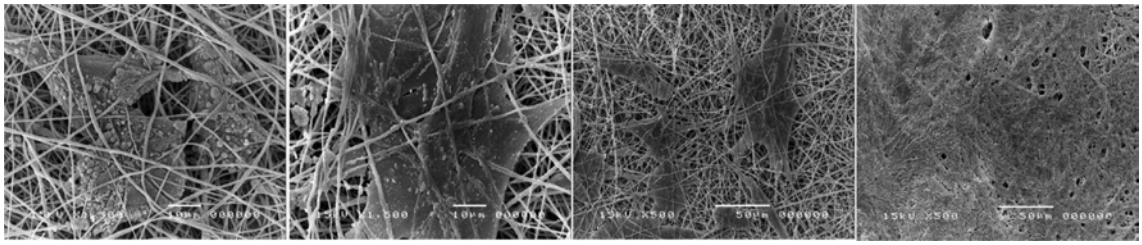
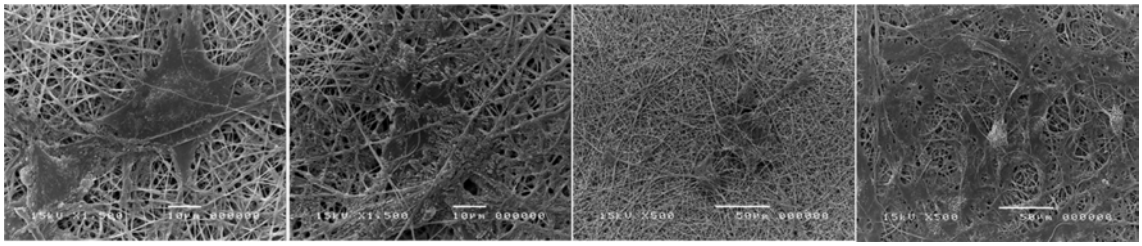
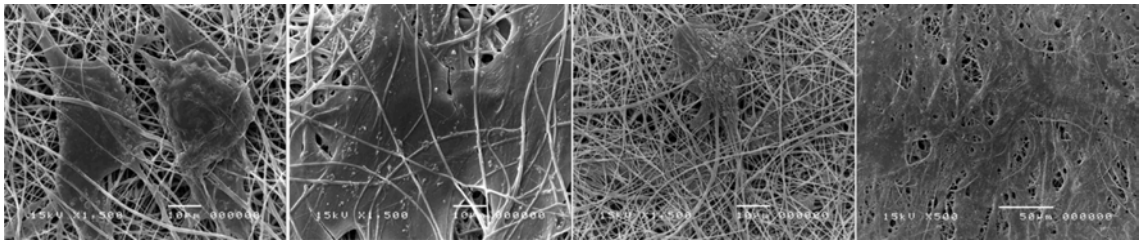
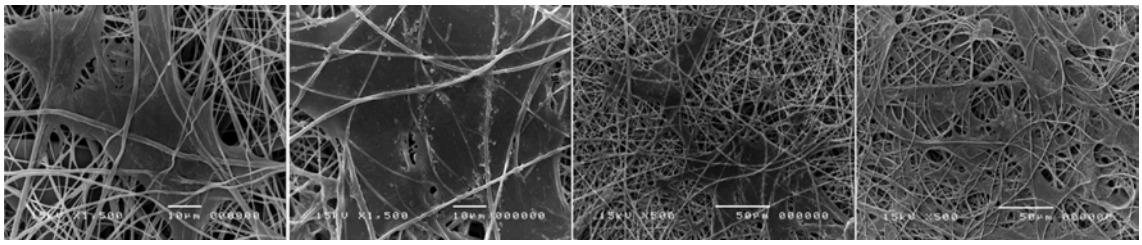
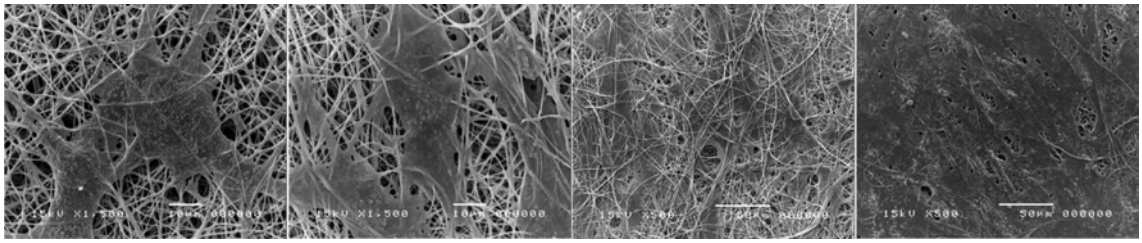
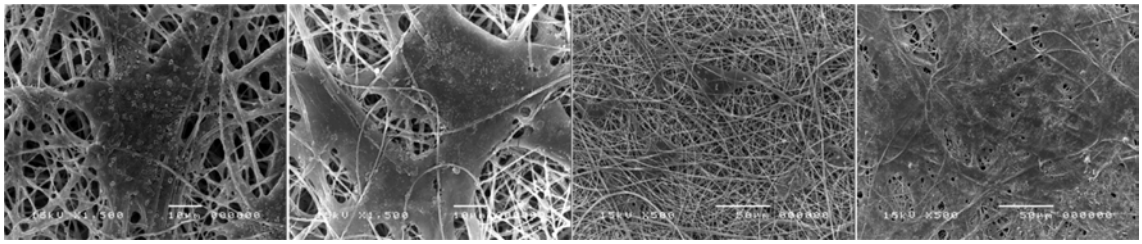
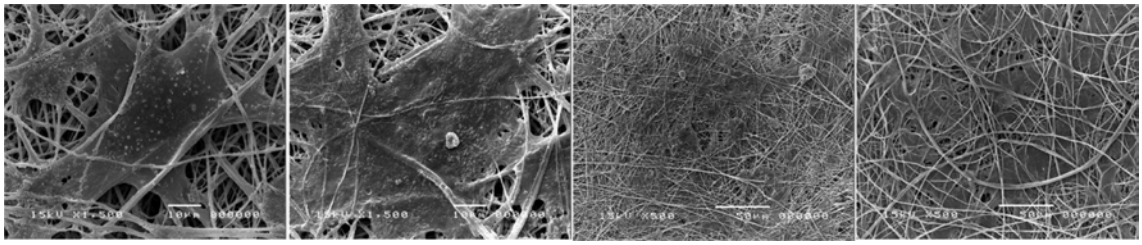
Materials	Incubation time			
	Magnification ×1500		Magnification ×500	
	2 h	5 h	1 d	7 d
TCPS				
Neat CA				
AC 10				
HPβCD 50				
HPβCD 100				
HPβCD 200				

Table 3. Continued

Materials	Incubation time			
	Magnification ×1500		Magnification ×500	
	2 h	5 h	1 d	7 d
FINC 50				
FINC 100				
FINC 200				

increase in the cell culturing time, which is evidenced by the cells covering most parts of the fiber mat surfaces on day 7, suggesting a good biocompatibility of these fibrous materials towards the tested cells.

Quantification of Synthesized Collagen: Synthesized by mature fibroblast cells, collagen is a structural ECM protein, responsible for providing strength of various tissues[38] and also plays an important role in the haemostasis and epithelisation phase of wound healing.[39,40] The ability of AC in promoting the differentiation of fibroblasts is of prime concern in this work. Therefore, the ability of HDFa that had been cultured on all of the fibrous substrates for 7 d, including TCPS, was used to assess the efficacy of these materials in promoting the cell differentiation. Figure 7 shows such results. Clearly, the normalized amounts of the synthesized collagen on the CA and the AC 10 fiber mats were not significantly different from that on TCPS. Clearly, the small amount of AC released from the AC 10 fiber mats was not enough to up-regulating the collagen production of the cells. As for the HPβCD and the FINC fiber mats, all but the FINC 50 fiber mats showed inferior collagen production of the cultured HDFa. The greatest ability of the FINC 50 fiber mats in promoting the differentiation of HDFa is in accord with the results on cell proliferation studies, in

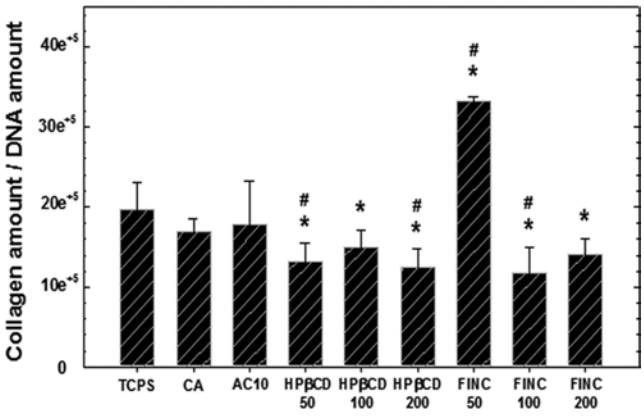


Figure 7. Synthesized collagen by HDFa that had been cultured on various fiber mat specimens on day 7 of cell culture was reported as the amount of synthesized collagen relative to the amount of extracted DNA. *$p<0.05$ and $^{\#}p<0.05$ as compared to the neat CA and AC 10 fiber mats, respectively.

which they were also the best, among all of the fibrous matrices.

Conclusions

The CA fiber mats containing AC/HPβCD inclusion complex at a mixture of AC/HPβCD of varying molar ratios (i.e., 1:0.5, 1:1, and 1:2; namely FINC 50, FINC 100, and FINC 200, respectively) were successfully prepared via the electrospinning technique. Smooth fiber mats without any kind of aggregation on their surfaces were obtained, with an average fiber diameter of 408, 483, and 515 nm, which corresponds to FINC 50, FINC 100, and FINC 200 fiber mats, respectively. DSC revealed the existence of AC/HPβCD inclusion complex loaded within those fiber mat specimens. The influence of the complex in CA fiber mats on release characteristics of AC from those specimens was evaluated. The cumulative amounts released from the AC/HPβCD complex-loaded CA fiber mats (i.e., FINC 50, FINC 100, and FINC 200 fiber mats) in comparison with AC 10 fiber mats were much greater. The maximum amounts of AC released from these materials were about 2.0, 4.8, and 4.9% (based on the actual weights of the fiber specimens), respectively. In the *in vitro* study, the FINC fiber mats (i.e., FINC 50, FINC 100, and FINC 200 fiber mats) were evaluated in terms of indirect cytotoxicity, for the ability to support both the attachment and the proliferation by HDFa cells and the ability of the cultured cells to synthesize collagen. These materials were proven as biocompatible materials. Among the various FINC fiber mats, they all seemed to promote both the adhesion and the proliferation at time points shorter than about 1 d. Only the FINC 50 fiber mats were found to be the better support for the proliferation and promotion of the fibroblast cells to synthesize collagen of the cultured cells on day 7.

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