

Preparation and Characterization of Caffeic Acid-Grafted Electrospun Poly(L-Lactic Acid) Fiber Mats for Biomedical Applications

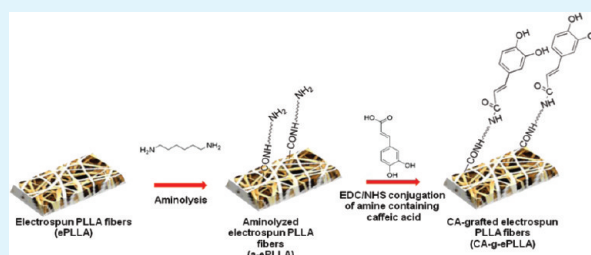
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S Supporting Information

ABSTRACT: Caffeic acid (CA) was chemically immobilized onto the surfaces of the individual electrospun poly(L-lactic acid) (PLLA) fibers to enhance the hydrophilicity and impart the antioxidant activity to the obtained fibrous membranes. This was done in two sequential steps. First, amino groups were covalently introduced onto the surfaces through the reaction with 1,6-hexamethylenediamine (HMD). In the second step, the amino moieties reacted with CA, which had been preactivated sequentially with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The success of the reactions was confirmed by the ninhydrin assay and X-ray photoelectron spectroscopic analysis (XPS). Indirect cytotoxicity evaluation with murine dermal fibroblasts (L929) and human dermal fibroblasts (HDFa) revealed that the neat and the modified PLLA fibrous matrices released no substances in the levels that were harmful to the cells. Direct culturing of HDFa on these fibrous substrates indicated that they supported the proliferation of the cells on days 2 and 3 very well and that the CA-immobilized substrates exhibited the highest cell viability. Lastly, the antioxidant activity of the CA-immobilized substrates, as revealed by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, was as high as 88% on average.

KEYWORDS: electrospinning, poly(L-lactic acid), immobilization, caffeic acid, antioxidant, radical scavenging



INTRODUCTION

Electrospinning is a simple and an efficient method for fabricating ultrafine fibers, the diameters of which range from a few micrometers down to tens of nanometers. Typically, an ultrafine stream of a polymeric liquid (solution or melt) is continuously drawn from the conical apex of its droplet by electrical forces and deposits randomly as a nonwoven fabric on a collection device.^{1,2} This is driven mainly by the Coulombic repulsive force between charges of the same polarity in the polymer liquid as well as the electrical force exerting on the charges by the electric field, which acts to destabilize the partially spherical droplet of the polymer liquid at the tip of the capillary to finally form a droplet of a conical shape. By increasing the strength of the electric field above a threshold value (which varies from one system to the next), the ultrafine stream of the polymeric liquid is drawn out from the tip of the cone, as previously mentioned.^{1,2}

Poly(L-lactic acid) (PLLA) is a biodegradable aliphatic polyester, the monomer of which, i.e., L-lactic acid or L-2-hydroxypropionic acid, is produced from a fermentation process of sugar or other related raw materials.³ The monomer then undergoes condensation polymerization into a high-molecular-weight PLLA or condensation dimerization into L-lactide, which is further processed into a high-molecular-weight PLLA by ring-opening polymerization.³ Due to its inherent biocompatibility, PLLA has been used extensively in medicine as surgical sutures (normally as a copolymer thread with

glycolic acid)⁴ and suture anchors.⁵ PLLA can be fabricated into fibrous matrices by electrospinning.^{6–14} Various solvent systems have been used to prepare electrospinnable PLLA solutions, some of which are chloroform,⁶ 3:2 w/w methylene chloride/*N,N'*-dimethylformamide (DMF),⁷ 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP),⁸ chloroform, dichloromethane (DCM), or tetrahydrofuran (THF),⁹ 9:1 v/v DCM/DMF,¹⁰ 3:1 v/v chloroform/acetone,¹¹ 7:3 v/v DCM/THF,^{12,13} and 7:3 v/v DCM/DMF.¹⁴

Approved by the U.S. Food and Drug Administration (USFDA) for various clinical applications in humans,¹⁵ applicability of electrospun PLLA fibrous matrices in biomedicine is an obvious choice. Kenawy et al.⁶ were the first group to report the use of electrospun fibrous matrices of poly(lactic acid) (PLA) and its blend with poly(ethylene-co-vinyl acetate) (PEVA) to incorporate an anti-infection drug, tetracycline hydrochloride, and to study the release of the drug therefrom. Although the PLA matrices showed a burst release of the drug, the PLA/PEVA blend counterparts showed a prolonged release characteristic. A burst release was also observed for a cephamycin antibiotic drug, cefoxitin, from electrospun fibrous poly(D,L-lactic acid) (PDLA) matrices containing the drug.⁷ Development of an antibacterial bandage based on electrospun

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PLLA fibrous matrices containing silver nanoparticles was reported by Xu et al.¹⁰ Li et al.,¹¹ on the other hand, developed a biosensor assembly based on electrospun PLA fibrous matrices that contained biotin. Though dispersed inhomogeneously within the mass of the PLA fibers, the incorporated biotin could immobilize streptavidin, which subsequently bound with biotinylated nucleic acid probes for the detection of a synthetic *E. coli* DNA. Finally, uses of electrospun PLLA fibrous matrices as substrates for cell/tissue culture were demonstrated on a number of different cell types, e.g., preosteoblastic and Schwann cells.^{8,12,13}

Previously, our group developed electrospun PLLA fiber matrices containing gallic acid or 3,4,5-trihydroxybenzoic acid, a natural phenolic acid indigenous to a variety of plants, and reported the release characteristic of the substance therefrom.¹⁴ In addition to its anticarcinogenic, antifungal, anti-inflammatory, and antityrosinase activities, gallic acid is one of the most potent antioxidants in its class.^{16,17} It was found that the cumulative amount of the substance released from the gallic acid-loaded PLLA fiber mats was greatest in the normal saline, followed respectively by those in the citrate-phosphate and the acetate buffer and that the free radical scavenging activity of the as-loaded and the as-released gallic acid remained active.¹⁴ Another compound of natural origins exhibiting a strong antioxidant activity is caffeic acid (CA) or 3-(3,4-dihydroxyphenyl)-2-propenoic acid.^{18–20} As a key intermediate in the biosynthesis of lignin, it can be found in all plants. Other biochemical activities of CA are anticarcinogenic, anti-inflammatory, and antimutagenic activities.^{19–21}

Here, it is of our interest to apply CA as the active therapeutic agent and electrospun PLLA fibrous matrices as substrates. However, to fabricate CA-incorporated PLLA fibrous matrices from a PLLA solution containing CA could lead to easy leaching of the substance into a liquid medium.¹⁴ To prolong the therapeutic effect of the substance, covalent immobilization of CA onto the surfaces of the individual PLLA fibers would be a logical choice. Among the various chemical pathways, grafting of CA onto an aliphatic polyester, such as PLLA and polycaprolactone (PCL), can be done in two sequential steps.^{22,23} First, some of the ester groups on the surfaces of electrospun PLLA fibers are reacted with 1,6-hexamethylenediamine (HMD), resulting in the introduction of the more reactive amino groups on the surfaces. Second, the carboxylic acid moiety of CA is activated successively with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), prior to being reacted with an amino group on the surface of an aminolyzed PLLA fiber. The introduction of the amino groups was assessed spectrophotometrically by the ninhydrin assay, while the presence of CA was by X-ray photoelectron spectroscopy (XPS). Biocompatibility of the surface-modified PLLA fibrous matrices was evaluated with live cultures with murine and human dermal fibroblasts. Lastly, the antioxidant activity of the CA-grafted PLLA fibrous matrices was evaluated based on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay.

EXPERIMENTAL SECTION

Materials. PLLA ($M_n = 80\,000$ g mol⁻¹), CA, HMD, EDC, and NHS were purchased from Sigma-Aldrich (USA). DCM (Carlo Erba, Italy), DMF [Lab-Scan (Asia), Thailand], and ethanol [Lab-Scan (Asia), Thailand] were of analytical reagent grade and used without further purification.

Preparation of Electrospun PLLA Fiber Mats. Electrospinning of PLLA was carried out based on a previously published method.¹⁴ Briefly, 10% w/v PLLA solution in 7:3 v/v DCM/DMF was fed into a glass syringe fitted with a blunt 20-gauge stainless steel hypodermic needle (OD = 0.91 mm), used as the nozzle. An aluminum sheet wrapped around a homemade rotating cylinder (width and diameter ≈ 15 cm; rotational speed ≈ 50 rpm) was used as the collector. The solution was charged with a Gamma High Voltage Research D-ES30PN/M692 at a fixed electric field of 20 kV/18 cm. After the solution had been electrospun continuously for 18 h, the thicknesses of the obtained fiber mats were measured to be 120 ± 15 μ m.

Surface Modification of Electrospun PLLA Fiber Mats. Aminolysis of the PLLA fiber mats was carried out based on a previously published method,²² with slight modification. Specifically, the fiber mats had been washed in an ethanolic aqueous solution (1:1 v/v) for 2 to 3 h, prior to being washed with a large quantity of deionized (DI) water. The aminolysis was then carried out by immersing the fiber mats in a HMD/isopropanol (IPA) solution of varying concentrations (i.e., 0.02, 0.04, 0.06, and 0.08 g mL⁻¹) for varying periods of the reaction time (i.e., 5, 10, 15, 20, and 30 min) at 50 °C. The aminolyzed PLLA fiber mats (hereafter, a-ePLLA) had been rinsed in DI water for 24 h at room temperature to remove unreacted HMD, prior to being dried in vacuo at room temperature until of a constant mass. Activation of CA was done based on a previously published method,²³ with slight modification. Specifically, 250 mg of CA, a priori dissolved in 10 mL of ethanolic aqueous solution (1:1 v/v), was reacted with 169 mg of EDC and later with 102 mg of NHS. The mixture was stirred in an ice–water bath for 1 h to finally obtain a solution of the activated CA. The aminolyzed PLLA fiber mats were subsequently immersed into the activated CA solution and stirred in the ice–water bath for 30 min. After 24 h of the reaction time at room temperature, the CA-grafted PLLA fiber mats (CA-g-ePLLA) were washed thoroughly with ethanol several times and finally dried in vacuo at room temperature.

Materials Characterization. The free amino (NH₂) groups on the surfaces of the neat and the modified PLLA fiber mats were quantified by ninhydrin or 2,2-dihydroxyindane-1,3-dione assay.²² The fiber mat samples were first immersed in 1 M ninhydrin/ethanol solution for 1 min, transferred into a glass tube, and then heated at 80 °C for 15 min to accelerate the reaction between ninhydrin and the NH₂ groups that might be present on the surfaces of the fiber mat samples. If there were enough NH₂ groups on the surfaces, they would turn blue. Upon complete evaporation of the adsorbed ethanol, 1,4-dioxane was added to dissolve the fiber mat samples. IPA was subsequently added to stabilize the blue compound. The absorbance of the obtained solutions were read at 538 nm using a Shimadzu UV-2550 ultraviolet–visible (UV–vis) spectrophotometer and the concentrations of the NH₂ groups in the sample solutions were calculated from a standard calibration curve of HMD solutions in 1,4-dioxane/IPA (1:1 v/v) (see Figure I in the Supporting Information).

Surface elemental chemistry of the neat and the modified PLLA fiber mats was analyzed by a Thermo Fisher Scientific Thetaprobe X-ray photoelectron spectroscopy (XPS).²⁴ Monochromatic Al K α X-ray was employed for the analysis of one spot on each sample with a photoelectron emission angle of 50° (with respect to the surface normal). The analytical area was approximately 400 μ m \times 400 μ m, and the maximal analytical depth was in the range of ~ 4 –8 nm. A few eV Ar⁺ ions, generated by an electron flood gun, were used for charge compensation. Electron and ion beams were focused and steered toward the analytical area. Further correction was made based on adventitious C 1s at 285 eV, using the manufacturer's standard software. Survey spectra were acquired for surface composition analysis with sensitivity factors from Scofield library.

The amounts of the immobilized CA on the surfaces of the CA-g-ePLLA specimens were also investigated. Specimens (circular disk; ~ 2.8 cm in diameter; cut from randomly selected areas of three different fiber mats) were individually dissolved in 10 mL of 7:3 v/v DCM/DMF solution. The absorbance of the sample solutions was spectrophotometrically read at 243 nm and the concentrations of the immobilized CA were calculated from a standard calibration curve of

CA solutions in 7:3 v/v DCM/DMF (see Figure II in the Supporting Information).

Static water contact angles of the neat and the modified PLLA fiber mats were measured at room temperature using a Krüss DSA 100 drop shape analyzer,²⁵ equipped with a Gilmont syringe and a 24-gauge flat-tipped needle. Five droplets of distilled water (10 μL) were placed randomly at different positions on each sample. The projected images of the droplets, after they had been settled on the surfaces until of no noticeable change in their shapes, were analyzed for the contact angles.

Morphological appearance and size of the individual fibers of the neat and the modified PLLA fiber mats were examined by a JEOL JSM 5410LV scanning electron microscope (SEM). At least 100 readings of the fiber diameters from various SEM images were analyzed using SemAphore 4.0 software.

Mechanical properties in terms of stress at maximum load, strain at maximum load, Young's modulus, and elongation at break of the neat and the modified PLLA fiber mats were tested on a Lloyd LRX universal testing machine (gauge length = mm and crosshead speed = 100 mm min^{-1}). The specimens were cut into a rectangular shape (10 mm \times 100 mm). The measurements were carried out on ten different specimens.

Biological Evaluation. Murine dermal fibroblasts (L929) and human dermal fibroblasts (HDFa) were used as reference cells. L929 and HDFa were cultured as monolayer in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (FBS; Biotech, UK), 1% L-glutamine (Invitrogen, USA), and a 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen, USA)]. The medium was replaced on every other day and the cultures were maintained at 37 $^{\circ}\text{C}$ in a humidified atmosphere containing 5% CO_2 .

In the indirect cytotoxicity evaluation, the fiber mat specimens were assessed using a procedure adapted from the ISO10993-5 standard test method. First, extraction media were prepared by immersing the fiber mat specimens (\sim 15 mm in diameter) in a serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1% lactalbumin, and 1% antibiotic and antimycotic formulation) for 1, 2, or 3 d in an incubator at the extraction ratio of 20 mg/mL. L929 or HDFa (\sim 40 000 cells/well) were separately cultured in wells of a 24-well tissue-culture polystyrene plate (TCPS; Biokom, Poland) in 10% serum-containing DMEM for 16 h to allow cell attachment onto the well surface. After starving the cells with SFM for 24 h, the medium was replaced with 0.5 mL of an extraction medium and the cells were reincubated for another 24 h. The viability of the cells cultured with each of the extraction media and the fresh SFM that had been preincubated for an equivalent time interval (i.e., control) was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of formazan crystals is supposedly proportional to the number of viable cells. First, each specimen was incubated at 37 $^{\circ}\text{C}$ with 300 μL /well of MTT solution at 0.5 mg mL^{-1} , without phenol red. After 30 min of incubation, the MTT solution was removed. A buffer solution containing dimethylsulfoxide (DMSO; 900 μL /well) and glycine buffer (pH 10; 125 μL /well) was added into the wells to dissolve the formazan crystals. After 10 min of agitation, the solutions were measured for their absorbance at 540 nm, using a Thermospectronic Genesis10 UV-visible spectrophotometer.

In the cell attachment and the cell proliferation studies, only HDFa were used. Specimens (\sim 15 mm in diameter) were placed in wells of a 24-well TCPS and subsequently sterilized in 70% ethanol for 1 h. The specimens, after having been washed successively with autoclaved deionized water and phosphate saline solution (PBS), were immersed in DMEM overnight. A stainless steel ring (\sim 12 mm in diameter) was placed on top of each specimen to ensure its complete contact to the bottom of the well. HDFa (\sim 40,000 cells/well) were seeded on the fiber mat specimens and empty wells of TCPS (i.e., control) and incubated at 37 $^{\circ}\text{C}$ in a humidified atmosphere containing 5% CO_2 . In the attachment study, the cells had been allowed to attach onto the

substrates for 2, 4, or 18 h, prior to being quantified for their viability by the MTT assay. Prior to the measurement, each cell-plated specimen had to be rinsed with PBS to remove unattached cells. In the proliferation study, the cells had been allowed to attach onto the substrates for 16 h, prior to being quantified for their viability by the MTT assay on days 1, 2, and 3 after cell culturing.

The morphology of the cells at some time points was observed by SEM. After removal of the culture medium, each cell-cultured specimen was rinsed twice with PBS and the cells were fixed with 3% glutaraldehyde solution [diluted from 50% glutaraldehyde solution (Sigma-Aldrich, USA) with PBS] at 500 μL /well. After 30 min, the specimen was rinsed again with PBS and underwent dehydration with ethanolic aqueous solutions of varying concentrations (i.e., 30, 50, 70, and 90 vol %) and pure ethanol for \sim 2 min each. It was then dried in hexamethyldisilazane (HMDS; Sigma-Aldrich, USA) for 5 min and finally in air. The specimens were then observed by a JEOL JSM-5200 scanning electron microscope (SEM). The morphology of the cells that had been seeded or cultured on glass substrates (12 mm in diameter; Menzel, Germany) was used as positive control.

Protein Adsorption. Specimens (\sim 15 mm in diameter) were placed in wells of a 24-well TCPS. A stainless steel ring (\sim 12 mm in diameter) was placed on top of each specimen to ensure its complete contact to the bottom of the well. A solution of type I collagen in PBS (0.1 mg mL^{-1} for 0.5 mL) was then added to each well. After 2, 4, 24, 48, and 72 h of immersion, each specimen was thoroughly rinse with DI water to remove excess collagen. The amount of adsorbed collagen on each specimen was carried out by first immersing the specimen in 2 mL of 1% w/v sodium dodecyl sulfate (SDS) aqueous solution for 30 min. The concentration of the dissolved collagen was then quantified by BCA protein assay kit (Thermo Fisher Scientific, USA). Specifically, 25 μL of each of the dissolved collagen sample solutions had been pipetted into a 96-well TCPS, before the BCA working solution of 200 μL was added. The plate was sealed and incubated at 60 $^{\circ}\text{C}$ for 30 min. The absorbance measurements were taken at 562 nm at room temperature. The collagen concentration was calculated from a standard calibration curve (see Figure III in the Supporting Information).

Antioxidant Activity. The antioxidative activity of CA after it had been grafted onto the surfaces of the PLLA fiber mats was assessed by the DPPH assay. The procedure was carried out based on a previously published method,^{14,26} with slight modification. Specifically, each specimen (\sim 2.8 cm in diameter) was dissolved in 10 mL of 7:3 v/v DCM/DMF solution. The sample solution was then diluted with 10 mL methanol. After that, 3 mL of an ethanolic solution of DPPH (100 μM) was added into 1 mL of the sample solution. The mixture had been incubated at room temperature in darkness for 30 min, prior to being spectrophotometrically read at 517 nm. The neat fiber mat specimens were used as internal control and the amount of the immobilized CA participated in scavenging activity of the DPPH radical species could be quantified against a standard curve of CA (see Figure IV in the Supporting Information). The antioxidant activity (% AA) was then expressed as the percentage of the absorbance value of the DPPH radical species that was decreased in comparison with that of the control condition (i.e., the testing solution without the addition of the dissolved specimens), according to the following equation

$$\%AA = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100 \quad (1)$$

where A_{control} and A_{sample} represent the absorbance values of the blank and the sample solutions, respectively.

Statistical Analysis. All values were presented as the means \pm standard deviations. Statistical analysis between two data sets was performed using One-Way Analysis of Variance (ANOVA) and Scheffe's post hoc test in SPSS (SPSS, USA). The values of p lower than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The purpose of chemical immobilization of CA onto the surfaces of the individual electrospun PLLA fibers is 2-fold. The

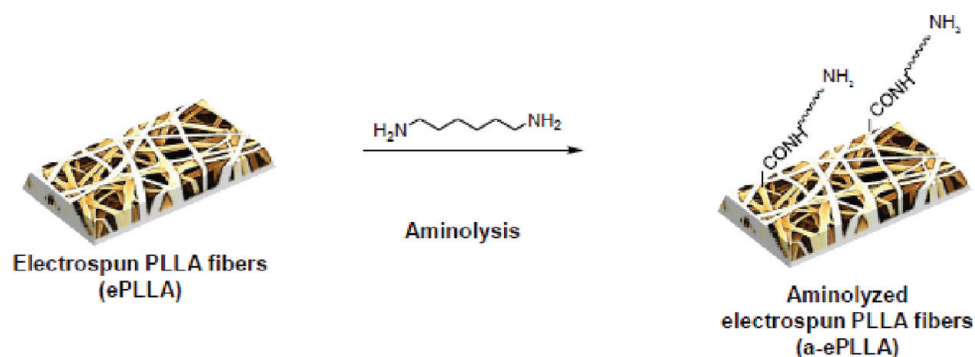


Figure 1. Aminolysis of the electrospun PLLA fiber surfaces through the reaction with 1,6-hexamethylenediamine (HMD).

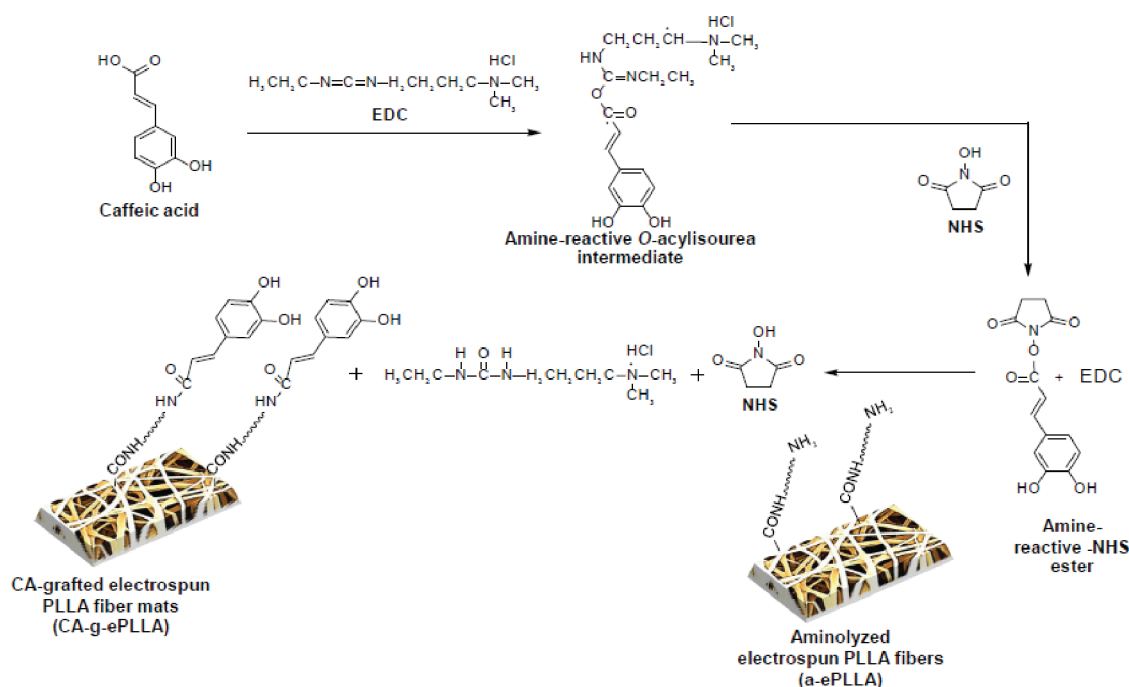


Figure 2. Chemical pathway detailing the conjugation reaction of CA with an amino moiety ($-\text{NH}_2$) on the aminolyzed electrospun PLLA fiber (a-ePLLA) surfaces via conjugating agents.

first is to increase the hydrophilicity of the surfaces, as the hydroxyl moieties of CA are more hydrophilic than the ester linkages indigenous to PLLA. Second, it is aimed at imparting the antioxidant activity to the modified PLLA fibrous matrices, as this activity is deemed important for an active wound dressing. According to the reaction schemes shown in Figures 1 and 2, the grafting of CA onto the surfaces of the individual electrospun PLLA fibers can be done in two sequential steps. In the first step, amino groups were covalently introduced onto the surfaces of the individual electrospun PLLA fibers through the reaction with HMD to obtain the aminolyzed PLLA fiber mats (a-ePLLA). Here, an amino moiety ($-\text{NH}_2$) of HMD would react with an ester linkage ($-\text{COO}-$) of PLLA to form the amide linkage ($-\text{CONH}-$), whereas the other amino moiety would be left available for further reaction. In the second step, the CA-grafted PLLA fiber mats (CA-g-ePLLA) were obtained through the reaction between CA, the carboxylic acid moiety ($-\text{COOH}$) of which had been activated sequentially with EDC and NHS, and the other amino moiety of HMD, which had been introduced on the surfaces of the a-ePLLA in the first step.

Characterization of Neat and Modified PLLA Fibrous Matrices. Quantification of Free Amino Groups. Quantification of the amino moieties that had been introduced on the surface of the a-ePLLA was evaluated with the ninhydrin assay. A number of factors would influence the amounts of the amino groups that would be introduced on the surface of the a-ePLLA. Some of these are the initial concentration of HMD, the reaction time, and the reaction temperature. Here, only the effects of the HMD concentration (see Table 1) and the reaction time (see Table 2) were studied. Within the

Table 1. Surface Density of $-\text{NH}_2$ Groups on the a-ePLLA as a Function of HMD Concentration at a Fixed Reaction Time of 10 Min^a

HMD concentration (g mL^{-1})	surface density of $-\text{NH}_2$ ($\times 10^{-7}$ mol cm^{-2})
0.02	0.82 ± 0.06
0.04	1.20 ± 0.03
0.06	1.65 ± 0.05
0.08	1.96 ± 0.04

^aThe reaction temperature was fixed at 50 °C.

Table 2. Surface Density of $-NH_2$ Groups on the a-ePLLA as a Function of Reaction Time at a Fixed HMD Concentration of 0.04 g mL $^{-1}$ ^a

reaction time (min)	surface density of $-NH_2$ ($\times 10^{-7}$ mol cm $^{-2}$)
5	0.76 \pm 0.04
10	1.20 \pm 0.03
15	1.77 \pm 0.07
20	1.82 \pm 0.06
30	3.10 \pm 0.04

^aThe reaction temperature was fixed at 50 °C.

investigated ranges of the HMD concentrations and the reaction times, the density of the amino groups was an increasing function of both independent parameters. Nevertheless, treating the neat PLLA fibrous membranes at a high HMD concentration and/or a long reaction time would jeopardize both the physical and the mechanical integrity of the modified fibrous membranes. As the aminolytic condition becomes harsher (i.e., a result of the increased HMD concentration, increased reaction time, etc.), increasing numbers of ester linkages would be cleaved and the carboxylic acid chain ends would react with HMD, resulting in greater numbers of shorter PLLA chains and this occurs from the surface in. Here, the optimized aminolytic condition that resulted in the a-ePLLA that did not disintegrate while undergoing further reaction and/or evaluation is 0.04 g mL $^{-1}$ of HMD/IPA solution at 50 °C for 10 min. At this condition, the surface density of the introduced amino groups was 0.120 \pm 0.003 μ mol cm $^{-2}$.

Elemental Composition of Surfaces and Quantification of Immobilized CA. XPS was used to examine the chemical composition at the surfaces of the neat and the modified PLLA fibrous matrices. Quantitative analysis of the obtained results is shown in Table 3. As expected, no nitrogen-based moieties

Table 3. Percentages of Area under the Peaks of High-Resolution C 1s, O 1s, and N 1s XPS Spectra, Including the Ratios of the Percentages of the Area under the Peaks O 1s/C 1s, N 1s/C 1s, and N 1s/O 1s, of the Neat and the Modified PLLA Fibrous Matrices

sample	C 1s	O 1s	N 1s	O 1s/C 1s	N 1s/C 1s	N 1s/O 1s
ePLLA	61.1	38.9	0	0.6367	0	0
a-ePLLA	64.4	34.8	0.8	0.5404	0.0125	0.0230
CA-g-ePLLA	60.6	39.0	0.4	0.6436	0.0066	0.0103

were observed on the surfaces of the neat PLLA fibrous membranes (i.e., the ePLLA). This was evidenced by the nonexistence of the N 1s spectra. In addition, the ratio between the surface oxygen atoms and the surface carbon atoms (i.e., O 1s/C 1s) was about 0.64. For the a-ePLLA, this ratio decreased to about 0.54 and, at the same time, surface nitrogen atoms could then be detected. This is because aminolysis introduced both the carbon and the nitrogen atoms on the fiber surfaces. For the CA-g-ePLLA, the O 1s/C 1s ratio increased again to about 0.64. This is because the grafting of CA introduced both the carbon and the oxygen atoms on the fiber surfaces. This resulted in the simultaneous decrease in the number of surface nitrogen atoms, as evidenced by the decrease in the N 1s/C 1s and the N 1s/O 1s ratios from those of the a-ePLLA.

The surface density of the immobilized CA on the CA-g-ePLLA was quantified spectrophotometrically to be 6.2 \pm 0.2

μ g cm $^{-2}$ or 0.034 \pm 0.003 μ mol cm $^{-2}$ (based on the projection area of the specimens and note that M_w of CA = 180.16 g·mol $^{-1}$) ($n = 5$). This implies that about 28% of the amino groups on the surface of the a-ePLLA participated in the immobilization reaction with CA. When calculated based on the actual weights of the specimens, the amount of the immobilized CA on the CA-g-ePLLA was 1.13 \pm 0.03 μ g mg $^{-1}$ or 0.0063 \pm 0.0006 μ mol mg $^{-1}$. To see whether the as-grafted CA could be released from the CA-g-ePLLA or not, a separate experiment was carried out. Here, the CA-g-ePLLA specimens (\sim 2.8 mm in diameter) were immersed in PBS at 37 °C for periods of 1, 3, and 7 days. After each time point, a sample solution was withdrawn and spectrophotometrically read at 243 nm. The obtained results confirmed nonexistence of CA in the sample solutions. This clearly implies that there was no physical adsorption of CA on the surfaces of a-ePLLA and that the cleavage of the amide linkages could not occur in PBS.

Wettability and Morphology. Static contact angles of water droplets on the surfaces of the neat and the modified PLLA fibrous membranes were used to assess the hydrophilicity of these surfaces. Such values for the neat PLLA fibrous matrices were 104.8° \pm 0.3°. The surfaces became much more hydrophilic after the aminolytic treatment in 0.04 g mL $^{-1}$ of HMD/IPA solution at 50 °C for 10 min (i.e., the water contact angles = 83.3° \pm 0.4°). The hydrophilicity of the surfaces increased even more after CA having been grafted onto the surfaces of the a-ePLLA (i.e., the water contact angles = 55.5° \pm 0.5°). The wettability of a surface depends not only on the chemical modification of the surface, but on the topographical nature of it as well. For an electrospun fibrous membrane, the wettability of its surface depends on the topography, the size, the arrangement,²⁵ and the surface chemistry of the individual, underlying fibers.²² To ascertain that the increase in the hydrophilicity of the modified PLLA fibrous membranes over that of the neat ones was not due to any change in their surface topography, the morphology of the neat and the modified PLLA fibrous membranes was investigated (see Figure 3). Evidently, there is no obvious discrepancy in the morphology of the modified fibers from that of the neat ones, as the surface of all of these individual fibers was smooth. Nonetheless, the diameters of the modified fibers (i.e., \sim 850 and \sim 920 nm on average after the aminolysis and the grafting of CA, respectively) were generally greater than those of the neat ones (i.e., \sim 620 nm on average). The increase in the electrospun fiber diameters after a surface modification was also reported on electrospun poly(D,L-lactic acid) (PDLLA) fibers that had been grafted with chitosan.²⁷ Recently, It has been demonstrated that an increase in the size of the individual fibers increased the hydrophobicity of the electrospun fibrous membranes.²⁵ Thus, the increase in the hydrophilicity of the modified PLLA fibrous membranes over that of the neat materials was certainly not due to the change in the size of these individual fibers.

Mechanical Integrity. The mechanical properties of the ePLLA and the CA-g-ePLLA were investigated. The stress at maximum load and the Young's modulus of the ePLLA were 3.6 \pm 0.2 and 351 \pm 5 MPa, respectively, whereas the strain at maximum load and the strain at break of the materials were 75.6 \pm 4.6 and 87.7 \pm 4.6%, respectively. On the other hand, the stress at maximum load and the Young's modulus of the CA-g-ePLLA were 0.89 \pm 0.29 and 154 \pm 14 MPa, respectively, whereas the strain at maximum load and the strain at break of the materials were 2.2 \pm 0.2 and 5.8 \pm 0.5%, respectively. Upon

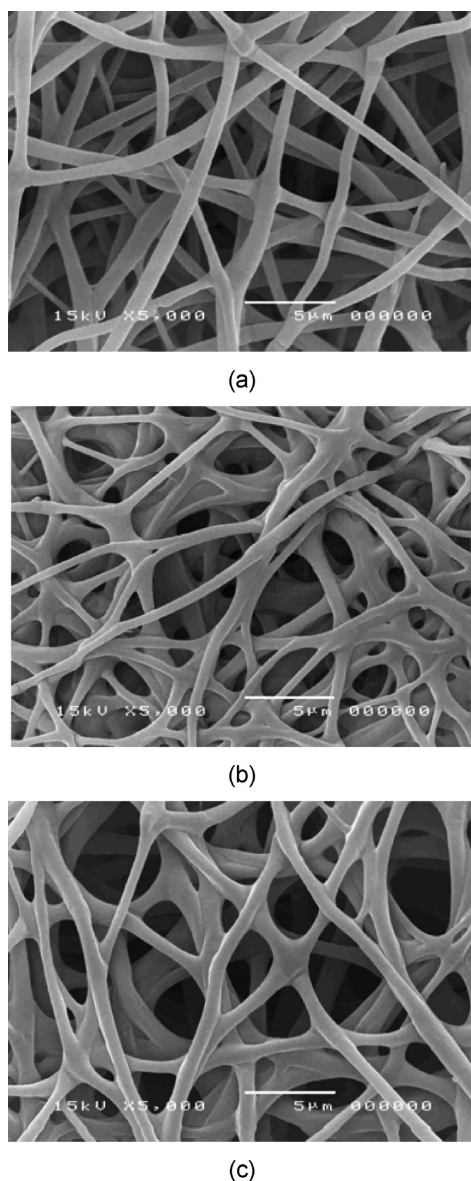


Figure 3. Representative SEM images (scale bar = 5 μm and magnification = 5000 \times) illustrating morphology of (a) neat electrospun PLLA fibers (ePLLA) and the one that had been modified by (b) aminolysis (0.04 g mL^{-1} of HMD/IPA solution at 50 $^{\circ}\text{C}$ for 10 min; a-ePLLA) and subsequently by (c) grafting with caffeic acid (CA) that had been a priori activated successively with EDC and NHS (CA-g-ePLLA). The size of these fibers were determined to be 618 ± 92 , 854 ± 181 , and 918 ± 186 nm, respectively.

the aminolysis and the EDC/NHS conjugation of CA onto the ePLLA surfaces, both the stiffness and the extensibility of the CA-g-ePLLA decreased significantly.

Biological Evaluation of Neat and Modified PLLA Fibrous Matrices. Indirect Cytotoxicity Evaluation. The potential for use of the neat and the modified PLLA fibrous membranes in biomedical applications was first assessed by the indirect cytotoxicity evaluation assay, using L929 and HDFa as reference cells. In the assessment, the extraction media were prepared by immersing each fiber mat sample in SFM for different periods of up to 3 days. The viability of the cells that had been cultured with each of these media, in comparison with that of the cells that had been cultured with the fresh SFM that had been preincubated for similar periods of time, for 1 day is

shown in Figure 4. The viability of the cells that had been cultured with the fresh SFM that had been preincubated for 1 d

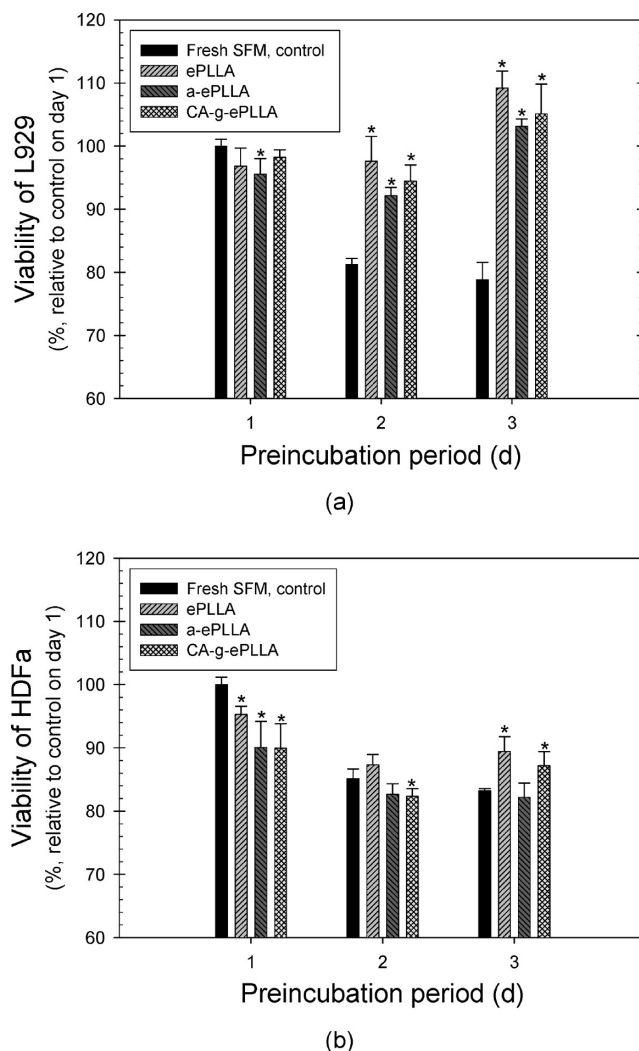


Figure 4. Indirect cytotoxic evaluation of ePLLA, a-ePLLA, and CA-g-ePLLA based on viabilities of (a) murine dermal fibroblasts (L929) and (b) human dermal fibroblasts (HDFa) that had been cultured with the extraction media from these materials against the viabilities of the cells that had been cultured with the respective serum-free media for 1 day as a function of the preincubation period of 1, 2, or 3 days. * $p < 0.05$: compared with the control group at any given time point.

was used as the basis to obtain the relative viabilities shown in the figure. For both cell types, the viability of the cells that had been cultured with the fresh SFM was found to decrease with an increase in the preincubation period, which may be due to the increase in the dissolved CO_2 level upon the prolonged incubation. Notwithstanding, the viabilities of either cell type were greater than $\sim 80\%$ on average. For L929 that had been cultured with the extraction media from both the neat and the modified PLLA fibrous matrices, the viabilities of the cells showed increasing trends, with the values ranging from ~ 92 to $\sim 109\%$ on average. For HDFa on the other hand, decreasing trends were observed, with the values ranging from ~ 82 to $\sim 95\%$ on average. Because the viabilities were greater than $\sim 80\%$, the obtained results indicated nontoxicity of all of the fibrous matrices, as none of the materials released substances in the levels that were detrimental to the cells.

Cell attachment and Cell Proliferation. All of the fibrous matrices were evaluated further for their potential for use as wound dressing materials. For this, HDFa were directly seeded or cultured on their surfaces in order to assess the ability of the materials in supporting the attachment and the proliferation of the cells. The viabilities of the cells that had been grown on the surfaces of TCPS at 2 h after cell seeding (for the attachment assay) and on day 1 after cell culturing (for the proliferation assay) were used as the bases to obtain the relative viabilities shown in Figure 5. For the attachment assay, the viability of the

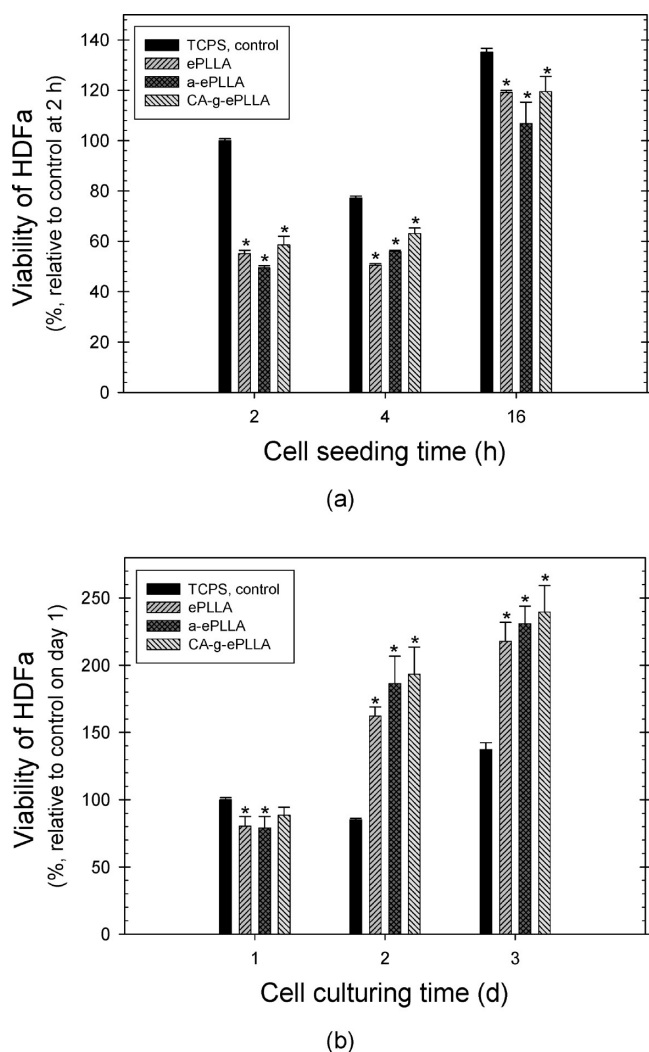


Figure 5. (a) Attachment and (b) proliferation based on viabilities of HDFa that had been seeded or cultured on the surfaces of ePLL, a-ePLL, and CA-g-ePLL in comparison with those of the cells that had been seeded or cultured on the surface of tissue-culture polystyrene plate (TCPS) as a function of the cell seeding or cell culturing time. * $p < 0.05$: compared with the control group at any given time point.

attached cells on the surface of TCPS at 4 h was lower than that at 2 h, whereas that of the attached cells at 16 h was greater. At any given time point, the viabilities of the attached cells on all of the fibrous matrices were lower than that on the control surface. At 2 and 4 h after cell seeding, the ability to support the attachment of the cells for a given type of the fibrous matrices was not of statistical difference. At 16 h, however, the viabilities of the cells attached on all types of the fibrous matrices

increased tremendously to approach that of the cells that could adhere onto the control surface. Noticeably, the CA-g-ePLL were better than the a-ePLL in supporting the attachment of HDFa at any given time point. On day 1 after cell culturing, the viabilities of the cells on all of the fibrous matrices were still inferior to that of the cells on TCPS. Interestingly, the profiling of viabilities was similar to that observed at 16 h after cell seeding. This result could be interpreted that at least 24 h was required for HDFa to completely adhere to these surfaces. Significant increase in the proliferation of the cultured cells on all types of the fibrous matrices was observed on days 2 and 3 after cell culturing. Though not statistically significant, the ability of the CA-g-ePLL in supporting the proliferation of HDFa was better than that of the a-ePLL, which in turn was better than that of the ePLL.

The inferiority of all of the fibrous matrices to TCPS in supporting the attachment of HDFa could be due to the lesser numbers of cells that were able to attach on the rougher and more hydrophobic surfaces of the fibrous matrices in comparison with the smoother and more hydrophilic surface of TCPS. Surfaces of different degrees of hydrophilicity (or hydrophobicity) would influence different types and amounts of proteins to adsorb. Even though a rough surface of the fibrous matrices possesses a greater specific surface area than a smoother surface of TCPS, the types and amounts of the proteins that are adsorbed on the smooth and hydrophilic surface of TCPS may mediate the adherence of the cells much better than do those adsorbed on the rough and hydrophobic surface of the fibrous matrices. The flat surface of TCPS also ensures that the adsorbed proteins are readily accessible to the cells. Unlike the flat surface of TCPS, only a limited part of the adsorbed proteins on the rough surface is accessible to the cells. Furthermore, the kinetics of the protein adsorption on the surfaces of different characters (i.e., different surface topographies, different effective surface areas, different surface wetting phenomena, etc.) play important roles in regulating the different time intervals for which the proteins require to adsorb in the amounts that are effective for mediating cellular behavior. Here, it is logical to hypothesize that a much longer time is needed for a much greater amount of proteins required to adsorb on the surfaces of the fibers to have a mediating effect to the cultured cells. Hence, on days 2 and 3, we see much greater increase in the proliferation of the cells grown on all types of the fibrous matrices and such an increase should be a result of the greater surface areas of the fibrous substrates. Among the three types of the fibrous matrices, the CA-g-ePLL were the best in supporting the attachment and the proliferation of HDFa (i.e., based on the average values of the viabilities shown in Figure 5). Because the surface of the CA-g-ePLL was more hydrophilic than those of the ePLL and the a-ePLL, the greater adsorption of proteins on their surface should be the main reason.

Here, a separate experiment was carried out to quantify the amounts of a model protein that were able to adsorb onto the surfaces of the ePLL and the CA-g-ePLL. Type I collagen was used as the model protein. The amounts of the adsorbed proteins were reported at various immersion time points and the results are shown in Figure 6. Obviously, increasing the immersion time in the protein solution caused the amount of the absorbed protein to increase, except for the CA-g-ePLL at 48 and 72 h that showed equivalent values (suggesting that the maximal adsorption was already reached at these time points). Regardless of the time points investigated, the adsorption of

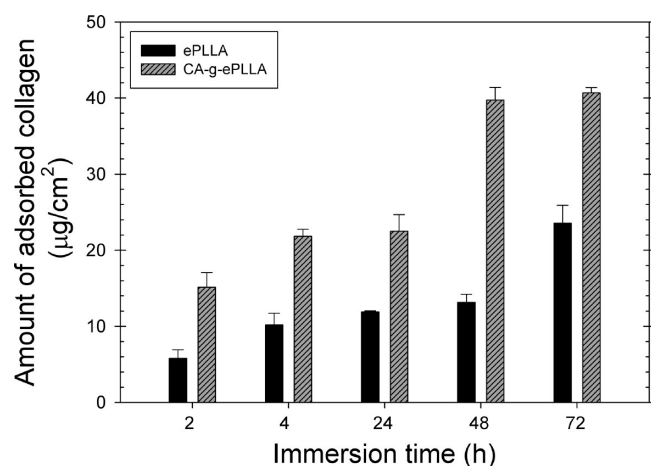


Figure 6. Areal amounts of type I collagen adsorbed on the surfaces of ePLLA and CA-g-ePLLA (i.e., the adsorbed amounts divided by the projection area of the specimens) at various immersion time points. All data sets at any given time point were significantly different ($*p < 0.05$).

type I collagen on the e-PLLA was in the range of 5.8–23.6 $\mu\text{g}\cdot\text{cm}^{-2}$ on average (based on the projection area of the specimens), which corresponded to 1.2–7.7 $\mu\text{g}\cdot\text{mg}^{-1}$ on average (based on the actual weights of the specimens) ($n = 5$). Significantly greater values were observed for the CA-g-ePLLA over the range of 15.1–40.7 $\mu\text{g}\cdot\text{cm}^{-2}$ or 2.8–8.6 $\mu\text{g}\cdot\text{mg}^{-1}$ on average. Evidently, the greater hydrophilicity of the

CA-g-ePLLA should be responsible for the greater amounts of type I collagen adsorbed on their surfaces.

Cell Morphology. The morphology of HDFa that had been seeded or cultured on the surfaces of the neat and the modified PLLA fibrous membranes was also investigated. Here, glass was used as the control substrate instead of TCPS because of the ease of taking the specimens for SEM observation. Representative SEM images are shown in Table 4. At 2 h after cell seeding, the majority of the cells on the glass surface was still round, while, at 4 h, the evidence of cytoplasmic process was already observed. The full cytoplasmic expansion of the cells on the glass substrate was realized after they had been cultured on the surface for 1 and 3 d. At 4 h after cell seeding, the majority of the cells on the surfaces of the ePLLA and the a-ePLLA was rather round, whereas that on the surface of the CA-g-ePLLA was more expanded. The full cytoplasmic expansion of the cells grown on these fibrous substrates was observed at 4 h after cell seeding, particularly for that of the cells grown on the CA-g-ePLLA. On day 1 after cell culturing, HDFa, in their fully expanded form, covered about 80% or more on the surfaces of all of the fibrous substrates. On day 3, the cell-covered areas were evidently increased, especially for that observed on the CA-g-ePLLA. The obtained results clearly confirmed the preference of HDFa toward the CA-g-ePLLA surface.

Antioxidant Activity of CA-g-ePLLA. The antioxidant activity of the CA-g-ePLLA was determined by the DPPH assay. DPPH• is a stable free radical, exhibiting a characteristic UV absorption at 517 nm, and has been extensively used to

Table 4. Representative SEM Images (magnification = 1500 \times ; scale bar = 10 μm) of Cultured HDFa on Glass, ePLLA, a-ePLLA, and CA-g-ePLLA at Four Different Time Points after Cell Seeding or Cell Culturing

Material	Cell seeding/culturing time point			
	2 h	4 h	1 d	3 d
Glass (control)				
ePLLA				
a-ePLLA				
CA-g-ePLLA				

evaluate the antioxidant activity of a variety of substances.^{14,28–30} CA, which can act as a donor of an H-atom or an electron in a similar manner to gallic acid, can transform DPPH• into its reduced form DPPH•-H.³¹ The antioxidant activity of CA, as the grafted species on the surface of the PLLA fibers, was determined to be $88.4 \pm 1.3\%$ ($n = 5$). The result confirmed that CA still retains its free radical scavenging ability, even though its mobility was limited because of its chemical immobilization onto the surface of the PLLA fibers. Interestingly, the amount of the as-grafted CA on the surfaces of the individual fibers of the CA-g-ePLLA that participated in the scavenging activity of the DPPH radical species was determined to be $2.73 \pm 0.10 \mu\text{g}\cdot\text{cm}^{-2}$ (based on the projection area of the specimens) or $0.49 \pm 0.17 \mu\text{g mg}^{-1}$ (based on the actual weights of the specimens) or about 44% (based on the amount of the immobilized CA).

CONCLUSION

Electrospun PLLA fiber mats, with the average fiber diameter of about 620 nm, were prepared from 10% w/v PLLA solution in 7:3 v/v DCM/DMF. The surfaces of the individual PLLA fibers were successfully modified by CA via a two-step grafting procedure and the reactions were confirmed by the ninhydrin assay and XPS. Amino groups were introduced first on the fiber surface and the optimal condition was 0.04 g mL⁻¹ of HMD/IPA solution at 50 °C for 10 min, resulting in the surface density of the introduced amino groups of $(1.20 \pm 0.03) \times 10^{-7} \text{ mol cm}^{-2}$. After this step, the wettability of the fiber mat surface improved significantly, as the water contact angles reduced from those of the neat PLLA fiber mats (i.e., $104.8^\circ \pm 0.3^\circ$) to $83.3^\circ \pm 0.4^\circ$. The wettability of the fiber mat surface improved even further after the chemical immobilization of CA (with the water contact angles being $55.5^\circ \pm 0.5^\circ$). Such the chemical modifications caused the fiber diameters to increase to ~850 and ~920 nm on average after the aminolysis and the grafting of CA, respectively. In the indirect cytotoxicity assay, none of the fibrous materials released substances in the levels that were detrimental to the cells. In the direct cell culturing assay, all of the fibrous matrices showed very good support for the proliferation of HDFa on days 2 and 3 after cell culturing, with the CA-modified fibrous substrates being the best. Lastly, the antioxidant activity of the grafted CA moieties on the PLLA fibers was determined to be $88.4 \pm 1.3\%$.

ASSOCIATED CONTENT

Supporting Information

Additional graphs: calibration curves (Figure I–IV) and an additional result (Figure V). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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