CHAPTER IV

PREPARATION AND CHARACTERIZATION OF CAFFEIC ACID-GRAFTED ELECTROSPUN POLY(L-LACTIC ACID) FIBER MATS FOR BIOMEDICAL APPLICATIONS

4.1 Abstract

Caffeic acid (CA) was chemically immobilized onto the individual fiber surface of electrospun poly(L-lactic acid) (PLLA) fiber mats to enhance the hydrophilicity and impart the antioxidant activity to the fibrous membranes. This was done in two sequential steps. First, amino groups were covalently introduced onto the surface of the individual electrospun PLLA fibers through the reaction with 1,6-hexamethylenediamine (HMD). In the second step, the amino moieties reacted with CA, which had been pre-activated 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 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 were the best. Lastly, the antioxidant activity of the CA-immobilized substrates, as revealed by the 1,1-diphenyl-2-picryldrazyl (DPPH) radical scavenging assay, was as high as 88% on average.

(**Key-words**: poly(L-lactic acid); Electrospinning; Caffeic acid; Grafted)

4.2 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 non-woven fabric on a collection device.[1,2] This is driven mainly by the Coulombic repulsion 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 US Food and Drug Administration (US-FDA) 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. While 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 anti-bacterial bandage based on electrospun 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., pre-osteoblastic 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 anti-carcinogenic, anti-fungal, anti-inflammatory, and anti-tyrosinase activities, gallic acid is one of the most potent anti-oxidants 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 anti-oxidant 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 anti-carcinogenic, anti-inflammatory, and anti-mutagenic 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 answer. Among the various chemical pathways, grafting of a substance 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 mouse 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.

4.3 Experimental

4.3.1 Materials

PLLA (M_n = 80,000 g·mol^{-1}), CA, HMD, 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.

4.3.2 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 home-made 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 μm.

4.3.3 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 were first washed in an ethanolic aqueous solution (1:1 v/v) for 2 to 3 h and then 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 concentration (i.e., 0.02, 0.04, 0.06, and 0.08 $g \cdot mL^{-1}$) 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.

4.3.4 Materials Characterization

The free amino (NH_2) groups on the surfaces of the neat and the modified PLLA fiber mats was 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 NH_2 groups that might be present on the surfaces of the fiber mat samples. If there were enough NH_2 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 NH_2 groups in the sample solutions were calculated from a standard calibration curve of HMD solutions in 1,4-dioxane/IPA (1:1 v/v).

Surface elemental chemistry of the neat and the modified PLLA fiber mats was analyzed by a Thermo Fisher Scientific Thetaprobe X-ray photoelectron spectroscope (XPS).[24] Monochromatic Al Kα 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 μm × 400 μm, 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 towards 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 disc; ~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.

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 = 50 mm and crosshead speed = 100 mm min^{-1}). The specimens were cut into a rectangular shape (10 mm × 100 mm). The measurements were carried out on ten different specimens.

4.3.5 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; Biochrom, 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 °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 (~15 mm in diameter) in a serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1 % lactabumin, 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 (~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 re-incubated 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 °C with 300 μL/well of MTT solution at 0.5 $mg \cdot mL^{-1}$, 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 (~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 de-ionized water and phosphate saline solution (PBS), were immersed in DMEM overnight. A stainless steel ring (~12 mm in diameter) was placed on top of each specimen to assure its complete contact to the bottom of the well. HDFa (~40,000 cells/well) were seeded on the fiber mat specimens and empty wells of TCPS (i.e., control) and incubated at 37 °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 ~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.

Specimens (~15 mm in diameter) were placed in wells of a 24-well TCPS. A stainless steel ring (~12 mm in diameter) was placed on top of each specimen to assure its complete contact to the bottom of the well. A solution of type I collagen in PBS (0.1 $mg \cdot mL^{-1}$ 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 °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.

4.3.6 Antioxidant Activity

The antioxidative activity of CA after it had been grafted onto the surface of the PLLA fiber mats was assessed by the 1,1-diphenyl-2-picryldrazyl (DPPH) radical scavenging assay. The procedure was carried out based on a

previously-published method (14,26), with slight modification. Specifically, each specimen (~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. A blank solution without the addition of the dissolved CA-modified PLLA fiber mat solution was used as control. The percentage of inhibition (%*AA*) was calculated as follows:

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

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

4.3.6 Statistical Analysis

All values were presented as the means ± 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 0.05 were considered statistically significant.

4.4 Results and Discussion

The purpose of grafting CA onto the surface of the individual electrospun PLLA fibers is two folds. The first is to increase the hydrophilicity of the surface, as the hydroxyl moieties of CA are more hydrophilic than the ester linkages indigenous to PLLA. Secondly, 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 Graphic to the Abstract, the grafting of CA onto the surface of the individual electrospun PLLA fibers can be done in two sequential

steps. In the first step, amino groups could be covalently introduced onto the surface of the individual electrospun PLLA fibers through the reaction with a diamine to obtain the aminolyzed PLLA fiber mats (a-ePLLA). Here, an amino moiety ($-NH_2$) of HMD would react with an ester linkage (-COO-) of PLLA to from the amide linkage (-CONH-), while the other amino moiety would be left available for further reaction. In the second step, the CA-grafted PLLA fiber mats (CA-g–ePLLA) could be obtained through the reaction between CA, the carboxylic acid moiety (-COOH) of which had been activated sequentially with EDC and NHS, and the other amino moiety of HMD which had been introduced on the a-ePLLA through the first step.

4.4.1 Characterization of Neat and Modified PLLA Fibrous Matrices

4.4.1.1 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 4.1) and the reaction time (see Table 4.2) were studied. Within the 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 more 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 $g \cdot mL^{-1}$ of HMD/IPA solution at

50 °C for 10 min. At this condition, the surface density of the introduced amino groups was (2.31 ± 0.02) $\times 10^{-7}$ mol·cm^{-2}.

4.4.1.2 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° ± 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°± 0.4°). The hydrophilicity of the surfaces improved even more after CA having been grafted onto the surfaces of the a-ePLLA (i.e., the water contact angles = 55.5° ± 0.5°). The wettability of a surface depends not only on the chemical integrity of the surface, but on the typographical nature of it as well. For an electrospun fibrous membrane, the wettability of its surface depends on the topography, the size, the arrangement (24), and the surface chemistry of the individual, underlying fibers (22). To ascertain that the improvement 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 4.1). According to these representative micrographs, there is no obvious alteration 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., ~850 and ~920 nm on average after the aminolysis and the grafting of CA, respectively) were generally greater than those of the neat ones (i.e., ~620 nm on average). It has been demonstrated just recently that an increase in the size of the individual fibers caused the hydrophobicity of the electrospun fibrous membranes to increase (24). Thus, the improvement in the hydrophilicity of the modified PLLA fibrous membranes over that of the neat materials was not due to the change in the size of these individual fibers.

4.4.1.3 Elemental composition of surfaces

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 4.3. As expected, no nitrogen-based moieties were observed on the surfaces of the neat PLLA fibrous membranes (i.e., the ePLLA). This was evidenced by the non-existence 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 of 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 of 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.

4.4.2 Biological Evaluation of Neat and Modified PLLA Fibrous Matrices

4.4.2.1 Indirect cytotoxicity evaluation

The potential for use of the neat and the modified PLLA fibrous membranes in biomedical applications was first assessed by the inidirect 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 d. 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 d is shown in Figure 4.2. The viability of the cells that had been cultured with the fresh SFM that had been preincubated for 1 d 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 ~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 ~109% on average. For HDFa on the other hand, decreasing trends were observed, with the values ranging from ~82 to ~95% on average. Because the viabilities were greater than ~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.

4.4.2.2 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 attached cells on the surface of TCPS at 4 h was lower than that at 2 h, while 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-ePLLA were better than the a-ePLLA 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-ePLLA in supporting the proliferation of HDFa was better than that of the a-ePLLA, which, in turn, was better than that of the ePLLA.

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 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-ePLLA was 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). Since the surface of the CA-g-ePLLA was more hydrophilic than those of the ePLLA and the a-ePLLA, the greater adsorption of proteins on their surface.

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 ePLLA and the CA-g-ePLLA. 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-ePLLA at 48 and 72 h that showed equivalent values (suggesting that the maximal adsorption was already reached at those time points). Regardless of the time points investigated, the adsorption of type I collagen on the e-PLLA was in the range of 5.8-23.6 $\mu g \cdot cm^{-2}$ on average (based on the projection area of the specimens), which corresponded to 1.2-7.7 $\mu g \cdot mg^{-1}$ 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 $\mu g \cdot cm^{-2}$ or 2.8-8.6 $\mu g \cdot mg^{-1}$ on average (n = 5). Evidently, the greater hydrophilicity of the CA-g-ePLLA should be responsible for the greater amounts of type I collagen adsorbed on their surfaces.

4.4.2.3 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 4.4. 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, while that on the surface of the CAg-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-gePLLA. 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 towards the CA-g-ePLLA surface.

4.4.3 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 evaluate the antioxidant activity of a variety of substances [14, 27-29]. CA, which can act as a donor of an H-atom or an electron in a similar to gallic acid, can transform DPPH· into its reduced form DPPH·-H [30]. The antioxidant activity of CA, as the grafted species on the surface of the PLLA fibers, was determined to be 88.4 ± 1.3% (n = 5). The result confirmed that CA still retains its free radical scavenging ability, even though its mobility was limited due to its chemical immobilization onto the surface of the PLLA fibers.

4.5 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 surface of the individual PLLA fibers was 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 $g \cdot mL^{-1}$ of HMD/IPA solution at 50 °C for 10 min, resulting in the surface density of the introduced amino groups of (2.31 ± 0.02) × 10^{-7} $mol \cdot cm^{-2}$. 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° ± 0.3°) to 83.3° ± 0.4°. The wettability of the fiber mat surface improved even further after the chemical immobilization of CA (with the water contact angles being 55.5° ± 0.5°). 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 ± 1.3%.

4.6 Acknowledgements

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Table 4.1 Surface density of amino groups ($-NH_2$) on the aminolyzed electrospun PLLA fibers (aePLLA) as a function of 1,6-hexamethylenediamine (HMD) concentration at a fixed reaction time of 10 min

HMD concentration ($g \cdot mL^{-1}$)	Surface density of $-NH_2$ ($\times 10^{-7}$ $mol \cdot cm^{-2}$)
0.02	0.36 ± 0.01
0.04	2.31 ± 0.02
0.06	4.37 ± 0.04
0.08	5.62 ± 0.04

The reaction temperature was fixed at 50 °C.

Table 4.2 Surface density of amino groups ($-NH_2$) on the a-ePLLA as a function of reaction time at a fixed HMD concentration of 0.04 $g \cdot mL^{-1}$

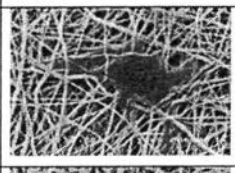
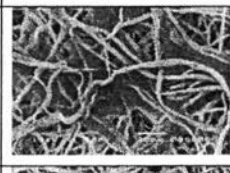
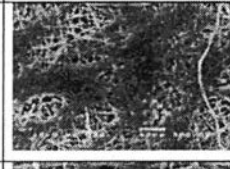
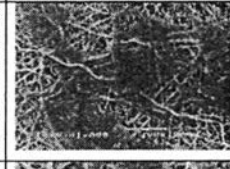
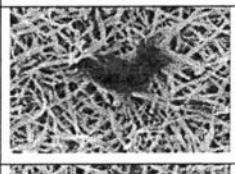
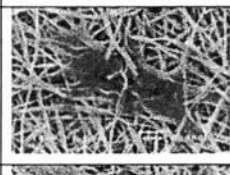
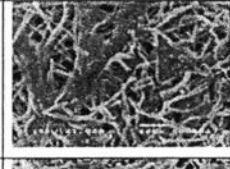
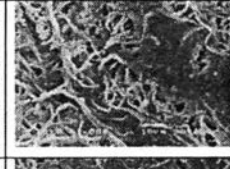
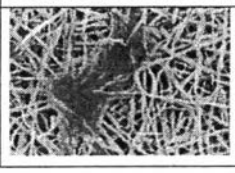
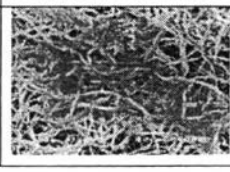
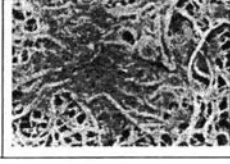
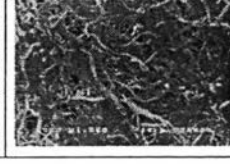
Aminolyzing time (min)	NH_2 conc. ($x10^{-7}$) mol/cm^2
5	0.58 ± 0.04
10	1.74 ± 0.04
15	4.45 ± 0.02
20	4.64 ± 0.03
30	9.70 ± 0.03

The reaction temperature was fixed at 50 °C.

Table 4.3 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

Table 4.4 Representative SEM images (magnification = 1500x; scale bar = 10 μm) of cultured HCFa 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				

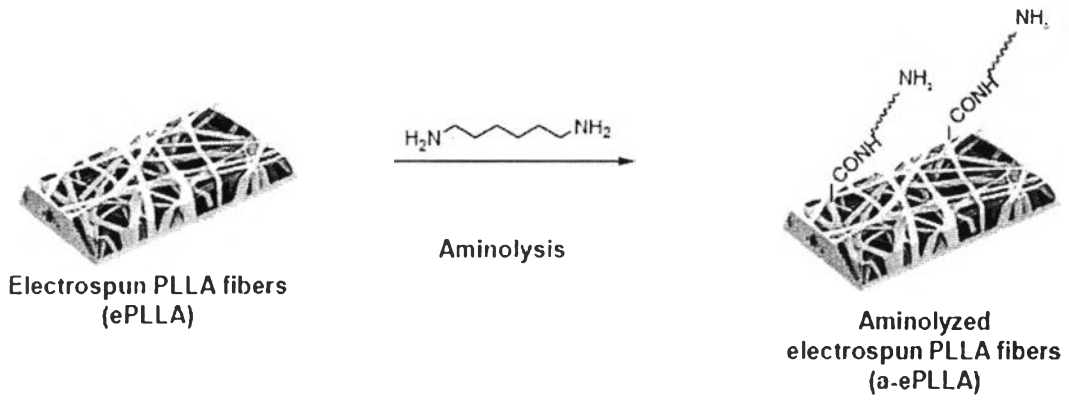


Figure 4.1 Aminolysis of the electrospun PLLA fiber surfaces through the reaction with 1,6-hexamethylenediamine (HMD).

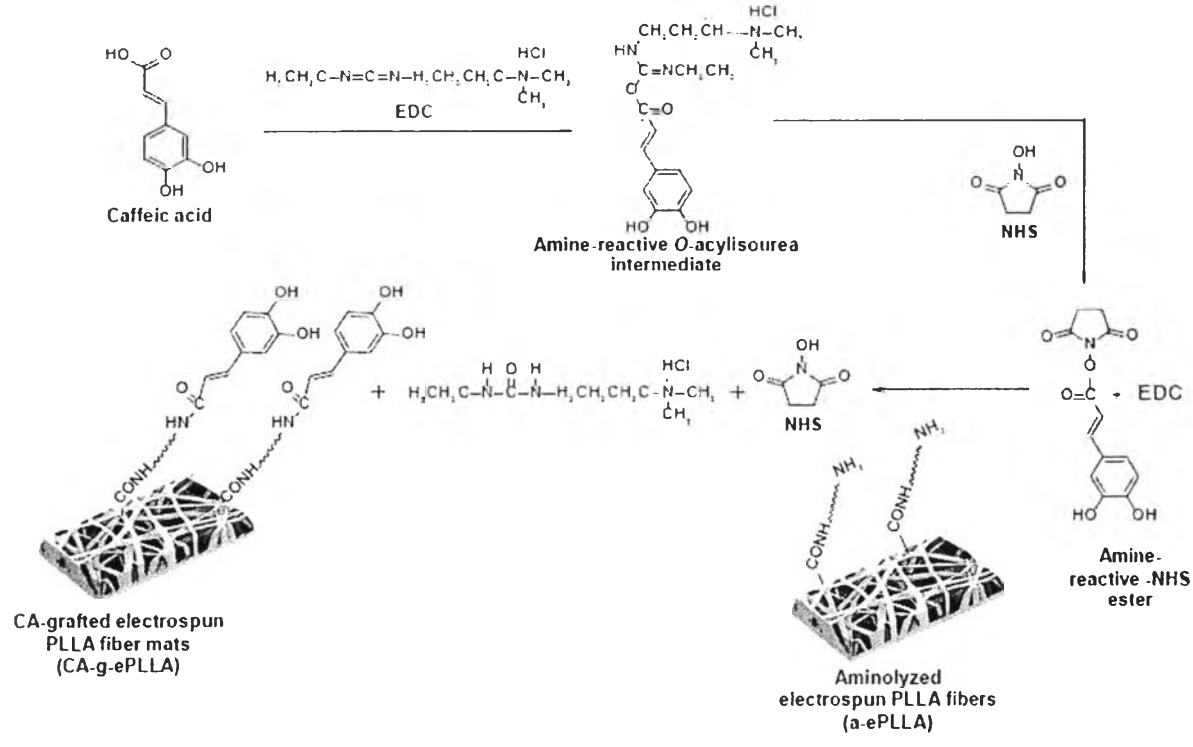
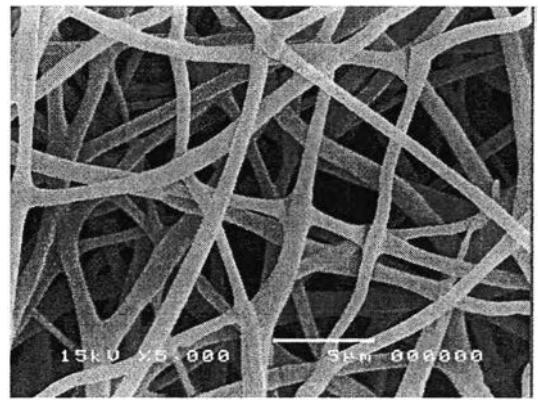
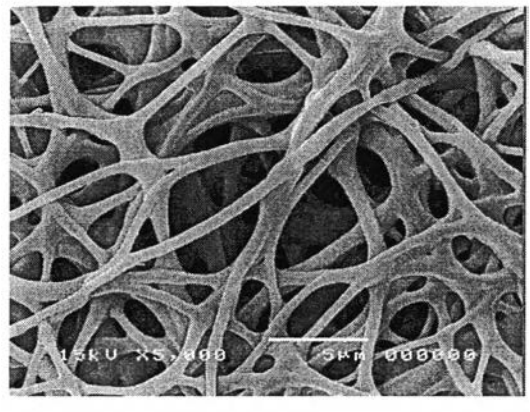


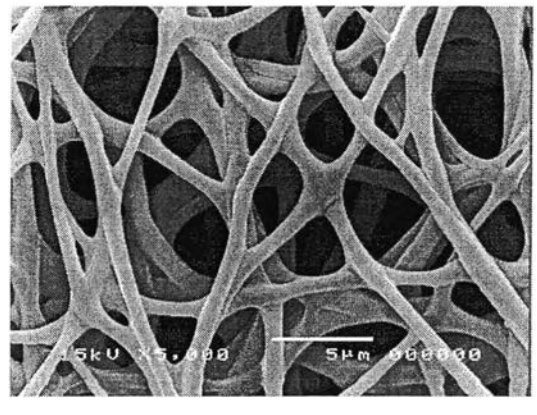
Figure 4.2 Chemical pathway detailing the conjugation reaction of CA with an amino moiety (-NH_2) on the aminolyzed electrospun PLLA fiber (a-ePLLA) surfaces via conjugating agents.



(a)



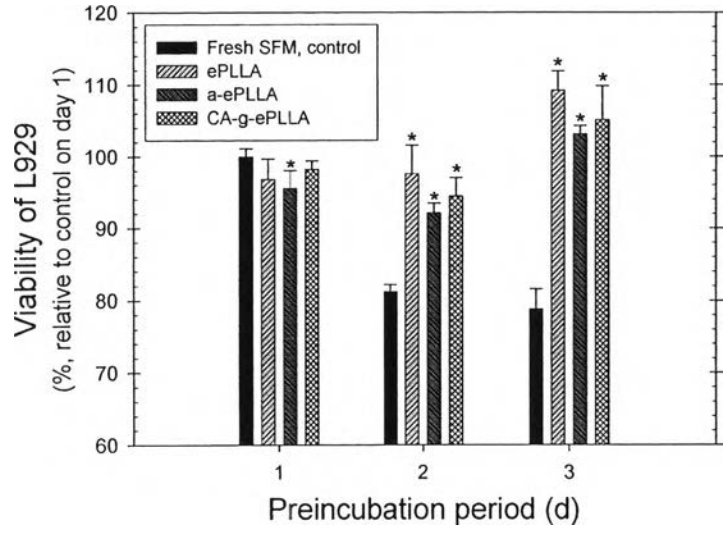
(b)



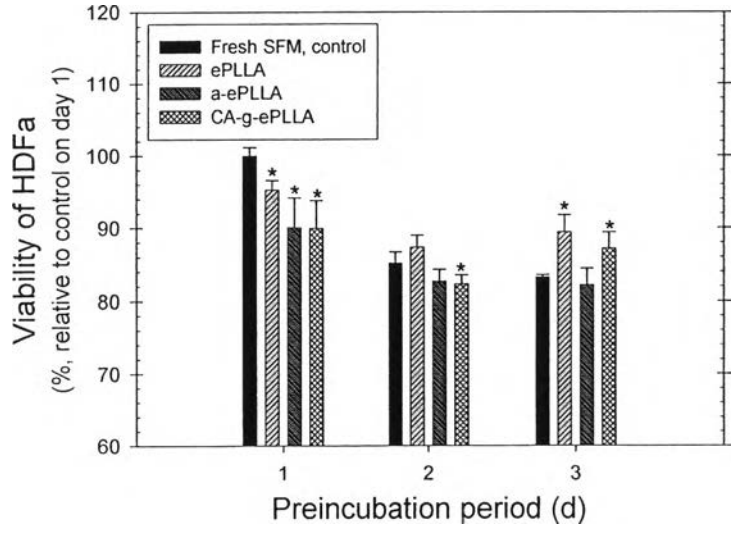
(c)

Figure 4.3 Representative SEM images (scale bar = 5 µm and magnification = 5000x) illustrating morphology of (a) neat electrospun PLLA fibers (ePLLA) and the one that had been modified by (b) aminolysis (0.04 $g \cdot mL^{-1}$ of HMD/IPA solution at 50 °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.

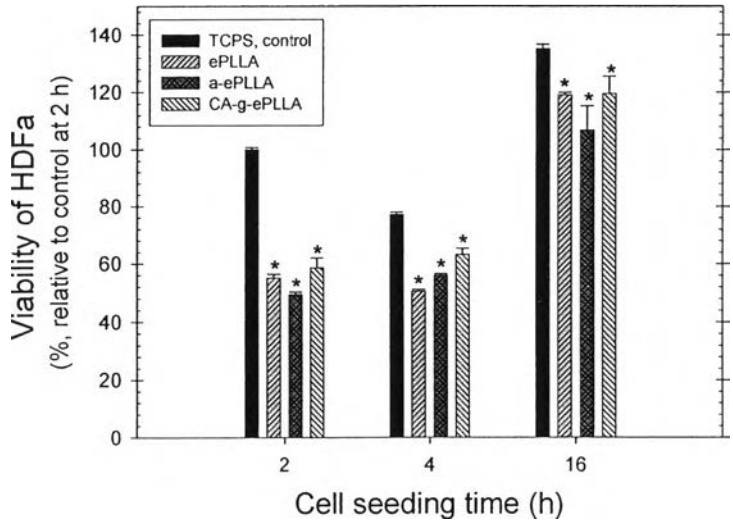


(a)

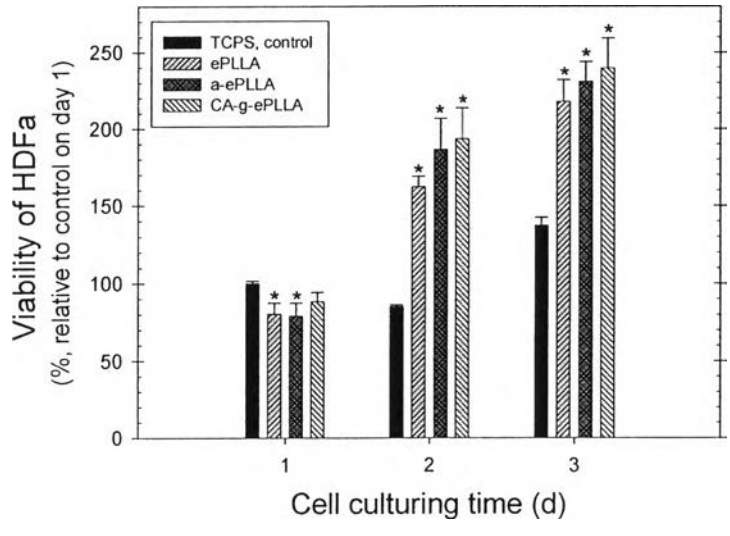


(b)

Figure 4.4 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 withthe respective serum-free media for 1 day as a function of the preincubation period of 1, 2, or 3 d. $*p < 0.05$: compared with the control group at any given time point.



(a)



(b)

Figure 4.5 (a) Attachment and (b) proliferation based on viabilities of HDFa that had been seeded or cultured on the surfaces of ePLLA, a-ePLLA, and CA-g-ePLLA 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.

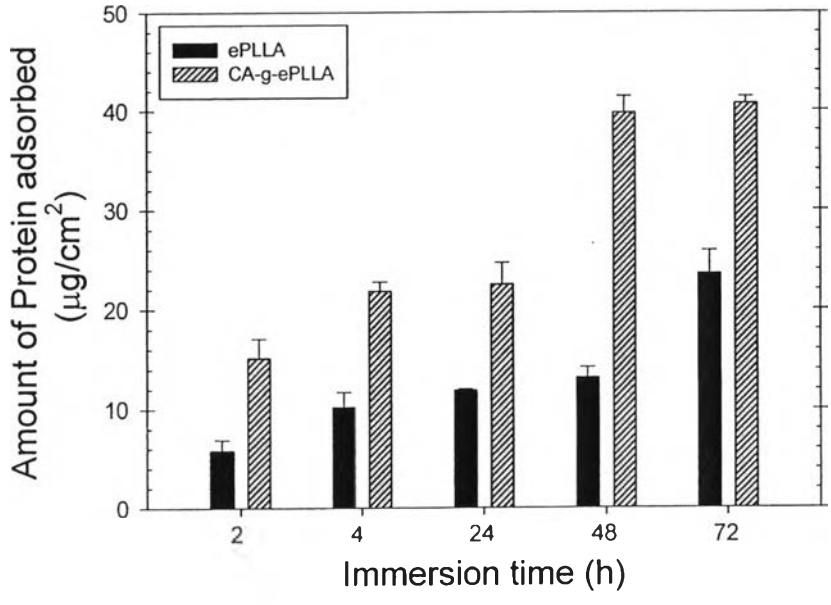


Figure 4.6 The areal amounts of type I collagen adsorbed on the surfaces of ePLLA and CA-g-ePLLA (i.e., the adsorbed amounts divided by the projection area of the specimens) at various immersion time points. All data sets at any given time point were significantly different ($*p < 0.05$).