Plasma-Treated and Collagen-Coated PLLA Nanofiber Membrane for Cartilage Tissue Engineering

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ABSTRACT

Biodegradable nanofiber membranes from poly-L-lactic acid (PLLA) were prepared by electrospinning and used as a scaffold for cartilage tissue engineering. Operating parameters during the electrospinning process were studied in details in terms of fiber diameter and pore size of the membranes. The nanofiber membranes were characterized by SEM, FESEM, BET, AFM, and TEM. In order to improve cell attachment and growth, nanofibers were subjected to DC-pulsed oxygen plasma treatment, acrylic acid grafting, and collagen coating by covalent binding of collagen to carboxylic moieties of the polyacrylic acid. The bioactive scaffolds could be used for culture of chondrocyte with improved cell viability, proliferation, and differentiation.

Keywords: nanofibers, collagen, electrospinning, plasma modification, tissue engineering

1 INTRODUCTION

Eletrospinning (ES) is a novel technique to produce nonwoven membrane composed of nanofibers with diameter ranging from several micrometers to several hundred nanometers [1]. It is a spinning method that can produce polymer nanofiber under a high-voltage electrostatic field operated between a metallic nozzle of a syringe and a metallic collector in air. The fibers are deposited in the form of a nonwoven fabric onto the target collector through a random deposition process of projected jet of polymer solution. The nanoscale diameter of the produced nanofibers and the structure of the nonwoven fabric often resemble the supermolecular feature of extracellular matrix (ECM) [2]. PLLA was selected to be electrospun into nanofibers in this study. This polymer is safe, biodegradable, and widely used *in vivo* as scaffolds for tissue engineering applications. To induce cell adhesion and tissue formation, the chemically and biologically inert PLLA polymeric material is further modified by plasma-induced grafting of acrylic acid (AAc) and surface immobilization of collagen, a natural occurring polymer extracted from the native ECM [3].

2 MATERIALS AND METHODS

2.1 Electrospinning

PLLA with an average molecular weight of 8.5×10^4~1.6×10^5 g/mol and a density of 1.24 g/ml was obtained from Sigma. A mixed solvent system of methlyene chloride and *N*, *N*-dimethylformamide with a weight ratio of 1.5:1 was used to dissolve the polymer at 60 ℃. The concentration of polymer solution was varied from 8 to 12 wt%. The apparatus for ES includes a glass syringe, a stainless-steel needle, a syringe pump (KD Scientific Corp.), a high-voltage power supply (Spellman), and a collector (area 21×29.5 cm^2). The syringe was mounted on the syringe pump horizontally. PLLA solution was drawn horizontally from the needle tip with a electrostatic force generated from the high voltage applied between the tip and a grounded collector. The PLLA polymer solution formed Taylor cone and jetted through the tip of needle to the collector (Figure 1). The flow rate of the PLLA solution, applied voltage, and distance between needle tip and collector were controlled from 3.0 to 5.0 ml/h, 12.0 to 18.0 kV, and 10.0 to 15.0 cm, respectively.

2.2 Characterization of Nanofiber Membrane

Scanning Electron Microscopy (SEM; JSM-5410, JEOL) was used to observe membrane morphology and estimate the diameters of nanofibers at an accelerated voltage of 20 kV. The surface property of nanofiber was measured by Atomic Force Microscopy (AFM; DI5000, Digital Instrument.). For pore size determination, the membrane was cut into 2.5×2.5 cm^2 squares and pore size was determined by Capillary Flow Poremeter (CFP-1500-AEX, Porous Materials Inc.). A sample was put into the Porewick (16 dyne/cm), and the wet sample was placed in the chamber, which was locked by pressing the cap. Nitrogen gas was blown from the bottom layer of the wet sample by increasing the gas pressure. Using the pressure value when the gas first penetrated through the wet sample, pore size of the membrane can be calculated.

2.3 Plasma-Induced AAc Grafting

The nanofiber membrane was washed with ethanol and dried in a vacuum oven overnight. A DC-pulsed O_2 plasma reactor was used for modification of the membrane. The operating conditions in the reactor were: pressure = 200 mTorr, voltage = 600 V, treatment time = 30 s. The treated membranes were subsequently shaken in AAc aqueous solutions at 60℃ for AAc grafting. The amount of AAc grated to the nanofiber membrane was determined by the TBO method.

2.4 Surface-Immobilization of Collagen

The AAc-grafted nanofiber membrane was reacted with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) at pH 6 (MES buffer) for 4 hr for activation of the carboxylic groups, followed by reacting with collagen solution in 0.5 M acetic acid for 24 hr. The collagen-coated membrane was washed entensively with PBS and dried. The amount of collagen immobilized was determined by *p*-dimethylaminobenzaldehyde.

3 RESULTS AND DISCUSSION

3.1 Fiber Diameter and Pore Size of the Nanofiber Membrane

The diameters of nanofibers generated from ES were studied under different operating conditions, including voltage of powder supply, needle to collector distance, concentration of polymer solution, needle diameter, and type of collector. The results are summarized in Figure 2. The most significant parameters that could influence fiber diameter are found to be distance from needle to collector, where diameter decreased with increasing distance; and polymer concentration, where diameter increased with increasing polymer concentration.

Figure 3 show the SEM micrographs for the nanofiber membranes collected on Cu and paper collectors, respectively, with the best parameters for fabricating nanofiber membrane, *i.e.* 8 wt%, 18 KV, 3 ml/hr, 15, cm, and 22 gauge. The fiber appears to be uniform in size and deposited randomly on the collector to form a thin nonwoven membrane with thickness around 70 μm. When surface ultra-structure of the nanofiber was studied with FESEM and AFM, surface roughness was evident and many cracks were found on the surface (Figure 4). This surface morphology could arise from the rapid solvent evaporation from the fibers, which are known to be dry when they reached the collector. The enormous surface area provided by the nanofiber membrane (~1500 m^2/g) from BET measurements (Table 1) can easily surpass that of silica gel (400 m^2/g), indicating a ideal candidate for cell adhesion, catalysis, filter, and fuel cell membrane applications.

The calculated porosity of the membrane from polymer density, weight and dimensions of membrane is *ca*. 85% in all cases. The pore size and pore size distribution of the nanofiber membrane were measured with a capillary flow porometer. The pore size is generally under 2 μm for all membranes tested and the pore size distribution is sharp. The pore size is the same irrespective of the types of collector used, conductive (Cu) or non-conductive (paper). It is, however, sensitive to polymer concentration among all ES operating parameters studied. The pore size increased with increasing polymer concentration and increased from 1.5 to 4.2 μm when the polymer concentration was increased from 8 to 12 wt% (Figure 5). Using a rotational collector could also increase the pore size but rotational speed appeared to have no effect on pore size (Figure 5).

3.2 Plasma-Induced AAc Grafting

Polyacrylic acid (PAAc) was grafted to the nanofiber surface by plasma-induced polymerization of AAc. The hydrophobic surface of PLLA nanofiber changed to a hydrophilic one after O_2 plasma treatment. The free radicals generated on the surface could be used for polymerization of AAc under UV radiation or at high temperature. We have found out that polymerization in hot water is more efficient than that under UV. The influence of polymerization time and AAc concentration on the amount of AAc grafted were studied and the results are shown in Table 2. The efficiency of AAc grafting increased with monomer concentration and grafting time. However, at 15% AAc concentration, self-polymerization of AAc occurred and grafting efficiency decreased. The maximum amount of carboxylic group in the nanofiber membrane is 0.78 $\mu mol/cm^2$. This value is high enough for introduction of a substantial amount of collagen molecule onto the fiber surface.

3.3 Surface Immobilization of Collagen

Collagen was surface immobilized to AAc-modified nanofiber membrane using the activation reagent EDC. EDC could activate the carboxylic groups of AAc and the active intermediate could be attacked by the primary amine groups in collagen, forming stable amide linkage between fiber and collagen. The amount of collagen immobilized in the membrane was estimated to be 9.20 ± 3.62 mg/cm^3 membrane or 20.4 ± 8.0 mg/mg PLLA. That this value is substantially higher than those reported in the literature for other collagen-modified 3-D scaffolds used in tissue engineering indicates the high efficiency of our method using DC-pulsed plasma AAc-grafting and collagen immobilization for surface modification of PLLA nanofibers.

The diameter of the nanofiber only slightly increased after modification and showed similar nanostructure when examined under SEM. However, significant changes of nanofiber surface ultra-structure were found after AAc

grafting and collagen immobilization. From Figure 6, it is evident the cracks observed in the original fiber diminished after AAc grafting due to the formation of a PAAc polymer film on the surface. Decreases in pore volume and pore diameter from BET measurement coincide with the change in surface morphology observed (Table 1). Collagen immobilization did not introduce a smooth surface coating of polymer on nanofiber surface, as is the case for PAAc. Indeed, scattered particles of collagen molecules were found to be distributed along the nanofiber surface and introduce surface roughness (Figure 6). The pore volume further decreased after collagen coating but the pore diameter remains the same (Table 1). Since collagen molecules were covalently bound to the surface, reconstitution of collagen into a natural fiber form could not occur in this case and the protein molecules were densely packed on the fiber surface. As can be seen under TEM for collagen-coated nanofiber, the collagen layer around the fiber is rather uniform with a estimated thickness around 10 nm (Figure 7).

4 TABLES AND ILLUSTRATIONS

Table 1: Surface area, pore volume, and pore diameter of electrospun nanofiber membranes measured by BET.

	original	AAc-grafted	Collagen-Coated
Surface area (m^3/g)	1484.69	1010.67	392.37
Total pore volume (cm^3/g)	1.0891	0.65	0.25
Average pore diameter (Å)	29.3426	25.97	25.96

Table 2: The amount of AAc grafted (μmol/cm^2) as a function of grafting time (hr) and monomer concentration (wt%).

.	**0.5 hr**	**1 hr**	**2 hr**	**4 hr**
1 wt%	0.038	0.05	0.054	0.068
5 wt%	0.048	0.046	0.067	0.073
10 wt%	0.062	0.065	0.069	0.078
15 wt%	0.069	0.061	0.054	0.052

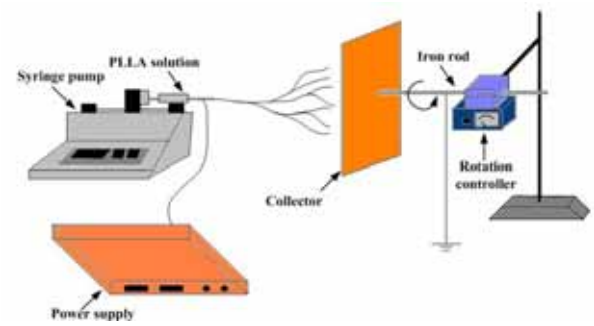


Figure 1: A schematic diagram of the apparatus for preparing PLLA nanofiber membrane by electrospinning.

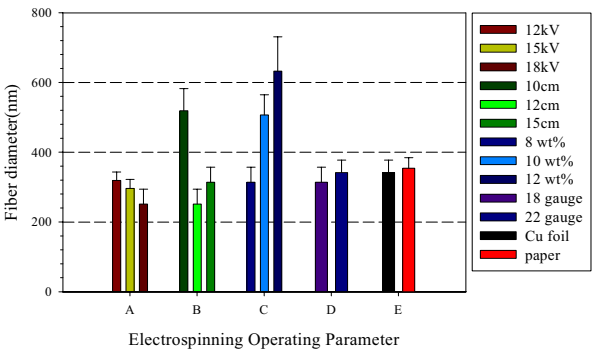
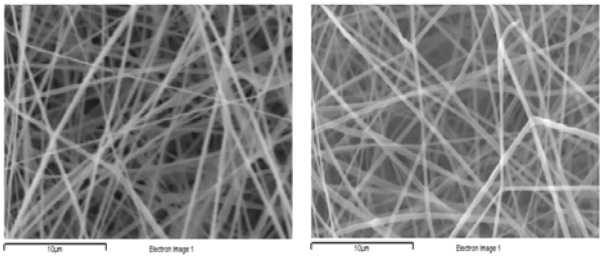
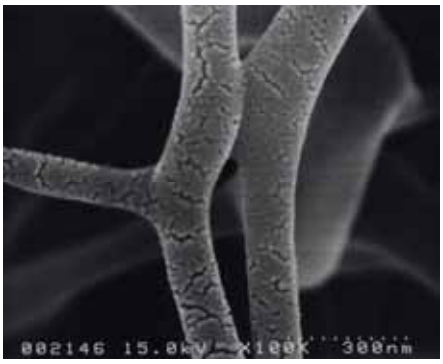


Figure 2: The effect of different electrospinning operating parameter on the diameter of PLLA nanofiber. A: voltage; B: needle to collector distance; C: concentration of polymer solution; D: needle diameter; E: type of collector.

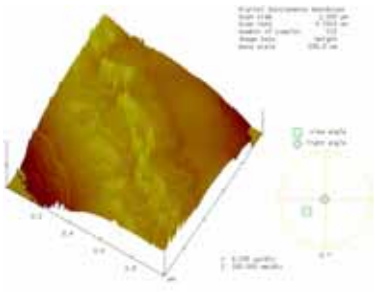


(a) (b)

Figure 3: SEM micrographs of nanofiber membranes with (a) Cu and (b) paper as collector. Magnification = 5000 x. Bar = 10 μm.



(a)



(b)

Figure 4: Surface ultra-structure of nanofiber surface from (a) FESEM (100,000 x) and (b) AFM observations.

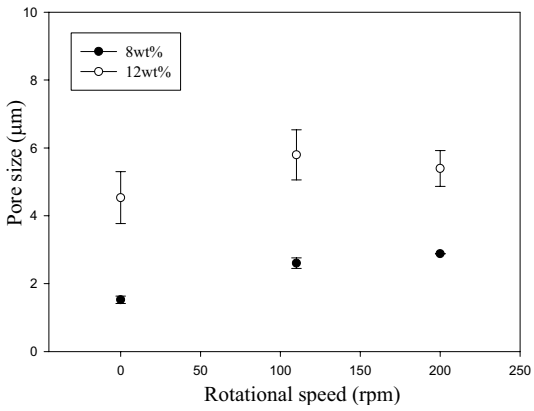
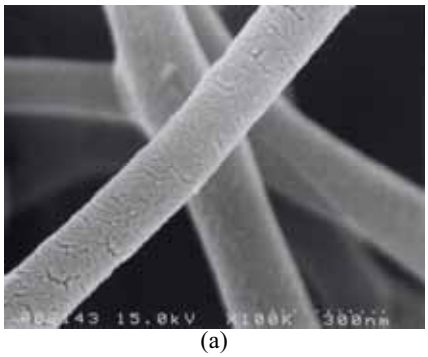


Figure 5: The effect of polymer concentration and collector rational speed on the pore size of nanofiber membrane.



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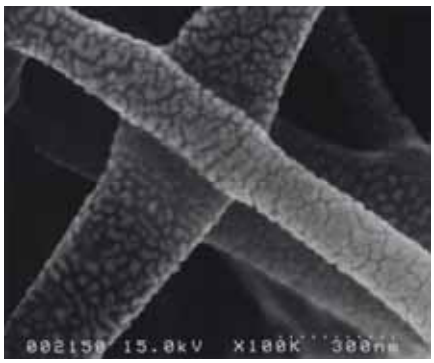
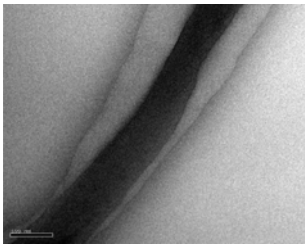
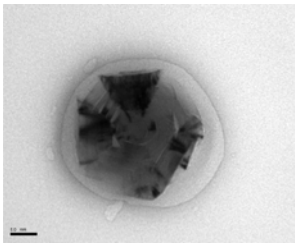


(b)

Figure 6: Surface ultra-structure of (a) AAc-grafted and (b) collagen surface-immobilized nanofiber from FESEM. Magnification = 100,000 x



(a)



(b)

Figure 7: TEM micrographs of ultrathin section of the collagen-coated nanofiber. (a) top view of fiber, magnification = 150,000 x. (b) cross-sectional view of fiber, magnification = 200,000 x.

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