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A N I N T E R N A T I O N A L J O U R N A L

Nanofibrous Scaffolds for Tissue Engineering Applications

Amit Jaiswal[1,2*]

[1]School of Bio Sciences & Technology, VIT University, Vellore 632104, India.[2]Centre for Biomaterials Cellular and Molecular Theranostics, VIT University, Vellore 632104, India.

ABSTRACT

The discipline of tissue engineering opens up the ways for repair and regenerate damaged organs and tissues. In the current work biomimetic nanofibrous scaffolds were fabricated by electrospinning. Poly-L-lactic acid (PLLA) was blended with collagen and gelatin to fabricate PLLLA-collagen and PLLA-gelatin fibrous scaffolds respectively. Pure PLLA and gelatin scaffolds served as controls. All the scaffolds displayed randomly oriented smooth fibers studied by SEM. Surface topography and roughness were studied by AFM and surface contact angle was also measured for all the fabricated scaffolds. Surface roughness was found to be higher in collagen and gelatin blended scaffolds in comparison to PLLA scaffold. Blending of collagen and gelatin reduced the surface hydrophobicity of the scaffolds. Human osteosarcoma cell lines MG-63 were cultured on all scaffolds up to 7 days and cell adhesion was studied through SEM and confocal microscopy. SEM and confocal results showed that gelatin blended PLLA scaffold showed better cell attachment and cell spreading.

Key words: Electrospinning, Biomimetic, Tissue Engineering

[1]Authors for correspondence: amitj@vit.ac.in

INTRODUCTION

Tissue engineering aims to restore or repair the function of damaged organ or tissues with the combination of cells, growth factor, materials and engineering methods (1). The major challenge in tissue engineering lies in the fabrication of scaffold which can mimic the structure and biological function of natural environment of body as close as possible(2). Several techniques including freeze drying, solvent casting, porogen leaching, electrospinning, solid free form fabrication have been explored to fabricate scaffold, which provide template and support for cell attachment and proliferation(3).

In last two decades, electrospinning has attracted tremendous attention because of its inherent specific characteristics of high surface area to volume ratio and high aspect ratio. Electrospinning method finds its application in various medical fields like drug delivery, sensors, wound dressing and tissue engineering etc. Many advantages are associated with electrospun scaffolds- such as electrospining technique is capable to produce fibers from micron to nano dimensions which mimic the extracellular matrix of almost all the tissues. It provides high surface area to volume ratio with porous structure, which enhances cell adhesion, nutrient and oxygen transport. It is simple, cost effective and versatile technique by which variety polymers can be electrospun to make fibrous matrix (4).

A large number of synthetic polymers have been successfully electrospun viz. poly-L-lactide (PLLA) (5), polycaprolactone (PCL) (6), poly(lactic-co-glycolic acid) (PLGA) (7), polyvinyl alcohol (PVA) (8) and polyethylene glycol (PEG) (9) etc. However, the lack of cellular recognition sites on synthetic polymers limits their application to tissue engineering. The natural polymers are biocompatible and possess cell recognition sites, but are difficult to electrospin in comparison to synthetic polymers and possess week mechanical properties (10). The composites of natural and synthetic polymers furnish advantages in terms of cell recognition sites, ease to electrospin and mechanical strength to the scaffold. Collagen is a natural protein present in extracellular matrix. The main practical problems with collagen are its antigenicity, high cost and difficult processing (11). Zeugolis et al. showed that electrospinning of collagen using certain solvents denatures the collagen (12). Gelatin on the other hand is a natural polymer derived from collagen with hydrolysis treatment and is denatured collagen. The merits associated with gelatin over collagen are biodegradability, low antigenicity, low cost and better shelf life.

Since gelatin has several advantages over collagen, the present study compares the potential of electrospun PLLA-collagen and PLLA-gelatin fibrous scaffolds for tissue engineering applications and explores gelatin as an alternative material to collagen for fabricating tissue engineered scaffold.

MATERIALS AND METHODS

A semi-crystalline biodegradable polymer of poly (L-lactide) (PLLA) with molecular weight (Mw) 300,000 g/mol. was purchased from Polyscience Inc (Warrington, PA). Gelatin (Type A, 300 Bloom) from porcine skin, collagen type–I and solvent for electrospinning 1, 1, 1, 3, 3, 3-Hexafluro-2-propanol (HFP) (analytical grade) with density 1.617 gm/ml was purchased from Sigma Aldrich (St.Louis, MO, USA).

Fabrication of Nanofibrous Matrix

The experimental procedure for collecting nanofibers was as followed. The solutions of polymers in various concentrations (Table 1) were prepared by mixing polymers in the solvent. For example solutions of PLLA and PLLA-collagen were prepared by dissolving PLLA and PLLA-collagen (3:1 ratio, w/w) respectively in HFP to make a 7.5 % final solution. Similarly solutions of gelatin and PLLA-gelatin (3:1 ratio, w/w) were also prepared followed by process of electrospinning. Prepared solution was kept under stirring for overnight at room temperature before electrospinning. Polymer solution was then filled in a 5 mL plastic syringe (BD, India) with a blunt ended metallic needle (24 Gauge) (BD, India). At the time of filling the syringe, air bubble entrapment in polymer solution was avoided. Polymer loaded syringe was fixed in a syringe pump, which dispensed solution at desired rate. Constant voltage of 10 kV was applied between the needle tip and collector using high voltage power supply (Gamma High Voltage, USA). The polymer droplet at the tip of needle stretched due to the electric field between the tip and collector and the resultant nanofibers were collected on an aluminium collector located below the needle tip. Only gelatin nanofibrous

scaffold was vapour crosslinked with 0.5% (v/v) glutaraldehyde dissolved in ethanol. Crosslinked scaffold were washed thoroughly with deionized water to remove excess of glutaraldehyde.

Table 1 - Operating parameters for electrospinning process

Sr. No.	Type of polymer/Blend	Solution Conc. w/v	Flow rate of (mL/hour)	Voltage (kv) & tip to collector Distance (cm)	Humidity (%)
1	PLLA	7.5%	0.4	10 kV, 15cm	70 %
2	PLLA-Collagen (3:1)	7.5 %	0.4	10 kV, 15cm	70%
3	Gelatin	7.5%	0.4	12kV 15cm	70%
4	PLLA-Gelatin(3:1)	7.5%	0.4	10kV 15cm	70%

Characterization of Nanofibrous Scaffold

Microstructure of scaffold by Scanning Electron Microscopy (SEM)

Samples were kept in vacuum drier for overnight before doing SEM (Hitachi, S-3400 N, UK) to avoid moisture gain by the samples. Samples (Dimension 0.5x0.5 cm) were mounted on stubs and sputtered coated with gold using SC7640 Sputter Coater (Quorum Technologies Ltd, UK). Samples were examined at an accelerated voltage of 10 kV under SEM.

Fiber Diameter Distribution

SEM images were analysed with image processing software (ImageJ, National Institute of Health, Bethesda, MD) to find out the fiber diameter distribution in PLLA, PLLA-collagen, gelatin and PLLA-gelatin scaffolds. A total of hundred fibers were randomly analysed to calculate the average fiber diameter.

Surface Topography by Atomic Force Microscopy (AFM)

AFM (Nanosurf easyScan 2 AFM, Nanosurf AG, Switzerland) provides three dimensional topographic structure of the scaffold by probing its surface structure with a very sharp tip. Fibrous scaffolds were analysed by atomic force microscopy in contact mode and root mean square roughness (Sq) of the electrospun nanofibrous mat was determined.

Contact Angle Measurement

The hydrophobicity of the nanofibers was measured by measuring the water contact angle on nanofiber scaffold using Digidrop GBX (GBX Instruments, Romans, France). The sample was placed on the holder of a contact angle meter and a drop of water (0.2 µl) was dropped on the sample surface. The contact angle of the drop on the surface of the nanofiber sheet was measured at room temperature. Three measurements were performed at different locations and the results were averaged.

Cell Culture

Human osteosarcoma cell line (MG-63) was purchased from National Centre for Cell Sciences (NCCS), Pune, India. MG-63 were cultured in Dulbecco's modified eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) added with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY) at 37 $^{\circ}$C in a humidified atmosphere of 5% CO_2. The scaffolds used for cell culture were sterilized by dipping in 70% ethanol and kept under short-UV-light for 30 min, and washed three times with phosphate buffer saline (PBS) (Invitrogen). MG-63 cells were grown in 25 cm^2 cell culture flask. Cells were seeded on the scaffolds at a seeding density of 1×10^4 cells/ml/well in 12-well tissue culture plates.

Cell Adhesion
For a scaffold to be successful tissue engineered construct, it is highly desirable that it facilitates cell adhesion on its surface. Adhesion of MG-63 cells was studied by SEM as well as by confocal microscopy.

Scanning Electron Microscopy (SEM)
MG-63 adhesion on PLLA, PLLLA-Collagen, Gelatin and PLLA-Gelatin scaffolds was studied by SEM. For sample preparation, at 7th day of culture on scaffolds, medium was discarded and scaffolds containing cells were washed with PBS. Cells were fixed in 2.5% glutaraldehyde (Merck) for 2 hour at room temperature, washed with PBS and dehydrated with graded ethanol from 10% to 100%. Samples were dried overnight, sputter coated with gold (Quorum Technologies Ltd, UK) and observed under SEM (Hitachi, S-3400 N, UK).

Confocal Microscopy
At 7th day of culture of cells on scaffolds, cell seeded scaffolds were harvested, washed with PBS, and fixed with 3.7% formaldehyde for 5 minutes at room temperature. A nuclear stain DAPI (4',6-diamidoino-2-phenylindole) (Sigma-Aldrich, St.Louis, MO, USA) was used to stain and visualize nuclei. Images were captured using a Zeiss-LSM 510 laser scanning confocal microscope (Carl Zeiss Meditec AG, Jena, Germany) under10x magnification.

Statistical Analysis
Quantitative data were collected in triplicate ($n = 3$) and reported as mean ± standard deviation where indicated. Statistical comparisons were made between groups using student's *t*-test and *p* values of less than 0.05 were considered significant.

RESULT AND DISCUSSION

Microstructure of the Scaffold by SEM
Electrospun nanofibers of PLLA, PLLA-Collagen, Gelatin and PLLA-Gelatin were uniform, smooth and without beads (Figure 1). All the scaffolds showed random, nonwoven architecture with interconnected pores which mimic the extracellular matrix (ECM) of the native tissues like bone, cartilage and skin. ECM components in tissues like bone, cartilage and skin are in fibrous form and present in nano dimensions (13). Thus fibrous architecture of scaffolds closely mimics the architecture of native tissue ECM. Performance of the fibrous scaffolds has been evaluated for several tissue engineering applications.

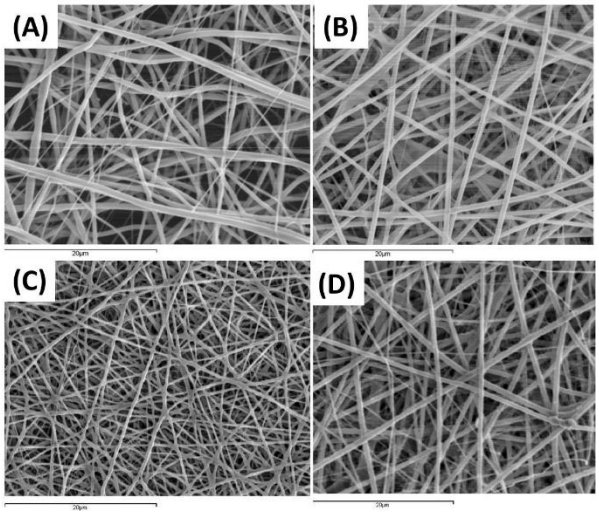

Figure 1 Morphology of electrospun (A) PLLA, (B) PLLA-collagen (C) Gelatin (D) PLLA-Gelatin scaffolds (Scale bar =2 µm).

For e.g. Venugopal et al. prepared electrospun fibrous scaffold of PCL/Hap/Gelatin with PCL/GEL 1:2 ratio (w/w) and achieved 411 ± 158 to 856 ± 157 nm fiber diameter (14). Similarly, Sambudi et al. prepared fibrous scaffold of chitosan/poly(vinyl alcohol) and showed fibrous morphology of scaffolds.
Scaffolds were also studied for *in vitro* behaviour of ATDC5 mouse chondrogenic cell line (15).

Fiber Diameter Distribution
The diameter range of PLLA, PLLA-Collagen and Gelatin, PLLA-Gelatin nanofibers is showed in figure 2 and 3 respectively. The average diameter of fibers in PLLA and PLLA-Collagen scaffold was 571.32±249.99 and 1062.14±498.83 µm respectively and of gelatin and PLLA-Gelatin was 403±137.43 and 681.81±177.80 µm respectively. The reason for getting larger diameter fibers in blended combination is the addition gelatin and collagen increased the viscosity of solution so the fiber diameter.

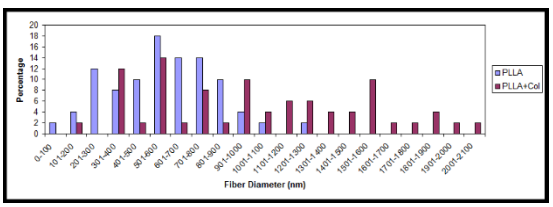

Figure 2 Fiber diameter range of electrospun nanofibers prepared from PLLA and PLLA-Collagen

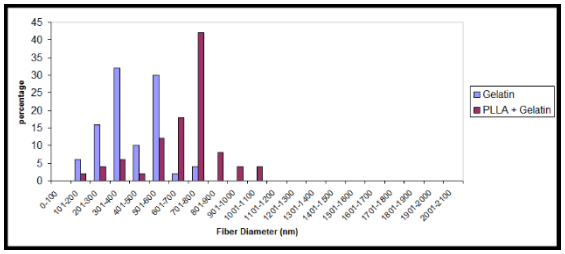

Figure 3 Fiber diameter range of electrospun nanofibers prepared from Gelatin and PLLA + Gelatin

AFM Analysis of Nanofiber Membrane

Cell attachment and proliferation is affected by scaffold surface topography (16). AFM images showed changes in topography of the scaffold after incorporation of collagen and gelatin in PLLA scaffold. Figure 4 are 3D images which clearly depicts uneven surfaces of all the scaffolds. The mean roughness value (Ra) was calculated with the help of AFM images. The roughness (Ra) of the four different electrospin nanofibrous sheet determined by AFM is shown in Table 2. Higher Sq value represents a rougher surface. PLLA-Collagen and PLLA + Gelatin exhibit a higher degree of roughness than the PLLA and gelatin respectively due to higher diameter of PLLA-Collagen and PLLA-Gelatin scaffold. Higher diameter scaffolds have been found with higher roughness in previous studies (17, 18).

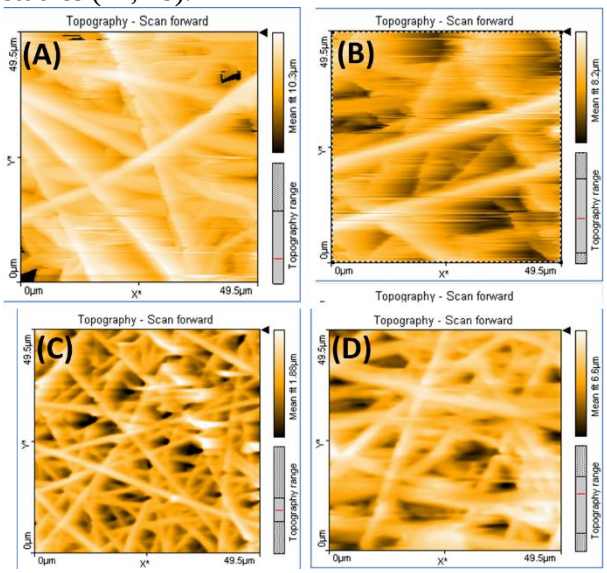

Figure 4 Mean fit AFM images of electrospun nanofibers of (A) Pure PLLA (B) PLLA-Collagen(C) Pure Gelatin and (D) PLLA-Gelatin

Table 2 - Mean root square roughness (Sq) of different nanofibrous scaffolds

S. No.	Sample	Roughness Sq (nm)
1	PLLA	1099.6
2	PLLA-Collagen	1409.5
3	Gelatin	334
4	PLLA-Gelatin	1171

Contact Angle Analysis

Cells attach in a better manner to hydrophilic scaffold surfaces. The results of water contact angle measurement on the different electrospun nanofibrous sheet showed that PLLA sheet is very hydrophobic in nature and gelatin and collagen is very hydrophilic. Addition of collagen and gelatin to PLLA changed the hydrophobicity of the PLLA surface to approximately same extent.

Table 3 - The initial contact angle values of electrospun fibrous scaffolds using distilled water

S. No.	Sample	Contact angle (in degree)
1	PLLA	120.6±1.1
2	PLLA-Collagen	86.2±0.42
3	Gelatin	19.0±1.6
4	PLLA-Gelatin	80.3±1.5

Cell Attachment

Contact angle data led to the speculation that blended scaffolds will facilitate cell attachment compared to PLLA scaffold. To observe this we studied cell attachment on all four types of scaffold by SEM and confocal microscopy. SEM images were used to assess MG-63 cells adhesion on surface of all types of scaffolds, which are shown in Figure 5. Adhesion of MG-63 cell lines to scaffolds was analyzed by SEM after 7 days of culture. SEM micrographs showed that the cells attached firmly on all scaffolds. PLLA scaffold showed least number of cells adhered compared to all other scaffold. This might have happened due to hydrophobic nature of PLLA scaffold as determined by contact angle study. Hydrophobic surfaces exhibit poor cell adhesion (19). PLLA-Gelatin (Figure 5 D) scaffold showed highest number of cells attached and cells were found to be attached all over the scaffold surface unlike that on PLLA-collagen and gelatin scaffold. This confirms that PLLA-Gelatin nanofibrous scaffold not only facilitated the cell attachment but also the cell spreading.

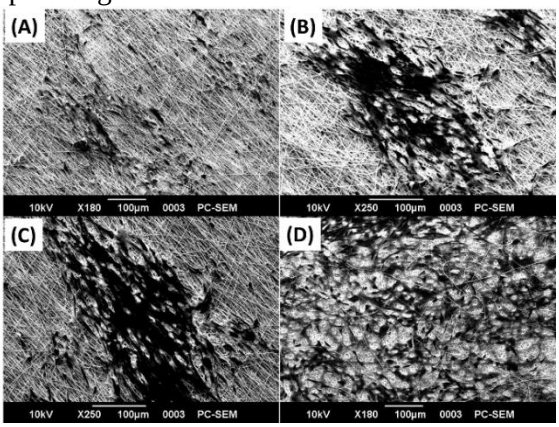

Figure 5 SEM micrographs of MG-63 cell lines attached on (a) PLLA, (b) PLLA-collagen, (c) Gelatin and (d) PLLLA-gelatin scaffolds after 7 days of culture.

Cell Attachment- Confocal Microscopy

Cell adhesion was also observed by confocal microscopy and the results were in concordance with cell attachment SEM study (Figure 6). PLLA scaffold showed least number of cells attached to scaffold surface. PLLA-Gelatin scaffold promoted highest cell adhesion which was found to be slightly more than cell adhesion on PLLA-Collagen scaffold (Figure 6 B and D). Hence, both the cell adhesion studies done by SEM and confocal microscopy proved that PLLA-Gelatin is a better scaffold for cell attachment and spreading. This might have happened due to the conformation denaturation of collagen present in PLLA-collagen scaffold during the entire process of fabrication of scaffold (20). A change in raw material structure may affect its properties (12). The triple helix structure of collagen might have denatured which resulted into its relatively lesser cell adhesion on PLLA-Collagen scaffold.

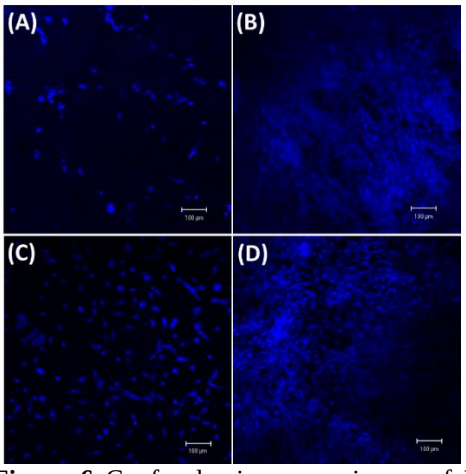

Figure 6 Confocal microscopy image of MG-63 cell lines attached on (a) PLLA, (b) PLLA-collagen, (c) gelatin (d) PLLA-gelatin scaffolds after 7 days of culture. Nucleus (blue) were stained with 4',6-diamidino-2-phenylindole (DAPI) are shown.

CONCLUSIONS

In the present study, different scaffolds of PLLA, PLLA-collagen, gelatin and PLLA-gelatin were fabricated via process of electrospinning. All scaffold displayed fibrous morphology as studied by SEM which mimic the native ECM architecture of skin, bone and cartilage. Blending of either collagen or gelatin increased the roughness and decreased the hydrophobicity of the PLLA scaffold, which rendered the surface a better surface for cell attachment and spreading.

Cell culture study showed that PLLA-gelatin scaffold showed improved cell adhesion and spreading compared to all other scaffolds. Our results indicate that gelatin could serve as an alternate material of collagen for blending into synthetic and natural polymers to prepare tissue engineering constructs.

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Erratum

In the 01 page, that read:
"*[1]School of Bio Sciences & Technology, VIT University, Vellore 632104, India.[2]Centre for Biomaterials, Cellular and Molecular, VIT University, Vellore 632104, India.*"

Read:
"*[1]School of Bio Sciences & Technology, VIT University, Vellore 632104, India.[2]Centre for Biomaterials Cellular and Molecular Theranostics, VIT University, Vellore 632104, India.*

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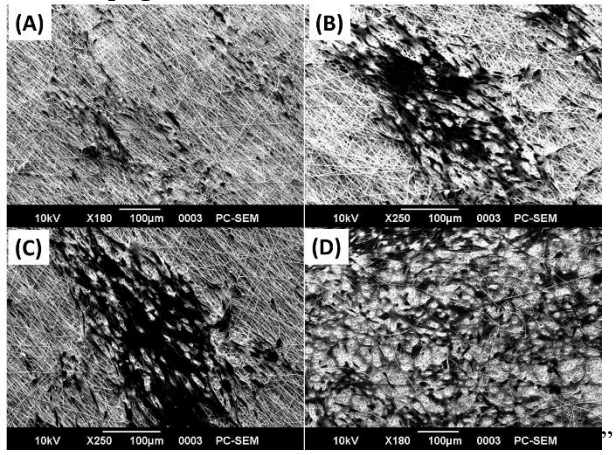
" 

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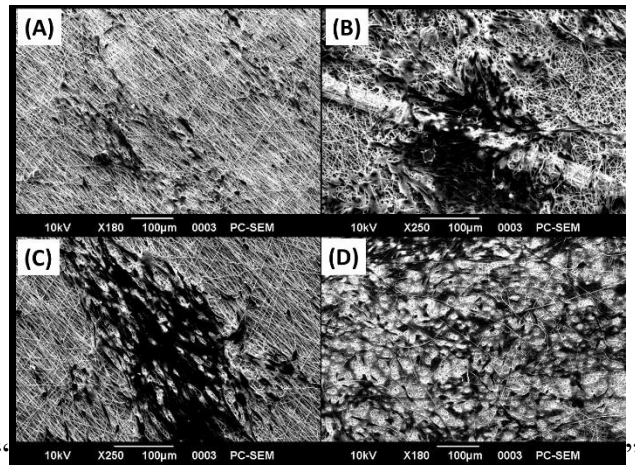
" 

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