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ORIGINAL ARTICLE

TERM

Chitosan-Poly(Vinyl Alcohol) Nanofibers by Free Surface Electrospinning for Tissue Engineering Applications

Parinita Agrawal, Krishna Pramanik*

Department of Biotechnology and Medical Engineering, National Institute of Technology, Rourkela, India

Deformities in tissues and organs can be treated by using tissue engineering approach offering the development of biologically functionalized scaffolds from a variety of polymer blends which mimic the extracellular matrix and allow adjusting the material properties to meet the defect architecture. In recent years, research interest has been shown towards the development of chitosan (CS) based biomaterials for tissue engineering applications, because of its minimal foreign body reactions, intrinsic antibacterial property, biocompatibility, biodegradability and ability to be molded into various geometries and forms thereby making it suitable for cell ingrowth and conduction. The present work involves the fabrication of nanofibrous scaffold from CS and poly(vinyl alcohol) blends by free-surface electrospinning method. The morphology and functional characteristics of the developed scaffolds were assessed by field emission scanning electron microscopy and fourier transformed infra-red spectra analysis. The morphological analysis showed the average fiber diameter was 269 nm and thickness of the mat was 200–300 μm. X-ray diffraction study confirmed the crystalline nature of the prepared scaffolds, whereas hydrophilic characteristic of the prepared scaffolds was confirmed by measured contact angle. The scaffolds possess an adequate biodegradable, swelling and mechanical property that is found desirable for tissue engineering applications. The cell study using umbilical cord blood-derived mesenchymal stem cells has confirmed the *in vitro* biocompatibility and cell supportive property of the scaffold thereby depicting their potentiality for future clinical applications. Tissue Eng Regen Med 2016;13(5):485-497

Key Words: Tissue engineering; Biocompatibility; Electrospinning; Chitosan; Poly(vinyl alcohol); Messenchymal stem cells

INTRODUCTION

A variety of clinical situations involves bone, cartilage and other tissue defects and lesions. Currently the patients are treated by three ways namely autograft, allograft or xenograft though each of these techniques have inbuilt disadvantages. There is always a chance that the grafted tissue may not work as expected in the patient, allograft and xenografts suffer additional problem of donor scarcity, disease transmission or contamination and immune rejection. Tissue engineering provides a lasting cure for such diseases by offering a biocompatible replaceable tissue having functional and mechanical integrity [1].

One of the important aspect of tissue engineering is the scaffold designing to have composition, structure, mechanical, biological and physiochemical features that mimic extra cellular matrix (ECM) of the tissue to be repaired [2-4]. Scaffolds as support to tissue regeneration should be non-immunogenic, non-toxic, biocompatible and biodegradable. Furthermore, scaffold should possess interconnected pores, channels and suitable surface properties which allow *in vitro* cell adhesion, ingrowth and reorganization and provide necessary space for neo-vascularization *in vivo*. The scaffold should have sufficient mechanical strength when cultured *in vitro* to maintain the spaces required for cell ingrowth and matrix formation and also have capability of bearing stresses and loading *in vivo* [5]. It is possible to design scaffolds with tailored physical, biological and mechanical properties by combining various bioabsorbable polymers and bioactive ceramics [6].

Numerous natural and synthetic polymers have been investigated for targeting specific tissue engineering applications. Chitosan (CS) has attracted attention of many researchers because of its biodegradable, biocompatible, and non-toxic properties and thus it is proposed as a safer material for use in biomedical applications [7-11]. Di Martino et al. [7] found that CS possesses intrinsic antibacterial activity and can reduce the infection rate of experimentally induced osteomyelitis by *Staphylococcus aureus* in rabbits. There cationic amino group associates with anions on the bacterial cell wall, suppressing biosynthesis and

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***Corresponding author:** Krishna Pramanik, Department of Biotechnology and Medical Engineering, National Institute of Technology, Rourkela 769008, Odisha, India.
Tel: 91-9437191611, Fax: 91-6612462299, E-mail: kpr@nitrkl.ac.in

disrupts the mass transport across the cell wall accelerating bacterial death. Due to antibacterial property CS has been applied in various biomedical related studies and also it can be easily blended with other polymers to serve the need of specific application [7]. CS has been reported to combine with a variety of delivery materials such as alginate, hydroxyapatite, hyaluronic acid, calcium phosphate, poly(methyl methacrylate), poly(L-lactic acid), and growth factors for potential application in orthopedic tissue engineering [12-18]. As a topic of great interest in the biomedical field, CS blended with poly(vinyl alcohol) (PVA) has been reported to have good mechanical and chemical properties [19]. PVA, is a water soluble synthetic resin which is obtained through polymerization of vinyl acetate monomer. By hydrolysis, the acetate groups are converted to hydroxyl groups. PVA is being used in controlled release systems and due to its biocompatible nature; it is employed in a variety of biomedical applications [20]. The enhanced property of CS/PVA blend has been attributed to the interactions between CS and PVA through hydrophobic side-chain aggregation and intermolecular and intra-molecular hydrogen bonds.

The other important factor in tissue engineering is the scaffold fabrication method. Recent research focuses on the electrospinning method for the development of nanofibrous scaffolds that are ideal for the desired three dimensional cell cultures for tissue regeneration [21]. Electrospinning is a simple and easy way to produce nanofibres that resembles the collagen component of ECM. The fibers produced by this method have the characteristics of large surface-to-volume ratio, high porosity and pore to pore connectivity that are desired for tissue engineering [10,11]. High surface area of electrospun nanofibers allow better cellular spreading and attachment and high porosity influences efficient nutrient supply to the cells. Electrospun nanofibres are mostly generated by conventional needle based electrospinning method [22]. But there are several limitations of this method the most important of which are frequent choking of nozzles and low productivity. Thus in this study we used a more effective and advanced free liquid surface electrospinning method which overcomes the limitations of conventional electrospinning method to fabricate nanofibrous polymer matrix [23].

Mesenchymal stem cells (MSCs) are fibroblast-like, adherent cells that can differentiate into a variety of cell types [24,25]. These are promising candidate as a cell source in tissue engineering as they are easy to obtain and has the ability to differentiate into osteocytes, chondrocytes and adipocytes on induction. MSCs can be isolated from variety of sources including umbilical cord blood (UCB), bone marrow (BM), placenta and other adult tissues [26]. In this study, the use of MSCs isolated from UCB is advantageous because UCB is considered as a biological waste, and thus there is no ethical issue involved for its use. The collection of UCB is non-invasive and less expensive procedure than collecting MSCs from other potential sources like BM aspirates [27] and adipose tissue [26].

Alhosseini et al. [28] reported a preliminary study that relates the fabrication of CS/PVA nanofibrous scaffold by the conventional needle based electrospinning method using 90:10 w/w of PVA:CS. In the present work, an attempt has been made to optimize the blending ratios of CS/PVA and to assess their nano-fibrous scaffold forming capability with desirable properties. The developed nanofibrous mats were further subjected to physico-chemical, mechanical and structural characterization. The scaffolds were evaluated for their cell supportive property in terms of cell attachment, proliferation and cell viability by performing cell culture study using human MSCs (hMSCs) seeded on the scaffold.

MATERIALS AND METHODS

CS (molecular weight 160000, deacetylation degree ≥75%) was purchased from HIMEDIA (Mumbai, India) and PVA (molecular weight 14000) from Otto Chemika (Mumbai, India). Acetic acid (M 60.05 g/mol) used of Merck (Mumbai, India).

Preparation of CS-PVA blends

10% aqueous solution of PVA (wt%) was prepared and 2% (wt) CS solution was prepared by dissolving CS in acetic acid water (90% v/v). The PVA solution was mixed with CS solution at different volume ratios of CS/PVA such as 10:90, 20:80, 30:70, 35:65, and 40:60.

Preparation of electrospun nanofibers

The CS/PVA nanofibrous mat was prepared in free liquid surface electrospinning machine (Elmarco, NanoSpider Lab 200, Liberec, Lzech). 50 mL each of CS/PVA blends was poured in a solution holder in which the cylindrical electrode rotates to form a thin film of polymer solution over it. On applying 50 kV numerous small Taylor cones were generated on the electrode surface, which then split into nanofibers and deposited into the collector near the upper static electrode. The electrospinning process was continued till the solution was utilized completely. The distance between the electrodes was maintained at 15 cm. The fibrous mat collected on collector was dried and stored for further characterization.

Characterization of polymer solution and nanofibers

Rheological property

The viscosity of polymer solutions were measured by Bohl-

inVisco 88 viscometer, manufactured by Malvern Instruments Ltd., (Malvern, United Kingdom) and necessary calculations were done by applying Moore Model [29].

Morphological study of electrospun nanofibrous scaffold

The morphology of the electrospun mats were evaluated using Field Emission Scanning Electron Microscopy (FESEM). The electrospun samples were coated with a thin layer of gold (Au) and their morphologies were observed under NOVA NanoSEM 450 microscope at (FEJ, Oregon, USA) 5000× magnification that operated at the acceleration voltage of 15 kV.

The roughness profile of nanofiber scaffolds surface were studied using a scanning probe atomic force microscope (NT-MDT system, NTEGRA Module, Moscow, Russia). The quartz cantilever with a tip radius of 10 nm was used in semi contact mode. Surface roughness analysis of the scaffolds was performed by WSxM 5.0 develop 7.0 image browser software. The arithmetic mean value of the surface roughness (Ra), which refers to the integral of the absolute value of the roughness profile height over the evaluated length approximated by a trapezoidal rule, was calculated.

XRD analysis

The electrospun mats were subjected to X-ray Diffractometer (XRD) to obtain X-ray diffraction pattern in order to reveal information about the crystallographic structure of the prepared nanofibres. The instrument used for scanning was XRD-PANalytical (Almelo, the Netherlands) with scanning range 15−55°.

FTIR analysis

Molecular structure of prepared nanofibres was characterized by Fourier Transform Infra Red (FTIR, Shimadzu, IR-Prestige-21, Kyoto, Japan) spectroscopy. The pellet was made by pressing the thin fiber sheets in between KBr powder layers in the KBr press Technosearch instrument. The pellet was then placed in instrument to record FTIR readings. The FT-IR analysis was based on the identification of absorption bands concerned with the vibrations of functional groups present in macromolecules [30].

Swelling behavior

The swelling property of the prepared scaffold was measured by the conventional gravimetric method following procedure described elsewhere [28]. The dry weight (Wd) of the sample was measured and then incubated for 48 h at 37°C in distilled water. The wet weight of the sample was measured (Ws) when excess water was blotted out with tissue paper. The equilibrium swelling percent (Es) of the scaffolds is defined as the ratio of weight increase (Ws-Wd) with respect to the initial weight (Wd) of dry samples and is calculated by the relation, Es=(Ws-Wd)/Wd. Each value was averaged from three parallel measurements.

Hydrophillicity

Water in air (WIA) contact angle measurement was done using DSA 4, Kruss (Germany) contact angle analyzer following sessile drop method [31,32]. Contact angles were measured, at room temperature, by subtending water droplets on the scaffold surface. An average of these values was then recorded as water contact angle for each scaffold sample.

Tensile strength

The tensile strength of the prepared electrospun nanofibers was measured using Universal Mechanical Tester (computer controlled Instron Electropuls E1000, Instron, MA, USA) with a 1 KN load cell under standard atmospheric condition. The samples were cut into 40×10 mm size and placed in grips of the testing machine. Tensile property of the scaffold samples were measured with grip separation. The samples were stretched with cross head speed as 2 mm/min, that attempted to pull the specimen apart and the test was run until the sample broke under the load. To ensure reliable result the process was performed twice for each sample.

Biodegradability

The scaffolds of known dry weights (W_i) were sterilized by immersing in 70% ethanol and then incubated in simulated body fluid (SBF, pH 7.4) at 37°C. The SBF solution was refreshed daily to ensure continuous degradation. Samples were removed from the solution, rinsed with distilled water and weighed in every 24 h for 5 weeks. The experiment was done in triplicates for each scaffold. The extent of degradation was expressed as a percentage of weight remained of the dried sample after degradation. The percentage weight loss was calculated by following equation:

$$\text{Weight } \% = (W_i - W_f)/W_i \times 100$$

Where, W_f is final weight of scaffolds.

In-vitro biocompatibility study

Isolation, culture and morphological characterization of hMSCs

UCB samples were collected from Ispat General Hospital, Rourkela, India with prior consent of the delivery patients and brought to laboratory for processing. The samples were collected in hemobag containing 10 mL citrate phosphate dextrose adenosine buffer and anticoagulant. The blood sample collection procedure was approved by the Institutional ethical committee.

UCB sample was diluted by mixing with equal volume of

phosphate buffer saline (PBS) and then added to RPMI 1640 in 2:1 v/v followed by centrifugation at 500×g for 20 min at 20°C. Buffy coat layer formed after density gradient centrifugation was collected and washed twice with PBS by centrifugation at 400×g for 10 min [27]. The cell pellet was suspended into complete culture media containing Dulbecco Modified Eagle Medium (DMEM, Gibco, Life Technologies, New York, USA), 10% v/v fetal bovine serum (HiMedia, Mumbai, India) and 1% v/v antibiotic solution (10K units/mL Penicillin and 10 mg/mL Streptomycin). Cell suspension was plated into a T75 culture flask for primary culture and incubated under humidified condition at 37°C, 5% CO_2. Morphology change during cell culture was constantly observed by phase contrast microscopy and media was changed every third day, till confluence was reached.

Scaffold neutralization

Nanofibrous mats were cut into small equal sized discs (5 mm diameter and 1 mm height, to accommodate into 96-well tissue culture plate) and sterilized by immersing in 70% ethanol for 3 h followed by 1 h Ultra violet rays treatment. Then the samples were neutralized by washing with PBS at regular intervals. The pH of the solution was checked every time when PBS was changed, until the pH reached 7.

Seeding and culturing of hMSCs on the scaffold

hMSCs isolated from UCB and cultured *in vitro* were seeded onto the neutralized scaffolds for biocompatibility study. For this purpose, cultured hMSCs of 4th passage were trypsinized and centrifuged to obtain concentrated cell mass which was suspended in the complete media. 40 μL cell suspension (containing approximately 5×10^4 cells/mL) was seeded directly onto the sterilized scaffolds and kept for incubation at 37°C in CO_2 incubator for 3 h after which 150 μL culture medium was added into each well and incubated.

Cell metabolic activity

The hMSCs seeded scaffolds were washed with PBS and 200 μL culture medium was added into each well. 5 μL (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide) solution (0.8 mg/mL) was added into the culture and incubated for 4 h in CO_2 incubator. The purple colored precipitate formed was dissolved in 200 μL DMSO and absorbance was measured at 595 nm using Perkin Elmer VICTOR™ X3 2030 Multilabel Plate Reader (Perkin Elmer, Shelton, CT, USA).

Cell attachment and spreading

hMSCs seeded scaffolds cultured for 7 days were washed twice with PBS and fixed with freshly prepared 2% glutaraldehyde solution for 15 min. The constructs were dehydrated with ethanol gradient of 35%, 50%, 70%, and 90%, 5 min each step. The samples were dried overnight, sputter coated with gold and observed under field emission scanning electron microscope.

Immunofluorescence study

The immunofluorescence study on the nanofibrous scaffold surface was performed by staining cellular components followed by the observation under fluorescence and confocal microscopes. Cell seeded scaffolds were stained with calcein-AM dye (Invitrogen, Eugene, OR, USA) and incubated in dark for 30 min. The constructs were counter stained with EtBr (Invitrogen, Eugene, OR, USA) and incubated in dark for 5 min. After incubation the constructs were examined under florescence microscope to check live and dead cells.

Cell spreading was further confirmed by observing under confocal microscope. hMSCs seeded nanofibrous scaffolds were fixed using 4% paraformaldehyde solution for 10 min and permeabilized by treating with 1% Triton X-100 for 5 min. The samples were then washed with PBS and stained with green-fluorescent Alexa Fluor 488 phalloidin (Invitrogen, Eugene, OR, USA) for staining cytoskeleton components (F-actin) and kept in dark for 20 min. Unreacted dye was removed by washing with PBS and then subjected to nuclear stain Hoechst-33258 (Sigma, St. Louis, MO, USA). The samples were mounted on coverslip and observed under laser scanning confocal microscope (TCS SP8, Leica, Microsystems, Wetzlar, Germany).

RESULTS

Rheological property of blend solution

The solution viscosity is a critical factor that affects solution

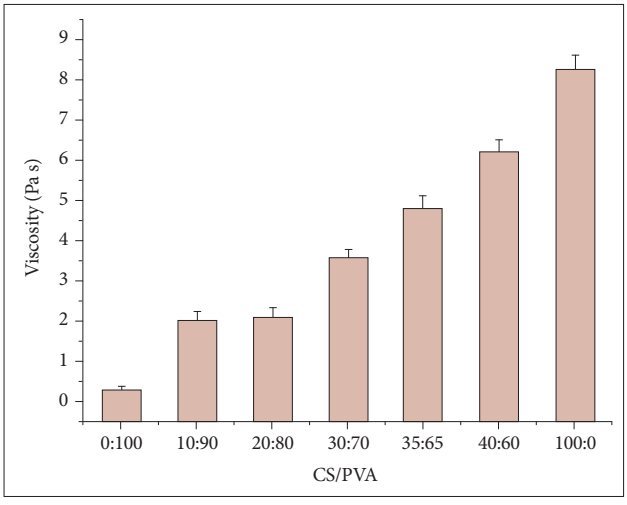

Figure 1. Rheology behaviour of CS:PVA blends solutions. CS: chitosan, PVA: poly(vinyl alcohol).

spinnability and morphology of electrospun fibers [33]. As indicated in Figure 1, the viscosity of blend decreased with increase in PVA (0.317 Pa sec) content as expected. The optimum CS/PVA ratio was found to be 35:65 showing excellent fiber formation. Furthermore, blend containing 40:60 CS/PVA was not found favorable for fiber formation due to its low spinnability characteristic. This is attributed to the high viscosity of blend containing higher CS [34].

Morphology analysis

Figure 2 shows the FESEM micrographs of the prepared electrospun CS/PVA nanofibers. The fiber diameter is largely influenced by the viscosity and charge of the polymer solution. The fibers obtained were randomly oriented and their diameter was in nanometer range. An average fiber diameter of 300 nm was achieved with CS/PVA blend ratio of 10:90. For blend ratios 20:80, 30:70, and 35:65, the average fiber diameter obtained was 282 nm, 264 nm, and 260 nm, respectively. A trend of decrease in fiber diameter with decreasing PVA concentration in the blends was observed, and the blend ratio of 40:60 resulted into fused fibers which emerged as thin sheet inspite of a fibrous mat like structure as shown in Figure 2E.

Roughness profile by AFM analysis

The roughness of the prepared CS/PVA scaffolds determined by AFM study is shown in Figure 3. The average Ra values for nanofibrous scaffolds were in the range 108 nm−0.786 μm, which is favourable for cell attachment and cell growth for successful tissue regeneration [36,37]. The maximum Ra was obtained with CS/PVA blend 35:65 (0.7859 μm), which is in the reported range for osteoblast proliferation [38]. The Ra value for CS/PVA blend ratio of 30:70 (108.314 nm) was in the reported range that supports endothelial [39] and neuronal cells growth and proliferation [40]. Thus, it could be inferred that, the prepared blends could be applied for various tissue engineering applications.

XRD analysis

X-ray diffractogram was studied to assess the phase change during blend formation and crystalline nature of the prepared scaffolds. The XRD patterns of the electrospun nanofibers are shown in Figure 4A. The crystalline peaks of CS were shown at 2θ of 20.1° and the PVA diffractions exhibited at 2θ of 32° and 46° [41]. For CS/PVA blend ratio 35:65 the peak was found at 2θ of 19.6°. Crystalline peak at 46° was observed with slight shift with decrease in PVA amount in the blends.

FTIR analysis

FTIR spectra of pure CS, CS/PVA and pure PVA are shown in Figure 4B. In pure CS, the characteristic absorption bands

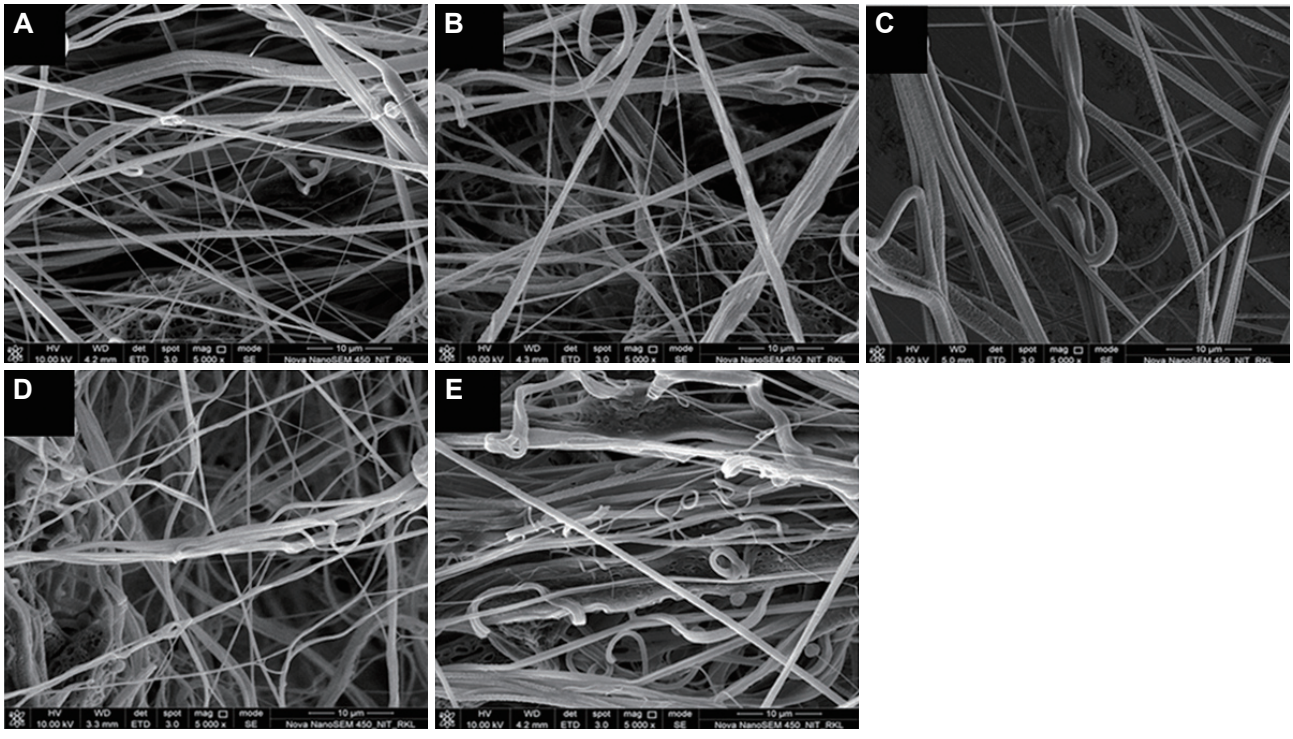

Figure 2. Field emission scanning electron microscopy study of 2% CS and 10% PVA solutions electrospun in the ratios (A) 10:90, (B) 20:80, (C) 30:70, (D) 35:65, and (E) 40:60 of CS:PVA. CS: chitosan, PVA: poly(vinyl alcohol).

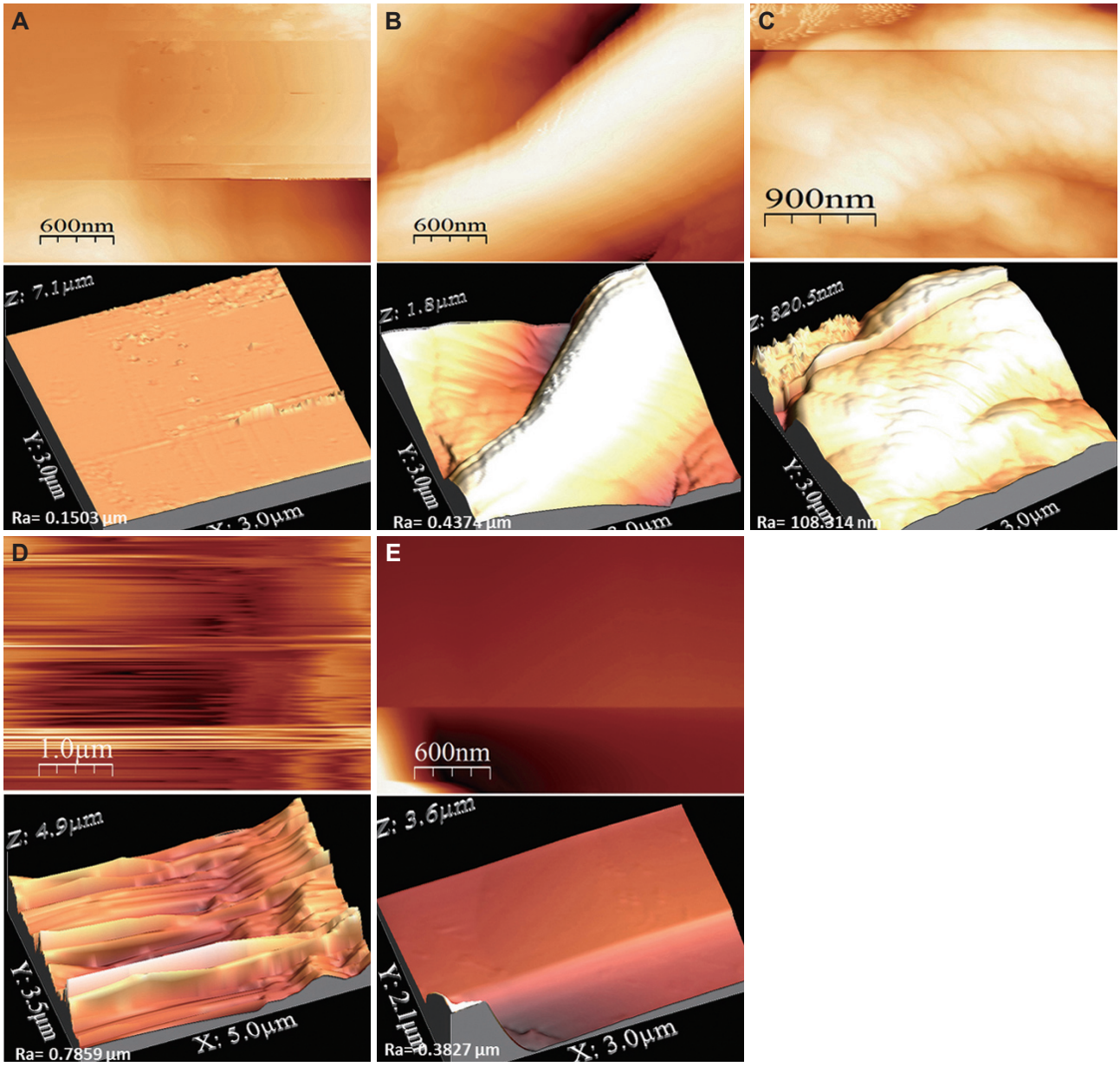

Figure 3. Surface roughness analysis by AFM for the CS:PVA nanofibrous scaffolds in the ratio (A)10:90, (B) 20:80, (C) 30:70, (D) 35:65, and (E) 40:60. CS: chitosan, PVA: poly(vinyl alcohol).

were observed at six locations. As indicated, the absorption band at 3440 cm^{-1} corresponds to the vibrations of hydroxyl groups present in pure CS. Whereas, the absorption bands at 1655 cm^{-1}, 1560 cm^{-1}, and 1358 cm^{-1} indicated C=O stretching, -NH2 bending and C-O stretching of primary alcohol groups, respectively. The band at 1143 cm^{-1} represents -C-O-C- glycosidic linkage between CS monomers [41,42].

PVA exhibited stretching vibration peak of its side hydroxyl groups at 3413 cm^{-1} [43]. With the addition of PVA, the absorption peak at 3440 cm^{-1} in the FTIR spectra of the electrospun CS/PVA membranes shifted to the lower wave numbers. The absorption peak at 1560 cm^{-1} of amino groups showed a gradual decrease as PVA content was increased in the blend from 40:60 to 10:90, due to decreasing amount of CS in blends. This interaction between CS and PVA macromolecules is by hydrogen bonding between amino groups or hydroxyl groups in CS and hydroxyl groups in PVA [44,45].

Swelling behaviour

Swelling behavior has a great influence on the structural stability of the scaffolds which is critical for their use in tissue engineering. Various reported *in vitro* cell culture studies have

confirmed that initial swelling is important that increases pore size thereby facilitating cell attachment and growth in a three-dimensional fashion [46]. However, continuous swelling would lead to the loss of mechanical integrity and moreover compressive stress is developed in surrounding tissue [47,48]. Therefore, the swelling behavior of the prepared CS/PVA scaffolds was tested for 48 hours duration. As indicated in Figure 5A, a higher swelling rate is observed with the blends containing CS <30% compare to blends with high CS content and the equilibrium state was achieved after 40 h. The low swelling rate observed with high CS content (>30%) could be attributed to the more rigid network formed by inter and intra polymer interactions.

Hydrophilicity

As it is observed from Table 1, the measured contact angle of

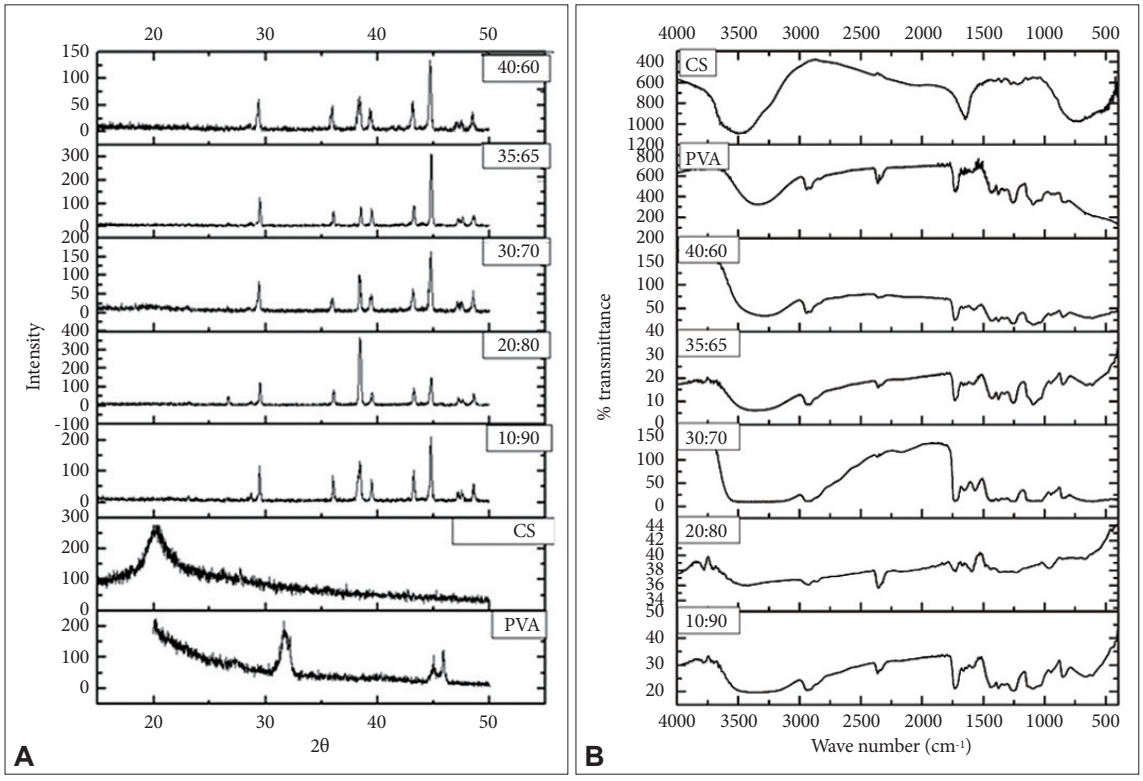

Figure 4. (A) XRD and (B) FTIR analysis of electrospun CS:PVA nanofibres. XRD: X-ray Diffractometer, FTIR: Fourier Transform Infra Red, CS: chitosan, PVA: poly(vinyl alcohol).

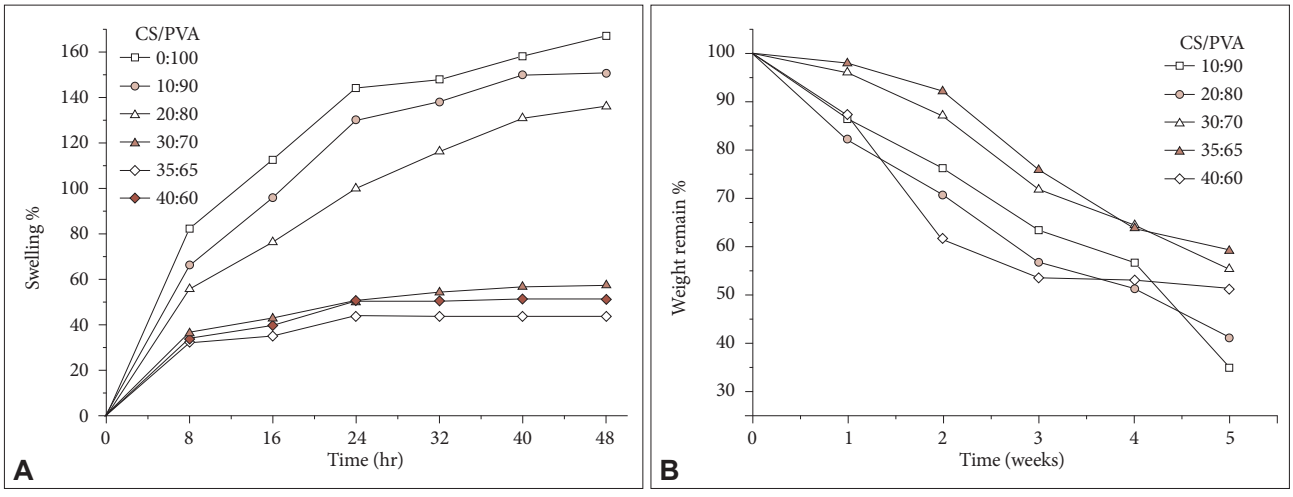

Figure 5. (A) Swelling ratio and water uptake capacity of CS:PVA nanofibrous scaffolds and their (B) *in vitro* biodegradation study. CS: chitosan, PVA: poly(vinyl alcohol).

all the scaffolds is found to be <80°, indicating their hydrophilic nature. Furthermore, the increase in hydrophilicity (decrease in contact angle) with high PVA content in the blends is because of the more hydrophilic characteristic of PVA in comparison to CS [49]. Previous studies indicated that cell culture substrates with contact angles between 60–80° depicted enhanced cell adhesion capability [49-51].

Table 1. Mean contact angle values for CS:PVA nanofibers

S. No.	Blend ratio (CS:PVA)	Mean contact angle (in degree)
1	10:90	48.32
2	20:80	57.14
3	30:70	61.90
4	35:65	64.70
5	40:60	70.30

CS: chitosan, PVA: poly(vinyl alcohol)

Tensile strength

Tensile strength of the CS/PVA blends was performed at varying load (Table 2). The tensile strength of pure PVA scaffold was measured to be 1.33 MPa. The increase in tensile strength of the CS/PVA blend was observed with decrease in PVA in the blend. A similar trend of tensile strength was also reported by earlier study [52,53]. However, the highest tensile strength (6.15

Table 2. Tensile strength of CS/PVA nanofibrous scaffolds

S. No.	Blend ratio (CS:PVA)	Thickness (mm)	Maximum load (n)	Tensile strength (MPa)	Load at break (n)	Tensile strain at break (%)	Modulus (kPa)
1	0:100	0.10	13.32	1.33 (0.08)*	1.33	0.39	3205.46
2	10:90	0.10	5.79	3.22 (0.24)	1.44	38.91	23408.32
3	20:80	0.30	13.98	4.66 (0.62)	4.77	109.52	7572.45
4	30:70	0.25	5.93	5.93 (0.62)	5.84	25.67	37687.91
5	35:65	0.31	18.44	6.15 (1.84)	10.49	83.44	12385.66

*values in parenthesis represent the standard deviation for tensile strength. CS: chitosan, PVA: poly(vinyl alcohol)

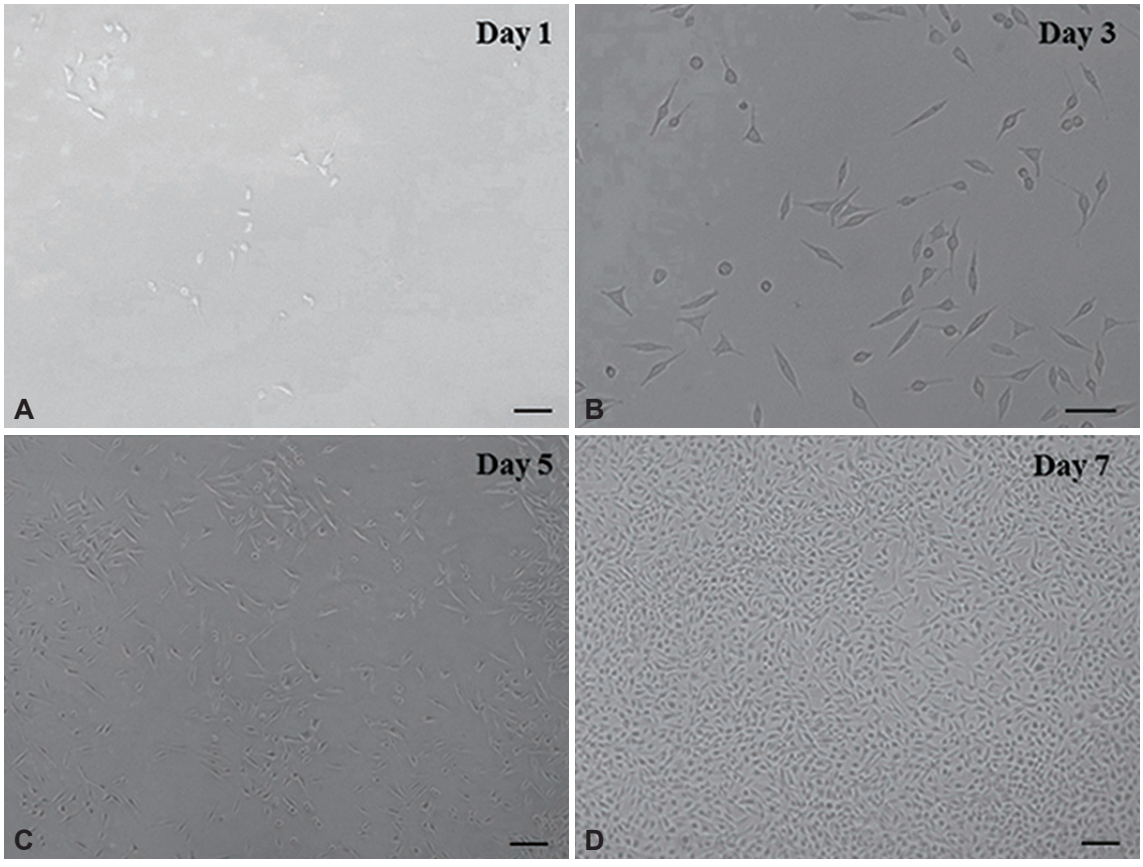

Figure 6. Morphology analysis of UCB derived hMSCs (50× magnification) by phase contrast microscopy showing (A) round cells in 1st day, (B) elongated morphology on 3rd day, (C) increase in cell number on 5th day, and (D) confluence achieved on 7th day during *in-vitro* culture. Scale bar=100 μm. UCB: umbilical cord blood, hMSCs: human mesenchymal stem cells.

MPa) was obtained with CS/PVA blend ratio of 35:65. CS/PVA blend containing higher CS content such as CS/PVA (40:60) was not favourable for nanofibers formation due to its high viscosity which resulted in insufficient thickness of the nanofibrous mat to be separated from the collector and measure the tensile strength. Similarly, no fiber formation was observed with pure CS polymer solution.

Biodegradation

The experimental result of biodegradation behavior of PVA and CS/PVA nanofibrous scaffolds is shown in Figure 5B. PVA scaffolds incubated in SBF had the highest weight reduction and were completely degraded after 30 min. However, the addition of CS controlled the degradation of scaffolds in SBF solution. The scaffolds showed rapid degradation, while weight remain for blend ratios 30:70 and 35:65 (CS:PVA) was 55% and 59% respectively, at the end of 5th week of observation. Thus these ratios were further used for *in vitro* cell studies that require scaffolds stability for longer period of time to support cell proliferation and tissue formation.

In-vitro biocompatibility study

Morphology of hMSCs

hMSCs were isolated from UCB following Ficoll-Hypaque density gradient centrifugation method and the morphology change was observed during *in vitro* culture. It was observed that round floating cells attained elongated morphology after attaching to the culture flask (Fig. 6A and B). The number of cells was gradually increasing with culture time indicating that the cells were proliferating (Fig. 6C) and the confluence was achieved on 7th day (Fig. 6D).

Cell attachment and spreading

Cell attachment and cell spreading on the scaffolds were assessed from FESEM micrographs (Fig. 7). On 3rd day, the cells

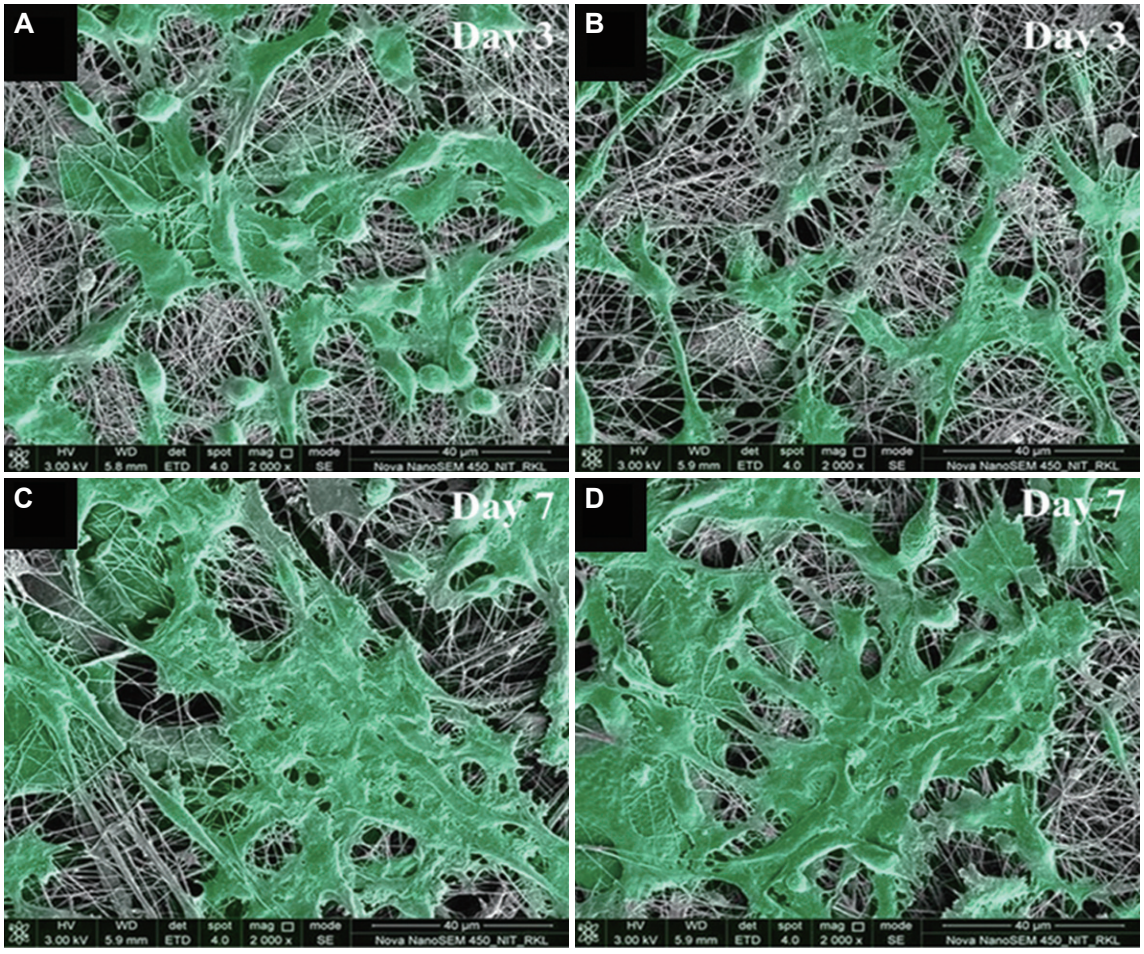

Figure 7. FESEM micrograph of hMSC seeded CS:PVA 30:70 (A and C), 35:65 (B and D) scaffolds. Increase in cell number shows successful cell growth and change in cell morphology confirming cell attachment and spreading on the scaffold. FESEM: Field Emission Scanning Electron Microscopy, hMSC: human mesenchymal stem cell, CS: chitosan, PVA: poly(vinyl alcohol).

were observed to be attached on the scaffold surface in small aggregates, and found to proliferate (Fig. 7A and B). On 7th day of culture, the size of the aggregates was found to increase and bridged across the scaffold connecting with other cell aggregates with abundant ECM secretion filling space (Fig. 7C and D). Some cells were shown to be migrated inside the fibrous layer and grown in between the nanofiber structure, confirming that the prepared scaffold supports cell penetration. The number of cells gradually increased with culture time and well spread covering the scaffold surface.

Metabolic activity

Nanofibrous scaffolds seeded with hMSCs were analyzed for the metabolic activity of cells on 1st, 7th, 14th, and 21st day of culture by MTT assay. The activity was assessed by checking absorbance at 595 nm and experimental data is shown in Figure 8. Scaffolds of all the blend ratios showed good compatibility for cell growth, though a significant increase in cell growth was observed for CS/PVA blend ratio of 35:65 as compared to other blends. This represents the better cell supportive property of the scaffold and thus was used for further *in vitro* cell-scaffold interaction study.

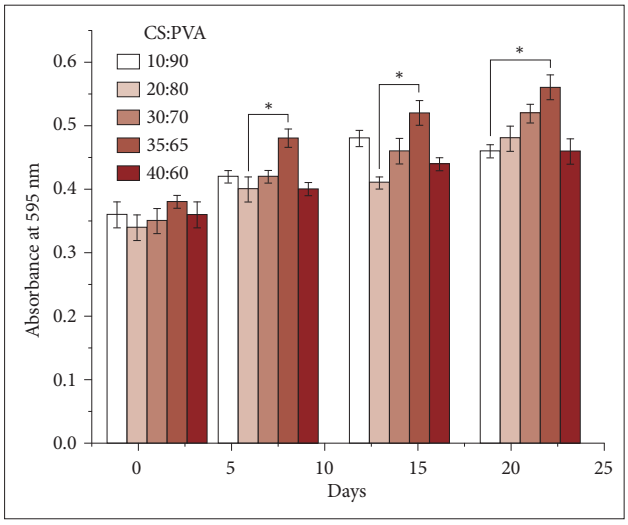

Figure 8. Metabolic activity test by MTT assay. Bar graph showing significant increase in cell growth for CS/PVA 35:65 as compared to other ratios. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, CS: chitosan, PVA: poly(vinyl alcohol).

Immunofluorescence assay

Immunofluorescence study was performed to assess cell proliferation, spreading and actin development on electrospun scaffolds. Cell seeded scaffolds (CS/PVA=35:65) were stained with florescent dye to check the presence of live and dead cells on the scaffold after 7 days of incubation. Figure 9 shows the immunofluorescence result. The scaffold surface was found to be covered with green fluorescence showing live cells.

Confocal micrographs show that the nuclei of the cells were distinct and rounded suggesting normal growth of hMSCs on the prepared nanofibrous scaffold (Fig. 10A). Cytoskeleton integrity plays a major role in cell viability and cell proliferation. Organization of F-actin, the main component in cytoskeleton, largely determines the morphology and surface movement [54]. Therefore, cytoskeleton integrity was assessed by confocal microscopic images (Fig. 10B). The organization of F-actin has shown no remarkable change and cells retained cell morphology with intact cell membrane. Cells were uniformly distributed on the surface as well as inside the scaffold. The scaffold was completely covered by cells forming a compact mat with well-developed actin filaments that were observed throughout the scaffold surface (Fig. 10C and D).

DISCUSSION

Fabrication of artificial nanofibrous scaffolds that mimics the

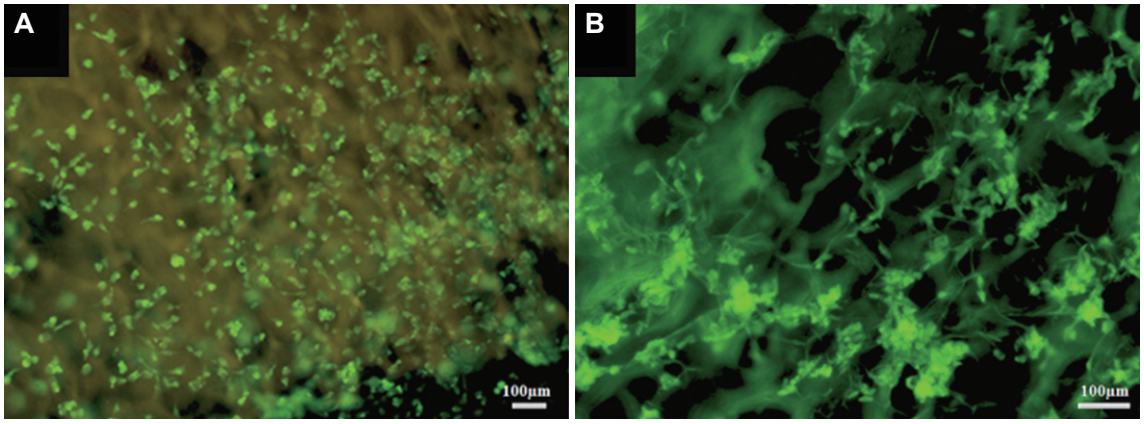

Figure 9. Fluorescence images of cell seeded CS:PVA 35:65 nanofibrous scaffold showing live cells stained green on the CS:PVA fibers at (A) 50× and (B) 100× magnifications. Scale bar=100 μm. CS: chitosan, PVA: poly(vinyl alcohol).

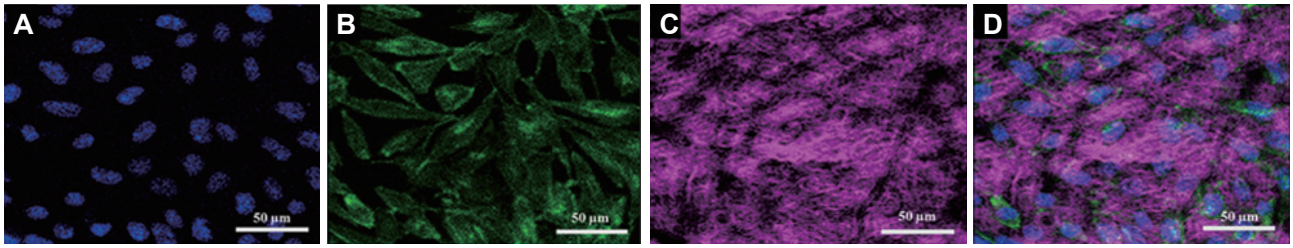

Figure 10. Confocal study of hMSC seeded CS:PVA (35:65) nanofibrous scaffold, showing cells spreaded on the scaffold surface with (A) nucleus stained blue, (B) cytoskeleton component stained green, (C) scaffold structure stained purple, and (D) compiled image of all frames. Image captured at 400×. Scale bar=50 μm. hMSC: human mesenchymal stem cell, CS: chitosan, PVA: poly(vinyl alcohol).

ECM is the major research area for various types of tissue regeneration. Nanofibrous scaffolds prepared by electrospinning provide good surface area for attachment and spreading of cells on the scaffold, which is an essential factor for tissue formation [10]. CS is a natural polymer that has intrinsic biological properties and has ability to bind to growth factors, thereby making it an ideal component for scaffold material [7,8]. In present study, CS and PVA solutions were blended in different ratios and CS/PVA nanofibrous scaffolds were fabricated by free liquid surface electrospinning technique. PVA is a biocompatible synthetic polymer. PVA was added to improve the viscosity of CS solution by interacting with CS through hydrogen bonding thereby rendering the polymer solution spinnable [28]. An advanced method of free liquid surface electrospinning was used in this study instead of conventional needle based electrospinning, since it provides nanofibrous mat formation in bulk amount leading to higher productivity [23]. The result for rheological property of the blend solutions is in good agreement with the study published by Paipitak et al. [34] reporting a linear increase in viscosity of PVA solution when blended with CS. From FESEM analysis of fiber morphology, it was observed that the fiber formation and diameter were influenced by blend ratio of the two polymers. The average fiber diameter increased with increase in viscosity of the CS/PVA blends. CS affects not only viscosity but also charge density at the surface of the ejected jet through its cationic polyelectrolytic property. It increases the charge density at the surface of the jet leading to increase in elongation force which ultimately decreases diameter of the generated fiber [35]. Surface roughness is an important property that facilitates cellular adhesion on the scaffold. The surface roughness profile of the blends by AFM analysis showed that the Ra values obtained for CS/PVA blends (35:65 and 30:70) were in the range reported by various researchers that signifies the different level of cellular attachment and proliferation [36-40].

The presence of CA and PVA components in the CS/PVA scaffolds after processing was confirmed by XRD and FTIR studies. The decrease in crystallinity of the electrospun CS/PVA blends is attributed to the hydrogen bond between CS and PVA macromolecules. The inter-molecular interaction is determined by FTIR spectra when two polymers are blended for nanofibers fabrication. The shifting of peak at 3440 cm^{-1} to lower wave numbers in the blends showed that PVA moderated the interaction between CS macromolecules and thereby improved the electro-spinnability of CS on blending with PVA [45].

The observed swelling behaviour of the CS/PVA scaffolds is in good agreement with the previous studies stating that CS decreases swelling rate when blended with PVA and the degree of reduction is dependent upon factors such as, weight ratio of components [47,48]. There is a wide variation in the values of contact angle which may be partly due to variation in blend composition and partly due to difference in surface roughness profile of the scaffolds as revealed by AFM study. Study by Vogler has shown that more hydrophilic surfaces (contact angle $\theta<60°$) do not necessarily possess high efficiency of cell attachment [51]. Among the prepared scaffolds, CS/PVA scaffolds of 30:70 and 35:65 ratio possess contact angles in desired range indicating their superior cell supportive properties which signifies their potentiality for tissue engineering applications [49,50]. Biodegradation is the process in which polymer mass is slowly reduced through solvation and depolymerization. It was observed that the degradation rate of CS/PVA scaffolds was much slower than PVA samples. This could be due to higher hydrogen bonding between PVA and CS that leads to slower depolymerization leading to prolonged biodegradation of CS/PVA scaffolds [48].

The *in vitro* cell scaffold interaction studies have shown that the prepared CS/PVA nanofibrous blends support hMSCs attachment, growth and proliferation as observed in FESEM study. The change in cellular morphology and appearance of interconnection indicates that the CS/PVA blends with 30:70 and 35:65 ratios provide an environment which is highly efficient for hMSCs proliferation. The quantitative analysis of cell metabolic activity by MTT assay indicated that all the blends showed a steady increase of absorbance representing the increased metabolic activity with culture period. However, among all the sets of experiment, maximum activity was observed with 35:65 ratio of CS/PVA scaffold and thus this ratio was used for further study. The immunofluorescence study showing green

fluorescence on the scaffold surface depicted the maintenance of high cell viability, which represents that the prepared scaffold were free from any adverse conditions that facilitated cell proliferation and growth on the scaffold. Thus the physico-chemical analysis and *in vitro* study proved that CS/PVA electrospun nanofiber scaffold with blend ratio 35:65 is superior to other CS/PVA blends and hence suitable for various tissue engineering applications.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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