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Fabrication of chitosan-based nanofibrous scaffold using free surface electrospinning for tissue engineering application

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Abstract. *Agrawal P, Pramanik K. 2017. Fabrication of chitosan-based nanofibrous scaffold using free surface electrospinning for tissue engineering application. Bioteknologi 14: 60-70.* Tissue engineering offers a promising approach for repair of defective tissues and organs. Developing scaffold from a variety of polymer blends or composites allows the adjustment of the properties aimed at duplicating required tissue. In recent years, considerable attention has been given to develop chitosan-based biomaterials for their applications in the field of tissue engineering due to its minimal foreign body reactions, an intrinsic antibacterial nature, biocompatibility, biodegradability, and the ability to be molded into various geometries and forms such as porous structures that are suitable for cell ingrowth and osteoconduction. The present work involves the preparation of nanofibrous mat from chitosan blended with other biopolymers such as silk fibroin, poly-vinyl alcohol, and polyethylene oxide by free surface electrospinning method. The morphology and functional characterization of the developed scaffolds was performed by SEM and FTIR studies. The average fiber diameter of 269 nm and 122 nm were obtained with chitosan/polyvinyl alcohol and chitosan/silk fibroin, poly ethylene oxide blends respectively. Crystalline nature of the scaffolds was confirmed by XRD studies. The scaffolds are also shown to have desired biodegradable and biocompatible properties. Chitosan-based polymeric scaffolds are thus proved to be potential materials for tissue engineering applications.

Keywords: biocompatibility, biodegradability, chitosan, free surface electrospinning, tissue engineering

INTRODUCTION

Bone and cartilage defects and lesions occur in a variety of clinical situations. Patients are treated in mainly three ways, namely by applying autograft, allograft, or xenograft. Each of these has inbuilt disadvantages, as there is always a chance that the grafted tissue may not work as expected in the patient. Allograft and xenografts have the additional problem of donor scarcity, disease transmission or contamination and immune rejection. Tissue engineering provides a lasting cure for this by offering a biocompatible and replaceable tissue having functional and mechanical integrity (Brahatheeswaran et al. 2011).

Tissue engineering involves scaffold designing including composition, structure, mechanical, biological, and physiochemical features analogous to natural bone and duplicating its extracellular matrix (ECM) (Li et al. 2002; Risbud and Sittinger 2002; Chang et al. 2015). Specific surface area and pore size are important for initial cell adhesion. Improved cell migration provided by scaffolds with pores above 300 microns is significant for scaffold, which was designed for bone or cartilage tissue growth. An added advantage of the larger pores is a reduction in cell aggregations that develop along the edges of the scaffolds. A study by Murphy et al showed that scaffolds with a mean pore size of 325 microns were optimal for bone tissue engineering (Gravel et al. 2006). By facilitating capillary formation, pores greater than ~300 μm lead to direct osteogenesis while pores smaller than ~300 μm can encourage osteochondral ossification. However, larger pores may compromise the mechanical properties of the scaffolds by increasing void volume. Scaffolds for osteochondral tissue regeneration should be non-immunogenic, non-toxic, biocompatible, and biodegradable. The scaffold should possess an interconnected and spread porosity (usually exceeding 90%) with a highly porous surface and microstructure. This would allow in vitro cell adhesion, ingrowth and reorganization and would provide the necessary space for neo-vascularization in vivo. The scaffold should have sufficient mechanical strength during *in vitro* culturing to maintain the spaces required for a cell's growth and matrix formation. Pore size and orientation are shown to influence the mechanical properties of chitosan (CS) scaffolds. Tensile testing of hydrated samples showed that porous membranes have greatly reduced elastic moduli (0.1-0.5 MPa) compared to non-porous membranes (5- 7MPa) (Ji et al. 2006). Moreover, a scaffold must provide sufficient temporary mechanical support and match the mechanical properties to the host tissue as closely as possible; to bear in vivo stresses and loading. It is possible to produce scaffolds with tailored physical, biological, and mechanical properties by combining bioabsorbable polymers and bioactive ceramic phases**.**

A many natural and synthetic polymers have been investigated previously. Chitosan has attracted attention of many researchers because of its characteristic as biodegradable, biocompatible, and non-toxic and thus it has

been believed as a safer material for use in biomedical applications (Hutmacher et al. 2001; Chen et al. 2002; Hutmacher et al. 2004; Cheung et al. 2007). Di Martino et al. (2005) found that CS possesses intrinsic antibacterial activity (Ji et al. 2006). Studies have shown that CS can reduce the infection rate of experimentally induced osteomyelitis by Staphylococcus aureus in rabbits. Its cationic amino group associates with anions on the bacterial cell wall, suppresses the biosynthesis, and disrupts the mass transport across the cell wall. Thus, it accelerates the bacterial death. Due to this antibacterial property, it has been mixed with other polymers in various biomedical related studies. CS has also been reported to be combined with a variety of delivery materials such as alginate, hydroxyapatite, hyaluronic acid, calcium phosphate, PMMA, poly-L-lactic acid (PLLA), and growth agents which are potentially applied in orthopedic tissue engineering.

In recent years, polymer blending has become a method for providing polymeric materials with desirable properties for practical applications. Chitosan blended with PVA has been reported to have good mechanical and chemical properties. Additionally, it has been studied in the biomedical field (Chen et al. 2002). The enhanced property has been attributed to the interactions between chitosan and PVA in the blend through hydrophobic side-chain aggregation and intermolecular and intra-molecular hydrogen bonds.

The other important factor is the fabrication method. While the fabrication of porous scaffold has been the choice of many researchers, the fabrication of scaffold from nanofibres generated by electrospinning is gaining importance in recent years. Electrospinning is a simple and easy way to control the morphology of ultrafine fibers. In this process, high voltage electric is used. The fibers produced by this method have some characteristics, such as very large surface-to-volume ratio and high porosity with a small pore size (Deitzel et al. 2001; Huang et al. 2003), pore distribution is irregular in the matrix. Therefore, there is a need for a systematic research effort to prepare electrospun nanofibres from polymeric blends of chitosan with other biopolymers.

The objectives of this research was (i) to prepare chitosan-based polymer blends of desired properties to develop tissue-engineered scaffold, (ii) to fabricate chitosan-based electrospun nanofibrous mat, (iii) to optimize key parameters of electrospinning process, (iv) To characterize the nanofibrous scaffold, and (v) to perform *invitro* study of cell scaffolding for biocompatibility and biodegradability were the aims of the research.

MATERIALS AND METHODS

Preparation of polymer blend

PVA was dissolved in distilled water (DW) at a concentration of 10 wt%, and chitosan was dissolved in acetic acid-water (AA-water) solution (2 wt%) at a concentration of 2 wt%. These solutions were mixed at different weight ratios of PVA/chitosan, i.e., 90/10, 80/20, 70/30, 65/35, 60/40 and 50/50 (5 mL each).

Preparation of chitosan-silk fibroin blends

Preparation of SF by degumming method

Silk Fibroin (SF) was obtained from *Bombyx mori* silkworm cocoons by the Degumming method, which includes cutting the cocoons into small pieces, cleaning and removing completely the traces of the silkworm and any other debris. The cocoons were then washed with distilled water and then boiled in 0.01 M sodium carbonate for 60 min; then they were washed under running distilled water thrice to remove sericin. After an overnight oven drying at 45°C, the resultant fibers were dissolved in 9.3 M Lithium bromide (LiBr) and heated at 50°C. LiBr residue was removed by dialysis process, using dialysis cassette (Thermo Scientific, slide-A-Lyzer 10K) against distilled water for three days, with the water being changed every 3h. The dialyzed solution was freeze-dried in a lyophilizer to obtain silk in dried powder form (now onwards referred as regenerated SF). The regenerated SF powder was kept in airtight container until it was needed.

Silk fibroin solution preparation and blending with CS

Regenerated silk fibroin powder was dissolved in the aqueous solution to form 1 wt% polymer solution. The solution was mixed and allowed to stir for 24 hours. CS/SF blend solutions were prepared by mixing CS and SF solutions in different ratios by volume (75:25, 50:50, 25:75 and 10:90) making final volume to 5 mL. These solutions were kept on magnetic stirrer overnight, after which they were electrospun.

Preparation of chitosan-SF-PEO blends

Polyethylene oxide (PEO) powder was added to the CS/SF blend solutions to modify them and enhance the fiber formation efficiency during electrospinning. Thus CS, regenerated SF and PEO powder was mixed in weight ratios 1:1:1, 2:1:1 and 2:2:1 (CS:SF:PEO) and stirred overnight , before being subjected to electrospinning.

Study of Rheological behavior of polymer blends

Prior to electrospinning, the viscosity of solutions was tested by Bohlin Visco 88 viscometer, manufactured by Malvern Instruments, U.K. To calculate viscosity, Moore Model was applied.

Preparation of nanofiber by electrospinning

Nanofibers were made by subjecting polymers to high voltage in electrospinning machine (Elmarco, Nanospider "NS Lab 200"). The samples were tested for fiber formation by keeping a drop of the sample on the sample space under various process conditions like changing electrode to collector distance (working distance), the voltage applied and electrode rotation speed. Those blend ratios which were able to form fibers were then electrospun in higher volumes for obtaining nanofiber sheets. The fibers were sorted and collected on the fabric after drying which was then stored for characterization.

Study of key electrospinning parameters

Ratio of polymers in the blend

Polyethylene oxide is added to the blend solution to make the CS:SF formulation electrospinnable. PEO is a synthetic polymer, thus its degradation and removal from the body is an issue when used in larger amounts. Lowering the amount of PEO will serve the purpose of decreasing immune reaction when the cell-scaffold construct is incorporated into the body.

Weight ratios of CS:SF:PEO were prepared to keep minimum possible ratio of PEO. CS:SF:PEO (1:1:0.4, 1:1:0.3, 1:1:0.2 and 1:1:0.1) solutions were prepared and kept for stirring overnight; then these were electrospun to check nanofiber formation.

Effect of process parameters

For testing optimum process parameters, the electrospinning was performed under various processing conditions, namely the applied voltage (working distance) and the speed of electrode rotation (rpm).

Characterization of nanofibrous scaffold

Morphology analysis

The SEM (Scanning Electron Microscopy) was used to evaluate the morphology and microstructure of the synthesized samples. The electrospun fiber samples were coated with a thin layer of platinum (Pt) and their morphologies were observed under a Scanning Electron Microscope (JEOL-JSM 6480 LV SEM), that was operated at the acceleration voltage of 15 kV. Images were taken at 5000X, 10000X and 20000X magnifications.

XRD analysis

The electrospun fibers were subjected to X-rays to obtain a X-ray diffraction (XRD) pattern to reveal detail information about the chemical composition and crystallographic structure of manufactured nanofibres. The instrument used for scanning was XRD- PANalytical and range were 10^{o}-50^{o} keeping the 2-theta step size.

FTIR analysis

Fourier Transform Infra-Red (FTIR) was used to characterize molecular structure of nanofibers. FT-IR analysis was based on the identification of absorption bands due to the vibrations of functional groups presented in macromolecules (Tangsadthakun et al. 2006).

To make a pellet, Nanofibrous polymer scaffold was grounded into a fine powder. A thin fiber sheet was then pressed in between the two KBr powder layers in the KBr press Technosearch instrument. This preparation was then pressed from 0-10 tons and then released. By this process, a pellet was formed. This pellet was then placed in IR-Prestige-21 to record the FTIR readings, and a plot of wavenumber (cm-1) versus percent transmittance (%T) is prepared.

Swelling ratio and water uptake capacity

The conventional gravimetric method was used to measure the equilibrium swelling ratio (Es). First, the dry weight (Wd) of the scaffold was measured and then, it was immersed in distilled water and incubated for 24h at 37^{o}C. The wet weight (Ws) of the scaffold was determined by weighing it after the excessive water from the immersion was blotted out with absorbent paper. The equilibrium swelling ratio of the scaffolds was defined as the ratio of weight increase (Ws-Wd) about the initial weight (Wd) of dry samples. Each value was averaged from three parallel measurements. Es was calculated using the following equation:

$$Es = (Ws-Wd)/Wd$$

And water uptake percentage (Wu) was measured using the equation:

$$Wu = (Ws-Wd)/ Ws \times 100$$

Mechanical strength testing

Sample preparation for tensile testing. Fiber sheets were cut into specific geometry, namely 20mm X 10mm, and a cardboard sheet was pasted at each end to provide support and grip of the clamp. The thickness of the sheets was measured using Digital Vernier Calpier (Absolute Digimatic, Mitutoyo).

Tensile test. Tensile strength of electrospun nanofiber sheets was measured using Universal Mechanical Tester. The fiber sample was stretched with a computer controlled Instron Electropulse E1000 to test its tensile strength. After a particular load and elongation, the sample breaks, and the program generates the result in the form of graph of Load v/s Extension. Depending on the fed information regarding dimensions of the sample and the generated raw data by the program, several parameters are also shown in the result: like load at break, Modulus, and tensile strength of the sample. To ensure a reliable result, the process was performed twice for each sample.

Biodegradation study

The scaffolds with dry weights noted were sterilized by immersing in 70% ethanol and then in stimulated body fluid (SBF) with pH 7.4 at 37°C. The SBF solution was replaced daily to ensure continuous degradation. Samples were removed from the medium, rinsed with distilled water and weighed in every 15 minutes for the first hour and then every 2h for 24h and then twice regularly for one month. 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 of weight loss was calculated using the following equation:

$$\text{Weight loss} = (Wi - Wf)/Wi \times 100$$

Where, Wi and Wf represent the initial and final weight of scaffolds, respectively.

***In-vitro* biocompatibility study**

To study the biocompatibility of electrospun nanofibres, the cells were seeded on the scaffold. Following steps were performed for this: (i) Scaffold sterilization- Electrospun

nanofibres were sterilized by immersing them in 70% ethanol for 1 h. (ii) Scaffold neutralization- Scaffolds were neutralized by washing them with PBS 3-4 times at regular intervals. The pH of the solution was also checked every time PBS was changed. When the pH was close to 7, the scaffold was neutralized. (iii) Cell preparation- Mesenchymal stem cells were trypsinized and suspended in DMEM and 10% FBS having broad-spectrum antibiotic was centrifuged to obtain individual cells in a suspension. (iv) Cell seeding- Cells were seeded on the sterilized nanofibers and kept for incubation at 37ºC for 72 hr.

RESULTS AND DISCUSSION

Preparation of polymer blend

Polymer solutions were mixed in different w/w and v/v ratios and stirred overnight on magnetic stirrer, to form clear blends, which were then characterized and processed to form nanofibres.

Study of rheological behavior of polymer blends

The viscosity of pure Chitosan was 0.317 Pa sec which increased several folds after being blended with PVA solution at various ratios, as shown in Figure 1. This is in line with the data published by Paipitak et al. (2011), who reported that a linear increase in viscosity of CS solution blending with increasing amounts of PVA. Blending CS with SF and PEO also showed increment in viscosity, as shown in Figure 1.

Experiments performed by Alhosseini et al. (2012) have established that the high viscosity increases the interaction of two polymers, mainly through hydrogen bonding, and decreases the effects of surface tension. This will result in formation of fibers with uniform morphology after electrospinning.

Study of key electrospinning parameters

Ratio of polymers in the blend

CS:SF:PEO blend solutions were prepared by keeping the minimum possible ratio of PEO and electrospun to check nanofiber formation. No fiber was formed for the blends containing less than 0.5 ratios of PEO. The result is shown in Table 1.

Effects of process parameters of electrospinning

The parameters studied for optimization are listed in Table 2.

Characterization of nanofibrous scaffold

Morphology analysis-Scanning Electron Microscopy (SEM)

CS/PVA nanofibres- Figure 2.A-J shows SEM micrographs of the electrospun CS/PVA nanofibers. An average fiber diameter of CS/PVA blend, weight ratio 90/10 was found to be 300 nm with a range of 240-349 nm. For blend ratios 70/30, 65/35 and 60/40, the average fiber diameter obtained was 282 nm, 264 nm, and 260 nm respectively. The trend of decrease in fiber diameter with decreasing PVA concentration in the blends was observed, and with the blend ratio 50/50 (CS/PVA) resulted in fibers with beads morphology (Figure 2.I-J).

CS/SF/PEO Nanofibres- Morphology analysis of CS/SF/PEO mixed scaffolds showed unaligned nanofibers formation as showed in Figure 3.A-D. Micrograph of ratio 1:1:1 scaffold at 20,000X magnification suggests that the diameter of the fibers was less various and was within the range of 122nm to 130nm. For combination ratio 2:2:1, the fiber diameter was found to be in the range of 120nm to 126nm.

In electrospinning, the fiber diameter is dependent on the viscosity and charge of the solution. It was discovered that fiber diameter increased as the viscosity rate increased. CS affects not only the viscosity, but also the rate density at the surface of the ejected jet through its cationic polyelectrolytic property. It increases the rate density on the surface of the jet, which in turn increases the elongation pressure and reduces the diameter of the fiber (Desai et al. 2009).

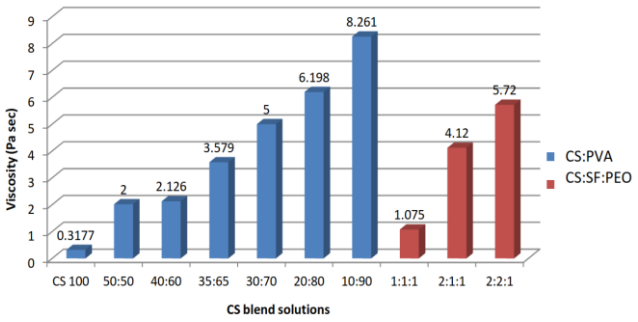

Figure 1. Viscosity measurement of CS blend solutions.

Table 1. Effect of composition of polymer blend on electrospinning.

CS:SF:PEO blend	Nanofiber formation
1:1:0.1	No
1:1:0.2	No
1:1:0.3	No
1:1:0.4	No
1:1:0.5	Yes
1:1:1	Yes

Table 2. Effect of Applied voltage, Working Distance, and electrode rotation on electrospinning.

Sample	Voltage applied (kV)	Working Distance (cm)	Electrode rotation speed (rpm)
CS:PVA	70	11.5	6.8
CS:SF	15 to 50	11.5	7.0
CS:SF:PEO	60	12.0	7.0

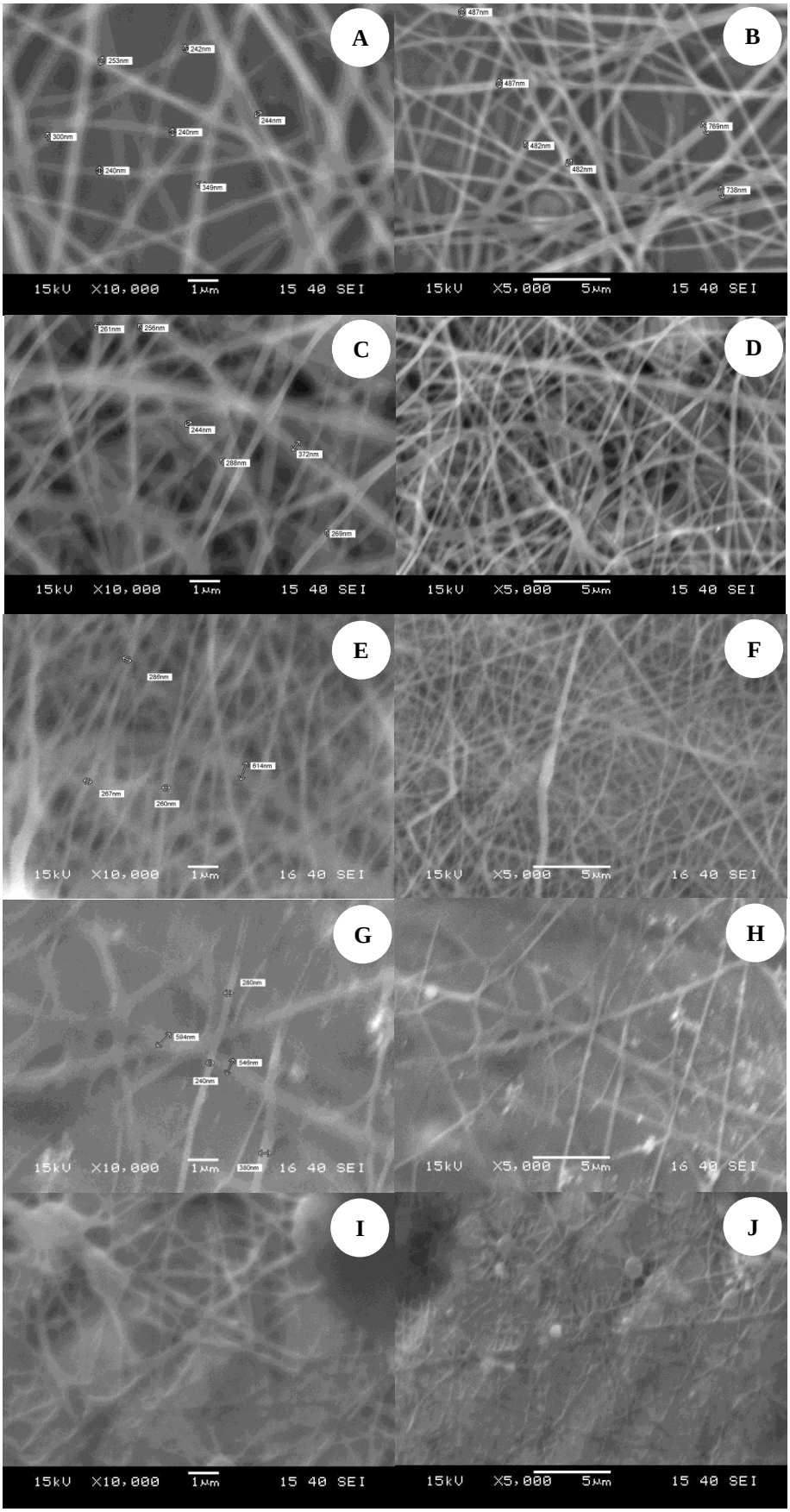

Figure 2. SEM micrograph of electrospun CS: PVA fibers of ratio 10:90 (A-B), 30:70 (C-D), 35:65 (E-F), 40:60 (G-H) and 50:50 (I-J).

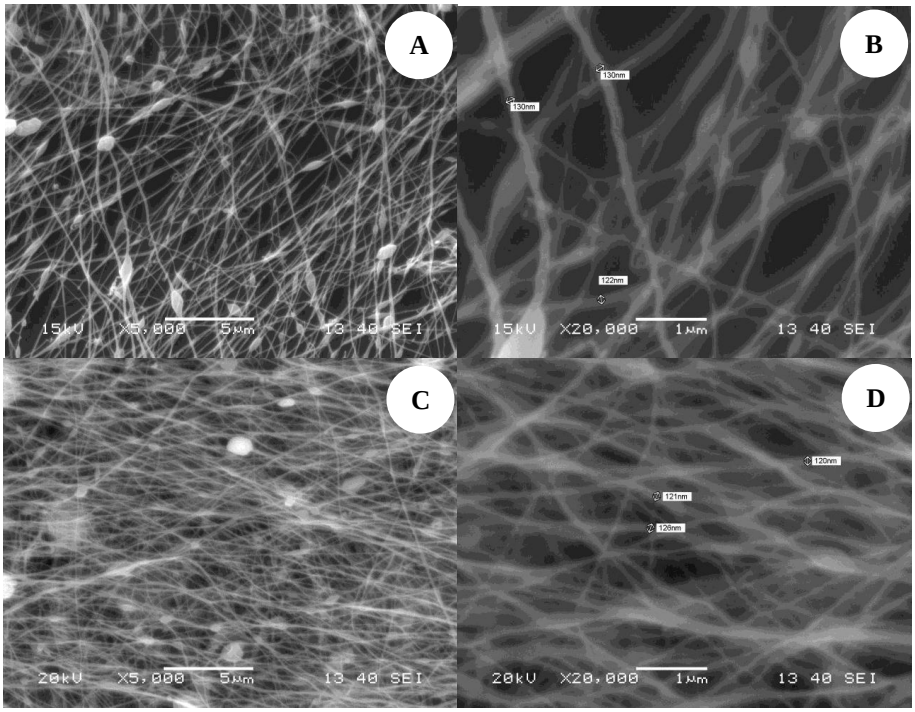

Figure 3. SEM micrograph of electrospun CS:SF:PEO fibers of ratio 1:1:1 (A-B) and 2:2:1 (C-D).

Table 3. Standard X-Ray Diffraction peak values for polymers used

Component	2 Theta value of	
	Major peak	Minor peak
Chitosan (CS)	19o	9.4o
Polyvinyl alcohol (PVA)	30o	48o
Silk Fibroin (SF)	20.4o	24.5o
Polyethylene oxide (PEO)	30o	40o

Phase analysis

X-ray diffractogram was obtained and shown in Figure 4 and Table 3. This was used for analyzing for accessing the crystallinity of the sample. Phase change during blend formation and electrospinning process were studied by XRD.

CS/PVA nanofibres- A dome-shaped curve at 20º in 35:65 (CS:PVA) sample depicted the presence of chitosan in it, while peaks at 30º and 48º confirmed the presence of PVA in all the prepared composites (Figure 4.A).

CS/SF/PEO Nanofibres- Rise at 20º confirmed the presence of chitosan in the sample and domes near 25º showed the presence of silk fibroin in the blends. Both the blends showed all the major and minor peaks of PEO in the X-ray diffractogram (Figure 4.B).

Hence, it was noted that the stage of the blends does not alter after processing, and the diffractogram also confirmed the availability of all the components in the blends along with their crystalline nature when processed into a scaffold by electrospinning. Crystallinity alludes to the degree of structural order in a solid. In a gem, the atoms or molecules are orchestrated in a regular, but by a regular, occasional way. Polymer shape crystalline regions have generally long lengths of molecules that usually prevent complete crystallization. Numerous polymers show semicrystalline behavior.

FTIR analysis

The inter-molecular interaction can be distinguished by FTIR, when two polymers are blended for nanofibres fabrication (Tangsadthakun et al. 2006). In the case of a CS-PVA blends and CS, SF and PEO composites used for electrospinning of nanofibers, the FT-IR analysis was based on the recognition of absorption bands concerned with the vibrations of functional groups present in macromolecules. FT-IR spectra obtained from pure chitosan, Chitosan/PVA, and pure PVA films are shown in Figure 5.A and for CS/SF/PEO films are shown in Figure 5.B.

For the spectrum of pure chitosan as seen in Figure 6, the characteristic absorption bands of chitosan were observed at six locations. The vibrations of hydroxyl and free amine groups appeared at 3439 and 3300 cm- 1, respectively. The absorption bands at 1655, 1560 and 1381 cm-1 indicated C=O stretching, -NH2 bending and C-O stretching of primary alcohol groups, respectively. The last one at 1152 cm-1 represented -C-O-C- glycosidic linkage between chitosan monomers (Miya and Iwamoto 1984).

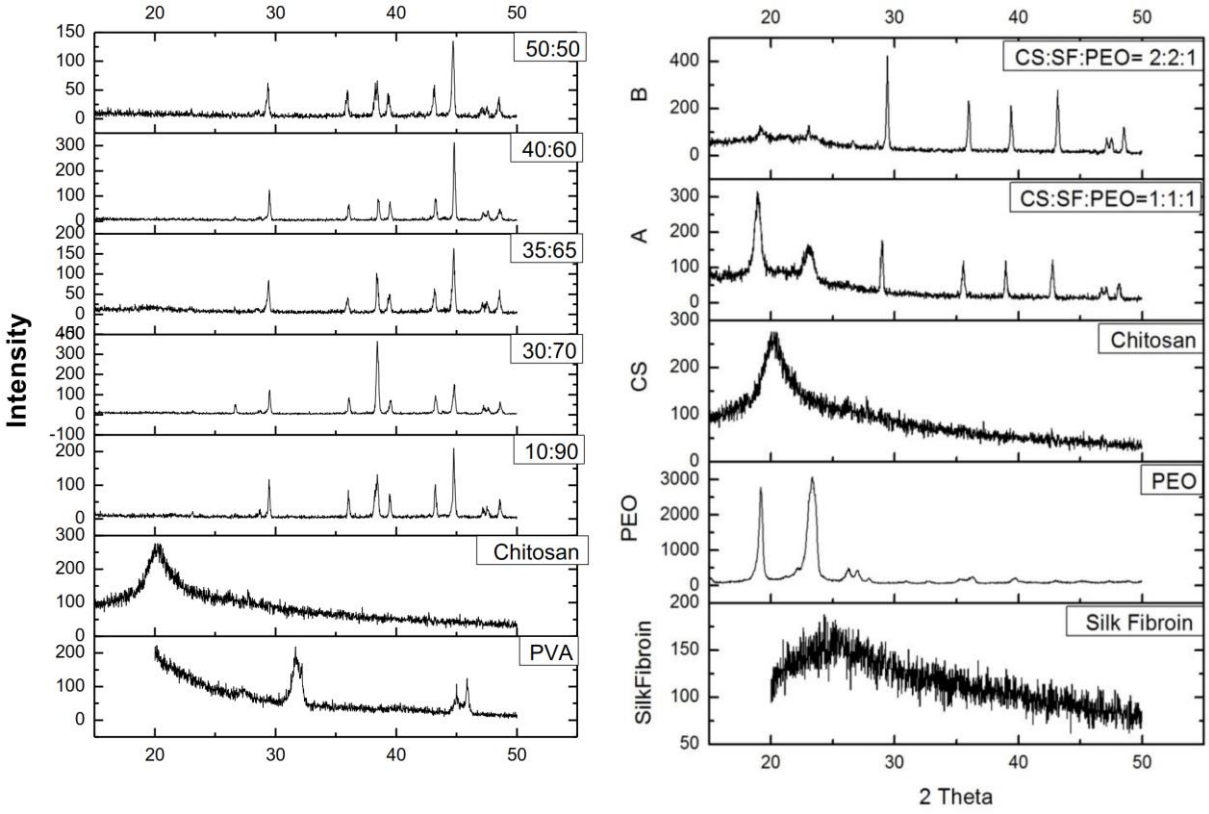

Figure 4. XRD analysis of electrospun CS:PVA blends (A) and CS:SF:PEO blends (B).

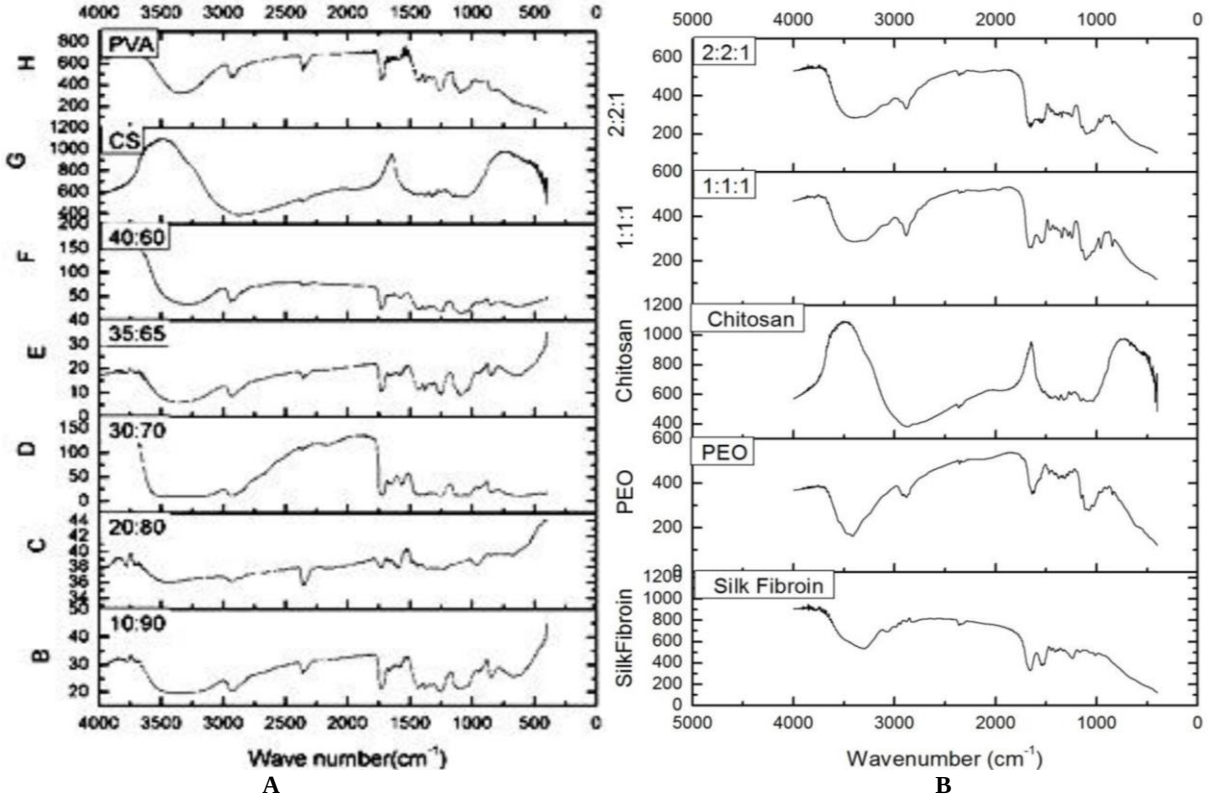

Figure 5. FTIR spectra of CS/PVA (A) and CS/SF/PEO (B) blends.

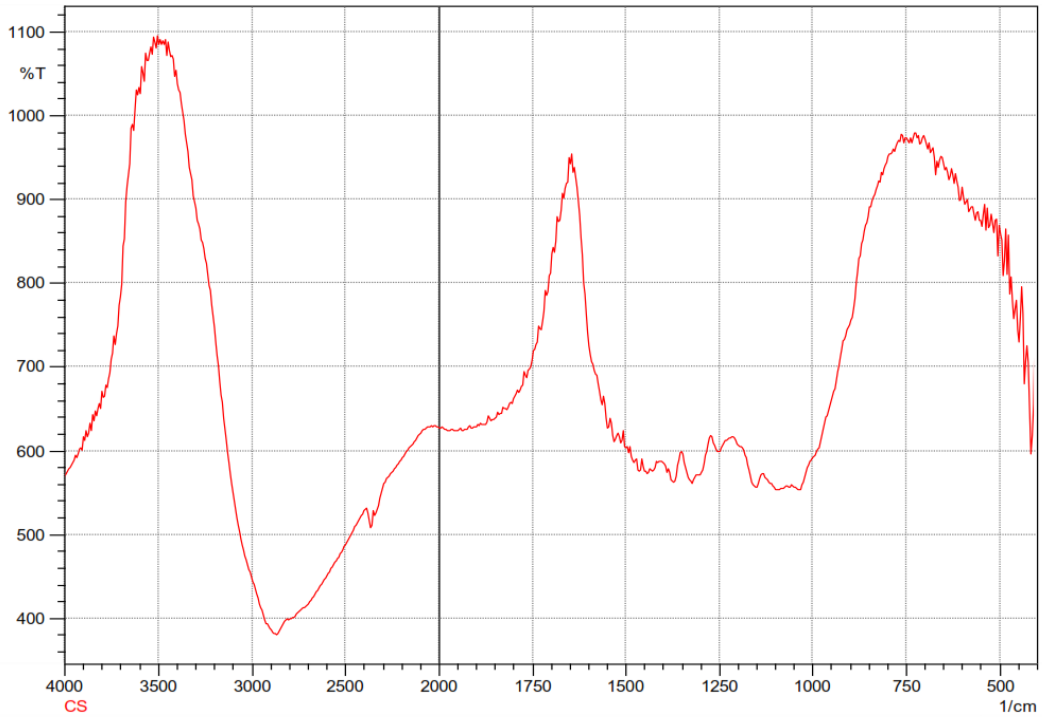

Figure 6. FTIR spectrum of Chitosan.

CS/PVA blends- In FTIR spectra of PVA, all major peaks related to hydroxyl and acetate groups were examined. Large bands between 3550 and 3200 cm-1 are linked to the stretching O-H from the intermolecular and intramolecular hydrogen bonds. The vibrational band between 2840 and 3000 cm− 1 refers to the stretching C-H from alkyl groups and the peaks between 1750-1735 cm− 1 are due to stretching C O and C-O from acetate group. The shift in the lower order of spectrum for the Chitosan/PVA blends is mainly due to primary alcohol and secondary alcohol interactions, which took place due to hydrogen bonding as it is earlier reported in studies of chitosan and PVA blends (Miya and Iwamoto 1984; Young et al. 1996).

CS/SF/PEO blend- FTIR spectra of pure SF illustrated four characteristic absorption bands for silk fibroin. At 700 cm− 1, it was due to amide-V group vibration while at 1260 cm− 1, it was due to amide III vibration and was presented in random coil of the structure. Amide II group present on beta-sheet conformation of SF showed absorption at 1525 cm− 1 position. While band at 1625 cm− 1 can be attributed to C O bond vibration or if the molecule is in β-sheet conformation, then this band depicts amide-I bond vibration. For the spectrum of pure PEO, various band locations that signify functional group are at 841 cm− 1 and 961 cm− 1 C-H2, O-C-O bond stretching is depicted at 1101 cm− 1 and band at 2891 cm− 1 was linked to C-H bond stretching.

Swelling ratio and water uptake capacity

CS/PVA blends- As shown in Figure 7, the swelling behavior of CS/PVA scaffolds with different ratios and time could be clearly distinguished. The blends containing CS less than 30% (w/w) showed good swelling. The other group of which the swelling ratios were as low as that of pure chitosan was the blends having chitosan composition more than 30%. This phenomenon can be attributed to the loss of gel-like structure after swelling. Water intake capacity of all the chitosan/PVA blends was approximately same, and the average value was 98.5576%.

CS/SF/PEO blends- Weight of CS/SF/PEO blends were found to be lesser than 30% of swelling. Water uptake percentage was also lesser than CS/PVA blends.

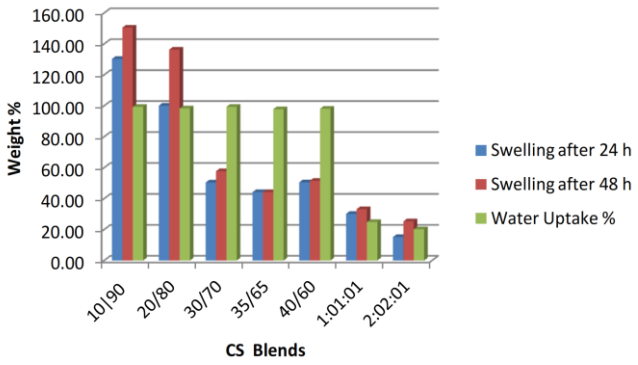

Figure 7. Swelling Ratio and Water Uptake Capacity of Chitosan composite scaffolds.

Mechanical testing

Due to the tiny dimension, the mechanical characterization of an individual nanofiber is a challenge for the existing test techniques. Figure 8 shows typical stress-strain curves of CS- composite nanofibers obtained by electrospinning for tissue engineering applications. Table 4 summarizes the tensile strengths obtained from different scaffolds composition at various loads. It was concluded that the tensile strength of the nanofibers largely depends on their geometry and composition.

CS/PVA blends- A trend of increase in tensile strength was in accordance with the increase in CS composition in the blends. The break at stretching which can be seen in each case, followed the trend made by non-uniform sheets. In the case of 40:60 nanofiber sheet, lesser thickness value (0.02mm) limited its tensile testing by this method.

CS/SF/PEO blends- Nanofiber sheets comprising the components in 1:1:1 ratio showed less tensile strength, and 2:2:1 composition of nanofiber sheet was so thin that its tensile testing could not be done by this method. It can be concluded that the presence of SF in the composite makes the fibers comparatively brittle, and thus decreases the tensile strength.

Non-uniform break on stretching can be explained by this; samples comprise of unaligned nanofibers, as revealed by SEM micrographs, thus the force gets distributed into many directions during stretching, while on the aligned fibers, the distribution of force is in a particular direction resulting into straight cut, as shown in Figure 9.

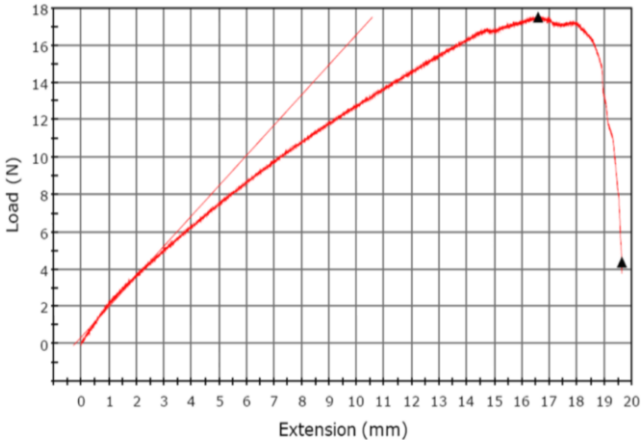

Figure 8. Stress-strain curve of CS composite Nanofibers.

Table 4. Tensile strength of composite scaffold at varying load.

Sample label	Sample thickness (mm)	Maximum load (N)	Tensile strength (MPa)	Load at break (N)	Tensile strain at break (%)	Modulus (kPa)
CS:PVA						
10:90	0.10	5.79	3.22	1.44	38.91	23408.32
20:80	0.30	13.98	4.66	4.77	109.52	7572.45
30:70	0.25	5.93	5.93	5.84	25.67	37687.91
35:65	0.31	18.44	6.15	10.49	83.44	12385.66
40:60	0.02	--	--	--	--	--
CS:SF:PEO						
1:1:1	0.055	2.89	5.79	2.77	1.36	425990.53
2:2:1	0.02	--	--	--	--	--

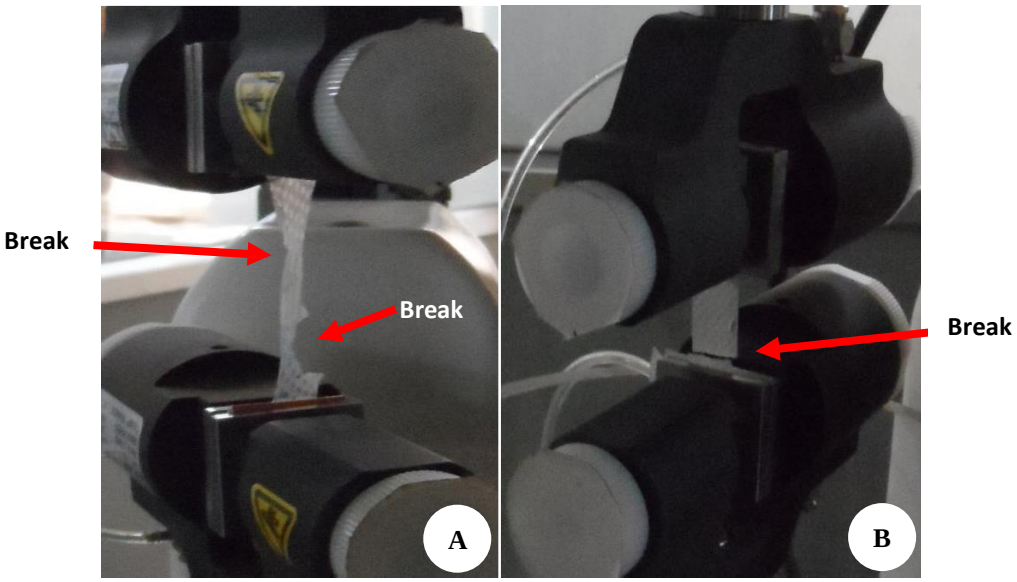

Figure 9. Stretching and break of un-aligned (A) and aligned (B) fiber sheets

The developed strategies and standards for figuring out the mechanical behavior of conventional fibers are insufficient in the case of manipulating or testing of nanofibers. It has been found that tensile strength of nanofibrous mat became less than that of cartilage, which should be near 40 MPa. This experiment was executed on non-aligned fibers, for fiber orientation plays an important role in determining tensile strength of any material. Fibers show good tensile strength when it is pulled along the path of fibers. Consequently, an attempt to form properly aligned nanofibers might comply the desirability in attaining the favored mechanical strength (Figure 10).

Biodegradation

The biodegradation results are shown in Figure 11 and 15.

CS/PVA blends- PVA scaffolds incubated in SBF had the highest weight reduction and were absolutely degraded after 30 mins. However, the addition of chitosan decreased the degradation of scaffolds in SBF solution. Concerning the stableness of scaffolds which was higher than that of pure PVA scaffolds, the obtained results proved to be the crucial feature, for it is known that the degradation rate of PVA scaffolds can be quick. Therefore, the addition of chitosan could extend the biodegradability of scaffolds. Most scaffolds showed complete degradation within 30 days of observation. While the blend ratio of 30:70 and 35:65 (CS: PVA) was disintegrated into smooth smaller pieces , of which weighing became difficult beyond 30 days.

CS/SF/PEO blends- Scaffolds were incubated in SBF, and it gave an understanding that degradation commenced immediately on the first hour and then there was no considerable change for the remaining 24hr. Their degradation was resumed after that and followed by weight loss, and the scaffolds were completely degraded on sixth day of degradation study.

I*n-vitro* biocompatibility study

Cells did not connect to the scaffold surface. Cell development was not observed in the arranged scaffolds. The media might lack adequate growth factors for cell attachment, growth, and proliferation. The scaffold might not have been neutralized since the utilization of acetic acid as solvent has made the formulation more acidic and thus it made the formulation unsupportive for cellular growth.

In conclusion, chitosan was mixed with PVA and SF polymers. The polymer mixes were effectively electrospun to manufacture CS/PVA and CS/SF/PEO nanofibrous platforms. The CS/SF/PEO scaffolds have been found to show superior physicochemical properties, compared with CS and CS/PVA platforms. Efforts to move forward mechanical properties of CS-based composites are basic for its application in bone tissue building. Cell study has affirmed that these mixes have beneficial biological properties, like biocompatibility and biodegradability. The spreading of cells on the scaffold surface was a bit nonuniform, as studied by preparatory cell culture research for which detail study is required to indicate their use for cell types. Chitosan can be mixed with numerous polymers as executed in present research; its mixing with ceramics can be studied to manufacture a more potent material which could be used in tissue engineering for precise tissues or cell types. Detail in vitro cell growth is required to ascertain the scaffold for precise tissue regeneration (Table 5).

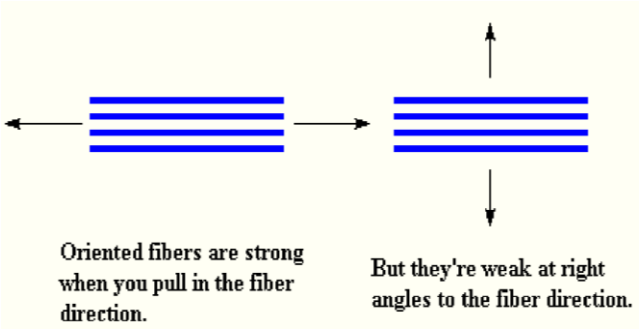

Figure 10. Relation between fiber orientation and tensile strength.

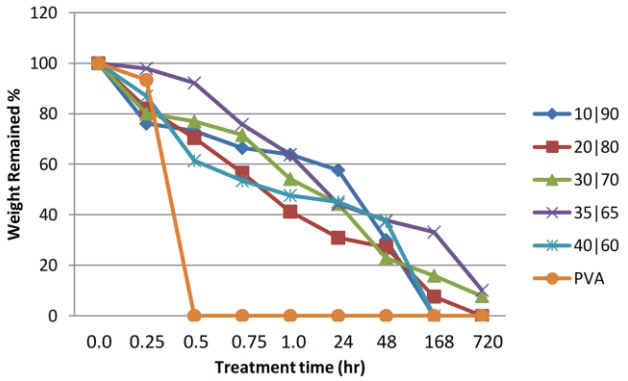

Figure 11. In vitro biodegradation of CS/PVA scaffolds with different blending compositions.

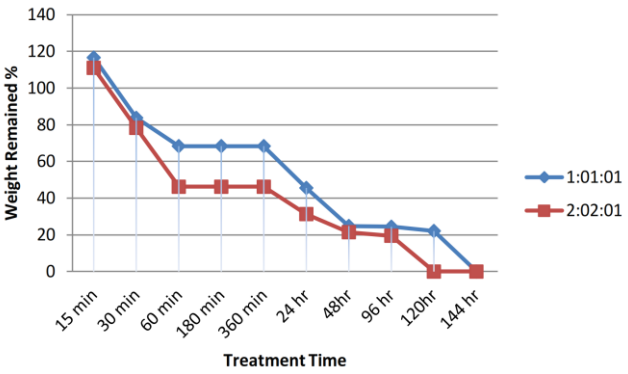

Figure 12. In vitro biodegradation of CS/SF/PEO scaffolds with different blending compositions.

Table 5. Combined results for various characterization techniques

Characterization Technique	Property of comparison	CS/PVA blends	CS/SF/PEO blends	Remarks
Rheology behavior	Viscosity Measurement	2 Pa sec - 8.261 Pa sec	1.075 Pa sec - 5.72 Pa sec	Increasing with increasing ratio of PVA
Morphology Analysis (SEM)	Fiber Diameter (Average)	300 nm	120 nm	Addition of SF results into finer nanofibers
X-ray Diffraction (XRD)	Diffraction pattern and phase change	No phase change	No phase change	Composites contain the components blended prior to electrospinning
FTIR	Functional group detection	Present	Present	Composites contain functional groups of their pure form components
Swelling Ratio	Swelling in weight %	Good swelling observed (~ 88.03%)	Lesser swelling (~29.202%)	Best result for CS/PVA 10:90 and 30:70
Water Uptake capacity	Water uptake Percent (Hydrophilicity)	(98.5576%)	Lesser uptake (22.53%)	CS/SF/PEO blends are less hydrophilic than CS/PVA blends
Mechanical Testing	Tensile strength	3.22- 6.15 MPa Average 4.99 MPa	5.79 MPa	Nanofibers formed by electrospinning possess considerable load-bearing capacity
Biodegradation	Weight loss in SBF wrt time	Complete Degradation after 30 days	Complete degradation in 6 days	Weight loss rate of CS/PVA scaffold is lower than that of CS/SF/PEO

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