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Evaluation of poly(L-lactide) and chitosan composite scaffolds for cartilage tissue regeneration

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ABSTRACT

The present study delineates the development of chitosan and poly(L-lactide) (PLLA) scaffolds cross-linked using a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), n-hydroxysuccinimide (NHS), and chondroitin sulfate (CS) for cartilage tissue engineering applications. Chitosan and PLLA were varied in concentration for developing scaffolds and prepared by freeze-drying method. The various scaffolds were studied using scanning electron microscopy (SEM), porosity by mercury intrusion porosimeter, and the molecular interactions among polymers using Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) studies. Differential scanning calorimetry (DSC) was used to predict the thermal properties of the scaffolds. The mechanical properties of the scaffolds were studied using static mechanical tester. The ability of the scaffolds to support chondrocyte proliferation was also studied. The microscopy suggests that the pore size of the scaffolds varied with the composition in the range of 38–172 μm and the porosities in the range of 73–93%. The XRD and the FTIR studies suggested that an alternation in the composition of the scaffolds altered the molecular interactions among the scaffold components. An increase in the chitosan content enhanced the swelling property. The degradation of the scaffolds was least when the proportion of chitosan and PLLA was in the ratio of 70:30. The *in vitro* cell proliferation study suggested that the developed scaffolds were able to support chondrogenesis, the glycosaminoglycan (GAG) content of the mature chondrocyte was 40 $\mu\text{g}/\text{ml}$ and the viability was approximately 90%. Hence, the so designed scaffolds may be tried for cartilage tissue engineering applications.

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PLLA; scaffold; freeze-drying; chondrocyte; chondrogenesis; glycosaminoglycans

Introduction

In the last two decades, the research on tissue engineering has exponentially increased. The field of tissue engineering allows replacement of damaged and injured tissues using a combination of material constructs and mammalian cells.[1] This helps in repairing and/or regeneration of the damaged and/or injured tissues. The constructs are used as a substitute for extracellular matrix (ECM) and act as a support for the cells.[2] These constructs are often regarded as scaffolds, which are technically defined as porous constructs allowing cell adhesion and growth.[3] The scaffolds may also support healing of the damaged or injured tissues by promoting cell differentiation in addition to cell adhesion and proliferation.[4] The scaffolds are of metal, ceramics, or polymers composites. Among these, polymeric scaffolds are the most commonly studied and evaluated for tissue engineering applications.[5,6] This may be attributed to the ease of handling and availability of various fabrication techniques, wide range of polymers

available with different chemistries, and existence of polymers with different physical properties. Blending of different polymers allows tailoring the physical properties of the scaffolds.[7,8] The polymers used for the fabrication of scaffolds are broadly classified either as synthetic or natural polymers. Some of the commonly used synthetic polymers are polylactic acid (PLA), poly(L-lactide) (PLLA), poly(D, L-lactide-co-glycolide) (PLGA), polyglycolic acid (PGA), and polycaprolactone (PCL), whereas, the natural polymers include collagen, chitosan, sodium alginate, gelatin, and pectin.[9,10]

In the current study, PLLA-and chitosan-based hybrid scaffolds were prepared. Both PLLA and chitosan are highly biocompatible and biodegradable.[11] Biodegradable polymers allow the resorption of the polymers as the cells grow to form a tissue. In addition to the above properties, chitosan has been reported to have antibacterial and antifungal activities.[12,13] This provides additional advantage during tissue repair by keeping the adjacent area sterile. Additionally, the

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Table 1. Composition of the scaffolds.

Sl. No.	Volume of chitosan solution (ml)	Volume of PLLA solution (ml)	Volume of cross-linking reagent ^a (ml)	Remarks
F1	20	0	50	Scaffold formed
F2	16	4	50	Scaffold formed
F3	14	6	50	Scaffold formed
F4	10	10	50	Scaffold formed
F5	6	14	50	Scaffold formed
F6	4	16	50	Scaffold formed

^aCross-linking reagent (ml): EDC, NHS, and chondroitin sulfate.

chemical structure of chitosan nearly resembles the chemical structure of GAG, a natural biopolymer found in tissues and ECM.[14] It has been reported to promote cell adhesion, growth, and subsequent differentiation. Chitosan-based scaffolds have been extensively studied for bone and cartilage tissue engineering applications.[15] The application of chitosan in cartilage tissue engineering is due to its ability to electrostatically attract GAGs.[16] Apart from this, chitosan-based polymeric constructs were found to sufficiently adhere to the cartilage surface and have been used for the rectification of the chondral articular defects.[17,18] To evaluate the effect of variation of chitosan and PLLA on the scaffold properties, the proportion of chitosan and PLLA was varied in this study. The preliminary study conducted in our lab suggested that cross-linking of the chitosan–PLLA hybrid scaffold using chondroitin sulfate-EDC-NHS cross-linking reagent improves the stability of the scaffolds as compared to the uncrosslinked one. Hence, the scaffolds were prepared by freeze-drying method and subsequently cross-linked using CS-EDC-NHS reagent. The developed scaffolds were characterized using scanning electron microscopy (SEM), porosimeter, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), DSC, and static mechanical tester. *In vitro* swelling and degradation properties of the scaffolds were also studied. The ability of the scaffolds to promote cell growth was studied *in vitro* by MTT assay, GAG quantification, and imaging the cell-scaffold constructs using scanning electron microscope.

Experimental details

Materials

Poly(L-lactide) (PLLA, MW: 1.0×10^5 Da) was a gift from PURAC, Gorinchem, Netherland. Chitosan, chondroitin sulfate, sodium chloride (NaCl), lysozyme, N-hydroxysuccinimide (NHS), 2-(N-morpholino) ethanesulfonic acid buffer (MES Buffer), trypan blue, dimethyl sulfoxide (DMSO), glutaraldehyde, and hexamethyldisilazane were obtained from Himedia, Mumbai, India. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was obtained from Thermo Scientific, Mumbai, India. Ethanol was obtained from Changshu Yangyuan Chemical, China. Acetic acid and chloroform were obtained from Merck Ltd. Mumbai, India. Dulbecco's modified eagle's

medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), fetal bovine serum (FBS), antibiotic antimycotic solution (100×), 5-[dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), collagenase (type II), 1,9-dimethyl methylene blue (DMMB), and trypsin were obtained from Sigma Aldrich Chemical, Mumbai, India. Double distilled water was used throughout the study. All the reagents were used as received.

Scaffold fabrication technique

Two percent (w/v) chitosan solution was prepared by dissolving 2 g of chitosan in 98 ml of 2% (v/v) acetic acid solution. Similarly, 2% (w/v) PLLA solution was prepared in chloroform. The solutions of chitosan and PLLA were mixed in different proportions (Table 1). Twenty milliliters of the mixture was poured in 25-ml cylindrical polystyrene bottles (inner diameter: 30 mm; height: 60 mm). The polystyrene bottles were stored in a 20 °C refrigerator for 24 h. After the end of specified period, the polystyrene bottles were put in the freeze dryer (Free zone 2.5 Freeze Dryer, Labconco, USA). The process of freeze-drying was carried out for 48 h at a temperature of 50 °C. The uncrosslinked scaffolds, so obtained, were immersed in 50 ml of cross-linking reagent. (The cross-linking reagent was made by dissolving 2 g of EDC, 1 g of NHS, and 0.2 g of 2% (w/v) aqueous solution of chondroitin sulfate in 80 ml of 0.1 M MES buffer. The final volume of the solution was made to 100 ml using 0.1 M MES buffer. This solution was used as the cross-linking reagent in this study. From the prepared stock solution of 100 ml of cross-linking reagent, 50 ml of the solution was taken for the cross-linking process.)

The scaffolds were allowed to cross-link for 24 h at room temperature (25 °C). The system was continuously agitated at 300 rpm in a shaking platform. Thereafter, the scaffolds were retrieved. The retrieved scaffolds were thoroughly washed in 50% hydroethanolic solution and 500-mmol NaCl solution. The washed scaffolds were allowed to dry in a closed chamber at room temperature for 48 h. Thereafter, the scaffolds were dried in vacuum (1.5 mBar) at 40 °C for 12 h. The dried scaffolds, so obtained, were collected, put into self-sealable polyethylene bags, and were stored in a desiccator for further characterization and use.

Scanning electron microscopy (SEM)

Scaffolds were cut into rectangular pieces having dimensions of 5 mm × 5 mm × 1 mm (L × B × H). The scaffold pieces were coated with gold by sputter coating technique. The coated scaffolds were then used for morphological analysis under scanning electron microscope (Quanta 200 F, FESEM, USA).

Porosity measurement

The porosity of the scaffolds was analyzed using mercury intrusion porosimeter (Porosimeter, Micromeritics, Model 9310, USA). The Washburn equation was used to calculate the percentage porosity of the scaffolds (Equation (1)).^[19] The mean pore sizes of the scaffolds were calculated by analyzing the SEM pictures using ImageJ software.

$$D = (1/P)4\gamma(\cos \varphi) \quad (1)$$

where D , pore diameter; P , applied pressure; γ , surface tension of mercury; φ , contact angle between mercury and the pore wall.

Fourier transform infrared spectroscopy

The chemical interactions among the components of the scaffolds were tested using FTIR by KBr pellet method (FTIR-8400S, Shimadzu, Japan).

X-ray diffraction

The X-ray diffractograms of the scaffolds were obtained from X-ray diffractometer (Rigaku-Ultima IV, X-ray diffractometer, USA) using Cu-K α radiation. The scanning was done in the 2θ range of 10–80° at scan rate of 2° 2θ per/min. The X-ray source was operated at 30 kV and 30 mA.

Differential scanning calorimetry

The thermal properties (DSC-200 F3 MAIA, Netzsch, Germany) of the scaffold were determined by heating the scaffolds in the temperature range of 25–200 °C at a heating rate of 2 °C/min. The scanning was done under nitrogen environment.

Mechanical analysis

The mechanical properties of the scaffolds were determined by compression and stress relaxation studies using a static mechanical tester (Stable Microsystems, TA-HDplus, UK). The scaffolds of cylindrical shape (diameter = 2 cm, height = 2 cm) were used for the studies. A 30-mm flat probe was used for the studies. Compressive strength of

the scaffolds was determined by compressing the scaffolds to 15 mm at rate of 1 mm/s. The stress relaxation properties were determined by compressing the scaffolds by a distance of 5 mm at a rate of 1 mm/s. After attaining the said distance, the probe was allowed to stay at the same position for 60 s. The decrease in the force profile was recorded and analyzed.

Swelling study

The swelling property of the scaffolds was measured in DPBS (pH 7.4) (Equation (2)). Scaffolds of cylindrical shape having dimension of 2 cm × 2 cm (diameter × height) were used for the study. The initial weights of the scaffolds (W_0) were accurately weighed. The scaffolds were immersed in 100 ml of DPBS (37 °C). The setup was then put into a temperature-controlled cabinet (37 °C). At regular intervals of time, the scaffolds were fetched out from DPBS, wiped with filter paper, and subsequently weighed (W_1). The study was conducted for 24 h.

$$\text{Swelling percentage \%} = \frac{W_1 - W_0}{W_0} \times 100 \quad (2)$$

where, W_0 and W_1 are the initial and final weights of the scaffolds, respectively.

In vitro degradation study

In vitro biodegradation test of the scaffold was done by immersing the scaffolds in DPBS solution containing lysozyme (10,000 µg/ml). The scaffolds having dimension of 2 cm × 2 cm (diameter × height) were immersed in 100 ml of the lysozyme solution. The setup was incubated in a temperature-controlled cabinet, maintained at 37 °C. At regular intervals of time, the scaffolds were taken out from the media, washed thoroughly with water, and subsequently freeze-dried. The weights of the freeze-dried scaffolds were noted down and the percentage decrease in the weight was calculated (Equation (3)).

$$\text{Weight loss \%} = \frac{W_0 - W_2}{W_0} \times 100 \quad (3)$$

where, W_0 and W_2 are the weights before and after the degradation study, respectively.

Biopsy collection and isolation of chondrocytes

Articular cartilage was isolated from the knee joint of a 24-month-old white rabbit by creating a defect of 3–4 mm in the lateral condyle under aseptic condition. (Articular cartilage was collected from Animal House, IMS, BHU and

the study was approved by the Ethical Committee of the Department of Orthopedics, IMS, BHU, Varanasi, India.) The tissues were carefully washed in DPBS and further minced into small pieces using tissue homogenizer. The small pieces of the tissue were treated with 10 ml of 0.25% trypsin, dissolved in DMEM for 24 h in a shaking flask, under humidified 5% CO₂ environment. This was followed by digestion with collagenase (type II) at 37 °C for 12 h in an agitator. After complete digestion, the solution was filtered through a 70-µm sterile nylon mesh and the filtrate was subjected to centrifugation at 3000 rpm for 10 min. After centrifugation, the supernatant was discarded and the cell pellet was dissolved in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic solution. Primary cell culture was done by maintaining the cell in 'T-75 flask' under humidified environment (5% CO₂). The culture medium was replaced after every two days and the cells were allowed to grow. The confluent monolayer of chondrocytes was obtained in 2 weeks of chondrocyte culture. The cell count and the viability test were done using haemocytometer and trypan blue dye test. Further, the cells were seeded on the scaffolds having a concentration of 1×10^6 cells per ml by static method. Before cell seeding, the scaffolds were sterilized by 70% ethanol.

Proliferation assay

Cell viability and functional activity of the porous scaffolds were determined by MTT assay. Cell-seeded scaffolds were incubated under humidified environment at 37 °C containing (5% CO₂) for 3, 5, and 7 days according to the method reported earlier.[20] Twenty microliters of MTT solution (5 mg/ml) was added to the cell suspension in 96U wells sterile tissue culture plate and mixed gently. Further, the plate was incubated for 4 h at 37 °C. To dissolve the formazan, 100 µl DMSO was added to each well plate and the absorbance of the colored solution was measured at 570 nm in a microplate reader (2030 multi label reader victor X3, Perkin Elmer, USA).

Cell attachment study

The morphology of the cell-seeded scaffolds was visualized under SEM (Quanta 200 F, FESEM, USA). The scaffolds, PLLA, and chitosan in various combinations were rinsed with DPBS and fixed with 2.5% (v/v) glutaraldehyde in 0.1 M DPBS. Thereafter, the scaffolds were dehydrated in ethanol and rinsed carefully with hexamethyldisilazane.

GAG quantification

GAG quantification was done for the cell-seeded F3 scaffold in 250 ml of shake flask. The flask was assayed twice

using the mixture of 1,9-dimethyl methylene blue solution (1 ml) and trypsin solution (30 µl), the optical density was measured at 570 nm and evaluated with the standard curve established by chondroitin sulfate.

Statistical analysis

The data are presented as mean $\pm$ standard deviation. ANOVA add-in of MS Excel was used for the statistical analysis of the results.

Results and discussion

Physical evaluation of the scaffolds

The prepared scaffolds appeared as spongy materials. The scaffolds were brownish-white in color. The brown color may be associated with the color of the chitosan, whereas, the whitish tinge is associated with the PLLA. An increase in the PLLA proportion within the scaffolds resulted in the increase in the whitish tinge. The scaffolds with higher proportion of chitosan were comparatively firmer as compared to the scaffolds with lower proportion of chitosan. In other words, an increase in the PLLA proportion decreased the apparent firmness of the scaffolds. The specific odor of acetic acid was absent from the scaffolds. The scaffolds are shown in Figure 1(a)–(f).

Scanning electron microscopy

The microstructure of the scaffolds was observed under SEM and has been presented in Figure 1(g)–(l). F1, F2, F3, and F4 showed the presence of interconnected pores, which were distinctly visible. Further increase in the PLLA proportion in F5 and F6 resulted in the formation of the microstructures, whose pores were nearly blocked. The micrographs suggested that an increase in the PLLA content drastically reduced the size of the pores. The distribution of the pores within the scaffolds varied significantly. [21,22] The shapes of the pores were not uniform (irregular shaped).

Porosity measurement

The porosity of the scaffolds increased with an increase in the chitosan proportion in the composites. An increase in the PLLA proportion resulted in the monotonous decrease in the pore size.[23] The average porosity of the F1, F2, F3, F4, F5, and F6 was $93\% \pm 2.26$, $88\% \pm 2.92$, $85\% \pm 3.21$, $83\% \pm 1.88$, $76\% \pm 2.21$, and $73\% \pm 2.65$, respectively (Figure S1). The average size of the pores of F1, F2, F3, F4, F5, and F6 was 56–172, 48–139, 54–158, 52–145, 41–116, and 38–108 µm, respectively. This characteristic increase in

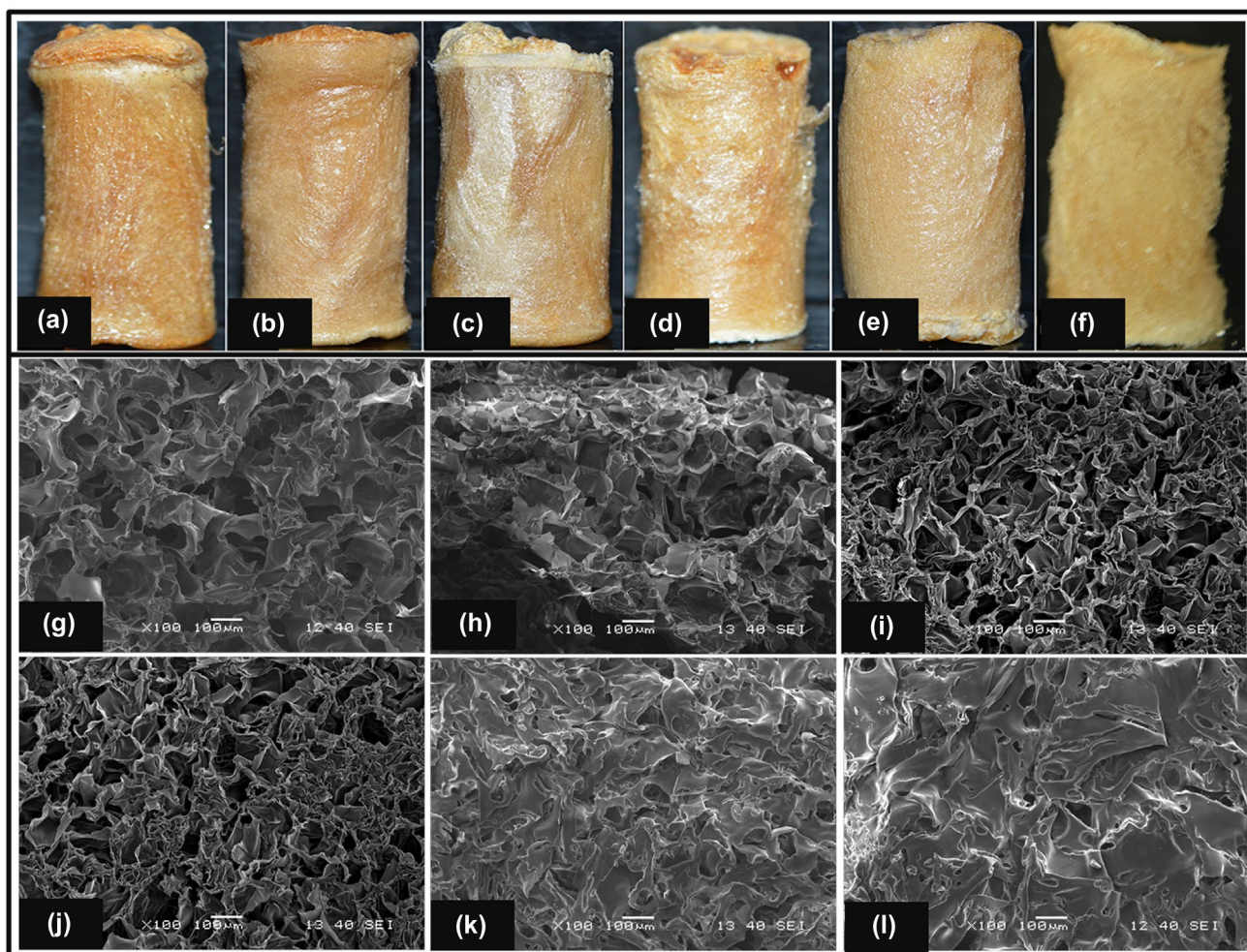


Figure 1. Pictographs of the scaffolds. (a) F1, (b) F2, (c) F3, (d) F4, (e) F5, and (f) F6 and scanning electron micrographs of the scaffolds (g) F1, (h) F2, (i) F3, (j) F4, (k) F5, and (l) F6.

the porosity is quite desirable, as it might cause an increase in the cell attachment in the scaffold.

Fourier transform infrared spectroscopy

The analysis of the functional group (present within the component of the scaffolds) and the chemical interactions among them was studied using FTIR spectroscopy. The scaffold showed a broad peak at $\sim 3500\text{ cm}^{-1}$. This peak may be explained by the presence of OH and NH stretching within the chitosan molecules and OH stretching of the PLLA molecules. F1 scaffold showed dual peak at ~ 1550 and $\sim 1650\text{ cm}^{-1}$, which may correspond to amide I and amide II peaks, respectively.[24,25] Introduction of PLLA within the scaffolds resulted in the shifting of the above-mentioned peaks to higher wave numbers (~ 1590 and $\sim 1735\text{ cm}^{-1}$). [26] An increase in the PLLA concentration resulted in the increase in the intensity of these peaks. The presence of dual peak at 2944 and 3000 cm^{-1} may be explained by C–H stretching of the CH and the

CH_3 groups of the PLLA molecules. These peaks were more predominant when the proportion of PLLA was increased. [27] However, at lower proportions of PLLA, these peaks were not resolved (Figure 2(a)–(f)).

X-ray diffraction

The XRD diffraction of the scaffolds is shown in Figure 2(g)–(l). The different peaks, which were overlapping in the diffractograms, were resolved using Origin (2015) software. F1 showed three distinct peaks at $\sim 11.5^\circ$, $\sim 18.5^\circ$, and $\sim 22.8^\circ$. Since F1 is chitosan-alone scaffolds, these three peaks may be explained by the presence of chitosan.[28] Incorporation of PLLA in F2 resulted in the disappearance of the peak at $11.5^\circ 2\theta$. Instead, a new peak at $17^\circ 2\theta$ was observed. The other two peaks were observed in F2, but there was a slight shift of the peaks toward higher diffraction angles. The appearance of the peak at $\sim 17^\circ 2\theta$ was due to the PLLA polymer. In F3, F4, F5, and F6, the peaks at $\sim 17^\circ 2\theta$ and $\sim 18.5^\circ 2\theta$ were only observed. The peak at $\sim 23^\circ 2\theta$

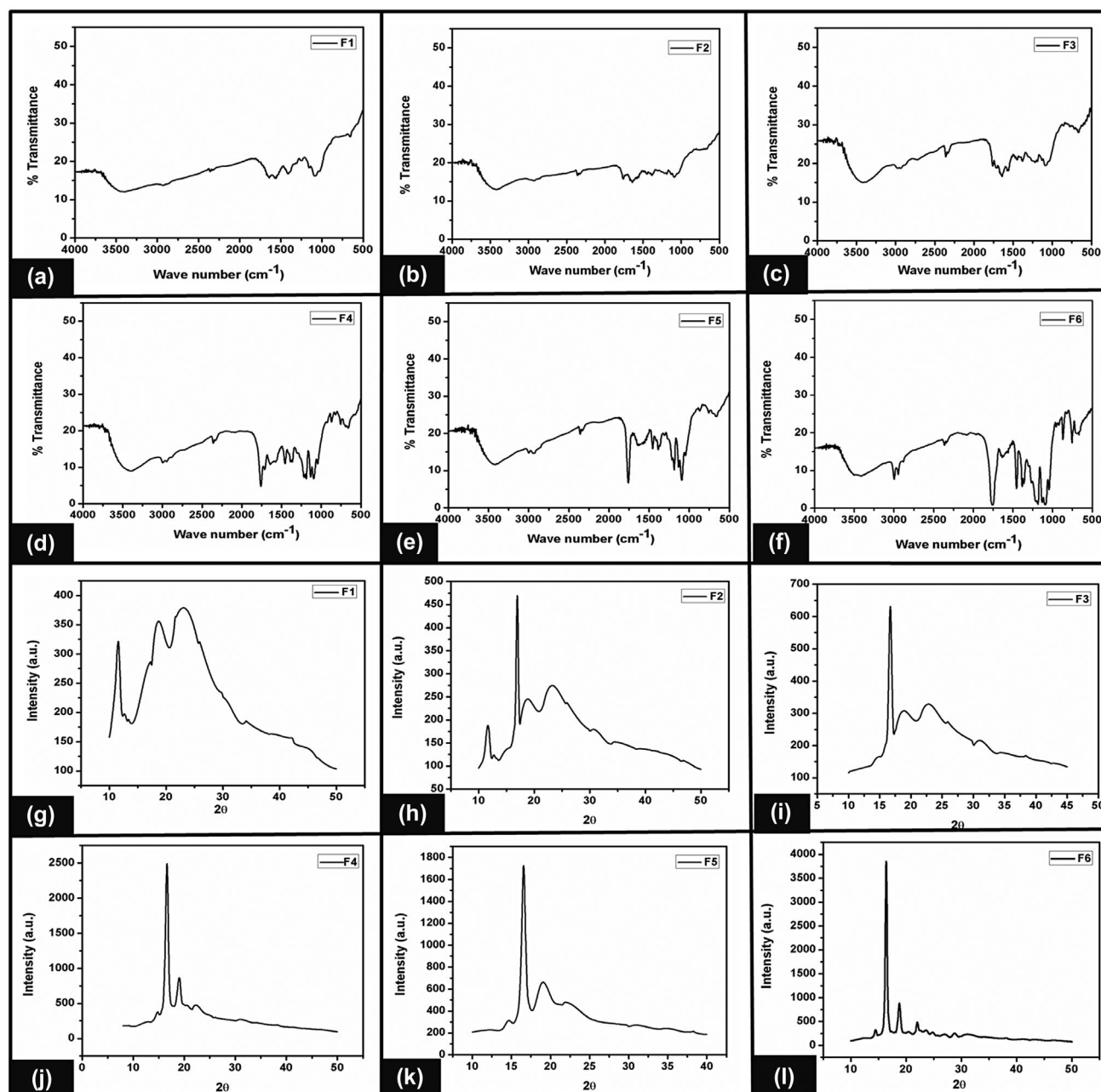


Figure 2. FTIR spectra of the scaffolds. (a) F1, (b) F2, (c) F3, (d) F4, (e) F5, and (f) F6 and X-ray diffraction patterns (XRD) of the scaffolds (g) F1, (h) F2, (i) F3, (j) F4, (k) F5, and (l) F6.

Table 2. XRD parameters.

	Intensity	Center	Width	d- Spacing	Crystallite size	Lattice strain
F1	284.29	11.4636	0.94443	7.712872	8.83	0.0411
	355.75	18.43064	1.3586	4.810012	6.19	0.0365
	200.27	22.84758	10.84803	3.88914	0.78	0.2342
F2	398.86	16.9498	0.35966	5.226762	23.34	0.0105
	178.125	18.58977	1.59047	4.769195	5.29	0.0424
	254.09	23.99163	9.39517	3.706212	0.9	0.1929
F3	462.32	16.70214	0.44107	5.303703	19.02	0.0131
	243.28	18.66124	1.67033	4.751091	5.04	0.0444
	2035.77	16.59587	0.56418	5.337425	14.87	0.0169
F4	571.76	18.96807	0.72562	4.674923	11.6	0.019
	1477.53	16.56159	0.50973	5.348395	16.46	0.0153
	450.7	19.1302	1.74542	4.635666	4.82	0.0452
F5	3530.99	16.39219	0.33882	5.403283	24.75	0.0103
	660.66	18.76638	0.44807	4.724709	18.78	0.0118

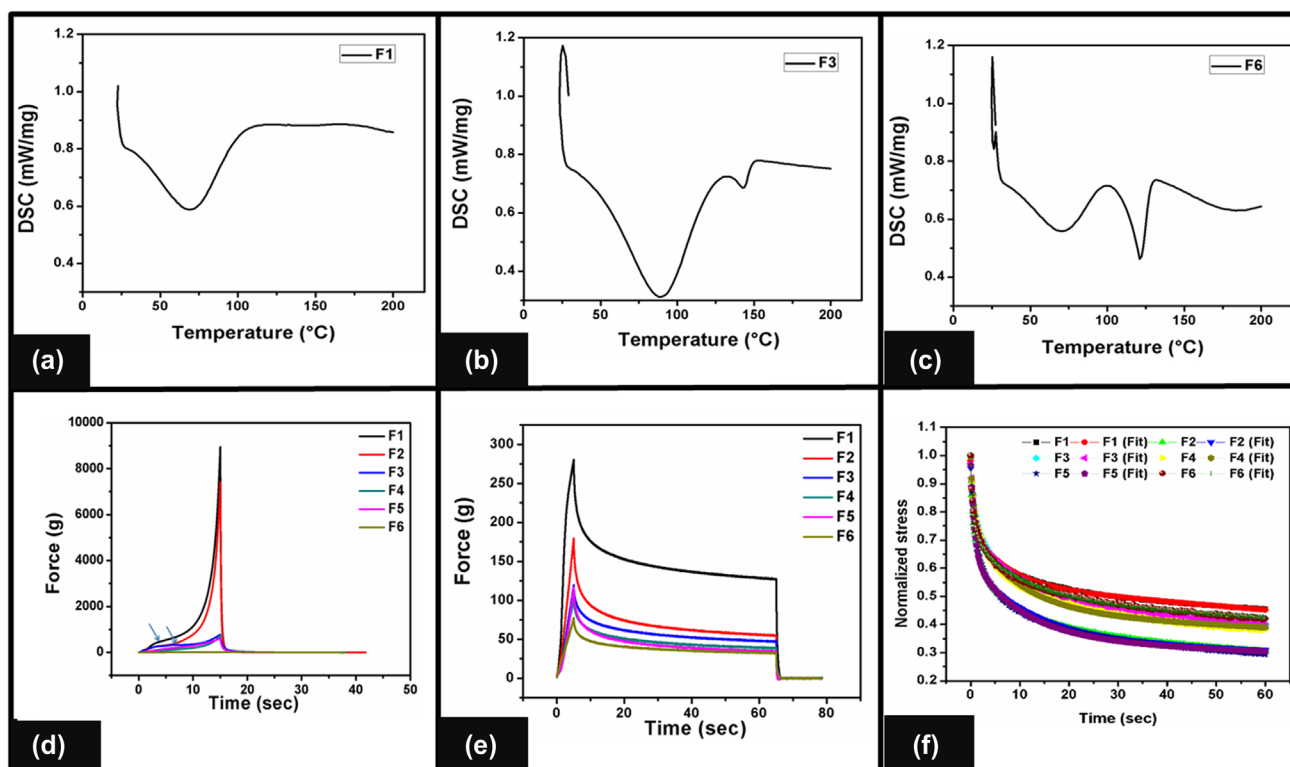


Figure 3. Thermal analysis of the scaffolds. (a) F1, (b) F3, and (c) F6, and mechanical analysis, (d) compressive profiles, (e) stress relaxation profiles of the scaffolds, and (f) Weichert model fitting.

was totally subsided. The PLLA containing scaffolds exhibit the peak intensity of the peak at $\sim 17^\circ 2\theta$, which further increased with the increase in the PLLA concentration (with an exception of F5). This suggested that the incorporation of PLLA in higher quantities resulted in the increase in the crystallinity of the scaffolds.[29,30] The peak at $18.5^\circ 2\theta$ was present in all the scaffolds. The d-spacing, crystallite size (D), and lattice strain (Table 2) were calculated using the peak details at $18.5^\circ 2\theta$. The d-spacing value suggested that incorporation of PLLA decreased the d-spacing values [31]. A decrease in the crystallite size was also observed when PLLA was incorporated in the scaffolds (Figure S2), the exception being F4, which showed highest crystallite size. In general, the lattice strain of the PLLA containing scaffolds were higher (except F4 and F6) compared to the chitosan-alone scaffolds. Interestingly, the lattice strain of F4 and F6 were much lower as compared to the lattice strain of the other scaffolds. This study suggested that the internal stability of F4 and F6 scaffolds were better, which contained chitosan and PLLA in the proportion of 50:50 and 20:80, respectively.

Differential scanning calorimetry

F1, F3, and F6 scaffolds were used as the representative scaffolds for the thermal analysis. The thermograms of

the scaffolds have been shown in Figure 3(a)–(c). A broad peak was observed in the temperature range of 70–90 °C. This broad peak may be explained by the evaporation of the bound water from the scaffolds.[32] The enthalpy of evaporation was calculated and was found to be 122, 238, and 70 J/g for F1, F3, and F6, respectively (Table S1). This suggested that the water holding capacity of the F3 scaffold was much better as compared to other scaffolds. PLLA containing scaffolds showed a second endothermic peak in the temperature range of 120–145 °C. The intensity of the peak was increased when PLLA fraction in the scaffolds increased. This sharp endothermic peak may be due to the melting of the PLLA molecules.[33]

Mechanical analysis

The compressive profiles of the scaffolds have been shown in Figure 3(d). It was observed that the compressive strength of the scaffolds was higher when the chitosan proportion was more. A close observation of the compression profiles exhibited discontinuity in the profiles (marked by arrow in the Figure 3(d)), when the chitosan proportion was higher. This suggested that the chitosan predominant scaffolds were brittle in nature, whereas, incorporation of PLLA within the scaffolds increased the ductility of the scaffolds.[34,35]

Table 3. Modeling parameters for mechanical study.

Model	Parameters	F1	F2	F3	F4	F5	F6
Weichert model	F_0	280.514	179.534	119.424	101.776	116.77	77.626
	F_r	127.12	54.67	47.77	38.746	34.766	32.244
	% relaxation	54.68	69.54	59.99	61.93	70.23	58.46
	P_0	0.463	0.323	0.399	0.377	0.287	0
	P_1	0.02	0	0.054	0.008	0.276	0.149
	τ_1 (s)	3.055	3.059	8.722	3.059	3.066	0.878
	P_2	0.302	0.56	0.309	0.307	0.21	0.333
	τ_2 (s)	141.145	16.867	223.006	7.95	15.098	20.638
	P_3	0.23	0.369	0.288	0.282	0.233	10.543
	τ_3 (s)	1971.45	1454.83	2665.99	95.4	94.523	1451.41
	R^2	0.99	0.99	0.99	0.99	0.99	0.99

The stress relaxation (SR) profile of the scaffolds has been shown in Figure 3(e). It was observed that as the scaffolds were compressed, there was an increase in the force value to a maximum limit (F_0). Thereafter, the probe was kept constant at a specified distance. This resulted in the decrease in the force profiles to a residual force (F_r). This decrease in the force values can be explained due to the polymer relaxation, breakage of polymer–polymer interactions and breakage of polymer chains.[36] However, the effect of these parameters cannot be resolved from the profiles directly. The estimation of the effect of the phenomenon may be predicted from the Weichert model (Figure 3(f)) (Equation (5)).[37]

The percentage of SR (%SR; Equation (4)) for F1, F2, F3, F4, F5, and F6 was 54.68, 69.54, 59.99, 61.93, 70.23, and 58.46%, respectively. The result suggested that, in general, there was an increase in the SR when PLLA was introduced into the scaffolds. Among the PLLA containing scaffolds, F4 and F5 showed 70% SR, whereas, F3 and F6 showed nearly 60% SR. The SR of chitosan-alone scaffold (F1) was the least (nearly 55%). It can be summarized that when the chitosan:PLLA proportions were 70:30 or 20:80, some sort of associative interactions might have happened which prevented the relaxation properties. The relaxation profile was modeled using Weichert model of viscoelasticity (Equation (5)).[38] The pre-exponent P_0 is a marker of inherent stability of the viscoelastic properties. The result indicated that the P_0 value was highest for F1. This suggested that the inherent stability of F1 was higher than the PLLA containing scaffolds.

Among the PLLA containing scaffolds, there was an initial increase in the P_0 values when PLLA content was increased from F2 to F3. Thereafter, there was a monotonous decrease in the P_0 values. It can be predicted that the inherent mechanical stability of F3 was highest among the PLLA containing scaffolds. A similar observation was also made from the percentage relaxation values, where it was found that F3 showed relatively lower %SR among the PLLA containing scaffolds. The parameters τ_1 , τ_2 , and τ_3 are regarded as the instantaneous, intermediate, and delayed relaxation times. The instantaneous

relaxation time is a marker of the molecular rearrangement of the polymer molecules when the material is subjected to stress.[39] The intermediate and the delayed relaxation times are associated with the breakage of the polymer–polymer interactions and the polymer chains, respectively (Table 3). The relaxation time of F3 was highest among all the scaffolds. This suggested that the chitosan–PLLA proportion might have reached the critical limit in F3, which resulted in the lower molecular rearrangement of the polymer, which in turn, resulted in the highest instantaneous relaxation time. Also, the highest, intermediate and delayed relaxation times suggest that the stability of F3 scaffolds was much better and did not undergo breakage of the polymer–polymer interactions and polymer chains easily.

$$\%SR = \frac{F_0 - F_r}{F_0} \times 100 \quad (4)$$

where F_0 , maximum force at the end of the compression stage; F_r , residual stress.

$$P(t) = P_0 + P_1 \exp(-t/\tau_1) + P_2 \exp(-t/\tau_2) + P_3 \exp(-t/\tau_3) \quad (5)$$

where P_0 , instantaneous elasticity; P_1 , initial elastic component; P_2 , intermediate elastic component; P_3 , delayed elastic component; τ_1 , initial relaxation time; τ_2 , intermediate relaxation time; τ_3 , delayed relaxation time; t , time.

In gist, from the SR study, it may be concluded that even though there is a decrease in the mechanical properties of the scaffolds as the proportion of PLLA was increased, the inherent stability of the scaffolds reached a critical limit when the proportion of chitosan:PLLA was 70:30 (F3), which may be further used for chondrocyte culture.

Swelling study

The swelling property of scaffolds is an important principle during the chondrocyte culture and cartilage regeneration. The swelling profiles of the scaffolds have been shown in the Figure 4(a). A preliminary analysis of the

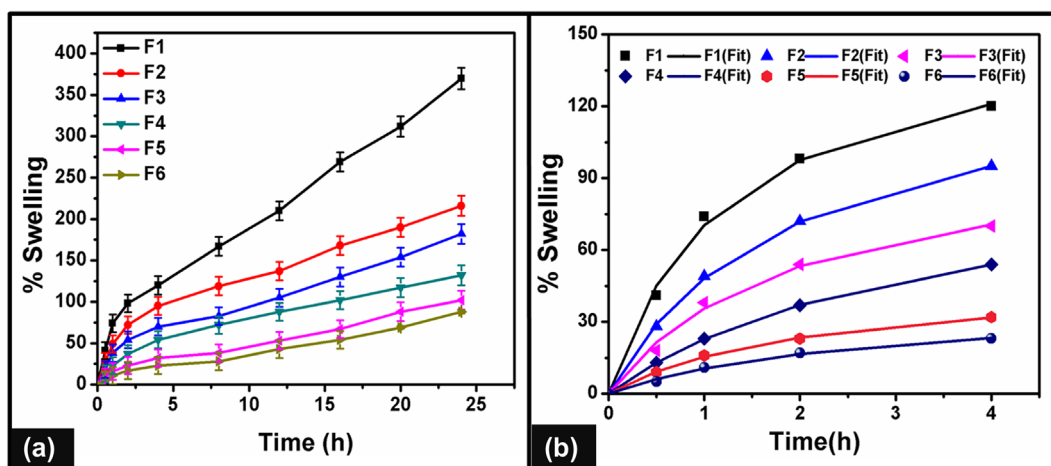


Figure 4. Swelling study. (a) Swelling profiles and (b) Peleg's model fitting F1, F2, F3, F4, F5, and F6.

Table 4. Swelling kinetics fitting using Peleg's model.

	F1	F2	F3	F4	F5	F6
K_1	0.007938	0.013653	0.018554	0.033514	0.044368	0.069406
K_2	0.006282	0.007092	0.009504	0.010158	0.020327	0.025667
R_0	125.9782	73.24258	53.8979	29.83803	22.53863	14.40789
R^2	0.998269	0.999838	0.997118	0.999989	0.999486	0.997697

swelling profiles of the scaffolds suggested a decrease in the chitosan proportion with a corresponding increase in the PLLA proportion resulted in the drastic decrease in the swelling of the scaffolds. This can be accounted to the fact that chitosan molecules are more hydrophilic in nature as compared to the PLLA polymer chains.[40] The swelling profiles of the scaffolds were fitted to Peleg's water sorption model (Figure 4(b)) (Equation (6)).[41]

Peleg's rate constant (K_1) and Peleg's capacity constant (K_2) are calculated from the initial duration of the swelling profiles. In our study, the swelling data up to 4 h were used for the calculation of the parameters of the Peleg's water sorption model. The parameters were calculated using non-linear fitting by minimizing the squared sum of differences of experimental data and model data. The fitting was done in MS Excel-2007 using solver add in Table 4. From the result, it was found that the K_1 values were increased with the increase in the PLLA content. The K_1 values are reported to be inversely related to the initial rate of water absorption within the polymeric architecture (Equation (7)). Hence, the initial rate of water absorption (R_0) was calculated. The initial rate of water absorption was found to be highest in F1 (chitosan-alone scaffold). Incorporation of PLLA in F2 drastically reduced the initial rate of water absorption. Thereafter, a further increase in the PLLA content decreased the initial rate of water absorption in a PLLA concentration dependent manner. Peleg's capacity constant (K_2) is a marker of water holding capacity of the polymeric architecture. It has been reported that the K_2

values are inversely related to the water holding capacity of the polymeric constructs. Similar to K_1 values, the K_2 values were also found to be higher in the scaffolds with higher proportion of chitosan. This suggested that an increase in the PLLA content within the scaffolds reduced the water holding capacity of the scaffolds,[42] which may not be desirable for being used as scaffold for chondrocyte culture.

$$M_t = M_0 \pm \frac{t}{K_1 + (K_2 \times t)} \quad (6)$$

$$R_0 = \frac{1}{K_1} \quad (7)$$

where M_t , moisture content at a known time (t); M_0 , initial moisture content; t , soaking time; R_0 , initial rate of water absorption.

In vitro degradation study

The biodegradation studies of the porous scaffolds have an effect on the cell viability and growth. To evaluate the biodegradation of the scaffolds, the scaffolds were incubated at 37 °C in lysozyme containing DPBS for 7, 14, 21, and 28 days, respectively. Lysozyme is the main enzyme, which promotes the hydrolysis of the linkages between *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid in peptidoglycan.[43,44] Chitosan-alone scaffold (F1) showed higher degradation rate as compared to the other

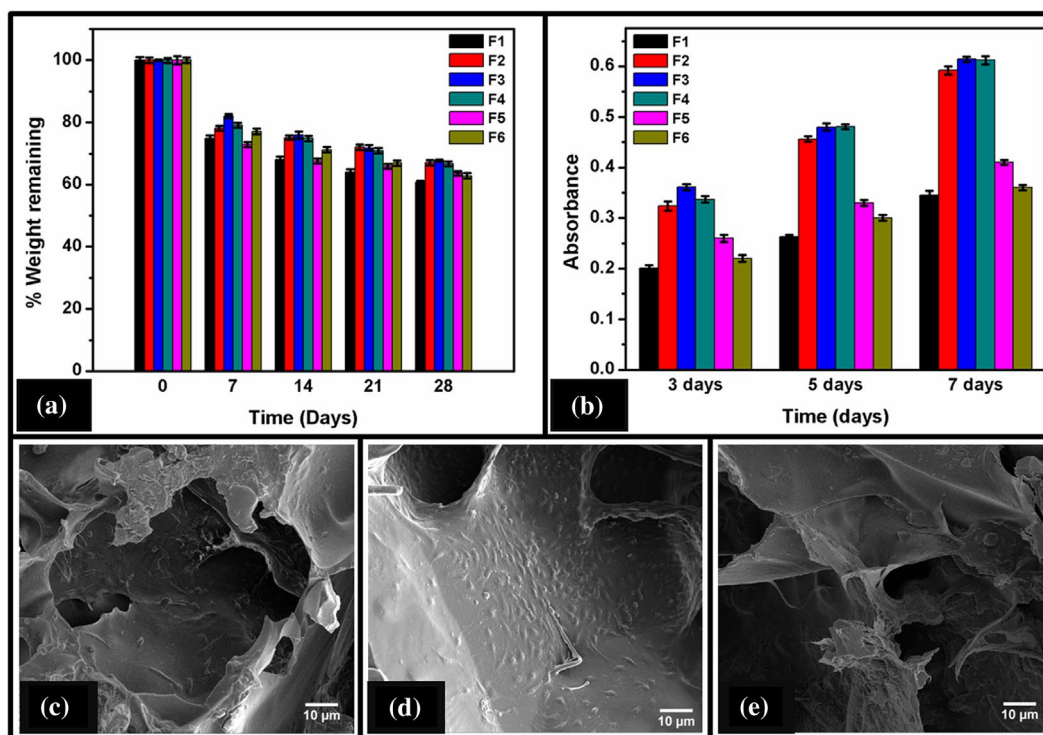


Figure 5. (a) Degradation profiles of scaffolds. F1, F2, F3, F4, F5, and F6. (b) Proliferation assay of cells on scaffolds after 3, 5, and 7 days of cell seeding and SEM images of cell-seeded scaffolds after 7 days. (c) F1, (d) F3, and (e) F6.

scaffolds (~39% of its initial weight). On the other hand, F3 showed lower degradation (~32% of its initial weight). Percent weight reduction of F2, F4, F5, and F6 were 33, 33.5, 36, and 37%, respectively. The result suggested that the addition of PLLA slowed down the degradation rate (Figure 5(a)).

Proliferation assay

The viability of the cells within the scaffolds was analyzed using MTT assay. The results showed that the cell proliferation in F1 (chitosan-alone scaffold) was least among all the scaffolds. Among the PLLA containing scaffolds, F3 showed better cell proliferation capability as compared to the other PLLA containing scaffolds. An increase in the experimental days resulted in the subsequent increase in the cell proliferation.[45,46] The extent of increase in the cell proliferation was highest in F3 and lowest in F1. Interestingly, at longer time durations, the cell proliferation in F3 and F4 was found to be similar (Figure 5(b)). The proliferation assays of different scaffolds as compared to F1 were statistically significant ($p < 0.05$). However, the proliferation assay between F3 and F4 was found to be statistically insignificant.

Cell attachment study

The attachment of the cells on the representative scaffolds (F1, F3 and F6) was analyzed under SEM. The images of the cell-scaffold constructs have been shown in Figure 5(c)–(e). The analysis was done after an incubation period of 7 days. The micrographs showed the presence of the cells attached on the scaffold surfaces. The attachment of the cells in F3 was highest. These results correspond to the results obtained from the MTT assay.[47]

GAG quantification

The total GAG content of the chondrocyte grown on F3 scaffolds in shake flask was evaluated at various time intervals and analyzed statistically. Total GAG content of the F3 scaffolds for 7, 14, 21, and 28 days growth time were 18, 22, 31, and 40 $\mu\text{g/ml}$, respectively (Figure S3). It is observed that GAG level using F3 is comparably higher than in shake flask without scaffolds and this might be due to supportive cell proliferation by the scaffold. Total GAG content observed at the end of 4 week is 40 $\mu\text{g/ml}$, which is higher for growth without scaffold (33 $\mu\text{g/ml}$ reported by the authors).[48] Also Table S2 depicts the optimized performance of F3 scaffold for chondrocyte culture as there

is enhanced proliferation, cell attachment and mechanical stability for the F3 combination. The viability for the chondrocyte culture during the growth was higher using F3 scaffold (around 90%).

Conclusions

The current study delineates the development of chitosan and PLLA-based scaffold for cartilage tissue engineering. The scaffolds were cross-linked with EDC-NHS-CS cross-linking reagent, which helped in improving the physical properties of the scaffolds. The prepared scaffolds behaved as spongy materials. Scanning electron microscopy has suggested composition-dependent alternation in the microstructures of the scaffolds, improved pore size, crystallinity, and optimal strength for chitosan and PLLA (70:30). The performance of the so optimized scaffold for cell proliferation, cell attachment, and GAG content on cell culture exhibited good results. The cell proliferation study using rabbit chondrocytes suggested that the cell proliferation was better when the ratio of chitosan:PLLA content was 70:30. *In vitro* degradation study using lysozyme showed that the scaffolds were biodegradable in nature. The GAG content obtained for the chondrocyte culture using so optimized-chitosan and-PLLA scaffolds exhibited a desired improvement in the GAG release quantification, which might be due to suitable modulation and maturation of the chondrocytes. The results are encouraging and further study under *in vivo* condition may be conducted for better evaluation of the developed scaffolds.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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