Tissue Engineering Application of Air Dried 3D – Collagen - Chitosan Bio – Composite Scaffolds

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Abstract:

Composite scaffolds of collagen–chitosan with different ratios were successfully prepared using emulsion air drying method. As collagen without any additive (e.g. cross linkers) has low tensile strength, 10–30 wt% of chitosan addition improved the mechanical properties of the composite scaffold but collagen–chitosan at 7:3 ratio was found to be a better composite having 13.57MPa ultimate strength with 9% elongation at break. The water uptake characteristics were performed at different pH and found to be ameliorated for the composite scaffolds compared with pure collagen and chitosan scaffold, respectively. The pores ranging from 100-300µm were well interconnected and their distribution was fairly homogeneous in the scaffold as observed in the scanning electron microscopic images. Furthermore, this collagen– chitosan composite scaffold decreased the bacterial counts and supported fibroblasts attachment and proliferation which demonstrate this composite to be a good substrate for biomedical application.

Key words: Collagen, Chitosan, Air-Dried, Cytoproliferative, Scaffold, Bio polymer, Cytotoxicity, Bio compatibility

INTRODUCTION

The regeneration of tissues at the site of injury is a challenging task in the field of biomedical science1. The need for suitable biomaterials, which influence the formation of an in vivo extra cellular matrix and support cellular proliferation for tissue regeneration, have motivated the researchers/scientists towards tissue engineering which led to the development of implantable porous scaffolds to be used at the damaged site. These scaffolds temporarily act as supporting materials for the growth of cells and they degrade along with in vivo production of extra cellular matrix leading to vascularization and tissue regeneration.[1] Primarily collagen is a naturally occurring material; it exhibits an extremely high level of biocompatibility and safety, eliciting low antigenicity and considered to be the most promising substitute for wound healing and skin regeneration. Secondly, collagen is biodegradable and bioreabsorbable. These properties, in turn, are controlled through regulation of the degree of crosslinking. Finally, collagen can be easily modified and can be combined with several synthetic polymers to produce a variety of systems. Different forms of reconstituted collagen materials like gel, film, sponge, powder, and fleece have found use in medical practices. [2–4] Different types of medicated dressing with antibiotic were developed to improve infected wound healing.[5–8] Chvapil put forth the combination of collagen sponge and antibacterial agents for treating chronic wounds.[9]

However collagen cannot be thermally processed because of the denaturation risk at temperatures higher than 50ºC and moreover, it cannot be stored for long time at room temperature.[10, 11] In many cases the bioabsorption of native collagen occurs before the implant could complete its function. Also, the chemical, physical and particularly the mechanical properties of collagen implants need to be adjusted according to the device requirements. Cross linking of collagen biomaterials is often applied to control or reduce the in vivo resorption rate or to improve mechanical properties of materials.[12-13]

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But crosslinker such as glutaraldehyde is known to induce local cytotoxicity and in addition, glutaraldehyde crosslinking of collagen-based biomaterials is associated with enhanced calcification of the implant, which has an adverse effect on the mechanical properties of the material.[14,15] Moreover, the drawback in collagen scaffolds lies that it may not facilitate the healing of an infected wound because of its protein nature and bacteria can use it as a substrate. At this stage, the imbalance between host resistance and bacterial growth leads to infection on the wound which leads to impaired healing. In severe wound infection, systemic administration of drugs may lead to insufficient drug concentration reaching the site of infection, drug associated side effect, and systemic toxicity. This has been overcome successfully by topical application of drugs, and collagen dressings with antibiotics have also been developed to control infection [16-18] but higher dosage of antibiotics in topical applications would also give associated side effects. Collagenchitosan 3D-scaffolds can overcome these drawbacks due to antimicrobial properties a well as good biocompatibility of chitosan. Moreover, these two naturally derived biodegradable polymers, which are most abundant, have also been explored clinically because they are biocompatible, easily modified, and easily processed into various structures. [20-24]

Three dimensional, porous collagen-chitosan composite scaffolds were prepared by air-drying blended solutions, and prospect as a promising biomaterial for tissue engineering has been evaluated by physiochemical and biological assay. The water uptake, thermal and mechanical properties of the scaffold was studied. The cytoproliferative potential of the scaffold was evaluated through fibroblast culture on this matrix.

Materials and Methods:

Preparation of pure Collagen

The collagen from bovine Achilles tendon was extracted following the patented procedure developed by CLRI. [25, 26] In brief, the bovine Achilles tendons, collected fresh from local slaughterhouse were cut into small bits of 3-4 mm. The bits were minced in a mincer (model 4612, Hobart, USA) and washed using a non-ionic surfactant. The washed tissues were suspended in sodium peroxide solution followed by washing with distilled water (qs). The obtained tissue was swollen again in distilled water (pH 2.5) followed by pepsin treatment. After the enzyme treatment, tissues were washed repeatedly in water to deactivate the enzyme. The coagulated collagen was dissolved in MilliQ water acidified to pH 3.5 using HCl to get pure collagen solution
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Preparation of Collagen-Chitosan scaffold

Four different ratio mixtures (9:1, 8:2, 7:3, 6:4 and 1:1 v/v) of collagen (10mg/ml) and chitosan (10mg/ml, medium molecular weight from Sigma Aldrich, USA) were taken and thoroughly homogenised using Ultra Trux mincer. The resulting homogenised mixture was degassed and poured into a Teflon Petri dish followed for air drying. Similarly collagen scaffolds were prepared using 10mg/ml solution.

Measurement of Tensile Strength

The Collagen-Chitosan scaffolds (1.7 x 1.2 x 0.3 cm) were used for its mechanical strength. Stress–strain measurement was carried out on an autograph (recorder) at a crosshead speed of 20 mm/min at 25°C and at a relative humidity of 65% and 85%.Percentage of elongation at break was measured using a universal testing machine (INSTRON model 1405) according to Vogel at an extension rate of 5 mm/min.

Water Uptake and Swelling Studies

The rectangular Collagen-Chitosan scaffold measuring 1.7 x 1.2 x 0.3 cm was dried under vacuum at 70°C for 4 hours and cooled in a desiccator at ambient temperature (25 ± 3ºC). Subsequently the weight of the dried scaffold was recorded and immersed in buffer solutions of pH 4, pH 7, and pH 9 for 24 hours to achieve complete saturation. The scaffold was removed from PBS, immediately weighed. Water uptake was determined according to the following equation and an average of four values was taken to find the percentage water uptake.
Water uptake = (Ww - WD)/Ww x100
Where Ww and WD represents the weight of wet and dry scaffold, respectively.

Fourier Transform Infra Red Spectra

Fourier Transform Infra Red (FTIR) spectra of lyophilized Collagen-Chitosan scaffold were obtained by using the ATR technique (Attenuated Total Reflection). Keratin-collagen scaffold pellet was prepared by mixing it with potassium bromide. An infrared spectrum of keratin was recorded from 400 to 4000 cm-1 using a Nicolet 20 DXB FT-IR spectrophotometer.

Circular Dichroism (CD) Spectra

Circular Dichroism (CD) spectrum of an aqueous solution of Collagen-Chitosan was taken on a JASCO J-20 spectropolarimeter in a thermostatically controlled 1 mm jacketed cell. Liquid nitrogen was circulated through the instrument for 30 minutes to maintain a constant temperature and protect the nativity of protein during the periodof experiment. A cell with 1 mm path length was employed. Baseline adjustment was carried out with 0.025 M acetic acid solution. The CD measurement of each of the samples was made from 195 to 250 nm. The CD data were expressed in terms of the mean residue ellipticity, θ in deg cm2/dmol.

Differential Scanning Calorimeter Analysis

Differential scanning calorimetry (DSC) analysis of collagen-chitosan scaffold after conditioning the samples at 24ºC, 65% R.H was performed for a range of temperature from 0ºC to 250ºC, at 10°C/min. using universal V4.4A TA instruments. The instrument was calibrated by an indium standard and the calorimeter cell was flushed with 50 mL/min liquid nitrogen.

Thermogravimetric Analysis

Thermogravimetric analysis (TGA) analysis of Collagen-Chitosan Scaffolds was performed using universal V4.4A TA instruments. About 3 mg of the sample was heated at 10ºC/min at a temperature range of 0ºC–600ºC using Al2O3 crucibles

Culture of Fibroblast

NIH 3T3 fibroblasts were obtained from National center for cells science (NCCS), Pune, India and the culture were maintained in Dulbecco's modified Eagles medium (DMEM) with 10% fetal calf serum (FCS) supplemented with penicillin (120 units/ mL), streptomycin (75mg/mL), gentamycin (160mg/mL) and amphotericin B (3mg/mL) at 37ºC humidified with 5% CO2.

MTT Assay for Viable Cell number Determination

Metabolic activity of the cells was determined calorimetrically by assaying the metabolic product (formazone) of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). [27, 28] The reaction product was extracted and read on a spectrophotometer (UV751GD, China) at 630nm. Fibroblasts were seeded in the wellsof 24-well plates at the density from 8× 103 cells/well to 7×106 cells/well and allowed to adhere for 6 h. The absorbance values were plotted against the exact cell numbers to establish a standard calibration curve.

Cell Proliferation on Collagen-Chitosan Scaffold

The scaffold and medium were placed in a 16 mm culture disc at 5°C overnight with interrupted stirring to remove air bubbles from the scaffold. Pre-wet collagen-chitosan scaffolds were blot dried by sterile filter papers and were transferred to 24 wells culture plates. Three hundred microlitres of cell suspension (2 × 106 cells/ml) was slowly dispersed over the top surface of of scaffold with a micropipette. The seeded scaffold was incubated in a 95 % humified incubator with 5% CO2 for 3 h and then washed with 500 µl medium to remove unattached and loosely attached cells. Seeded cells were cultured for 24 and 48 hrs and the medium was changed every day. The number of viable cell in the scaffold was determined using MTT assay. The medium of each well was replaced with MTT solution and the plates incubated at 37°C for 3 hr. After aspirating the MTT solution, acid isopropanol (0.04N HCl in isopropanol) was added to each well and pipetted up and down to dissolve all of the dark blue crystals of formazone and then left at room temperature for a few minutes to ensure all crystals are dissolved. Finally, absorbance was measured at 630 nm using UV spectrophotometer.

Scanning Electron Microscopy

The scaffolds were coated with gold prior to SEM ((JSM-T330, JEOL Co., CLRI, Chennai) analysis. Fibroblasts cultured scaffolds were fixed with 1% of glutaraldehyde followed by freeze-dried. **Histology** Cell seeded collagen-chitosan scaffold were fixed in 10% Neutral Buffered Formalin (NBF, Fisher Scientific) for 24 h, dehydrated in increasing concentrations of ethanol (Fisher Scientific) and embedded in paraffin (Fisher Scientific). Cross-sections (5 mm thick) were done using microtome (Ultramicrotome RSP 1250, JOEL Microsystems Inc.,), mounted on StarFrost glass slides (Engelbrecht, Edermunde, Germany), and air dried. Sections were stained with hematoxylin and eosin (Fisher Scientific).

Statistical Analysis

The statistical analysis was performed using SPSS (version 4.01). Data was expressed as mean ± SD of three independent experiments. The mean values were compared by Student's t-test. $P < 0.05$ was considered as significant.

RESULTS

Measurement of Tensile Strength

The collagen sponges prepared by addition of 10 to 30% of chitosan were found with increased mechanical strength. The collagen-chitosan scaffolds in the ratio of 9:1, 8:2, 7:3, 6:4 and 1:1 were found to have tensile strength of 8.85, 10.71, 13.57, 5.19 and 4.92 Mpa respectively while collagen scaffold was found to have 6.20 Mpa (Table I).

Measurement of Water Uptake

The water uptake studies were carried out at different pH (4, 7 and 9) and the collagen-chitosan composite scaffolds were found to have reduced water uptake in comparison to collagen scaffold at all the three pH. The data for the same were shown in table 2. Water uptake activities of collagen scaffold at pH 4, 9 and 7 was found to has 2391.605%, 1439.015% and 1915.71% respectively while collagen-chitosan composite of 7:3 ratio was found to has respectively 1134.7025%, 664.315% and 822.6525%.

FTIR Spectra

The degree of interaction between the two components of the scaffold was determined by FTIR as shown in the figure 1. The spectrum confirms alpha helical structure with β turns with characteristic absorption bands assigned mainly to the peptide bonds (-CONH-) for amide I, II, and III at 1687.1, 1544.5, and 1244.2 cm-1, respectively. The amide A band, which falls at 3371 cm-1 is because of the stretching vibration of N—H bonds The amide I mode was resolved in Gauss shaped bands corresponding to alpha helix (1650 cm-1) beta-turn (1620 cm-1). On the basis of literature data, the absorption at 1650 cm-1 suggests the presence of alpha helix structure, whereas the bands related to beta turn structure fall in the 1631–1515 cm-1 range. The Collagenchitosan scaffold might contain combination of alpha helix and beta turn. All collagen
FTIR spectra exhibited absorptions at 1,035 to 1,090 cm−1, which arise from the v(C–O) and v(C–O–C) absorptions of the carbohydrate moieties and in our results the peak at 1091 cm-1 confirms the existence of collagen in hybrid scaffold.

CD Spectra

The CD spectrum of aqueous solution of collagen-chitosan also confirms alpha helical structure with negative minimum absorption band at 200 and a weak positive maximum absorption band at 225nm (figure 2).

DSC analysis

The DSC thermogram of Collagen-Chitosan scaffold are shown in figure 3 .The first endothermic peaks seen as a sharp at 107.05ºC is due to evaporation of water. Another peaks fall in higher regions, peak at 137.5ºC attributed to glass transition, exothermic shoulder at 223.7ºC reflects crystallization temperature and endothermic peak at 229.3ºC shows the melting temperature of the composite material.

TGA Analysis

The thermogravimetric curve for collagen-chitosan scaffold sample (4 mg) is shown in Figure 4. The initial weight loss at 100ºC–150ºC is because of moisture loss. The second and third weight losses took place in the temperature range 300ºC– 460ºC. The fourth weight loss took place in the temperature range of 500 to 600ºC. About 74% weight losses were observed between 225ºC to 600 ºC.

Scanning Electron Microscopic Analysis

The SEM observation of the collagen-chitosan scaffold reveals heterogeneous porous microstructures with pore size ranging from 100-200 µm (Figure 5A and 5B). Scanning electron micrographs are shown in Figure 5C and 5D represents scaffolds seeded with NIH 3T3 fibroblast at 24 and 48 hrs respectively. All the cells were in contact with the Collagen –Chitosan scaffold surface and at 48 hrs the pores of the scaffold were found to be occupied with grown cells and final cell density reached upto 107cells/cm3 as observed by MTT assay. Cells have grown uniformly all over the scaffold including the entrance of the pores and the deep interior of the pores. This shows that the nutrition of the cells was good in all parts of the scaffold. All the cells showed equally good cell growth. The scaffold maintained its original shape throughout the cell culture. Therefore, all the cells could be alive on the original scaffold structure during the entire culture period.

Histology

Cell distributions were evaluated from the histological cross-sections of the cellseeded scaffolds (Figure 6). It reveals the ability of all scaffolds to allow cells topenetrate the scaffold structure. After initial seeding of cells, the cells quickly adhered onto the edges of the pores and at the end of 24 hrs there was dense cell spread densely along the pores (Figure 6B). The cells invaded the scaffold structure after 48hrs of the culture (Figure 6C).

Table 1: Tensile strength of collagen and collagen-chitosan composite scaffold

S.NO.	Biopolymer	COMPOSITION	Strength (Mpa)
1	Collagen	Alone	6.20
2	Collagen: Chitosan	9:1	8.85
3	Collagen: Chitosan	8:2	10.71
4	Collagen: Chitosan	7:3	13.57
5	Collagen: Chitosan	6:4	5.19
6	Collagen: Chitosan	1:1	4.92

Table 2: Water uptake of collagen and collagen-chitosan composite scaffold at different pH

S. NO.	Biopolymer	Composition	pH 4.0	pH 9.0	pH 7.0
1	Collagen	Alone	2391.605	1439.015	1915.71
2	Collagen: Chitosan	9:1	1826.36	1192.54	1490.065
3	Collagen: Chitosan	8:2	2255.85	509.51	584.73
4	Collagen: Chitosan	7:3	1134.70	664.32	822.65
5	Collagen: Chitosan	6:4	545.35	435.93	446.42
6	Collagen: Chitosan	1:1	497.24	392.35	413.57

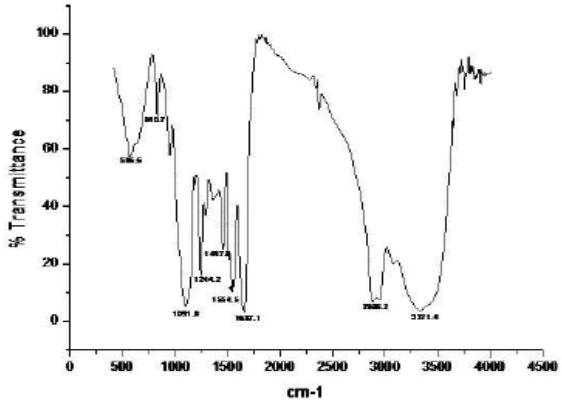

Fig 1: FTIR spectra of collagen-chitosan composite 254x190mm (96 x 96 DPI)

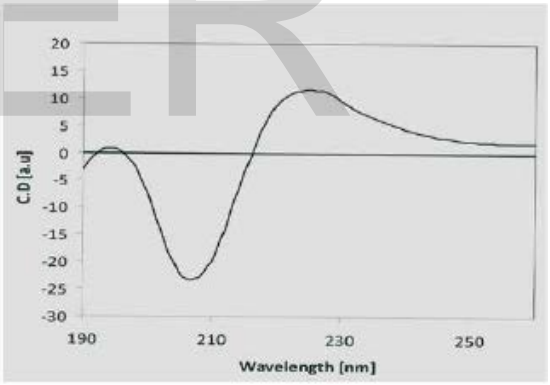

Fig 2: CD spectra of collagen-chitosan composite254x190mm (96 x 96 DPI)

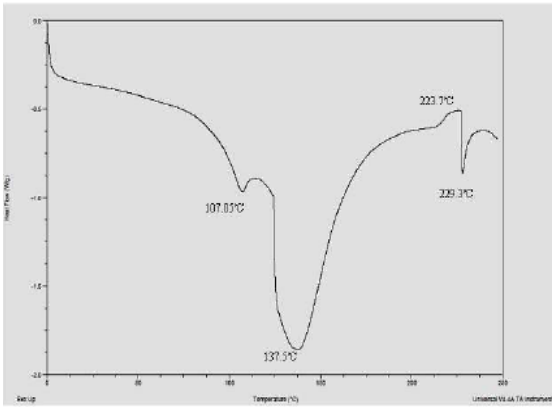

Fig 3: DSC thermograms of collagen-chitosan composite. 254x190mm (96 x 96 DPI)

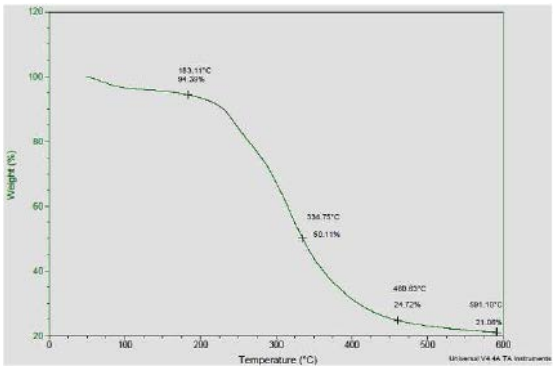

Fig 4: TGA thermograms of collagen-chitosan composite254x190mm (96 x 96 DPI)

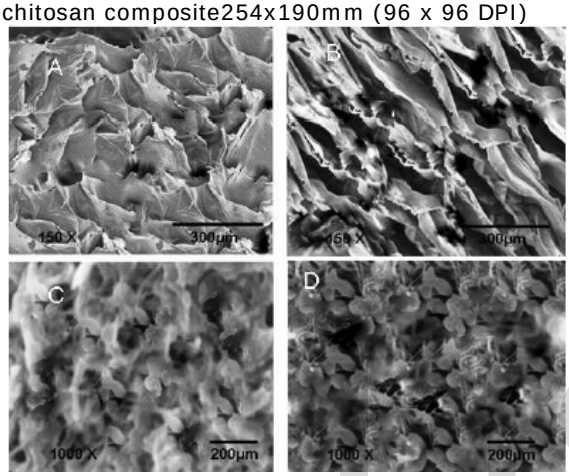

Fig 5: SEM images of collagen-chitosan composite scaffold: (A) surface view, (B) section view, (C) at 24 hrs of NIH3T3 fibroblasts culture and (D) at 48 hrs of the culture.254x190mm (96 x 96 DPI)

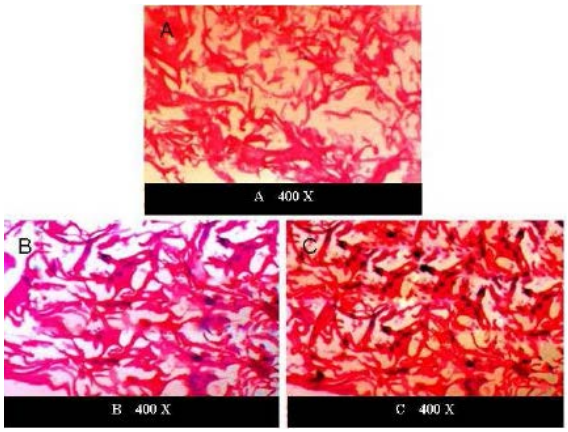

Fig 6: Light microscopic micrographs of H&E stained collagen-chitosan composite scaffold (A), cell seeded at 24 hrs (B) and 48hrs (C). 254x190mm (96 x 96 DPI)

DISCUSSION

A major goal of tissue engineering is to synthesize or regenerate tissues and organs.[29] The porous nature of the scaffold is a prerequisite for high-density cell culture and optimal scaffolding material should elicit minimal inflammatory response while and promoting cell binding and proliferation. Though chitosan is ideal biomaterials for their low price and good biocompatibility, cross-linking is an indispensable procedure for the preparation of biomaterials. A number of techniques have been used to cross-link these materials however the relatively high cytotoxicity of above chemical cross-linking agents, investigators began to pay attention to make
the scaffold without cross-linking agents. In this study, the fabrication and characterization of a novel collagen-chitosan composite scaffold was carried out without cross-linkers.

One of the most important properties of the scaffold is the mechanical strength and the scaffold has to be strong enough in order to support extensive vasculatures, the lymphatic system, nerve bundles and other structure in the skin while it is being used as skin substitute. Therefore, the scaffold should have an appropriate compressive modulus to absorb forces when they are implanted into the wounds. The compressive modulus of collagen, collagen-chitosan scaffolds, elucidated in Table 1, this was reflected by the structure of both biomaterials. Good water up-taking and mechanical property of any scaffold is a desirable feature to be good biomaterials for
most applications. In our current study, although, the composite scaffolds were foundto have reduced uptake activities but significant enhancement in tensile strength with good water uptake in the collagen-chitosan composite of ratio 7:3 make this scaffold to be an ideally improved biomaterial which could be applicable for skin tissue engineering.

The FTIR spectrum confirms alpha helical structure with β turns with characteristic absorption bands assigned mainly to the peptide bonds (-CONH-) for amide I, II, and III at 1687.1, 1544.5, and 1244.2 cm-1, respectively. The amide A band, which falls at 3371 cm-1 is because of the stretching vibration of N--H bonds The amide I mode was resolved in Gauss shaped bands corresponding to alpha helix (1650 cm-1) beta-turn (1620 cm-1). On the basis of literature data, the absorption at 1650 cm-1 suggests the presence of alpha helix structure, whereas the bands related to beta turn structure fall in the 1631–1515 cm-1 range. The Collagen-chitosan scaffold might contain combination of alpha helix and beta turn.**[30]** All collagen FTIR spectra exhibited absorptions at 1,035 to 1,090 cm−1, which arise from the v(C–O) and v(C–O–C) absorptions of the carbohydrate moieties and in our results the peak at 1091 cm-1 confirms the existence of collagen in hybrid scaffold. [31]

The CD spectrum of aqueous solution of collagen-chitosan also confirms structural nativity of alpha helix with negative minimum absorption band at 200 and a weak positive maximum absorption band at 225nm**.** [32] The thermo analytical (DSC) investigations show an endothermic peak in the temperature range of 230ºC– 240ºC which has been indexed as the helix peak and the
area under the peak represents a measure of the relative helix content of the sample.[33] The thermal stability of plain collagen was found to be in the range of 72.10C-750C[34] but our current findings for collagen-chitosan in

which the peak that fall in the 200ºC–230ºC temperature range reflect significantly increased thermal stability of the hybrid Collagen-Chitosan scaffold which would be valuable for storage even at higher temperature which is opposed to previous reports as most of the protein based biomaterials are thermo labile in nature.

Chitosan was reported to inhibit the growth of bacteria through binding of its positively charged amino groups to negatively charged bacterial cell wall.[35] Therefore, we have investigated the collagen-chitosan composite scaffolds whether the scaffold containing chitosan reduced the number of bacteria by immersing scaffold into the bacteria suspension. The bacteria number has been prominently reduced when there is an increase in the treatment time or the amount of Scaffold material (Data not shown). Fibroblasts were seeded on the porous scaffold and their adhesion and proliferation efficiency were visualized by H&E staining and SEM. Fibroblasts being ubiquitous cells were used to determine the overall efficiency of the scaffold in supporting cell growth and proliferation. The porous hybrid collagen-chitosan scaffold showed improved and almost homogeneous cell growth on the internal pore surface as revealed by H&E staining and SEM studies. H&E staining and SEM analysis also demonstrated the ability of the scaffold to allow the cells to penetrate into the interior of the scaffold structure which has been contributed by swelling property of the hybrid scaffold makes the pores easily accessible and allows the cells to invade homogenously. This approach thus exploits the additive cell binding
properties of collagen-chitosan along with the structural properties of collagen whilst avoiding the toxicological concerns. The cells on the scaffold surface were found

CONCLUSION

The use of composite scaffolds can provide unique biomechanical and biological properties for the development of functional tissue engineering scaffolds. This study demonstrated that a composite scaffold can be fabricated with two completely different natural polymer systems using emulsion air drying method. The composite scaffolds fabricated using collagen and chitosan are biocompatible; possess adequate physical and structural characteristics to support fibroblast attachment and proliferation, demonstrating it to be a good substrate for biomedical application. These data further suggested that mechanical properties of collagen-chitosan composite scaffold are adjustable by appropriately adding chitosan and the collagen, chitosan ratio at 7:3 was found to be a better composite for the above applications. The results of our study suggest that the collagen chitosan composite scaffold is a promising biologically functional material for skin tissue engineering, with prospects for clinical applications.

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