CHAPTER 4

PHYSICAL, THERMAL AND MECHANICAL
EVALUATION OF CELLULOSE – COLLAGEN –
POLYVINYL ALCOHOL – SILICA COMPOSITE

4.1 INTRODUCTION

Collagen is the fibrous protein which is abundant in most of the organs of the human body. There are 28 different types of collagen, which is classified based on its structure as fibrillar and non fibrillar (Blum 2011). The collagen types I, II, III, V and XI is classified under fibrillar. Other types of collagen forms non fibrillar structure (Exposito et al. 2010, Shoulders et al. 2009). The most common types of collagen found in human body are types I, II, III, IV and V (Ruys 2013). Type I collagen is found in tendon, bone, skin and ligament. Type I collagen is the major organic component of bone. Type II collagen is found in cartilage and vitreous, type III collagen is found in reticulin, type IV collagen is found in basement membrane and type V collagen is present in hair and placenta. Among these, type I collagen covers 90\% of collagen in humans. Hence, type I collagen is extracted from goat tendon and utilized for the synthesis of scaffolds. Type 1 collagen in the scaffolds can accelerate the wound healing process by inducing the deposition of natural collagen (Chattopadhyay & Raines 2014).

Collagen is generally applied as biomaterials for tissue regeneration, as it is widely found throughout the body. It is found to be the ideal material for skin regeneration as it is the main component in maintaining
the texture, strength and elasticity of collagen. But, in the purified state, collagen tends to degrade faster and mechanically weaker. Hence, it should be made as composites by combining with other biocompatible polymers. Unique properties can be obtained from the composites of polymers and ceramics. Polymers impart flexibility, mechanical strength, conductivity, compatibility to the scaffolds. While, ceramic fillers exhibit its thermal stability and rigidity. In addition, the ceramic fillers possess high surface area which is greatly favorable for biomedical applications. Collagen based biomaterials were proposed for various biomedical applications which includes, sutures, wound dressings, tissue engineering and many. Collagen is mixed with polymers such as polyvinyl alcohol, chitosan, cellulose, PLA, PLGA and tested for its ability to regenerate tissues. Highly porous structured collagen/chitosan/PLA with high stiffness was developed for articular cartilage repair (Haaparanta et al. 2014). PLGA/HA/collagen scaffold with regulated pore size and porosity was synthesized and applied successfully for bone regeneration (Mou et al. 2011). Surface activated PLA reinforced collagen sponges with improved mechanical properties was synthesized and found to be compatible for fibroblast cells (Liu et al. 2010). Li et al. (2015) incorporated basic fibroblast growth factor loaded gelatin microspheres into collagen/cellulose composites and observed improved proliferation of endothelial cells. Zulkifli et al. (2015) developed HEC/PVA and HEC / PVA / collagen nanofibres by electrospinning technique and proved that with the addition of collagen to HEC/PVA scaffolds, enhanced cell attachment and proliferation of human fibroblast cells was attained in the presence of collagen.

Inclusion of collagen with other natural or synthetic biopolymers will further improve its compatibility, elasticity and also non immunogenic scaffolds which mimics extracellular matrix can also be synthesized (Steele et al. 2013). In spite of these advantages, collagen could not be used individually
as scaffolds, because of its very poor mechanical properties and poor stability under aqueous conditions similar to PVA. Hence, collagen is blended with PVA and cellulose particles to improve its mechanical and aqueous stability. PVA imparts mechanical properties, while cellulose improves the aqueous stability and mechanical strength. The scaffolds should also possess good bioactivity to stimulate rapid cell attachment onto scaffold and to maintain long term bonding without any inflammatory response. This can be achieved with the incorporation of ceramics to the polymer composites (Baino et al. 2015). Mesoporous silica is proved to possess good bioactivity in bone regeneration (Sun et al. 2007, Whitehead et al. 2008). Hence, cellulose-collagen-PVA-mesoporous silica composite was synthesized and its physicochemical properties were tested to analyze the suitability of the composite scaffold for biomedical applications.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Chloroform, diethyl ether, acetic acid and NaCl were purchased from Merck. Iodoacetic acid, EDTA and phenylmethylsulfonyl fluoride were procured from sigma and were used without any further purification.

4.2.2 Collagen Extraction and Purification

The collagen solution was extracted from goat Achilles tendon collected from local slaughter house. Tendons were carefully removed from goat legs and cleaned with cold distilled water. The tendons were then cut into small pieces and allowed in 0.9% NaCl solution for 5 hours. NaCl treatment helps in removing the muscles and soluble proteins on the tendon surface. The NaCl treated tendons were then washed twice with cold distilled water. The washed tendons were then treated with chloroform: diethyl ether for 5 hours. Chloroform-diethyl ether combination aids in solubilizing the fat and its
further separation. Defatted tendons were washed with cold distilled water and allowed for swelling for overnight in 0.5 M acetic acid along with iodoacetic acid, phenylmethylsulfonyl fluoride and EDTA. The tendons were then homogenized and filtered with muslin cloth. The residue was collected, homogenized and filtered in muslin cloth. The filtrate was centrifuged at 18,000 rpm for 40 minutes. 0.5 % NaCl solution was added to supernatant and left undisturbed overnight. The solution was further centrifuged at 18,000 rpm for 20 minutes. The pellet was separated and dissolved in 0.5 M acetic acid and centrifuged at 18,000 rpm for 40 minutes. The supernatant was then dialyzed against 0.02 M Na$_2$HPO$_4$ for 3 days. The dialyzing solution was changed every 12 hours. After dialysis, the solution was centrifuged at 18,000 rpm for 20 minutes. The pellet was dissolved in 0.5 M acetic acid and centrifuged at 18,000 rpm for 40 minutes. The supernatant was then dialyzed against 0.05 M acetic acid for 3 days by changing the dialysis solution every 12 hours. The resultant collagen solution was stored at 4 °C for later use. The whole extraction and purification process was carried at 4 °C in order to prevent collagen denaturation. The concentration of the collagen solution was determined by lyophilizing the predetermined volume of solution and the concentration was found to be 2 mg/mL.

4.2.3 Preparation of Scaffolds

Composite of Cellulose-Collagen-PVA-silica of varying weight fractions was prepared by lyophilization method. Mesoporous silica, cellulose solution and PVA solution were synthesized as described in the chapter 2.

0.5% cellulose, 7.5% PVA solution and 0.2% collagen solution were mixed together in 1:2:2 volume ratio and allowed to stir, to obtain a homogenous solution, which hereafter will be denoted as Cellulose – Collagen – PVA solution. Mesoporous silica (0.5, 1 and 2 wt %) was added to
Cellulose – Collagen – PVA solution and stirred continuously until uniform mixing is attained. 15 mL of the final mixture was added to polypropylene petridish and subjected to lyophilization, as explained in the chapter 2. The free standing Cellulose-Collagen-Polyvinyl alcohol-Silica composite scaffold with about 300 µm thickness was obtained. The scaffold was also prepared using the same procedure without the addition of silica particles. Hereafter cellulose – collagen – polyvinyl alcohol – silica composite with different silica ratios (0, 0.5, 1 and 2) will be denoted as CCP0, CCP0.5, CCP1, and CCP2 respectively.

4.2.4 Characterization of Scaffolds

FTIR, SEM, DSC, TGA and tensile properties of the synthesized scaffolds were characterized as discussed in chapter 2.

4.2.5 Swelling Test

The swelling ratio of the synthesized scaffolds was carried by soaking the scaffolds in PBS solution for predefined time period, as discussed in chapter 2.

4.2.6 In vitro degradation

In vitro degradation assay was carried out by using the enzymes collagenase and lysozyme as explained in chapter 2

4.2.7 Porosity

The percentage porosity of the scaffolds was analyzed by liquid displacement method, using ethanol as the solvent and the analysis was carried out as described in chapter 2.
4.3 RESULTS AND DISCUSSION

4.3.1 Scanning Electron Microscopy (SEM)

Figure 1 shows the morphology of the synthesized composite scaffolds. From the SEM images it is very clear that all the scaffolds possess porous morphology. But, the structure of the scaffolds and the range of pore size were tuned with the varying concentration of incorporated silica. In the absence of silica (CCP0), interconnected fibrous porous network was observed. With the addition of low concentration of silica, the lamellar morphology with less number of pores was obtained (CCP0.5). Further, increasing the ratio of silica has greatly increased the pore size and number of pores. In the sample with 2% silica content, highly interconnected porous morphology was obtained. These results clearly indicate that with the increase in the silica content, highly organized, homogeneous and interconnected porous structure was obtained. Comparing the results in the presence and absence of silica and collagen, it was observed that, in both chapters 2 and 4, in the presence of silica, there was significant improvement in the porosity and pore size. However, highly organized structure was observed in the presence of collagen. Silica and collagen along with the freezing profile and ice sublimation helps in maintaining the structure of the scaffolds. Other reasons includes, collagen tend to form fibrous structure and when silica is added, it interacts with the amine groups of collagen and acts as the bridge between the polymer moieties leading to highly porous structure. This result is in good agreement with Alvarez et al. (2014). Hence, silica and collagen maintains the structure with the desired pore size and porosity which will aid in good cell attachment and proliferation.
Figure 4.2 shows the FTIR spectrum of composite scaffolds with varying silica compositions. The characteristic peak of the $-\text{OH}$ bond is observed in between 3700 – 2980 cm$^{-1}$. The characteristic peaks of cellulose, PVA and silica which were discussed in Chapter 2 are present in the scaffolds modified with the addition of collagen. In addition, due to the addition of collagen, the peaks corresponding to amide I, amide II and amide III are observed at 1659, 1555 and 1235 cm$^{-1}$. The amide I band is due to the carbonyl (C=O) stretch of amide group, amide II corresponds to N–H stretching and C–N bending vibrations. While, amide III band attributes to N–H in plane bending and C–N stretching of amide linkages. Also, $-\text{C}=\text{O}$, $-\text{NH}_2$ and $-\text{OH}$ groups in collagen will enhance the hydrogen bonding with cellulose, PVA and silica molecules. The peak seen at 1335 cm$^{-1}$ attributes to CH$\text{}_2$ wagging of proline side chains of collagen (Jackson et al. 1995) and this peak along with amide II band confirms the integrity of the ordered structure.
of collagen (Cheheltani et al. 2012; West et al. 2004). The peak at 1737 cm\(^{-1}\) corresponds to the carbonyl group of Asp or Glu in collagen (Furutani et al. 2011). With the addition of silica particles, the peak is shifted towards 1721 cm\(^{-1}\), indicating the interaction of C=O group of collagen with silica particles. The carboxyl group of polymer reacts with the silanol groups of the silica particles, which is much favored by higher electronegativity of oxygen and the lone pair of electrons present in the carbonyl group of polymers resulting in the homogeneous highly interlinked composite structure. The symmetric stretching of primary and secondary alcohol in cellulose structure was observed at 1062 cm\(^{-1}\) and the crystalline peak of PVA was observed at 1091 cm\(^{-1}\). But, with the addition of collagen, the common peak at 1077 cm\(^{-1}\) was observed. This, band is shifted to 1088 cm\(^{-1}\) with the addition of silica.

**Figure 4.2** FTIR spectra of cellulose-collagen-PVA-silica composites
The incorporation of 0.5% of silica has resulted in the increase in peak intensity. This could be due to the increase in number of bonds with the interaction of silica with polymers. But, with further addition of silica, the broadening of Si-O-Si peak was observed, which may perhaps be due to the reaction of functional group of silica with the hydroxyl and carboxyl groups of polymers. In addition, the overall decrease in intensity of the peaks was observed with the addition of silica particles. This might be due to the facts that, the overall crystallinity of cellulose and PVA might have decreased with the addition of silica particles (Pandey et al. 2009) and also due to the peak broadening, as the consequence of intramolecular hydrogen binding between cellulose, PVA, collagen and silica (Kuhn & Woste 2007; Yamashita et al. 2007).

4.3.3 Porosity

Figure 4.3 shows the percentage porosity of cellulose-collagen-PVA-silica composites. With the increase in the ratio of incorporation of silica, the percentage porosity increased gradually. The porosity in the range of 58 – 88% was observed with the increase in silica content. The increase in number of pores as observed in Figure 4.1 could have facilitated the increase in solvent uptake. Also, silica particles act as capillaries to take up the solvent. CCP0 has the porosity of 58% with unimodal pore size distribution. While, with the incorporation of silica, bimodal pore size distribution (mesopores (2-50 nm) of silica and macropores of the scaffolds) was observed. Addition of collagen to cellulose-PVA-silica composites, has improved the porosity by about 4%. Comparing the porosity of CP2 and CCP2, about 6% increase in porosity was observed with the addition of 2% silica along with collagen.
4.3.4 Swelling

As discussed in chapter 2, the swelling ability of the three dimensional construct is very essential in determining its biological importance. The degree of swelling of the composite scaffolds is as shown in Figure 4.4 and 4.5. The swelling efficiency of the scaffolds depends on the hydrophilic nature, water uptake and the porosity of the scaffolds. The scaffolds gained 0.4 (CCP0), 0.8 (CCP0.5), 1.7 (CCP1) and 1.9 (CCP2) times the initial weight of the scaffolds in about 1 h and attained maximum swelling within 1st hour (Figure 4.4). Later, for a period of 1 week, steady increase in swelling was observed (Figure 4.5). The saturation begins within a week, beyond which the degradation starts (Figure 4.6). Comparing with the results of chapter 2, it is clearly evident that in the presence of collagen the swelling ratio has increased by about 245 %. This result substantiates that, the addition of collagen and silica to cellulose reinforced PVA, have significantly enhanced the swelling ratio.
Figure 4.4 Swelling ratio of composite scaffolds in 1 hour

Figure 4.5 Swelling ratio of composite scaffolds in the presence of collagen and silica for 1 week
The improved swelling ratio will favor the transfer of nutrients from the cell medium to the growing cells. Also, the water retention capacity of the scaffolds will be much favorable for skin regeneration/wound healing. Moreover, it will also assist in the efficient drug encapsulation and release.

4.3.5 In vitro degradation

Figure 4.6 reveals the in vitro degradation profile of lysozyme and collagenase treated CCP samples. The efficiency of the intra molecular interaction/crosslinking of the scaffolds and its degradation ability was understood by carrying out in vitro degradation study using the enzymes. The scaffolds without silica degrades faster in both lysozyme and collagenase solution. With the incorporation of silica and its further increase in concentration, the rate of degradation has decreased. This clearly evidences, that the silica incorporated scaffolds are highly crosslinked which limits the contact of enzyme within the scaffold, decreasing the bond breakage. As the scaffold becomes compact with the addition of silica, the hindrance to enzyme diffusion was developed. With the increase in the ratio of silica, the degradation has decreased. Other factors which influence the degradation of polymers includes; chemical bond linking the polymers, pH, composition, water uptake, physical and chemical changes unlike crystallization of the monomers, molecular weight and the mechanical strength of the scaffolds (Gopferich 1996). Apart from these basic material properties, the diffusion of the solvent plays a major role in the degradation of polymers. The polymers may degrade either by bulk degradation or surface erosion based on the solvent diffusion rate (Lyu & Untereker 2009). From Figure 4.3 and 4.5, it is very clear that before the initiation of degradation, the solvent is diffused throughout the scaffold, hence, it could be understood that the scaffold experiences bulk degradation with the treatment of both lysozyme and collagenase.
Comparing the degradation rate between two enzymes, there is no significant change in the degradation percentage. After the period of 28 days, approximately 60 and 65% of degradation was observed in the scaffolds treated with collagenase and lysozyme solution. While, in the absence of...
collagen, 45 and 50% degradation was observed (Figure 2.5). \( \beta \) (1-4) glycosidic linkage of cellulose is more susceptible to lysozyme degradation and as cellulose plays the vital role in crosslinking, the breakage of glycosidic linkage would have disturbed the whole linking, which might be the reason for increased degradation rate in the lysozyme solution compared to collagenase in both CP and CCP groups. Modification of the scaffolds with collagen and silica, could tune the rate of degradation of the scaffolds. Hence, these degradable scaffolds satisfying the basic requirement of ideal tissue engineering scaffold could be utilized for drug delivery and tissue engineering applications.

4.3.6 Thermo Gravimetric Analysis (TGA)

Figure 4.7 shows the TGA thermograph of the scaffolds. As the temperature is applied in the range of 0 – 900 °C, distinct stages of weight loss was observed. In the scaffold preparation, it is very essential that the individual components used in the synthesis should endure high temperature, in order to avoid decomposition during processing and synthesis. Comparing Figures 4.7 and 2.6, significant difference was not observed in the thermal degradation pattern of scaffolds with and without collagen. The percentage weight loss below 250 °C does not show much difference even with the addition of collagen. The initial weight loss observed below 100 °C corresponds to loss of \( \text{H}_2\text{O} \) molecules and collagen denaturation. The measure of temperature required to convert triple helix structures to peptide sequences, determines the thermal stability of collagen. In the absence of collagen (CP0), the denaturation begins at 110 °C, whilst in CCP0 it begins at 80 °C, due to combination of collagen denaturation and loss of water molecules. With the incorporation of silica, the stability of the structure has improved greatly and the denaturation temperature has increased. In addition, the percentage weight loss has slightly decreased in the samples with silica, which proves that, with
the inclusion of silica particles, the thermal stability of the scaffolds has improved. The second transition of the CCP0 in between 110-278 °C with 65% weight loss corresponds to mass loss of PVA side chain and removal of structural water molecules from collagen. Whereas, in CCP0.5 and CCP2, the second transition is shifted to higher temperature at 281 and 286 °C with respect to increase in silica. The third transition from 300 to 400 °C corresponds to the degradation of main chain of PVA, decomposition of amorphous content of cellulose and collagen structure degradation resulting in almost 70-75% of weight loss varying with the ratio of silica in the scaffold. The final transition above 400 °C represents the degradation of crystalline regions of cellulose and PVA and complete degradation of collagen, thus resulting in the carbon residue.

Figure 4.7 TGA thermograph of CCP0, CCP0.5 and CCP2

Analyzing the overall degradation pattern of CP and CCP samples, the results could be concluded as, with the addition of collagen, the degradation temperature has shifted to lower temperature and on
incorporating silica particles, the degradation temperature has shifted to higher temperature and the percentage weight loss has decreased. With these results, it is clearly evident that the thermally stable homogeneous composites with good interaction were obtained.

4.3.7 Differential Scanning Calorimetry (DSC)

Figure 2.8 shows the DSC thermograph of the collagen modified samples and it reveals that the phase transitions of CCP samples are comparatively identical to scaffolds without collagen except with few modifications. In CCP0 the peaks were observed at 132, 220 and 278 °C. The split peak at 132 °C represents the collagen denaturation, dehydration and decomposition of amorphous content of silica.

![DSC thermograph of CCP0, CCP1 and CCP2](image-url)

Figure 4.8 DSC thermograph of CCP0, CCP1 and CCP2
Also, due to good intra molecular interaction of individual components, the glass transition temperature of PVA is shifted to high temperature and merged together to form a broad band at 138 °C. The melting of PVA chains is found at 220 °C. The sharp peak at 278 °C attributes to transition of crystalline structure of cellulose. At this temperature, the cellulose molecules undergo pyrolysis, resulting in levoglucoson. With the incorporation of silica particles, the peaks were shifted to higher temperature, due to the enhanced interaction among cellulose, collagen, PVA and silica particles.

4.3.8 Tensile Stress-Strain

The mechanical response of the scaffolds includes the essential requirement of the ideal scaffold. The mechanical properties of the material depend on the pore distribution, porosity, properties of the individual components. The degree of hydrolysis and concentration of PVA, effect of cellulose linking and elastic nature of collagen incorporates the effect on the mechanical property of the composite scaffolds. CCP0 composite shows an average tensile strength of 2.4 MPa and the average tensile strain of 178% (Figure 4.9). After incorporation of silica the tensile strength has drastically decreased to 0.212 MPa and the tensile strain decreased to 51%. The decrease in tensile stress and strain is due to the increase in pore size, number of pores and porosity. In spite of increased interaction with the addition of silica the tensile stress – strain has decreased considerably. The incorporation of fillers has loosened the structure and increased the brittleness of the scaffolds, resulting in decreased mechanical behavior. These results suggest that all the scaffolds are found to be highly elastic. But, the elasticity of the scaffolds decreased with the addition of collagen and silica. The mechanical properties also propose that these scaffolds can be further applied for wound healing, non load bearing areas of bone regeneration, vascular and cartilage implants.
4.4 CONCLUSIONS

Highly porous, interconnected scaffolds were obtained with the addition of collagen to cellulose-PVA-Si scaffolds. The crosslinking density
of the scaffolds has improved by blending collagen with cellulose-PVA-Si composite and was observed as peak shift of silanol and carboxyl groups in FTIR analysis. Also, the effect of peak broadening reveals the strong interaction of silica with the polymer backbone and these results attributes the homogeneous mixing of collagen and silica with the cellulose incorporated PVA matrix. Swelling ratio, porosity and in vitro degradation of CCP scaffolds has augmented greatly. With the addition of collagen, the enzymatic degradation of the scaffolds has increased by about 5%. The degradation rate of the scaffolds can be tuned by adjusting the silica content. The result of thermal analysis was in clear agreement with FTIR analysis proving that the interaction between the organic and inorganic phase has significantly improved by blending collagen with cellulose, PVA and silica. Highly elastic, porous, and thermally stable scaffolds with tunable degradation rate, ideal for non loading bearing and soft tissue regeneration was fabricated successfully by lyophilization technique.