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Functional Enhancement of Chitosan Nanoparticles and Silk Fibroin Binary Blended Scaffolds using MC3T3-E1 Cell Line for Tissue Engineering Applications

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Abstract
Novel materials with promising properties can be used to achieve scaffold-based tissue engineering goals. Nanochitosan and Silk polymer has remarkable thermal, morphological and biomedical properties as a material for bone tissue engineering scaffolds. The biocompatibility and bioactivity of these scaffolds are evaluated. NCS/SF blended scaffolds were fabricated using the freeze-drying technique. Thus, reinforced silk fibroin, is a bioactive and biocompatible alternative for bone tissue engineering applications. The blended scaffolds were fabricated and characterized. TGA and DSC studies reveal that the thermal stability of the blend is enhanced to carry out biomedical applications. The fabricated scaffolds showed pore sizes in the range of 100–160 µm as revealed by scanning electron microscope (SEM), good interconnectivity and high porosity. Nanochitosan incorporation had an antibacterial effect when incorporated at the higher levels in the blends. In vitro cytocompatibility results demonstrated that the blended scaffolds supported the growth and adhesion of MC3T3-E1 cell line as osteoblasts. Taken together, the Nanochitosan/Silk fibroin scaffolds might be a promising biohybrid material for tissue engineering.

Keywords
Silk fibroin, Nanochitosan, biomaterial, scaffolds, cell culture, *in vitro* studies.

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INTRODUCTION:
Bone tissue engineering is focused on fabrication of three-dimensional biodegradable porous scaffolds that possess ability to enhance adhesion, proliferation and differentiation of osteoblast cells, therefore supporting bone regeneration and functional bone tissue formation [1]. Scaffolds serves as an artificial extracellular matrix (ECM) which mimics the extracellular environment by providing appropriate environmental conditions for intercellular contact and signaling. With high porosity and interconnected network, scaffolds allow cell penetration and adhesion and support structure [2]. They are always embedded with growth factors and have an appropriate degradation rate which matches with bone formation [3]. With advanced technology, common materials such as synthetic polymeric materials, naturally derived polymers and

composite materials have been under extensive investigation as artificial scaffolds [4].

Among a vast range of biomaterials, Chitosan (CS) is counted as an excellent candidate for bone grafts as it resembles the structure of the glycosaminoglycans of natural bone. It has several outstanding properties including exceptional biocompatibility, biodegradability, osteoconductivity and antibacterial properties [5]. Being a polysaccharide, it contains reactive amine and hydroxyl groups that accelerate osteoblast growth and in-vivo bone formation. In addition, it is non antigenic and exhibit antitumor properties and thus has been extensively used for drug delivery, wound healing and tissue engineering [6].

However lower mechanical strength and solubility has limited its application in the biomedical field. It has been found that modification of chitosan as nanochitosan using TPP as cross-linking can successfully overcome these limitations [7]. Nanochitosan is an antibacterial, biocompatible, environment friendly, biodegradable material and has great potential for biomedical applications due to amino and hydroxyl groups in its chemical structure [8]. In order to provide most promising applications, it could be combined with another natural polymer called silk-fibroin.

The silk fibroin (SF) is a semi-crystalline natural polymer biocompatible and biodegradable produced by the silkworm. In solid-state, SF is found in two different crystalline modifications (silk I and silk II) as well as in a random coil form, depending on the conditions of sample preparation [9]. This biopolymer has a high periodicity with simple repeats session, separated by complex regions containing twenty amino acids with bulk side chains. The structure presents the sequence [Gly-Ala-Gly-Ala-Gly-Ser] $_n$ and allowing the folding of the crystalline region of the protein through hydrogen bonds in a secondary structure called antiparallel-β-sheet [10].

Moreover, SF has a tripeptide sequence of arginine (Arg), glycine (Gly) and aspartate (Asp), also known as RGD, which promotes cell attachment, especially osteoblasts, fibroblasts and stem cells [11]. SF is brittle on its own, which causes it to get easily deformed. In order to improve performance of such natural polymers and expand the applied range [12-14], they has been blended with various polymers which include both natural macromolecules like collagen, gelatin, elastin, chitosan, hyaluronic and sodium carboxymethyl cellulose as well as synthetic molecules like polyacrylamide, polystyrene, polyurethane, poly (L-lactide), polyvinylalcohol, poly ethylene glycol [15-16]. Those blend scaffolds with more superior properties were employed for various tissue engineering applications. Meanwhile, natural polymers have shown superiority in biomedical applications since they have proven to be most compatible with the native ECMs. SF and Nanochitosan porous scaffolds also supported the growth and adhesion of osteoblast. The blended scaffolds enhanced significantly MC3T3-E1 osteoblasts cell adhesion and cell proliferation.

2. MATERIALS AND METHODS

2.1 Materials

Chitosan was purchased from India Sea Foods, Cochin, Kerala 10×10^5 Da. Degummed Silk-fibroin in which sericin were removed was obtained from the sericulture farm in Vaniyambadi, Vellore District. MC3T3-E1 cell line was purchased from National Cell Science Centre Pune. The crosslinking agent sodium tripolyphosphate and the solvent formic acid and glacial acetic acid were procured from Finar chemicals, Ahmedabad and Thomas Bakers chemicals Pvt. Ltd., Mumbai respectively. All the chemicals utilized in this present study were of analytical grade.

2.2. Methods

2.2.1. Preparation of nanochitosan

As per the ionic gelation method, the nanochitosan was synthesized by the interaction of negatively charged sodium tripolyphosphate (TPP) with positively charged chitosan biopolymer. In order to prepare it, initially a homogeneous viscous chitosan gel was prepared by completely stirring known amount of chitosan (1g) dissolved in 200ml of 2% acetic acid for a period of 20 minutes. Sodium tripolyphosphate (0.8 g of sodium tripolyphosphate dissolved in 107 ml of deionized water) was then added dropwise to the above prepared homogeneous chitosan solution with rapid stirring for over a period of 30 min. A milky emulsion like appearance of nanochitosan obtained was then allowed to stand overnight to settle as suspension. The supernantant solution was decanted and finally the thick suspension of nanochitosan settled at the bottom of the beaker was washed several times with deionised water and preserved in the refrigerator for further use.

2.2.2. Preparation of silk fibroin

Silk fibers of 3 mm length were cut and 0.5g of it was dissolved in 100ml of 10% LiCl in formic acid. This silk fibroin solution was then stirred well under magnetic stirrer for a period of 2hrs. After this process is over, finally the thick emulsion of silk was preserved in the refrigerator.

2.2.3. Fabrication of nanochitosan/ silk fibroin scaffold

The above prepared nanochitosan and silk fibroin solutions were mixed, neutralized and stirred well for 2 hours to remove the air bubbles completely. This prepared solution was then freeze- dried to -80 ^{0}C for overnight, then lyophilized for 1 day and the scaffold was subjected to further studies.

2.3. Physico-chemical characterization.

2.3.1. **Thermo Gravimetric Analysis**

Thermogravimetric analysis was conducted to measure the thermal weight loss of the Nanochitosan / silk blends on a SDT Q600 V8.0 Build 95 instrument at a heating rate of 100°C per minute in nitrogen atmosphere. The weight losses at different stages were analysed.

2.3.2. Differential Scanning Calorimetry

The differential scanning calorimeter (DSC) was used to examine the thermal property of the Nanochitosan / silk fibroin blends. The measurements were performed with NETZSCH DSC 200 PC in a pan Al, pierced lid in the N_2 atmosphere at a heating rate of 100 K /min. The results were recorded and analysed.

2.3.3. **Scanning Electron Microscopy (SEM)**

The surface morphology and cross sectional morphology of the Nanochitosan / silk fibroin blends were observed with scanning electron microscopy to verify the compatibility of the mixtures of Nanochitosan and silk. For the analysis, the samples were cut into pieces of various sizes and wiped with a thin gold – palladium layer by a sputter coater unit (VG – microtech, UCK field, UK) and the cross section topography was analysed with a Cambridge stereoscan 440 scanning electron microscope (SEM, Leica, Cambridge, UK).

2.3.4. **Anti-Microbial studies**

The antimicrobial present in the synthesized compounds were allowed to diffuse out in the medium and interact in a plate freshly seeded with the test organisms. The resulting zone of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters. SDS nutrient broth for fungal strain of one liter, was prepared by dissolving 10g of commercially available peptone type1, bacteriological and 40g of dextrose in 1000mL distilled water and boiled to dissolve the medium completely. The pH was maintained at 6.

2.4. Cell culture

Cell types were mainly used to assess the effect of the scaffold composition onto the different stages of cell differentiation within the osteoblast lineage. MC3T3-E1 cells were cultured in Iscove's Medium (Sigma, USA) and MC3T3-E1 cells were added in the culture media to induce osteoblastic differentiation. The cell-line was then washed, hydrated for 2 h with PBS prior to cell seeding and thereafter the scaffolds were placed in a 24-well cell culture plate. After this process, then 2×10^4 cells/scaffold was seeded in a volume that soaked the scaffold and were incubated for 3 h. Five hundred µL of culture medium was added into each well. After 24 h, the scaffolds were changed to new culture wells in order to analyze only the cells growing into the scaffolds. Empty scaffolds (without cells added) were treated in the same manner and used as controls, to obtain proliferation and differentiation data.

2.4.1. Alkaline Phosphatase Assay (ALP)

Cell differentiation was evaluated by alkaline phosphatase (ALP) activity. The samples were permeabilized with 0.5% Triton X-100 and incubated with a 20nM p-nitrophenyl phosphate (Sigma, USA) solution. According to the manufacturer's instructions, the ALP activity of the MC3T3-E1 cells was then evaluated by a standard procedure. . ALP activity was measured by mixing 50 µL of supernatant with 50 µL p-nitrophenyl phosphate (5 mM) in 150 mM 2-amino-2-methyl-1-propanol buffer solution. After 30 min incubation at 37°C, the reaction was stopped by the addition of 50 µL of 0.2 N NaOH and the OD was measured at 520 nm using an ELISA reader (Bio-Rad Model 550, USA).

2.4.2. Fluorescent Assay-Cell viability analysis by live and dead assay

The scaffolds were rinsed with 0.1M PBS for 20 min. The samples were later stained using calcein-AM dye and kept under incubation for a period of 30 min. The samples were removed, washed with PBS and

observed under fluorescence microscope (Carl Zeiss, Axiovert 40CFL).

3. RESULTS AND DISCUSSION

3.1. Thermogravimetric Analysis (TGA)

TGA has been used to investigate the thermal degradation, phase transition and crystallization of the polymers. In order to ascertain the thermal stability, the prepared scaffold as NCS/SF were subjected to TGA analysis. The physical process like evaporation and the chemical process like loss of volatile gases due to degradation are studied thoroughly using thermogravimetry [17] The thermogram shows the relationship between a sample's mass and its temperature. Where polymeric scaffold of binary blend as NCS/SF and pure nanochitosan were given below.

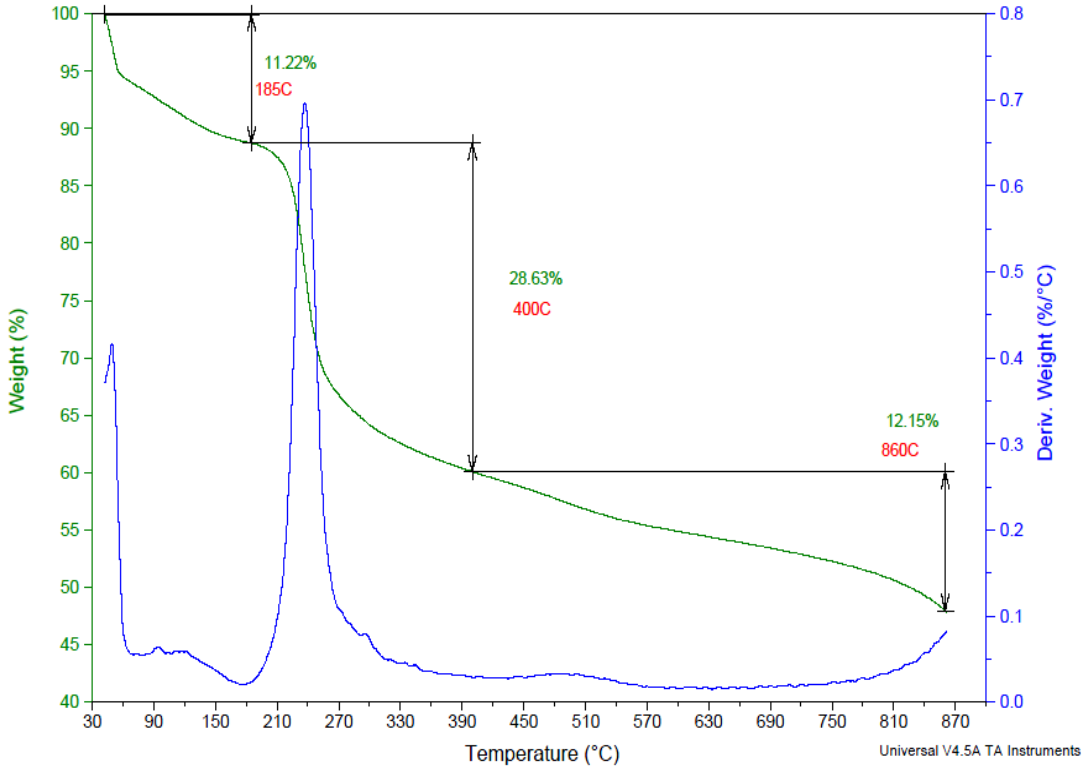


Figure-1: Represents the TGA thermogram of nanochitosan

The TGA thermogram of nanochitosan indicates that the first thermal loss occurs at temperature range 50-150ºC and it was accompanied by the weight loss ranging from 8% to 10% which may be due to the loss of residual water present in the sample. The maximum weight loss in nanochitosan occurred after 150ºC, may be due to the decomposition of polymer matrix interactions among the polymers and crosslinking agent. At the end of the experiment, nearly 45.63% of the sample remained as residue which indicates the formation of highly thermal stable nanochitosan as a result of ionic crosslinking [18].

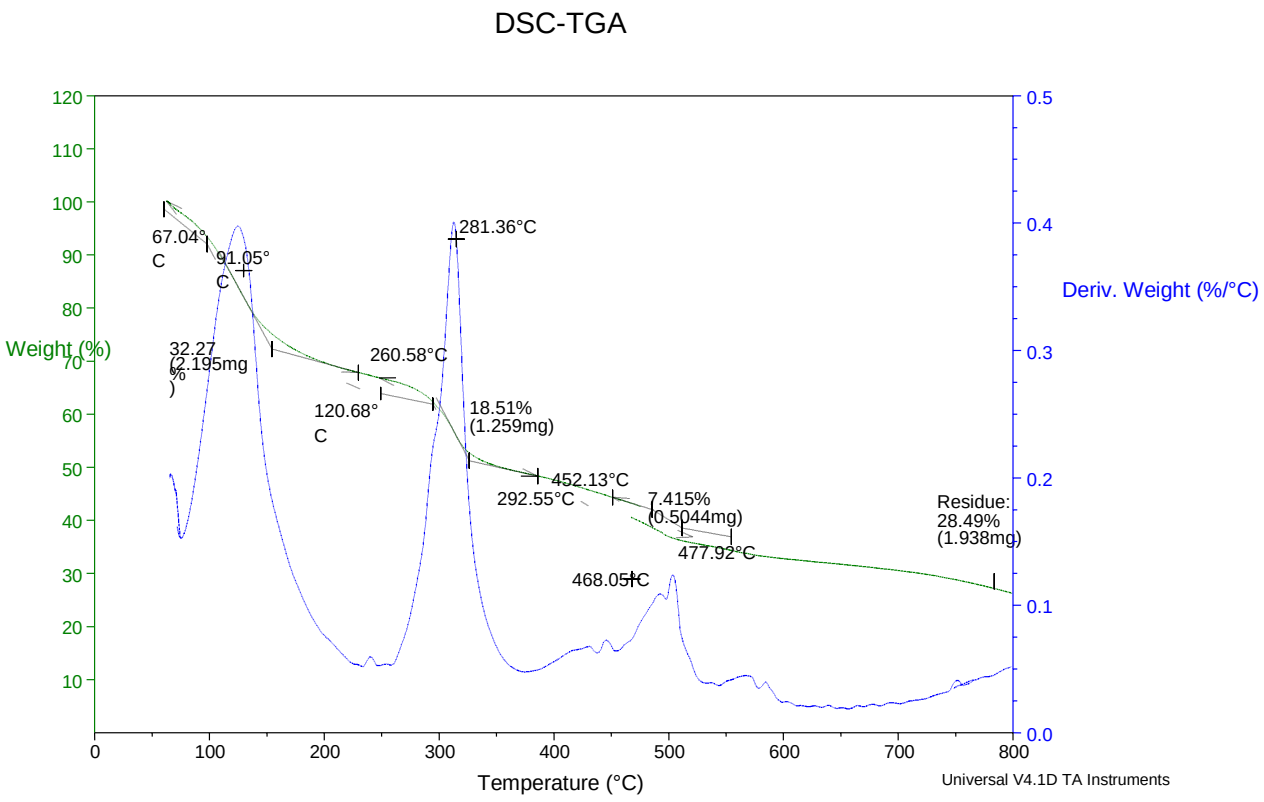


Figure-1a, represents the TGA thermogram of binary blended scaffolds NCS/SF, in MC3T3-E1 Cell line.

From the **Figure 1a** it was evident that decomposition had occurred at four stages at the temperature range of 170, 250, 370, 510 dues to moisture loss, breaking of side chains, decomposition of polymer linkage and decomposition of polymer backbone.

The TGA results shows that Nanochitosan /Silk Fibroin binary blended Scaffolds was found to have high thermal stability, which was owing to the conformation transition of SF from α-helix structure to β-sheet structure [19]. Generally, the silk fibroin increases the thermal stability of the composite. Around 71.57 % the sample gets disintegrated leaving behind 28.49% of the sample as a residue showing higher thermal stability, which confirms the slower degradation rate.

3.2. Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is the best analytical technique to find the polymer crystalinity, which measures the physical nature of the sample. DSC can also be used to study oxidation reaction as well as other chemical reactions. Differential scanning calorimetric is a convenient tool to determine the physical and chemical changes such as glass transition temperature (Tg), melting point (Tm) and Crystallization temperature (Tc) [20]. From DSC thermogram the glass transition temperature of CS-AL nanocomposite was calculated.

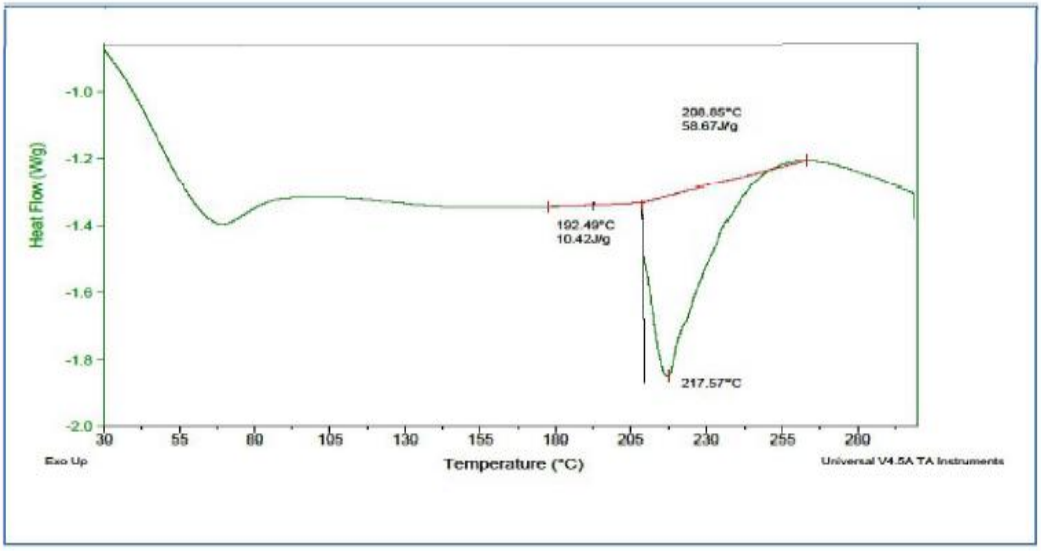


Figure 2: DSC Thermogram of Nanochitosan.

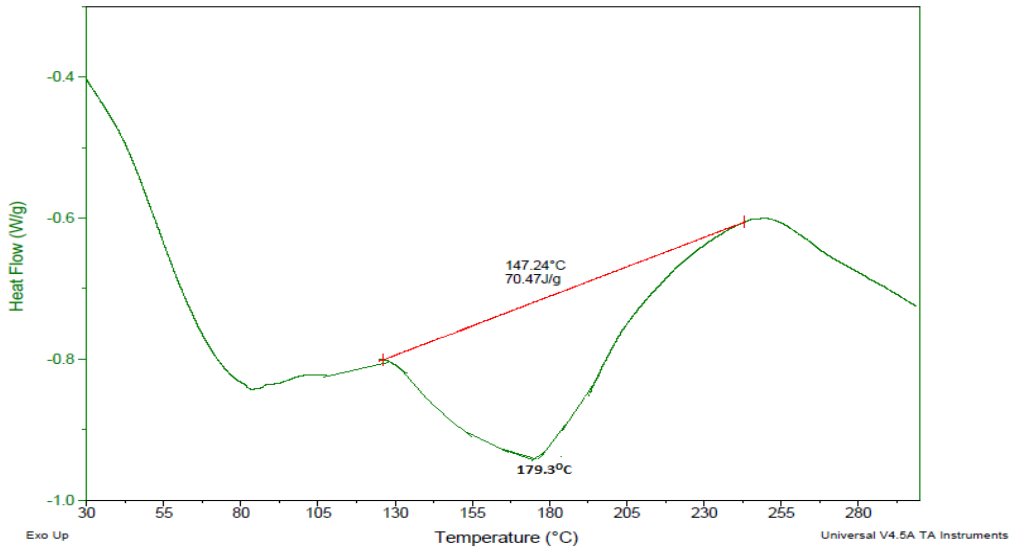


Figure 2a: DSC Thermogram of binary blended scaffolds NCS/SF, in MC3T3-E1 Cell line.

The DSC thermogram details of nanochitosan prepared from chitosan by utilizing the ionotropic gelation method was shown in **Figure 2**. A broad endothermic peak obtained below 80°C is due to the removal of absorbed water [21] and a sharp endothermic peak at 217°C is associated with the breakage of Nanochitosan phosphoric acid cross linkage. The glass transition temperature of the nanochitosan was found to be 205°C and the higher value of glass transition temperature is due to the presence of crosslinking agent [22].

Figure 2a represents the DSC thermo gram details of NCS/SF Scaffold. The initial weight loss, below 100 °C, was due to water evaporation. Increasing the SF content in the blends increased the temperature of thermal degradation, indicating that SF provided a thermal stability in the blended films. One broad endothermic peak was observed in the temperature range of 179.3°C. The glass transition temperature was found to be 147.24°C. The observation of a single glass transition in the DSC heating curves indicates that nanochitosan, and silk-fibroin are highly compatible, with higher thermal stability [23]. From the obtained higher onset temperature and higher value of glass transition temperature, it was concluded that the NCS/SF Scaffold was also found to be highly thermally stable and it shows most promising materials to carry out tissue engineering.

Scanning Electron Microscopy (SEM)

SEM is a widely used technique to study morphology and surface characteristics of the prepared blend. In the present study, SEM is used to assess morphological changes in modified chitosan with crosslinking agent as TPP, considerably modified chitosan morphology, [24] and also its physical, chemical and biodegradable characteristics, which varied with respect to the nature of the NCS/SF Scaffold.

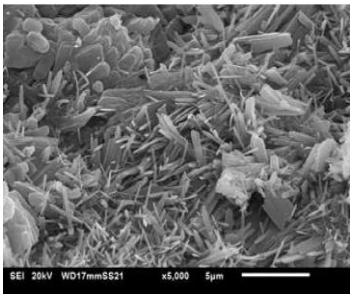


Figure 3

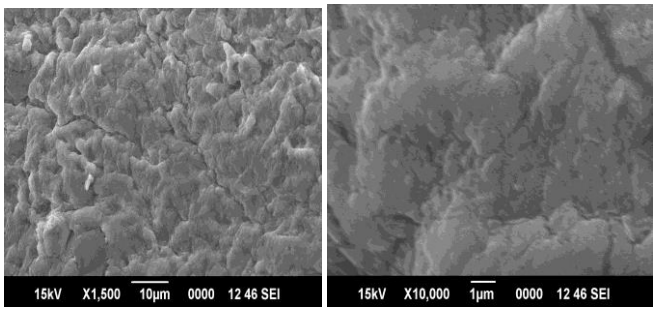


Figure 3a

Figure 3 and 3a Shows Scanning **Electron microscopy images of Nanochitosan and binary blended scaffolds NCS/SF in MC3T3-E1 Cell line.**

The SEM picture of nanochitosan **(Figure-3)** demonstrates the good dispersion of the nanoparticles which was found to be having a rod-shaped structure. Nanochitosan has relatively a rough surface with uneven structure which exhibited amorphous feature. The scaffold material used for tissue engineering should have an appropriate pore size for cells to attach and migrate [25]. In the present study, mixing NCS and SF offered a network structure with pore sizes ranging from 10 µm to 150 µm with interconnected pores. It has been reported that uniform and regular pores with high porosity can improve the mechanical properties of the scaffold [26]. When NCS/ SF content was in the ratio (1:1), the internal structure of the scaffold material had laminar pore walls. Thus, by blending of the two different polymers, the morphology and size of the pores can be controlled.

The binary NCS/SF scaffolds showed well-oriented pores, from the surface to the inside, which might be beneficial for cell seeding and distribution. NCS/SF scaffold with a fully porous structure could facilitate uniform cell seeding and nutrient delivery, which are important for cell growth in a porous 3D scaffold. This finding implied that NCS/SF are compatible very well even in nano-scale and it could be combined to generate a composite scaffold with the dual advantages of these two naturally-derived biomaterials [27].

Antifungal activity

The antifungal straining is an essential test to be done to check whether the prepared sample is effective against fungi. The antifungal activities of the prepared samples were analyzed by Agar Well Diffusion method. The zone of inhibition was calculated to find out the antifungal activity. The plates of the antifungal activity of the samples are given below [28].

Figure-4: Antifungal Activity of binary blended scaffolds NCS/SF against *Aspergillus niger, Aspergillus flavus and Mucor Mycosis.*

A better antifungal activity by the parent compound was correlated with that of water insolubility of chitosan which precipitates and stacks on the microbial cell surface as the physiological pH in microbial cells is around neutral. The formation of impermeable layer will block the channels on the cell surface and hence prevent the transportation of essential nutrients which are crucial for survival of microbial cells [29]. Contrary to that, the water-soluble chitosan derivatives are unable to form such layer, and therefore they exert no antimicrobial activity.

Particle surface charge plays a role in the inhibitory effect of chitosan nanoparticles by contributing a positive charge to improve the interaction between nanoparticles and negatively charged microbial cell surface [30]. This in turn alters fungi cell membrane permeability which eventually induces leakage of intracellular material.

In order to confirm antifungal activity against pathogenic bacteria and fungi such as *Aspergillus niger, Aspergillus flavus and mucor mycosis* on agar plates of the samples, binary NCS /SF Scaffolds were exposed to the selected fungi. Hence, zone of inhibition values of NCS/SF Scaffolds prepared are observed as 21cm, 23cm and 24cm. It was concluded the prepared scaffolds are active against the selected, fungal species and hence it can be considered as very good antimicrobial agents and it will effectively kill the microbes [31].

Alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) catalyzes the hydrolysis of phosphate esters in alkaline buffer and produces an organic radical and inorganic phosphate. Alkaline phosphatases are a group of enzymes that split off a terminal phosphate group from an organic ester in alkaline solution [32]. Alkaline phosphatase is widely distributed in almost every tissue in the body and serum ALP levels are of interest in the diagnosis of hepatobiliary disorder and bone disease.

Alkaline phosphate (ALP) activity is a widely established marker of osteoblast cells and initiator of matrix mineralization [33]. Figure-4 illustrates the *in-vitro* ALP activity of osteoblasts cultivated in Nanochitosan /Silk Fibroin binary blended Scaffolds – at various concentration.

Osteoblast differentiation in vitro and *in vivo* can be characterized in three stages: (a) cell proliferation, (b) matrix maturation, and (c) matrix mineralization [34]. In vitro, matrix maturation and mineralization are usually enhanced by growing the cells to complete confluency and by adding specific osteogenic factors [35]. (a) During proliferation, several extracellular matrix proteins (procollagen I, TGF-β, and fibronectin) can be detected. The matrix maturation phase (b) is characterized by maximal expression of alkaline phosphatase (AP).

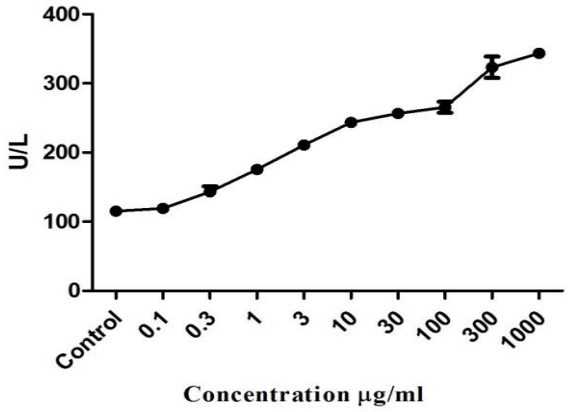


Figure-5: Alkaline phosphatase (ALP) activity in MC3T3-E1 cells culture assessed by 3D porous scaffold of NCS/SF binary blend.

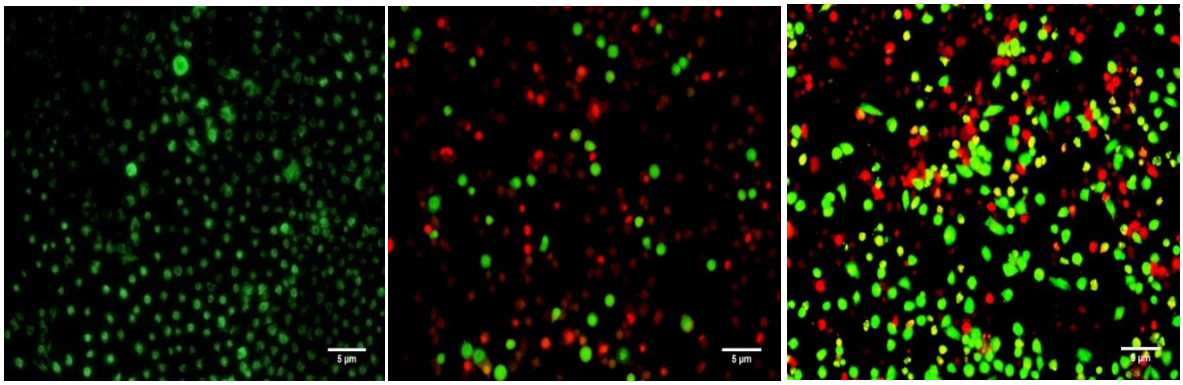
As shown in **(figure-5),** ALP activities of Nanochitosan /Silk Fibroin binary blended Scaffolds got increased with time. However, there is significant difference in ALP activity was noticed among increase in concentration. While in general, a higher ALP activity was observed for Nanochitosan /Silk Fibroin Scaffolds on account of its relatively rougher surface denoting first check-point for osteogenic differentiation [35].

The maximum toxicity of 18.34% was found for 100 µg/mL and cell viability for this concentration was 81.66%. Therefore, even at high tested concentration, Nanochitosan /Silk Fibroin Scaffolds show minimum toxicity to MC3T3-E1 cells. This confirms that the prepared Nanochitosan /Silk Fibroin binary blended Scaffolds can be biocompatible and good bone tissue engineering material.

FLUORESCENT-ASSAY

The fluorescent assay shows viability by the uses of two-color fluorescence—green fluorescence from calcein dye (marker of live cells) and red fluorescence from EthD-1 (marker of dead cells). Calcein-AM is enzymatically converted, producing fluorescent living cells, since live cells have intracellular esterase activity. Ethidium homodimer-1 is only able to enter dead cells and after binding to nuclei acids producing a red fluorescent signal [36]. Calcein AM (acetoxymethyl ester of calcein) and EthD-1 (ethidium homodimer), that measure known parameters of cell viability, the intracellular esterase activity and plasma membrane integrity [37]. Based on the simultaneous staining of live (green labeled) and dead (red labeled) cells, the cell behavior in terms of viability, proliferation, morphology and distribution was qualitatively investigated. The *in vitro* cell attachments, cell colonization and cell proliferation of the NCS/SF scaffold on MC3T3-E1 Cell culture assessed was analyzed at various concentrations [38].

Cell viability study is crucial to determine the biocompatibility of the developed scaffold matrices. Increased growth of cells over culture time signifies the non-cytotoxic behavior of the cell supportive material [39]. Figure 5.10 represents the live/ dead assay of NCS/SF blend scaffolds. The blended scaffolds showed increased cell viability which is due to the increased percentage of cell supportive domains in the blend scaffolds. Patrício et al [40] showed that the blending method of PCL/PLA had an influence on the surface roughness of plotted scaffolds and so possibly on the cell performance.



Control **25µg/ml** **50µg/ml**

Figure-6: Fluorescence microscopic images of MC3T3-E1 Cell culture assessed by living(green-labeled) and dead (red-labeled) scaffolds, NCS/SF.

Figure- 6 shows fluorescence images are observed at two days of culture the cell viability assay of NCS/SF binary scaffolds can be observed, where red cells are displayed on a dark background and of a rounded shape, because they did not extend to adhere to the substrate, indicating cell death. one can see that in the positive control cells are elongated, because they adhered to the substrate, and also maintain their integrity allowing the polyanionic calcein to be retained inside living cells producing a uniform green fluorescence (striped green) indicating that the cells are viable [41]. The results indicate that the proposed scaffold sample shows integrity and the study of cell viability, were found to be well attached and spread throughout the scaffolds representing better cell-material interaction the number of viable cells observed is more on NCS/SF representing the higher cell proliferation are observed due to the presence of building blocks of amino acids in silk -fibroin [42]. It is noteworthy that during cell culture that the scaffold is stable and shows no ruptures, showing an appropriate crosslinking between Nanochitosan and Silk-Fibroin which is indicative of cell viability.

Most importantly, these NCS/SF scaffolds improve MC3T3-E1 cells' growth, attachment and proliferation and had no negative effect on the cytocompatibility of cells. The number of dead cells in all of the groups was clearly statistically non-significant in comparison to the number of live cells. The results indicated that the insertion of silk-fibroin into the scaffolds had an optimum promotion effect for cell attachment and growth of MC3T3-E1 cells and presented nontoxicity to the cells [43]. All the above results suggest that these binary NCS/SF porous scaffolds have great potential to be used for bone tissue engineering and wound dressing materials.

CONCLUSION

The present study was mainly aimed to synthesize and characterize the Nanochitosan and Silk-Fibroin binary scaffolds. The TGA and DSC studies of the nanaochitosan/Silk-Fibroin clearly indicate that the prepared scaffold was found to be highly thermally stable. In addition to this, the surface morphology of the scaffold also identified from the SEM studies. This type of work could encourage the synthesis of new polymers, where some functionality is required, for specific purposes such as cell line culture of the osteoblast MC3T3-E1are carried out to study the invitro studies of assays like ALP and Fluorescent assay. SF is a natural biomaterial with unique biomedical and mechanical properties which make it favorable for a wide range of bone TE applications. The scaffold was found to be superior scaffold in terms of cell supportive property. The addition of SF enhanced specific ECM formation ability of scaffold has shown a slightly higher cell proliferation. Therefore, the scaffold is proven to promote cellular differentiation leading to ECM formation. However, the future of SF in bone TE, where mechanically stable and long-term degradable biomaterials are needed, is promising and has great potential tobring viable strategies and innovations.

REFERENCES

1. Hannink G, Arts CJJ. Bioresorbability, porosity and mechanical strength of bone substitutes. what is optimal for bone regeneration? Injury, 42: 22–25, (2011).
2. Navarro M, Aparicio M, Charles-Harris M, Ginebra MP, Engel E, Planell JA. Development of a Biodegradable Composite Scaffold for Bone Tissue Engineering. Physicochemical, Topographical, Mechanical, Degradation, and Biological Properties. Advances in Polymer Science, 200: 209-231, (2006).
3. Yun YR, Jang JH, Jeon E, Kang W, Lee S, Won JE, Kim HW, Wall I. Administration of growth factors for bone regeneration. Regen. Med, 7: 369-385, (2012).
4. Qi XN, MO ZL, Zhang J, Z hang ZQ. Preparation of chitosan/silkfibroin/hydroxyapatite porous scaffold and its characteristics in comparison to bi-component scaffolds. J. Biomed. Mater. Res, 102 (2): 366–372, (2014).
5. Levengood SKL, Zhang M. Chitosan-based scaffolds for bone tissue Engineering. Journal of Materials Chemistry, 2: 3161–3184, (2014).
6. Lin K, Xia L, Gan J, Zhang Z, Chen H, Jiang, X, et al. Tailoring the nanostructured surfaces of hydroxyapatite bioceramics to promote protein adsorption, osteoblast growth, and osteogenic differentiation. ACS Applied Materials & Interfaces. 5: 8008–8017, (2013).
7. Mattioli-belmonte M, Cometa S, Ferretti C, Iatta R, Trapani A, Ceci E, et al. Characterization and cytocompatibility of an antibiotic/chitosan/cyclodextrins nanocoating on titanium implants. Carbohydrate Polymers, 110: 173–182, (2014).
8. McMahon R E, Wang L, Skoracki R, Mathur AB. Development of nanomaterials for bone repair and regeneration. Journal of Biomedical Materials Research− Part B Applied Biomaterials, 101 (B(2)): 387–397, (2013).
9. Bhattacharjee P, Naskar D, Kim HW, Maiti TK, Bhattacharya D, Kundu SC. Non-mulberry silk fibroin grafted PCL nanofibrous scaffold: Promising ECM for bone tissue engineering. Eur. Polym. J, 71: 490-509, (2015).
10. Naskar D, Nayak S, Dey T, Kundu SC. Non-mulberry silk fibroin influence osteogenesis and osteoblast-macrophage cross talk on titanium-based surface. Sci Rep, 4: 4745, (2014).
11. Patra C, Talukdar S, Novoyatleva T, Velagala SR, Mühlfeld C, Kundu B, Kundu SC, Engel FB, Silk protein fibroin from *Antheraea mylitta* for cardiac tissue engineering. Biomaterials, 33: 2673-2680, (2012).
12. Lv Q, Hu K, Feng Q, Cui F. Fibroin/collagen hybrid hydrogels with crosslinking method: preparation, properties, and cytocompatibility. J. Biomed. Mater. Res, A 84: 198–207, (2008).
13. Mandal BB, Kapoor S, Kundu SC. Silk fibroin/polyacrylamide semi-interpenetraing network hydrogels for controlled drug release. Biomaterials, 30: 2826–2836, (2009).

14. Bhardwaj N, et al. Silk fibroin–keratin-based 3D scaffolds as a dermal substitute for skin tissue engineering. Integrative Biology, **7:** 53–63, (2015).
15. Sharabi M, Benayahu D, Benayahu Y, Isaacs J, Haj-Ali R. Laminated collagen-fiber bio-composites for soft-tissue bio-mimetics. Compos. Sci. Technol. 117: 268–276, (2015).
16. Murakami K, *et al.* Hydrogel blends of chitin/chitosan, fucoidan and alginate as healing-impaired wound dressings. Biomaterials, 31: 83–90, (2010).
17. Samal S, Fernandes E, Chiellini F, Chiellini E. Silk microgels formed by proteolytic enzyme activity. J. Therm. Anal, Calorim. 97: 859, (2009).
18. Mudigoudra BS, Masti SP, Chougale RB. Thermal Behavior of Poly (vinyl alcohol)/ Poly (vinyl pyrrolidone)/Chitosan Ternary Polymer Blend Films. Research Journal of Recent Sciences, 1(9): 83-86, (2012).
19. He J, Qin Y, Cui S, Gao Y, Wang S. A novel chitosan-tussah silk fibroin /nanohydroxyapatite composite bone scaffold platform with tunable mechanical strength in a wide range. J. Mater. Sci, 46: 2938–2946, (2011).
20. Gómez-Pachón EY, Sánchez-Arévalo FM, Sabina FJ, Maciel-Cerda A, Campos RM, Batina N, Morales-Reyes I, Vera-Graziano R. Characterization and modelling of the elastic properties of poly (lactic acid) nanofibre scaffolds. Journal of Materials Science, 48 (23): 8308-8319, (2013).
21. Koh LD, Cheng Y, Teng CP, Khin YW, Loh XJ, Tee SY, Han, MY. Structures, mechanical properties and applications of silk fibroin materials. Progress in Polymer Science, 46: 86-110, (2015).
22. Sivakami MS, Gomathi T, Venkatesan J, Jeong HS, Kim SK, et al. Preparation and characterization of nanochitosan for treatment wastewaters. Int J Biol macromol, 57: 204- 212, (2013).
23. Carrillo F, Colom X, Sunol JJ, Saurina J. Structural FTIR analysis and thermal characterization of lyocell and viscose-type fibres. Eur Polym J, 40: 2229-2234, (2004).
24. Han KS, Song JE, Tripathy N, Kim H, Moon BM, Park CH, Khang G. "Effect of pore sizes of silk scaffolds for cartilage tissue engineering". Macromol Res, 1-7, (2015).
25. Chattopadhyay DP, Inamdar MS. Improvement in the properties of cotton fabric through synthesized nano chitosan application. Ind J Fibre Text Res, 38: 14-21, (2013).
26. Hopkins AM, De Laporte L, Tortelli F, Spedden E, Staii C, Atherton TJ, Hubbell JA, Kaplan D. "Silk hydrogels as soft substrates for neural tissue engineering". Adv. Funct. Mater, 23(41): 5140-5149, (2013).
27. Zhang QP, Xia WS. Physico chemical properties of chitosan-based films. Food Technol Biotechnol, 46: 262-269, (2008).
28. Mandal BB, Park SH, Gil ES, Kaplan DL. "Multilayered silk scaffolds for meniscus tissue engineering". *Biomaterials*, 32(2): 639-651, (2011).
29. Ai H, Wang FR, Xia YQ, Chen XM, Lei CL. Antioxidant, Antifungal and Antiviral Activities of Chitosan from the Larvae of Housefly, *Musca domestica.* L. Food Chemistry, 132: 493-498, (2012).
30. Wang LS, Wang CY, Yang CH, Hsieh CL, Chen SY, Shen CY., Wang JJ, Huang KS. Synthesis and anti-fungal effect of silver nanoparticles-chitosan composite particles. Int. J. Nanomedicine, 10: 2685–2696, (2015).
31. Lopez-Moya F, Colom-Valiente MF, Martinez-Peinado P, Martinez-Lopez JE, Puelles E, Sempere-Ortells JM, Lopez-Llorca LV. Carbon and nitrogen limitation increase chitosan antifungal activity in *Neurospora crassa* and fungal human pathogens. Fungal Biol, 119:154–169, (2015).
32. Chatterjee S, Chatterjee BP, Guha AK. A study on antifungal activity of water-soluble chitosan against Macrophomina phaseolina. Int. J. Biol, Macromol, 67: 452–457, (2014).
33. Simonaitiene D, Brink I, Sipailiene A, Leskauskaite D. The effect of chitosan and whey proteins-chitosan films on the growth of *Penicillium expansum* in apples. J. Sci. Food Agric, 95: 1475–1481, (2015).
34. Kasperk C. et al. Human bone cell phenotypes differ depending on their skeletal site of origin. J Clin Endocrinol Metab, 80(8): 2511-7, (1995).
35. Jamuna-Thevi K, Saarani NN, Abdul Kadir MR, Hermawan H.MTriple layered PLGA/nanoapatite/lauric acid graded composite membrane for periodontal guided bone regeneration. Mater Sci Eng C Mater Biol Appl. 43: 253–263, (2014).
36. Augustine R, Malik HN, Singhal DK, Mukherjee A, Malakar Kalarikkal N, Thomas S. Electrospun polycaprolactone/ZnO nanocompositemembranes as biomaterials with antibacterial and cell adhesion properties. J Polym Res, 21: 347, (2014).
37. Chen L, Hu J, Ran J, Shen X, Tong H. A novel nanocomposite for bone tissue engineering based on chitosan–silk sericin/hydroxyapatite: biomimetic synthesis and its cytocompatibility. RSC Adv, 5: 56410–56422, (2014).
38. Shor L, Guceri S, Wen X, Gandhi M, Sun W. Fabrication of three- dimensional polycaprolactone/hydroxyapatite tissue scaffolds and osteoblast-scaffold interactions invitro. Biomaterials, 28: 5291–5297, (2007).
39. Pati F, Kalita H, Adhikari B, Dhara S. "Osteoblastic cellular responses on ionically crosslinked chitosan-

tripolyphosphate fibrous 3-D mesh scaffolds". J.Biomed.Mat.Res.Part A, 101(9): 2526-2537, (2013).

40. Han KS, Song N, Tripathy H, Kim BM, Moon CH, Park H, Khang G. "Effect of pore sizes of silk scaffolds for cartilage tissue engineering", Macromol Res, 1-7, (2015).
41. Zeng S, Liu L, Shi Y, Qiu J, Fang W, Rong M, Gao W. "Characterization of Silk Fibroin/Chitosan 3D Porous Scaffold and In Vitro Cytology". PloS one, 10(6): e0128658, (2015).
42. Luangbudnark W, Viyoch J, Laupattarakasem W, Surakunprapha P, Laupattarakasem P. "Properties and biocompatibility of chitosan and silk fibroin blend films for application in skin tissue engineering", Sci World J, 23: 45-48, (2012).
43. Varghese S, wang NSH, Canver AC, Theprungsirikul P, Lin DW, Elisseeff J. "Chondroitin sulfate based niches for chondrogenic differentiation of mesenchymal stem cells". Matrix.biol, 27(1): 12-21, (2008).