Digest Journal of Nanomaterials and Biostructures Vol. 14, No. 4, October-December 2019, p. 963-972

FABRICATION OF NANOCHITOSAN BASED BIOCOMPATIBLE POLYMER BLEND FOR BONE TISSUE ENGINEERING APPLICATIONS

V. SANGEETHA[a*], P. N. SUDHA[b], T. GOMATHI[b], J. JAYAPRABAKAR[a]
[a]*Research Scholar, Sathyabama Institute of Science and Technology, Chennai 600119, India*
[b]*PG and Research Department of Chemistry, D.K.M College for Women, Vellore 632001, India*
[c]*School of Mechanical Engineering, Sathyabama Institute of Science and Technology, Chennai 600119, India*

In vitro cytocompatibility of nanochitosan blends was evaluated with three different assays. SEM analysis revealed the highly interconnected porous structure of the blends. Addition of Silk to nanochitosan/PVP increased both pore diameter and porosity. FTIR and XRD analysis confirm the formation of ternary blends by indicating that the two polymers interact yielding a miscible blend with strong electrostatic interaction. MTT assay, Fluorscent Spectroscopy, NRR and ALP assay on MC3T3-E1 cells studies indicated that the material is non toxic, cells are attached and proliferated on the pore surfaces of the scaffold.

(Received August 9, 2019; Accepted October 28, 2019)

Keywords: NCS/PVP/SF blends, Bone tissue engineering, In vitro studies, Biocompatibility

1. Introduction

Tissue engineering is a multidisciplinary field, in which the therapeutic products were developed as scaffolds and combined with viable human cellular systems, for repair, restoration or regeneration of cells or tissues damaged by injury or disease [1,2]. In this bone tissue engineering has emerged as a promising alternative in cases of bone loss, overcoming problems of rejection and donor scarcity associated to the clinical used bone grafts [3-4]. Bone primarily consists of living cells embedded in a mineralized organic matrix or extracellular matrix (ECM) composed of a collagen and hydroxyapatite (HA) [7,8]. It has an intrinsic capacity for regeneration, cell adhesion, migration, growth, differentiation, and apoptosis are all controlled, in part, by the transmission of signals between the cell nucleus and the ECM.

The field of tissue engineering focuses on the use of 3D scaffolds to encourage regrowth and tissue regeneration at the wound site. Therefore, many attempts have been made to develop suitable 3D bone constructs scaffold consisting of natural biomaterials in order to increase bone regeneration capacity. The key role of the 3D scaffold is to mimic natural extracellular matrix (ECM) in tissue engineering [9-12].

Biomaterials exist with precise architectural order over several length scales with unique properties such as biodegradability, non-toxicity, anti-bacterial effect and biocompatibility. Chitosan is one of them, which is widely used in wound healing because it stimulates haemostasis and accelerates tissue regeneration. Chitosan is metabolized by certain human enzymes, such as lysozyme, thus, it is biodegradable. Chitosan is an attractive material for a tissue engineering scaffold because it has structural similarities to glycosaminoglycans and is hydrophilic. Chitosan's monomeric unit, N-acetylglucosamine, occurs in hyaluronic acid, an extracellular macromolecule that is important in wound repair [13,14]. Many notable advances in technology have followed

* Corresponding author: sanchan2007@gmail.com

exploitation of the properties offered by new polymeric materials like blends and composites [15-18]. Therefore, to overcome the drawback of chitosan, in this study, we had chosen the biocompatible polymers such as polyvinyl pyrolidone and silk fibroin to prepare the chiosan scaffold material with modified properties suitable for bone tissue engineering applications.

Bombyx mori silk fibroin (SF), a naturally occurring biopolymer, has excellent tuneable mechanical properties which make it an important scaffold entity for hard as well as soft tissue engineering applications [19-21]. SF, a core protein element of silk fiber, has been used as a biomaterial for medical applications because of its biocompatibility and biodegradability [22,23]. Another advantage of SF as a bone substitute is its high mechanical strength [24-26]; it can bear the force produced *in vivo* [27]. The ultimate tensile strength of SF extracted from *Bombyx mori* (*B. mori*) is 300–740 MPa [28,29] and it also has great breaking strain and high toughness more than synthetic fibers such as Kevlar [30,31]. SF scaffold can support several cell types such as osteoblast-like cells, bone marrow stromal cells [22], keratinocytes, and dermal fibroblast cells [32,33] via excellent permeability for oxygen and water [34]. These properties of SF have attracted many researchers to use this biomaterial in bone tissue engineering applications. The disadvantage of SF scaffold is the high brittleness, which makes it difficult to handle.

In order to avoid the disadvantage and limitation of pure SF and pure CS, in this study chitosan was modified into nanochitosan and mixed with silk fibroin and PVP to exhibit the blending utility in physiological compatibility and mechanical pliability. Polyvinylpyrrolidone (PVP), is a good alternative material for blending due to amide groups with strong hydrophilicity rather than hydroxyl and carboxyl groups. However, the internal relationship between the technical parameters of preparing the NCS/PVP/SF scaffolds and their subsequent morphology was systematically studied.

2. Materials and methods

2.2. Materials

Chitosan (deacetylation 92%) was procured from India Sea Foods, Cochin, India. Cocoons of *Bombyx mori* were obtained from the sericulture farm in Vaniyambadi, Vellore District, India. PVP, Sodium tripolyphosphate and acetic acid of AR grade was purchased from Finar chemicals, Ahmadabad and Thomas Bakers chemicals Pvt. Ltd., Mumbai and used without any further purification.

2.3. Preparation of Nanochitosan

Nanochitosan was prepared using sodium tripolyphosphate through ionic gelation method. By dissolving 1g of chitosan in 200 ml of 2% acetic acid solution under magnetic stirring, a homogeneous viscous chitosan solution was prepared. To this chitosan solution about 0.8g of sodium tripolyphosphate dissolved in 107 ml of water was added dropwise with continuous stirring. At room temperature the above solution was allowed to react for 30 minutes, which results in the formation of nanochitosan suspension and the suspension formed was allowed to settle for about 24 hours. The solution was decanted to isolate nanochitosan and was rinsed with distilled water and poured in petridish for drying.

2.4. Preparation of ternary blends

About 1 g of prepared nanochitosan was dispersed in the minimum amount of deionized water and blended with the equal proportion of polyvinyl pyrollidone solution under magnetic stirring. With this 0.5g of silk fibroin cut into 1 mm length was added. The stirring was continued for about 20 minutes at room temperature to get the homogeneous mixture of ternary blend. Then the ternary blend (1:1:0.5) solutions were poured into the cleaned petridish and left for air drying. The same procedure was followed to prepare various ratio such as 1:1:0.5 blend with crosslinker by adding 4 ml of glutaraldehyde and mixing for half an hour using hand stirring.

2.5. Characterization

The FT-IR spectra of prepared samples were recorded by using the Perkin Elmer 200 FTIR Spectrophotometer. The X-ray diffraction patterns of the above prepared samples were tested by Shimadzu XD-DI Diffractometer using Ni filter Cu Kα radiation source (λ=0.154 nm), set at scan rate = 10˚/min, using a voltage of 40kV and a current of 30 mA. The morphologies of pure nanochitosan and binary blends were examined using a scanning electron microscopy (HitachiS3400N).

2.6. Cell viability studies

2.6.1. MTT assay

The MTT assay was performed according to the method of Mosmann (1983). After treatment, the medium was removed and replaced with 100 µL/well of the MTT solution (0.5 mg/mL in cell culture medium without phenol red). At the end of 3 h incubation, 100µL/well of DMSO was added to dissolve the purple formazan while shaking for 10 min atroom temperature. The absorbance of the resulting solutions was read at a wavelength of 550 nm in a Bio-Rad 550 microplate reader.

2.6.2. NRR assay

The neutral red test solution was prepared (4g/L in PBS, stored in the dark at 4°C) with medium to a concentration of 50 µg/mL and stored in the dark at 4°C. After incubation of the plates for 4 days and removal of medium, cells were then incubated with 100µl of neutral red test solution for 3 hours. The test solution was then replaced with fresh medium and cells washed with 200 µL PBS. After treatment of the cells for one minute with either 50 µL test chemical concentration or control medium, the cells were again washed with 200 µL PBS. To fix the cells and release the remaining neutral red into solution 100 µL of 1% acetic acid in 50% ethanol was added to each well. The absorbance of the resulting solutions was read at a wavelength of 540 nm in a Bio-Rad 550 microplate reader.

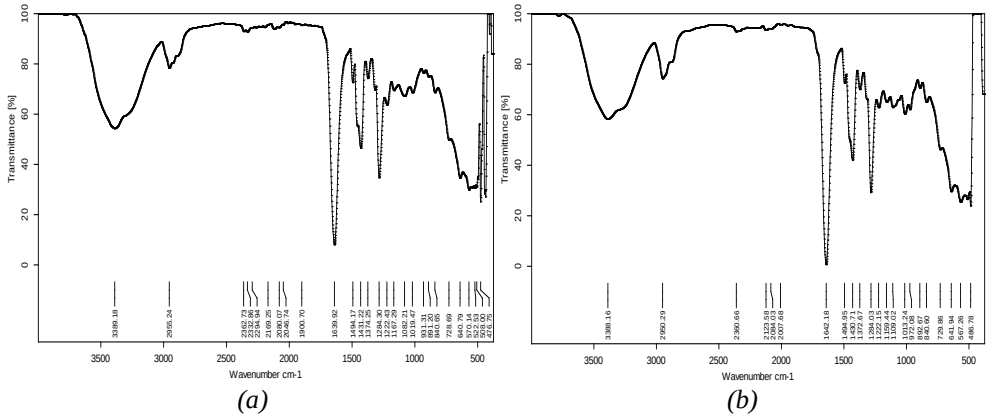
2.6.3. Alkaline phosphatase activity

Cells were seeded on the scaffold material for the estimation of alkaline phosphatase (ALP) activity. After the adequate incubation on the respective days, the cells were rinsed with PBS buffer, homogenized in 25 mM carbonate buffer (pH = 10.3) containing 0.1% Triton X-100. The pretreated cells of ALP activity was measured by incubating for 30 min at 37°C in 250 mM carbonate buffer containin g 1.5 mM $MgCl_2$ and 15 Mm para-nitro phenyl phosphate (p-NPP). In the presence of ALP, p- NPP is transformed to p-nitro phenol and inorganic phosphate. From the absorbance at 405 nm spectrophotometer (Systronics, India) ALP activity of the scaffold material was determined [35].

3. Results

3.1. FTIR studies

FT-IR provides specific information about chemical bonding and molecular structure. In this study, FT-IR analysis was applied to examine the possible interactions between the components of NCS/PVP/SF ternary blends. Figs. 1a and 1b represents the FT-IR spectrum of NCS/PVP/SF (1:1:0.5) ternary blends prepared in the absence and presence of the crosslinker glutaraldehyde.



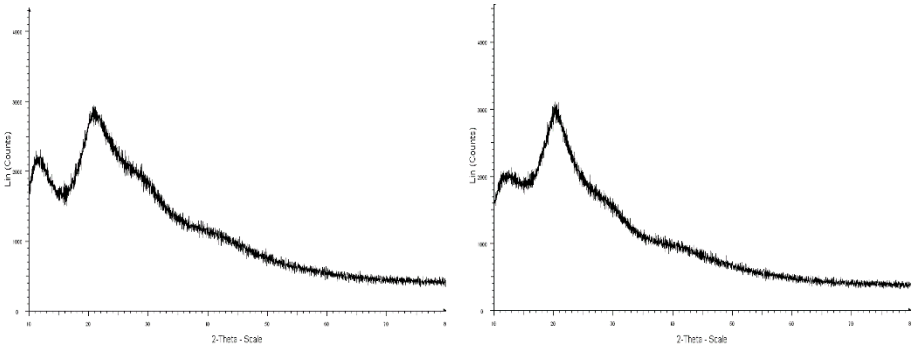
(a) *(b)*

Fig. 1. (a) FTIR spectrum of NCS/PVP/SF blend; (b) FTIR spectrum of NCS/PVP/SF blend with glutaraldehyde.

The FTIR spectrum of NCS/PVP/SF (Fig. 1a), shows a broad band around 3389 cm^{-1} due to NH stretching and OH stretching and the bands at around 1639 cm^{-1} and 1494 cm^{-1} due to amide I and amide II groups. The characteristic C-O stretching vibration appears at 1082 cm^{-1} and the P=O stretching vibration at 1284 cm^{-1}. The aliphatic (CH) stretching vibration shows a band at 2955 cm^{-1}. From the Figs. 1b, it is evident that the broadband obtained at around 3388 cm^{-1} indicates the presence of intermolecular hydrogen bonded OH stretching, NH stretching and polymeric association. The prominent bands observed at various wave numbers such as around 2950, 1642, 1494 and 1430 cm^{-1} were attributed to asymmetric and symmetric CH stretching, carbonyl stretching, NH bending, and CH deformation respectively. The presence of OH in plane bending in alcohols, P=O stretching, alcoholic C-O stretching, C-N stretching and C-C bending was confirmed by the appearance of bands at around 1372, 1222, 1159, 1109, 1013 and 486 cm^{-1} respectively.

3.2. XRD studies

XRD patterns were recorded to determine the structural phases in NCS/PVP/SF blends. The XRD patterns of NCS/PVP/SF blends without and with crosslinker were shown in Fig. 2a and 2b.



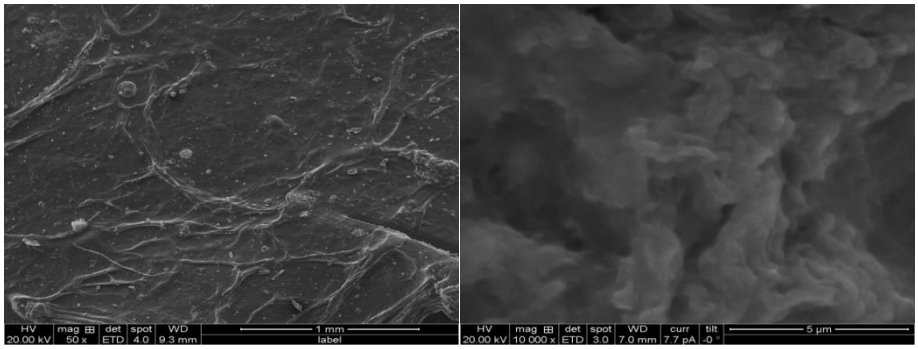
(a) *(b)*

Fig. 2. (a) XRD pattern of NCS/PVP/SF blend; (b) XRD pattern of NCS/PVP/SF blend with glutaraldehyde.

The XRD pattern of the NCS/PVP/SF blend without glutaraldehyde indicated the presence of broad major characteristic peaks at 2θ = 12, and 21°. The results revealed that the blend was amorphous in nature. The broad XRD pattern of NCS/PVP/SF blend with glutaraldehyde also shows less crystalline nature. The diffraction peaks at 2θ = 12°, and 20° were seen merged with the broad amorphous peak, which is related to the interaction between nanochitosan, PVP and SF.

3.3. SEM analysis

SEM observations of the prepared NCS/PVP/SF blend and NCS/PVP/SF blend with glutaraldehyde were shown in Fig. 3a and 3b. The images show the rough morphologies with porous structure with is essential for cell adhesion and proliferation.



a) *b)*

Fig. 3. SEM images of (a) NCS/PVP/SF blend and (b) NCS/PVP/SF blend with glutaraldehyde.

SEM analysis revealed that there were significant changes on the surface of prepared blends after interaction between the polymers. Compared with the blend prepared in the presence and absence of glutaraldehyde, the blend prepared in the presence of glutaraldehyde showed the increased porous structure.

3.4. Cytotoxicity studies

Cytotoxicity assays are widely used in in vitro toxicology studies. The LDH leakage assay, a protein assay, the neutral red and the MTT assay are the most common employed for the detection of cytotoxicity or cell viability following exposure to substances. In this study MTT assay, ALP assay and NRR assay are studied for the prepared novel NCS/PVP/SF ternary blends.

3.5. MTT assay

The metabolic activity which represents the potentiality of cells to progress in the cell cycles was quantitatively estimated during 14 days of MC3T3-E1 cells cultured over the scaffolds by MTT assay [36]. The reduction of MTT is thought to mainly occur in the mitochondria through the action of succinate dehydrogenase, therefore providing a measure of mitochondrial function [37]. The seeded cell films were incubated and selected for MTT assay on days 1,3,5,7 and 14. And then the cells are washed with PBS, incubated for 4 hours. According to the calibration curve the cell viability was calculated by converting OD values to cell number [38]. The cell proliferation of MC3T3-E1 cell line on the prepared nanochitosan/PVP/SF ternary blends was observed to be trice (Figure 4).

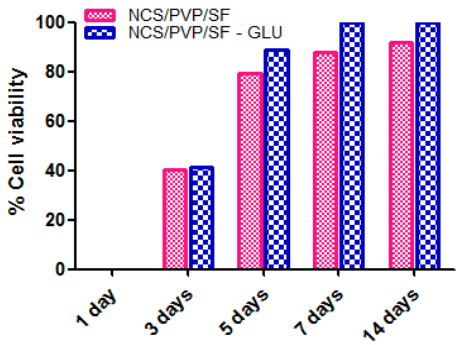
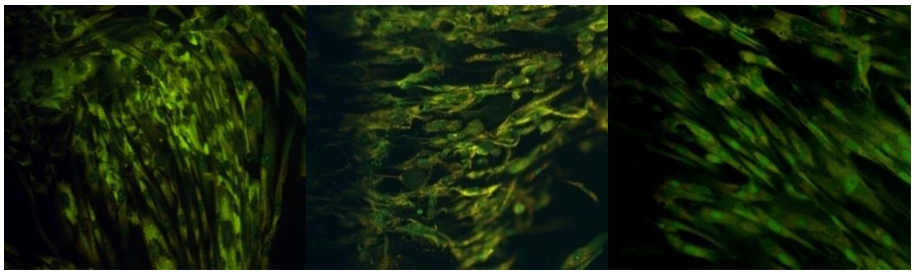


Fig. 4. MC3T3-E1 cells proliferation studies on NCS/PVP/SF ternary blends via MTT assay.

As can be seen from Figure 4 that on day 3 the optical density (OD) measured with MC3T3-E1 cells seeded NCS/PVP/SF - GLU ternary blend was higher than NCS/PVP/SF ternary blend. The cell viability was gradually increased with time irrespective of the type of material. However, maximum viability was obtained with blend up to 7 days of culture. On day 7, higher cell viability was shown by NCS/PVP/SF - GLU ternary blend followed by SF and NCS/PVP/SF ternary blend.

3.6. Cell viability studies

Cell Viability and growth of cells on NCS/PVP/SF and NCS/PVP/SF – GLU ternary matrices were examined using Live/Dead staining (Fig. 5b and 5c) and compared with the control (Fig. 5a). From the distribution of green colored cells in images taken on day 7, the NCS/PVP/SF – GLU ternary blend appeared to have the most viable cells, followed by NCS/PVP/SF ternary blend matrix.



a) *b)* *c)*

Fig. 5. Images of cell viability studies after 7 days (a) Control; (b) NCS/PVP/SF ternary blend and (c) NCS/PVP/SF – GLU ternary blend.

3.7. Alkaline phosphatase assay

The alkaline phosphatase enzyme was assessed to evaluate the early osteogenic differentiation of MC3T3-E1 cells. The characteristic marker of early osteoblastic differentiation is attributed to the cellular secretion of ALP. ALP enzymes catalyse the hydrolysis of extracellular pyrophosphates and increase the local concentration of inorganic phosphates which facilitate biomineralization [39]. The ALP activity of MC3T3-E1 cells over the scaffolds was studied during 1, 3, 5, 7 and 14 days of culture period. An increase in enzyme activity was observed with all the scaffold with varied activity level with increasing culture period from 1 to 14 days. This increase in ALP activity represents the cells in a more differentiated stage (Fig. 6).

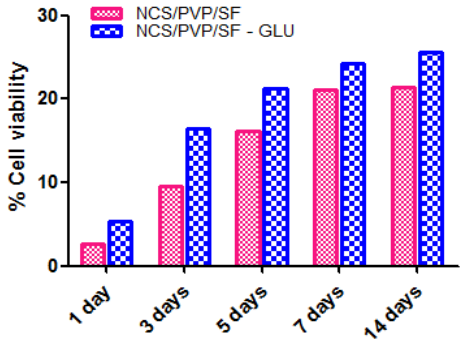


Fig. 6. MC3T3-E1 cells proliferation studies on NCS/PVP/SF blends via ALP assay.

As compared to NCS/PVP/SF blends, MC3T3-E1 cells show significantly higher ALP activity on NCS/PVP/SF blend prepared in the presence of glutaraldehyde. Hence, NCS/PVP/SF with GLU blend is confirmed to provide a superior supportive platform for osteogenic differentiation of MC3T3-E1 cells.

3.8. NRR assay

The neutral red release assay is also used to measure cell viability. Living cells take up the neutral red, which is concentrated within the lysosomes of cells. The uptake of neutral red depends on the cell's capacity to maintain pH gradients, through the production of ATP. In this analysis NCS/PVP/SF and NCS/PVP/SF with GLU blends were tested using the MC3T3-E1 cell lines. After treatment with samples, NR dye-loaded cell membrane gets damaged and release NR dye. The percentage release of neutral red dye gives the treatment-related cell survival rates, which was measured at 24 h and 48 h incubation period. The result revealed that the percentage cell viability of NCS/PVP/SF with GLU blend (Figure 7), being greater than NCS/PVP/SF blend.

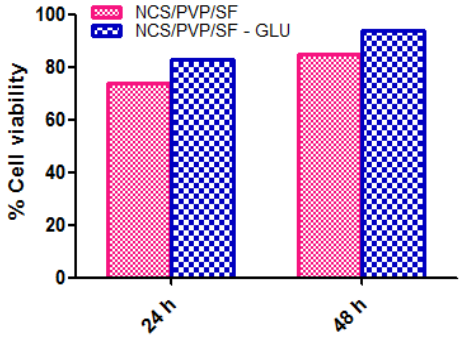


Fig. 7. MC3T3-E1 cells proliferation studies on NCS/PVP/SF ternary blends via NRR dye uptake assay.

4. Discussion

The main purpose of this study is to develop a functional scaffold material for bone tissue engineering. From the bone tissue engineering point of view, a good scaffolding material should have osteocompatibility and osteoconductivity. Cell adhesion and cellular morphology are the important indicators which reflect the affinity of the cells to the scaffold thereby forming the cell scaffold construct and finally neo tissue regeneration [40]. Cell viability depends on material architecture, especially pore size, porosity and their interconnectivity [41]. Metabolic activity signifies the potentiality of cells to progress in the cell cycle [36]. To some extent, these bioactivities are determined by the chemical structure or components. Many research works proved that either raw chitosan or many chitosan derivatives are osteoconductive or even osteoinductive. Application of the nanomaterials in a wide range of biomedical applications has compelled many studies [42]. Chitosan is a positively charged polysaccharide and therefore, it is not suitable for cell attachment when it is used by itself.

Therefore, we converted chitosan into chitosan nanoparticles with their large surface area have a strong tendency to agglomerate [43] and interact with biomolecules such as proteins and DNA in the biological environment [44-46]. After conversion also nanochitosan carries a positive charge. Further for the enhanced cell affinity and to improve the physico-chemical properties of nanochitosan, in this study we had prepared the ternary blends of nanochitosan with PVP and SF in the presence and absence of glutaraldehyde crosslinker. This blended material is expected to have a good environment for cell growth experiments. Glutaraldehyde crosslinked three dimensional materials made of nanochitosan, PVP and SF provides much better material for tissue engineering purposes.

From the comparison of FT-IR spectra of NCS/PVP/SF ternary blends prepared in absence and presence of glutaraldehyde, it can observe that after the reaction of nanochitosan with PVP and SF, the stretching vibration of –OH and NH groups observed at around 3400 cm^{-1} in nanochitosan [47,48] were shifted to lower wavenumbers in the prepared blends. Also, in addition the intensity of C=O group is increased due to the addition of PVP and SF to nanochitosan. These observed shifts in the wavenumbers and intensity for NCS/PVP/SF ternary blends conclude that the nanochitosan, PVP and SF were effectively bound to form a biomatrix in the presence of

crosslinker. The proper microstructure of pores is the key point to exert the blends efficacy, which includes the pore size, porosity, interconnection between the pores, and surface/volume ratio. The scaffold should be highly porous with proper pore size to support cell migration, cell proliferation, and vascularization deep inside the pores and permeable to facilitate the ingrowths of blood vessels, the transportation of nutrients and the removal of waste products [49]. The results obtained from FTIR, XRD and SEM analysis showed the interconnection between the polymers from the porous structures. From the XRD and SEM studies it was confirmed that the prepared blends were amouphous in nature with rough surface morphology. The change in cellular morphology may be attributed to the enhanced binding sites on the material surface thereby favoring enhanced cellular adhesion and proliferation.

These cell supportive properties of the NCS/PVP/SF and NCS/PVP/SF with GLU blend were evaluated in term of cellular attachment, spreading and proliferation of MC3T3-E1 cell cultured on the scaffold surface. As can be seen from the results, the MC3T3-E1 cells attached to the scaffolds attained a more or less elliptical shape after 12 h of culture thereby demonstrating their initial signs of spreading. The images were taken to observe the viability of MC3T3-E1 cells cultured on the developed NCS/PVP/SF and NCS/PVP/SF with GLU blend. The cell viability of MC3T3-E1 cells seeded over scaffolds was done by live/dead staining solution, where the green signal indicates viable cells and red signal indicate dead cells. Fig. 4 shows that the MC3T3-E1 cells are viable and healthy over the entire scaffold irrespective of their composition. However, cells were observed more elongated over NCS/PVP/SF with GLU blend in comparison of NCS/PVP/SF blend.

The penetration and proliferation of MC3T3-E1 cells within the blends were determined from the MTT, NRR and ALP assays. The images (Fig. 5-7) indicate that the cells were not only proliferated well over the blends, but also penetrated inside the blends at varying depth of penetration of MC3T3-E1 cells colonization. The highest penetration was occurred in NCS/PVP/SF with GLU blend.

MTT assay (Fig. 5) shows the relative cellular metabolic activity of MC3T3-E1 cells grown over the developed scaffolds during 3, 5 and 7 days of culture. The number of cells is found to increase with culture time on NCS/PVP/SF and NCS/PVP/SF with GLU blend with a varying rate of proliferation. After 7 days of exposure, NCS/PVP/SF with GLU blend supported 100% cell viability for MC3T3-E1 cell lines, which suggested good affinity and biocompatibility for cells. Incomparison with previous studies [50,51], it can be concluded that the NCS/PVP/SF and NCS/PVP/SF with GLU blend showed absolutely non-toxic effects in vitro, and also its properties can be controlled by synergetic effect of PVP and SF. The alternative cytotoxicity-based test method such as NRR assay uses MC3T3-E1 cells to identify potential cell viability of NCS/PVP/SF blends. In this assay, the cells are incubated with a water-soluble weak cationic dye, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red; NR), which selectively accumulates within lysosomes (due to the pH difference in lysosomes and cytoplasm) of healthy cells. Subsequent exposure of the NR dye-loaded cells to potential ocular irritants results in cell membrane damage and release of the NR dye which is quantified to correlate to the cell viability [52 – 54]. The NRR assay was included in several inter-laboratory studies and external validation studies, including an ECVAM retrospective validation study [55].

ALP activity is a critical tool for the assessment of cellular differentiation. ALP is an early marker which represents the differentiation and mineralization of the osteoblast phenotype [56, 51]. ALP assay of MC3T3-E1 cells seeded blends was done to evaluate the osteogenic potential of the scaffolds as shown in Fig. 6. During the initial days of incubation, an increase in ALP activity is evident upto 14 days since ALP is an early osteogenic marker. These results indicate that the NCS/PVP/SF and NCS/PVP/SF with GLU blend can stimulate matrix formation and enhance osteoblast cell differentiation.

Indeed, systematically three different cytotoxic assays were studied for the prepared ternary blends. We believe that the these assays are widely applicable for the purpose of determining cytotoxicity markers for the evaluation of xenobiotic cytotoxicity. To avoid overestimation or underestimation of the toxicity, more than one assay should be used to determine cell viability in in vitro studies with many time incubations points and this would increase the reliability of the results obtained.

5. Conclusion

In the present study, novel nano polymer blends using nanochitosan, polyvinylpyrollidone and silkfibroin were prepared by solution gelation method to mimic the function of extracellular matrix of bone. Fluroscent spectroscopy,NRR and ALP assay on MC3T3-E1 cell studies indicated that the material is non toxic, cells are attached and proliferated on the pore surface of the scaffold. Thus the present work concluded that the proposed nano ternary blends have great potential application in the field of bone tissue engineering.

Acknowledgement

The authors wish to express their sincere acknowledgement to the Department of Chemistry, DKM College for Women, Vellore, India and Jeppiaar Research Park, Sathyabama Institute of Science and Technology, Chennai, India for the extensive support rendered towards completion of this study.

References

[1] J. Venugopal, S. Low, A. T. Choon et al., J. Biomed. Mater. Res. B Appl. Biomater. **84**, 34 (2008).
[2] J. Venugopal, M. P. Prabhakaran, S. Low et al. Curr. Pharm. Des. **14**, 2184 (2008).
[3] R. Murugan, S. Ramakrishna, Compos Sci Technol. **65**(15-16), 2385 (2005).
[4] J. F. A. Valente, T. A. M. Valente, P. Alves et al., Mater Sci Eng C-Mater **32**(8), 2596 (2012).
[5] A. K. Bajaj, A. A. Wongworawat, A. Punjabi, J. Craniofacial Surg. **14**(6), 840 (2003).
[6] J. Clavero, S. Lundgren, Clin. Implant. Dent. Relat. Res. **5**(3),154 (2003).
[7] V. Karageorgiou, D. Kaplan, Biomaterials **26**(27), 5474 (2005).
[8] L. Meinel, R. Fajardo, S. Hofmann et al. Bone **37**(5), 688 (2005).
[9] M. M. Stevens, J. H. George, Science. **310**(575), 11135 (2005).
[10] B. Dhandayuthapani, Y. Yoshida, T. Maekawa et al., Int. J. Polym. Sci. **19**, (2011).
[11] B. P. Chan, K. W. Leong, Eur. Spine J. **17**(4), 467 (2008).
[12] Q. L. Loh, C. Choong, Tissue Eng. Part B Rev **19**(6), 485 (2013).
[13] C. K. Lim, N. S. Yaacob, Z. Ismail et al., Toxicology In Vitro **24,** 721 (2010).
[14] L. C. Keong, A. S. Halim, Int. J. Mol. Sci. **10**, 1300 (2009).
[15] W. J. Li, R. Tuli, X. Huang et al. Biomaterials. **26,** 5158 (2005).
[16] F. S. Kittur, K. V. Harash Parashant, K. Udaya Sankar et al.. Carbohydr. Polym. **49**, 185 (2002).
[17] Y. Qin, R. Xing, S. Liu et al., Carbohydr. Polym. **87**, 2664 (2012).
[18] Z. Rui, L. Xiang, S. Bolun et al., Int. J. Biol. Macromol. **68**, 92 (2014).
[19] L. Meinel, R. Fajardo, S. Hofmann et al., Bone **37**(5), 688 (2005).
[20] N. Minoura, M. Tsukada, M. Nagura, Polymer. **31**(2), 265 (1990).
[21] F. G. Omenetto, D. L. Kaplan, Science **329**(5991), 528 (2010).
[22] G. H. Altman, F. Diaz, C. Jakuba et al.. Biomaterials **24**(3), 401 (2003).
[23] Y. Cao, B. Wang, Int. J. Mol. Sci. **10**(4), 1514 (2009).
[24] B. Kundu, R. Rajkhowa, S. C. Kundu et al., Adv. Drug Deliv. Rev. **65**(4), 457 (2013).
[25] F. Mottaghitalab, H. Hosseinkhani, M. A. Shokrgozar et al., J. Control. Release. **215**, 112 (2015).
[26] J. Nourmohammadi, F. Roshanfar, M. Farokhi, Mater. Sci. Eng. **76**, 951 (2017).
[27] R. Nazarov, H. J. Jin, D. L. Kaplan, Biomacromolecules. **5**(3), 718 (2004).
[28] P. M. Cunniff, S. A. Fossey, M. A. Auerbach et al., Polym. Adv. Technol. **5**(8), 401 (1994).
[29] Z. Shao, F. Vollrath, Nature **418**, 741 (2002).
[30] N. Du, Z. Yang, X. Y. Liu et al.. Adv. Funct. Mater. **21**(4), 772 (2011).
[31] J. Gosline, P. Guerette, C. Ortlepp et al., J. Exp. Biol. **202**(23), 3295 (1999).
[32] I. Dal Pra, A. Chiarini, A. Boschi et al., Int J Mol Med. **18**(2), 241 (2006).

[33] T. L. Liu, J. C. Miao, W. H. Sheng et al., J Zhejiang Univ Sci B. **11**(1), 10 (2010).
[34] R. E. Unger, A. Sartoris, K. Peters et al., Biomaterials. **28**(27), 3965 (2007).
[35] Jayachandran Venkatesan, Ira Bhatnagar et al., Marine drugs. **12**, 300 (2014).
[36] C. G. Havens, A. Ho, N. Yoshioka et al. Mol. Cell. Biol. **26**, 4701 (2006).
[37] T. F. Slater, B. Sawyer, U. Sträuh, Biochim. Biophys. Acta **77**, 383 (1963).
[38] I. Guidi, D. Galimberti, S. Lonati et al., Neurobiol. Aging. **27**, 262 (2006).
[39] A. C. Allori, A. M. Sailon, S. M. Warren, Tissue Engineering Part B: Reviews **14**(3), 275 (2008).
[40] Z. Li, H. R. Ramay, K. D. Hauch et al. Biomaterials **26**(18), 3919 (2005).
[41] H. I. Chang, Y. Wang, InTech: Rijeka, Croatia, 569 (2011).
[42] S. Vandghanooni, M. Eskandani, Bioimpacts **1**(2), 87 (2011).
[43] L. K. Limbach, Y. Li, R. N. Grass et al., Environ Sci Technol. **39**(23), 9370 (2005).
[44] P. Thevenot, W. Hu, L. Tang, Curr Top Med Chem. **8**, 270 (2008).
[45] T. Xia, M. Kovochich, M. Liong et al., ACS Nano **2**, 85 (2008).
[46] X. Mu, Z. Li, X. Li et al. J Phys Chem. **113**, 5390 (2009).
[47] Sajid Bashir, Imran Nazir, Hafeez Ullah Khan et al., Trop.J.Pharm.Res. **13**, 1047 (2014).
[48] Thandapani Gomathi, P. Supriya Prasad, P. N. Sudha et al., Int. J. Biol. Macromol. Part B **104**, 1794 (2017).
[49] J. H. Lee, T. G. Park, H. S. Park et al., Biomaterials **24**, 2773 (2003).
[50] J. P. Zheng, C. Z. Wang, X. X. Wang et al., Reactive and Functional Polymers. **67**, 780 (2007).
[51] J. Li, Y. Dou, J. Yang, Y. Yin et al. Mater. Sci. Eng. C. **29**, 1207 (2009).
[52] S. Raja, Settivari, Ricardo Acosta Amado, Marco Corvaro, R. Nicolo et al., Regul. Toxicol. Pharmacol. **81**, 407 (2016).
[53] S. J. Reader, V. Blackwell, R. O'Hara, ATLA **17**, 28 (1989).
[54] S. J. Reader, V. Blackwell, R. O'Hara et al., Toxicol. In Vitro Int. J. Publ. Assoc. BIBRA **4**(5), 264 (1990).
[55] V. Zuang, Altern. Lab. Anim. **29**(5), 575 (2001).
[56] B. M. Chesnutt, A. M. Viano, Y. Yuan et al., J Biomed Mater Res A. **88**, 491 (2009).