Indian Journal of Geo Marine Sciences
Vol. 51 (05), May 2022, pp. 396-403
DOI: 10.56042/ijms.v51i05.65570

In-vitro biocompatibility studies of nanochitosan/ polyvinylpyrrolidone blends on MC3T3-E1 cells showing beneficial effect in bone tissue engineering

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Received 03 February 2022; revised 08 May 2022

Chitosan is a naturally occurring polymer with numerous biological properties that can be used in the biomedical field. Chitosan was modified to nanochitosan in this study. The polycationic nature of chitosan interacts with polyanions (TPP) to form nanoparticles. The prepared nanochitosan was mixed with polyvinyl pyrollidone to create binary blends with ratios of 1:1, 1:2, and 2:1. The polymeric blends were interpreted by using the analytical tools FTIR, XRD and SEM. The results of the FTIR and XRD confirm the formation of binary blends with strong electrostatic interaction. The study aims to investigate the compatibility of the prepared blend as tissue regenerative material. The blends were evaluated *in-vitro* using fluorscent spectroscopy, MTT assay, NRR assay and ALP assay to study its biocompatibility and cell viability. The results revealed that the quick multiplication followed by a moderate increment in cell number of MC3T3-E1 as cells reacted to the prepared material and hence the materials can be used in the regenerative medicine.

[**Keywords**: Cell viability, MTT assay, Nanochitosan, PVP]

Introduction

The use of polymers in pharmaceutical sectors and the field of polymer technology have grown significantly during the last few decades. Research on biopolymers, which provide the necessary functions for optimal utility, was particularly active worldwide. It is essential to emphasise the potential application of chitosan in the biomedical sector among a variety of widely used and biocompatible polysaccharides.

Chitosan is a naturally occurring polysaccharide that is cationic and nitrogenous in invertebrates and is also found in the exoskeletons of crustaceans. It is made up of hydrophilic N-acetyl glucosamine, and the amino group it contains is beneficial to absorption. Chitosan is presently in the form of gels, beads, fibres, membranes, microspheres, microparticle, tablets and capsules, and scaffold are used in different therapeutic and clinical frameworks, especially in orthopedic composites and tissue regeneration[1,2]. Because of its biocompatibility and osteoconductivity, it is a popular biopolymer in tissue engineering[3-5]. At the point, when chitosan is used, the amine functionality in the matrix advantageous for cell development, attachment and water absorption in cell culture[6].

Tissue engineering developed as a reaction to the issues related to the substitution of tissues lost because of infection or injury. Auto-grafting is a regular technique generally utilized as a part of treating the harmed tissue, in any case of organ deficiency, risk of organ transplant, and high expenses have constrained its application in medicinal field[7,8] and severe organ donor shortages[9]. The primary motivation for tissue engineering is to avoid these problems by developing biological substitutes that can replace damaged tissue[10].

As a result, numerous studies have been conducted to develop biodegradable and biocompatible materials for tissue engineering applications. Nanotechnology has been used in regenerative medicine because of its unique surface properties and small size. Different properties of nanoparticles can be obtained depending on the method of preparation. Ionotropic gelation, emulsification, solvent evaporation, spray drying, and other methods can be used to create chitosan nanoparticles[11]. Bodmeier and colleagues were the first to use ionic crosslinking to prepare chitosan nanoparticles. When nanochitosan was combined with tripolyphosphate[12], its zeta potential decreased. When chitosan is combined with polyanions, it can form

polyelectrolyte complexes. This complex formation was caused by the polycationic nature of chitosan[13,14].

Prabaharan *et al.*[15] created a new poly(L-lactic acid) (PLLA)-chitosan hybrid scaffold by combining PLLA as tissue-engineering scaffolds and drug release carriers. Further, Tanase *et al.*[16] investigated the enzymatic degradation and mechanical properties of chitosan (CS) and calcium phosphate-based composite scaffolds (CP). According to the findings, the scaffold material has a high potential for use in bone tissue engineering applications. Poly(lactic acid-co-glycolic acid) (PLGA) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanocapsules /chitosan scaffolds mimicking the natural healing process, according to Yilgor *et al.*[17]. Chitosan sponges containing platelet-derived growth factor stimulated bone formation in rat calvarial defects[18,19]. Venkatesan *et al.*[20] used MG63 cell lines to investigate the mineralization and protein adsorption properties of a chitosan-Alginate-Fucoidan composite[20].

To investigate the bone tissue engineering applications of nanochitosan, the current study blended nanochitosan with an ideal polymer such as polyvinylpyrrolidone (PVP), which enhances the properties of nanochitosan. PVP possess good bio compatibility and excellent adhesive property. PVP have low toxicity and high water solubility properties, therefore, its aqueous solutions are used various fields such as pharmacy, medicine, cosmetics, and so on[21,22]. When mixed with chitosan, PVP can easily interact through strong hydrogen binding with chitosan[23].

As a result, the current study sought to prepare nanochitosan/PVP binary blends in various ratios. FTIR, XRD, and SEM analyses were used to characterise the prepared materials. MTT, NRR, and ALP assays were used to determine cell viability. The prepared material is expected to provide good support and compactness for the culture of MC3T3-E1 cells. To anticipate the cytotoxicity of the prepared material, i*n-vitro* cytotoxicity tests can be utilized[24,25]. Cytotoxicity results will be depends on the test agent and the methods employed[26].

Materials and Methods

Preparation of nanochitosan

To create a homogenous solution, 200 ml of 2 % acetic acid was used to dissolve 1 g of chitosan. This chitosan solution was mixed for approximately 30 minutes at room temperature with a sodium tripolyphosphate solution (0.8g of TPP in 107 ml of water) to complete crosslinking. The resulting nanochitosan suspension was let about 24 h to settle. After separating the nanochitosan from the solution, it was washed and dried with distilled water.

Preparation of binary blend

Under magnetic stirring, 1 g of produced nanochitosan was dissolved in a tiny amount of deionized water and mixed with an equivalent amount of polyvinyl pyrollidone solution. For about 20 minutes at room temperature, the stirring was continued to produce a homogenous binary solution blend. The cleaned petridish was then filled with the binary mix (1:1) solutions, which were then let to dry by air. The same method was applied to create several ratios, including 1:2 and 2:1.

Characterization

The FT-IR spectrum was recorded using a Perkin Elmer 200 FTIR Spectrophotometer, and the XRD was recorded using a Ni filter Cu K α radiation source (= 0.154 nm), set at scan rate = 10 /min, with a voltage of 40kV and a current of 30 mA. The HitachiS3400N was used for SEM analysis.

Cell viability studies

MTT assay

The Mosmann's (1983) method was used to measure the MTT assay. After treatment, the media was withdrawn, and 100 L/well of MTT solution (0.5 mg/mL in cell culture medium without phenol red) was added in its place. After 3 h, 100L/well of DMSO was added, and the mixture was shaken for 10 min at room temperature in order to dissolve the purple formazan. A Bio-Rad 550 microplate reader set to 550 nm was used to gauge the solutions' absorbance.

NRR assay

By diluting medium to a concentration of 50 g/ml and storing it in the dark at 4 °C, the neutral red test solution (4 g/L in PBS) was prepared. Cells were incubated for 3 h with 100 l of neutral red test solution after 4 days of incubation and medium removal. The test solution was then replaced with new medium, and the cells were washed with 200 l PBS. The cells were washed with 200 l PBS after one minute of treatment with either 50 l test chemical concentration or control medium. Each well received 100 l of 1 % acetic acid in 50 % ethanol to fix the cells and release the remaining neutral red into solution. The absorbance of the resulting solutions was using a read Bio-Rad 550 microplate reader at 550 nm.

Alkaline phosphatase activity

To test the alkaline phosphatase's activity, cells were sown on the scaffold material (ALP). The cells were rinsed with PBS buffer after sufficient incubation on the designated days, and then they were homogenised in 25 mM carbonate buffer (pH = 10.3) containing 0.1 percent Triton X-100. A 30 minute incubation at 37 °C in a 250 mM carbonate buffer containing 1.5 mM $MgCl_2$ and 15 mM para-nitro phenyl phosphate was used to assess the ALP activity of pretreated cells (p-NPP). In the presence of ALP, p-nitro phenol and inorganic phosphate are produced. The ALP activity of the scaffold material was assessed using a 405 nm spectrophotometer (Systronics, India).

Results and Discussion

FTIR studies

The Fourier Transform Infrared (FT-IR) spectral technique is primarily used to evaluate polymer chemical groups and to investigate the formation of crosslinked networks between added polymeric components through potential interactions[27]. Figures 1 – 3 show the FTIR spectra of the NCS/PVP binary blends prepared in various ratios.

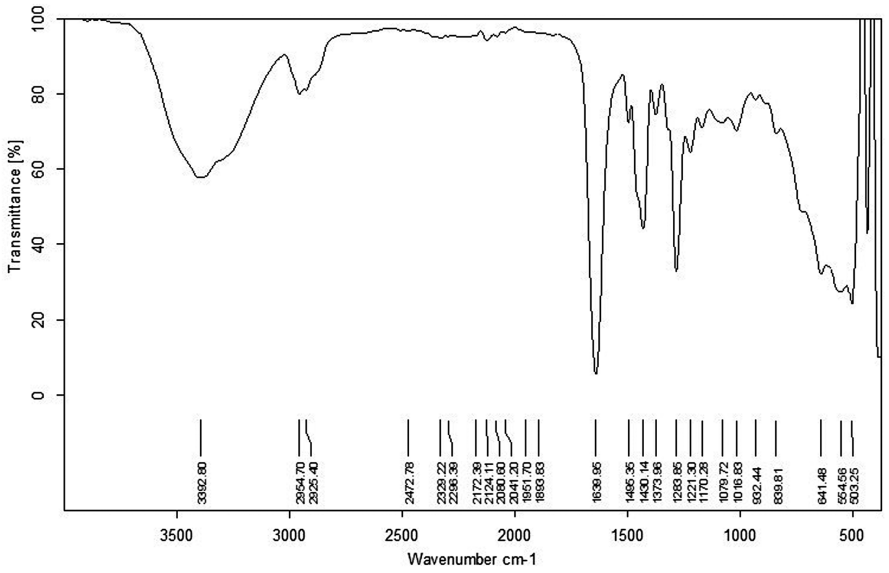

Fig. 1 — FTIR spectrum of NCS/PVP (1:1) binary blend

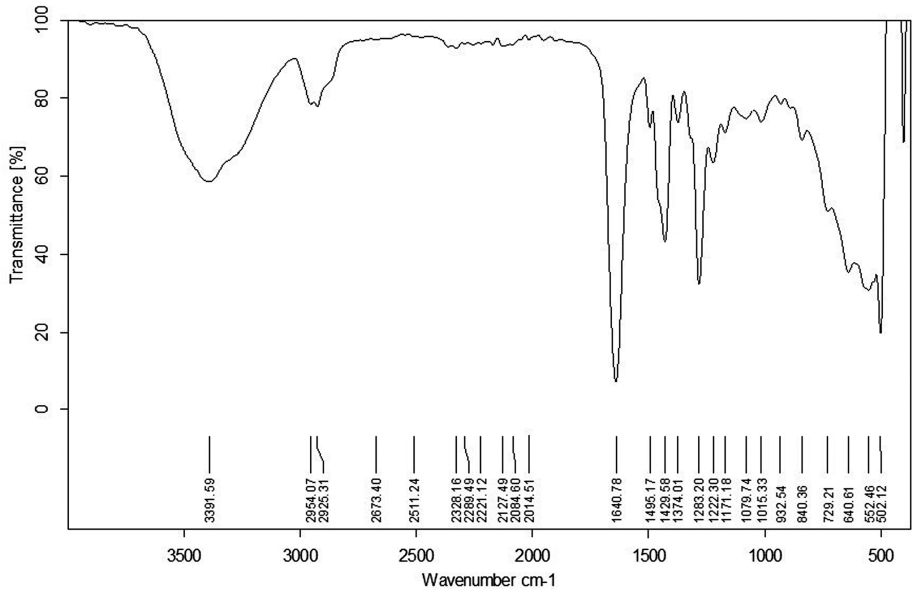

Fig. 2 — FTIR spectrum of NCS/PVP (2:1) binary blend

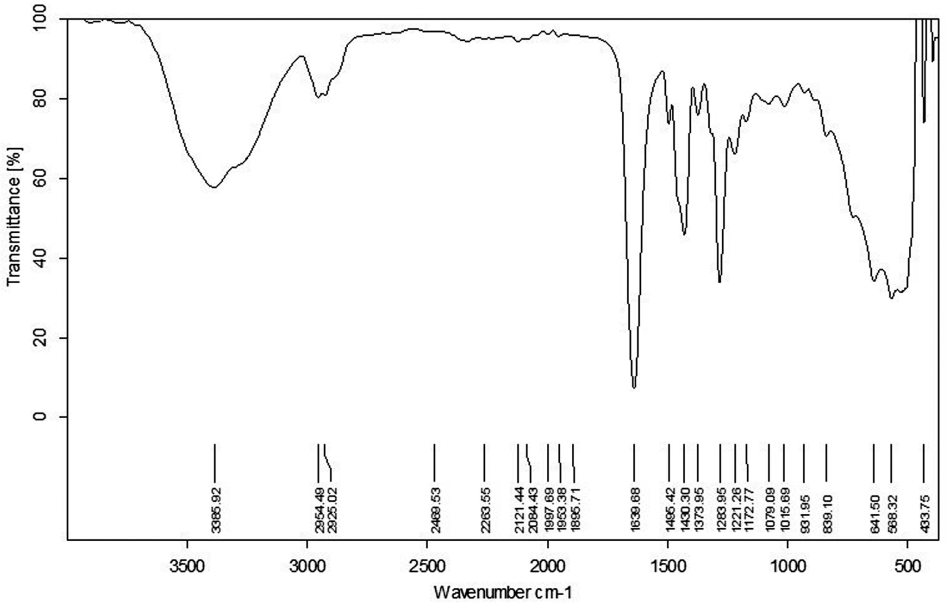

Fig. 3 — FTIR spectrum of NCS/PVP (1:2) binary blend

The FT-IR spectra of NCS/PVP binary blends prepared in 1:1, 2:1, and 1:2 ratios are depicted in Figure 1(a – c). According to the figures, OH and NH stretching modes are obtained between 3392 and 3385 cm^{-1}. Asymmetric and symmetric CH stretching, carbonyl stretching, NH bending[28], and CH deformation were attributed to the prominent bands observed around 2954, 2925, 1640, 1495, and 1430 cm^{-1}, respectively. The appearance of bands at around 1370, 1230, 1150, 1070, 1030, and 472.38 cm^{-1} confirmed the presence of OH bending, C-N stretching, P=O stretching, alcoholic C-O stretching, and C-C bending.

The comparison of FT-IR spectra of NCS/PVP binary blends prepared in different ratios reveals that after the reaction of nanochitosan with PVP, the OH and NH stretching modes found at various wavenumbers, such as 3400 cm^{-1} in nanochitosan[29], were shifted to lower wavenumbers. Furthermore, the addition of PVP to nanochitosan increases the intensity of the C=O group. The observed shifts in wavenumbers and intensity for NCS/PVP binary blends prepared in different ratios indicate that the nanochitosan and PVP were effectively bound to form a biomatrix.

X-Ray Diffraction (XRD)

XRD techniques predict polymer states such as crystalline or amorphous, and are used to calculate percentage crystallinity and identify polymers[30]. The structure of chitosan molecules is regular, and thus it forms crystalline regions to some extent; however, after crosslinking with tripolyphosphate, it exhibits a decrease in crystallinity due to the formation of nanochitosan. This could be attributed to the involvement of hydroxyl and amino groups[31] in electrostatic interactions with polyanions like TPP, which effectively destroyed the regularity[29]. There was also a peak shift during the blending of nanochitosan with polyvinylpyrrolidone due to the formation, rearrangements, and increased electrostatic interaction between the polymers.

Figures 4 – 6 show the X-ray diffraction spectra of NCS/PVP blends. The XRD pattern of the nanochitosan/polyvinylpyrrolidone (1:1) blend revealed a broad peak at around 2 = 12°, 23°, while the peaks for the 2:1 and 1:2 ratios were found at around 2 = 11° and 30°. The two broad peaks seen for the binary blends demonstrate a molecular miscibility of the polymer blend and support the FTIR findings by demonstrating a strong interaction between nanochitosan and PVP molecules.

MTT assay

The cytotoxicity and cell proliferation of nanochitosan/PVP binary blends were investigated using the MC3T3-E1 cell line and the MTT assay. Nanochitosan/PVP binary blends were found to be non-cytotoxic in the MC3T3-E1 cell line. The material used for bone tissue regeneration must have an extremely permeable structure to ensure that the natural environment is conducive to cell connection, expansion, tissue development, and adequate

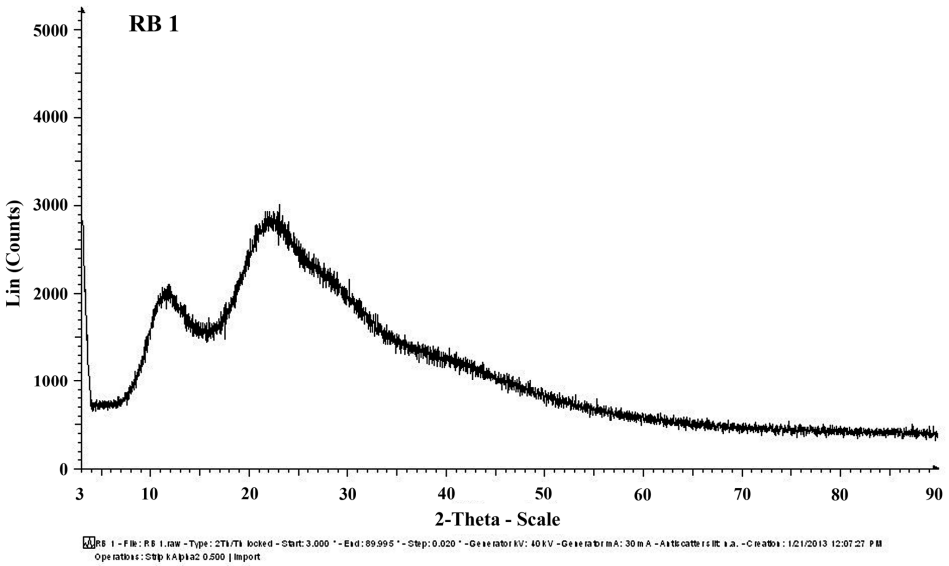

Fig. 4 — XRD pattern of NCS/PVP (1:1) binary blend

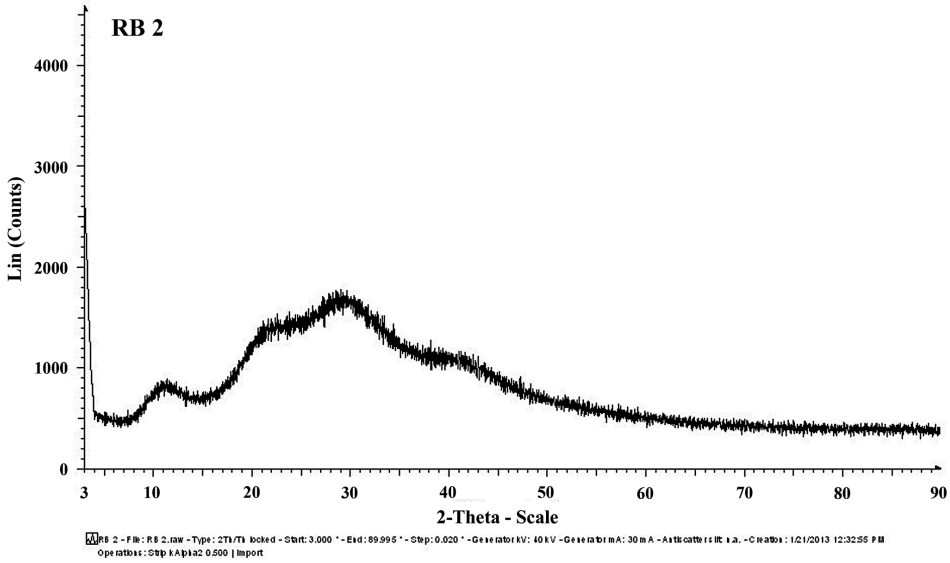

Fig. 5 — XRD pattern of NCS/PVP (2:1) binary blend

supplement flow. According to the characterization results, the nanochitosan/PVP binary blends were highly amorphous in nature and thus expected to have a high porous structure of the conductive nature.

The activity of mitochondria and the cellular viability on the cross linked films were studied through MTT assay (methyl thiazolyltetrazolium). Active mitochondria from viable cells that reduce a specific amount of MTT's tetrazolium ring. A measure of mitochondrial function[32] is provided by the activity of succinate dehydrogenase, which is assumed to occur largely in the mitochondria. The seeded cell films were incubated for 1, 3, 5, 7, and 14 days, respectively. The cells are then washed with PBS and incubated for 4 h. Cell viability was calculated using the calibration curve by converting OD values to cell number[33]. Cell proliferation of the MC3T3-E1 cell line was observed to be threefold on the prepared nanochitosan/PVP binary blends (Fig. 7). The cell viability of MC3T3-E1 osteoblastic cells cultured on the blends was demonstrated.

The percentage cell viability was determined by varying the concentrations of nanochitosan and PVP in the ratios of 1:1, 2:1, and 1:2. In comparison, cell

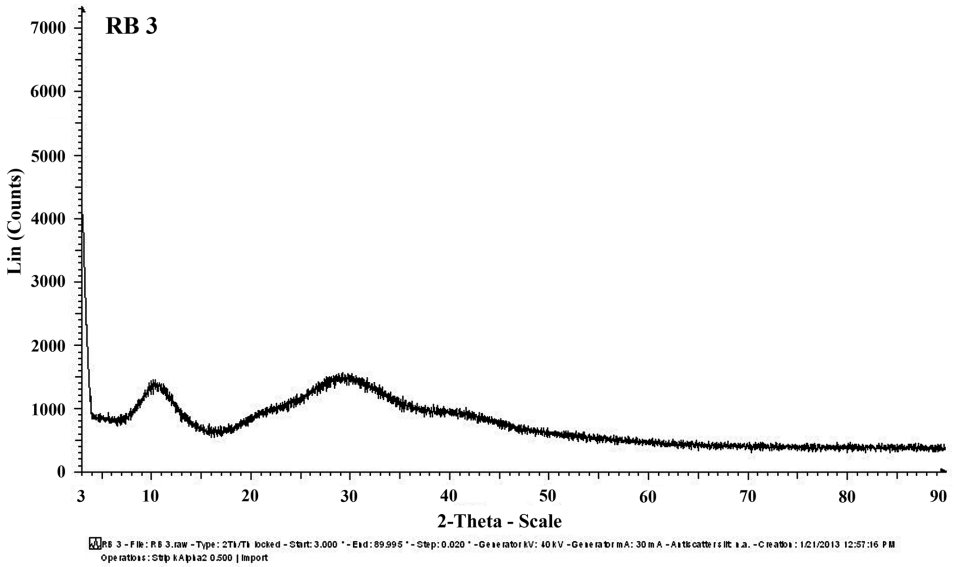

Fig. 6 — XRD pattern of NCS/PVP (1:2) binary blend

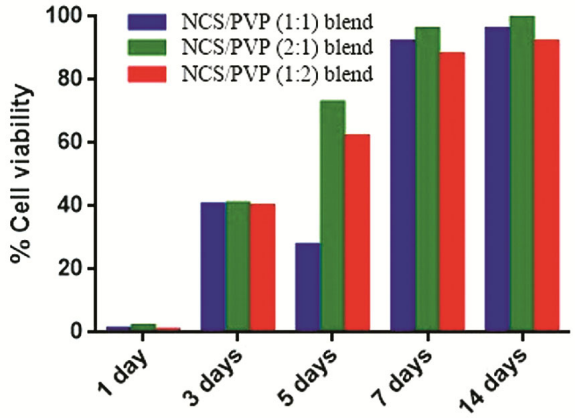

Fig. 7 — MC3T3-E1 cells proliferation studies on NCS/PVP binary blends via MTT assay

proliferation was high in the case of a 2:1 ratio, demonstrating that the presence of nanochitosan in higher concentrations increases cell proliferation through its active functional groups. On day 3, more or less equal proliferation was observed, whereas on the fifth day of incubation with the binary blends NCS/PVP (2:1) ratio showed an increased proliferation rate compared to 1:1 and 1:2 NCS/PVP binary blends.

These findings indicated that binary blends of NCS/PVP with varying polymer matrix ratios have high cell viability, which is required for bone tissue engineering applications. Cell attachment and cell proliferation are significantly aided by functional groups such as NH and OH. Dead cells were extremely rare, and viability remained very high throughout the 24 h incubation period, confirming the prepared material's biocompatibility.

Neutral Red Release (NRR)

Cell viability is also assessed using the neutral red assay[34–37]. It quickly passes through the cell membrane and builds up inside the lysosomes, where it interacts with the lysosomal matrix via anionic sites[38]. In comparison, the NR dye was easily dissolved in water and ethanol, whereas the MTT formazan was insoluble in water. The penetration of this weak cationic dye was mostly due to nonionic passive diffusion. Through electrostatic hydrophobic connections, it adheres to the lysosomal matrix[39,40]. Cell membrane damage and NR dye release, which is measured to correlate with cell survival, happen after NR dye-loaded cells are exposed to potential ocular irritants.

The neutral red release assay results show that cell viability and toxicity are time dependent. Cell viability increases with increasing time. The mean and standard deviation of the A_{570} and A_{540} esteems from triplicate cultures were estimated, and the percentage of viable cells was calculated and shown in Figure 8 to demonstrate the inconsistency of the strategy. The findings revealed that all of the NCS/PVP binary blends were non-toxic and had good cell viability for proliferation.

Alkaline phosphatase assay

The alkaline phosphatase activity (ALP) is widely used because it is thought to be a critical marker of osteoblast cell differentiation during the bone

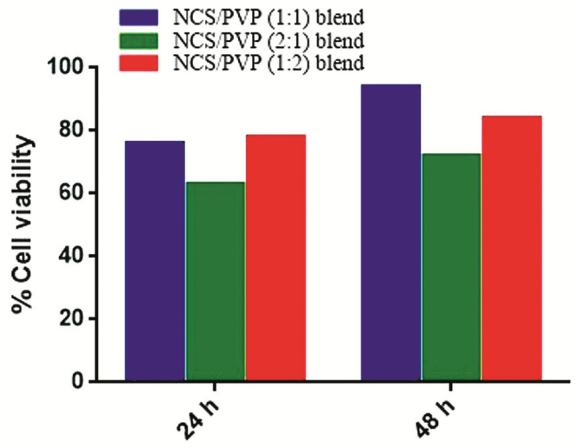

Fig. 8 — MC3T3-E1 cells proliferation studies on NCS/PVP binary blends via NR dye release assay

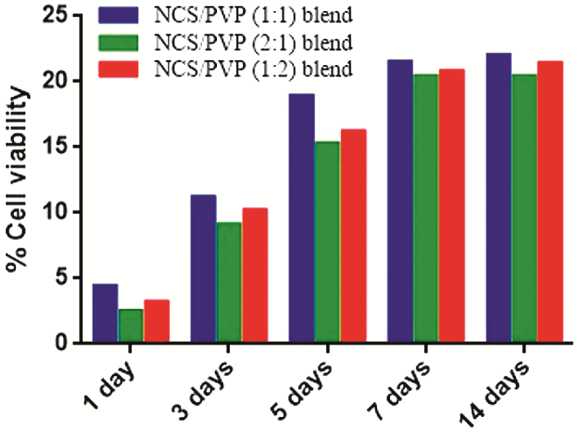

Fig. 9 — MC3T3-E1 cells proliferation studies on NCS/PVP binary blends via ALP assay

formation stage. The ALP activity of the compounds was measured to see if the NCS/PVP binary blends increased bone turnover and later bone arrangement (Fig. 9). The observation that under all settings up to day 14, rapid cell proliferation was followed by a more moderate increase in cell number suggests that the MC3T3-E1 cells responded to this material by stimulating proliferative activity.

The total protein content was determined after 1, 3, 7, and 10 days of culture. Figure 9 depicts the progression of ALP activity in MC3T3-E1 cells cultured on NCS/PVP binary blend surfaces. The results for day 1 and 3 were significantly different. Furthermore, a 1:1 binary NCS/PVP blend has higher activity than others. The ALP activity of NCS/PVP binary blends peaked on day 7. These findings suggest that binary NCS/PVP blends can improve osteoblast cell separation and network development.

Conclusion

A novel nanochitosan/polyvinyl pyrrolidone binary blend was created using the sol-gel technique to mimic the capacity of bone's extracellular matrix. Based on the physiochemical and biological characteristics of the produced blends, the biometrics were amorphous in nature and showed higher porosity, cell proliferation, and alkaline phosphatase activity than expected. The current study came to the conclusion that binary NCS/PVP blends offer a great deal of promise for use in bone tissue engineering.

Acknowledgments

This work was supported by Department od Chemistry, DKM College for Women, Vellore, India.

Conflict of Interest

The authors wish to declare that this work has no competing financial and potential interest.

Ethical Statement

The authors declare that no live organism harmed in this study.

Author Contributions

VS: Experimentation, writing - original draft and editing; PNS: Conceptualization, methodology, review & validation, and JJ: Type setting and review.

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