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3D Bio-Plotted Tricalcium Phosphate/Zirconia Composite Scaffolds to Heal Large Size Bone Defects

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Abstract: β-TCP-Zirconia scaffolds with different architectures were fabricated by means of 3D-Bioplotting in order to enhance the mechanical and in-vitro ability of the scaffold to heal large size bone defects. In the present study scaffold architecture with different strand orientations (0^o-90^o, 0^o-45^o-135^o-180^o, 0^o-108^o-216^o and 0^o-72^o-144^o-36^o-108^o) were fabricated, characterized and evaluated for mechanical strength and cell proliferation ability. β-TCP powder (25 μm) and PVA (Polyvinyl Alcohol) was acquired from Fisher Scientific, India. Zirconia (18 to 32 μm) was procured from Lobachemie, India. In brief 7.5%, PVA in distilled water was used as a binder and was mixed with 10 grams of (70/30) TCP-Zirconia ratio to make the ceramic paste. The paste was further sieved through a 100-micron sieve and was filled in a 30 ml syringe. With 400 microns needle, the scaffold architectures were printed layer by layer and were allowed to dry at room temperature. The dried samples were sintered at 1500oC in a silicon carbide furnace and were allowed to remain at this temperature for 5 hours. The sintered samples were then characterized by X-Ray Diffraction, Scanning Electron Microscopy, Uniaxial Compression Tests, Fourier transform infrared spectroscopy and cell proliferation by XTT assay using MG-63 human osteosarcoma cell line. It was revealed that all samples maintained their structure and functional groups after sintering. Also, it was found that the architecture with (0^o-72^o-144^o-36^o-108^o) strand orientation had the best strength and cell proliferation ability. Jointly these properties are required for scaffold fabrication in the field of bone tissue engineering.

Keywords: 3D-Bioplotter, in-vitro, β-TCP, Zirconia & Bone Tissue Engineering.

1 Introduction

Attempts to repair bone using implanted materials started long ago. Ancient Egyptians healed the bones using implants. Modern era work on repairing the bone defects started when a Dutch surgeon named Job van Meekeren, who made an attempt to repair a soldier's

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broken skull by implanting skull fragments from a dog. In 1911, Fred Albee successfully discovered a method for autologous bone grafting to achieve spinal fusion using parts of tibia bone [Sarkar and Lee BT (2015)]. Tissue engineering is a discipline which is based on the fact that human body has the potential to regenerate and by combining cell biology and engineering we can form new concepts which can be used to regenerate hard tissues efficiently [Mohamad Yunos, Bretcanu and Boccaccini (2008); Sapkal, Kuthe and Kashyap et al (2016)]. Research is being done all over the world to apply tissue engineering in the fields of orthopedics, artificial organ development and dentistry [Wang (2007)].

Bone tissue has got considerable importance in the body as it protects the body essential organs and holds body structure. Further, it has a high osseointegration property. Although, when the defects are too large such as in cases of trauma complete healing by self is not possible because of with there is a relative movement between the fractured bones [Sapkal, Jaiswal and Kuthe (2016)]. Current treatments available in the clinics are Autograft and Allograft implants. Autografts because of its biocompatible and bioactive nature is considered to be the gold standard. However, after implantation, it hampers the functioning at the original site. Another technique used is Allografts, where the graft of the tissue is taken from the donor of the same species but not the patient. This technique provides many options regarding shapes and sizes of the tissue used. But the drawbacks of these techniques are limited revascularization, risks of infection, immunological rejection and long-term mechanical failure [Jariwala, Lewis and Bushman et al. (2015); Sapkal, Kuthe and Kashyap et al. (2016)]. Scaffold-based regeneration has enhanced the ability to repair bone defects. Traditional methods which are used to fabricate the scaffolds are gas foaming, solvent casting, fiber meshes/fiber bonding, particulate leaching, phase separation, emulsion freeze drying, membrane lamination and melt molding. Nevertheless, these methods do not commit continuous interconnected porous structure which is necessary for the effective vascularization and tissue establishment [Sapkal (2016)].

Beta-TCP is a bioresorbable synthetic bone substitute which has been used in applications like augmentation of the alveolar ridge, sinus reconstruction and general bone reconstruction. But, because of its increase in fragility along with porosity, it cannot find applications which require reconstruction of large size bone defects [Gao, Deng and Feng et al.(2014); Mohamed, Mohamed and Beherei (2011)]. It is established that without any considerable adverse effect on the cell culture ability of the scaffold, the compressive strength can be significantly increased with the introduction of zirconia up to 30% in β-TCP matrix [Sapkal, Kuthe and Kashyap et al. (2017)]. This increase may be due to the martensitic transformation, i.e. conversion of ZrO_2 from tetragonal to cubic structure [Rapacz-Kmita, Ślósarczyk and Paszkiewicz (2006)]. In the present study, scaffolds with (30/70) Zirconia-βTCP ratio were printed using 3D Bioplotter system for (0^o-90^o), (0^o-45^o-135^o - 90^o), (0^o-72^o -144^o-36^o -108^o) and (0^o-108^o-216^o) lay down patterns. Further, the scaffolds were evaluated for its mechanical stability and cell proliferation ability.

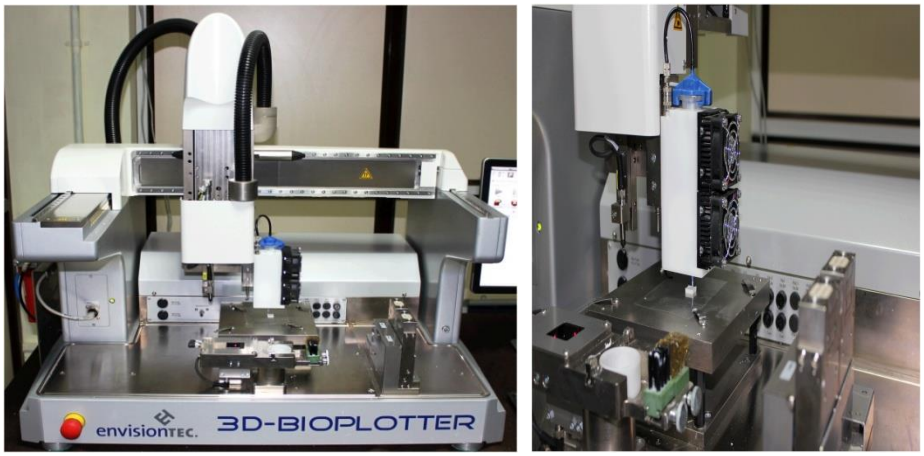
2 Materials and methods

2.1 Materials

β-tri-Calcium phosphate (25 µm particle size), Polyvinyl alcohol (PVA) were acquired from Fisher Scientific, India and Zirconium dioxide (25 µm particle size) was acquired from Loba Chemie, India.

2.2 3D-Bioplotting of TCP/zirconia scaffolds

A mesh size of 500 was used to sieve the β-TCP and Zirconium dioxide powders to obtain a uniform particle size of 25 µm of each. Afterward, 15 grams of TCP/Zirconia powder was prepared by adding Zirconia to TCP at a concentration of 30%. 37 grams of water was mixed with 3 grams of polyvinyl alcohol (PVA) for the preparation of binder solution. The solution was stirred for 30 min and then sieved with mesh size 500 to get a binder solution free of suspended particles. Finally, a uniform ceramic paste is formed by adding 15 grams of TCP/Zirconia to 8 grams of the binder solution. 3D-Bioplotter, Developer Series, Envision TEC, Germany was used to carry out solid freeform process. The system consists of (a) air pressure system to control the flow of the ceramic paste; (b) paste dispensing unit having syringe and nozzle, and (c) control unit which is connected to a computer having software (Visual Machines) to regulate the fiber deposition path. A 30ml PE (Polyethylene) syringe was filled with the ceramic paste and was placed inside the low-temperature head, which is fixed on the vertical axis of the machine. The paste was pushed outside the nozzle by applying air pressure to the plunger. The (Bioplotter RP) software of the machine was loaded with rectangular models of size 15 mm x 15 mm x 5 mm. Four different architectures having lay down pattern as (a) 0^o-90^o (b) 0^o-72^o - 144^o - 36^o - 108^o (c) 0^o - 108^o -216^o (d) 0^o- 45^o - 90^o -135^o-.



(a) (b)

Figure 1: 3D-Bioplotting of β-TCP/Zirconia ceramic: (a) Front view of 3D-Boplotter. (b) Isometric view of scaffold printing.

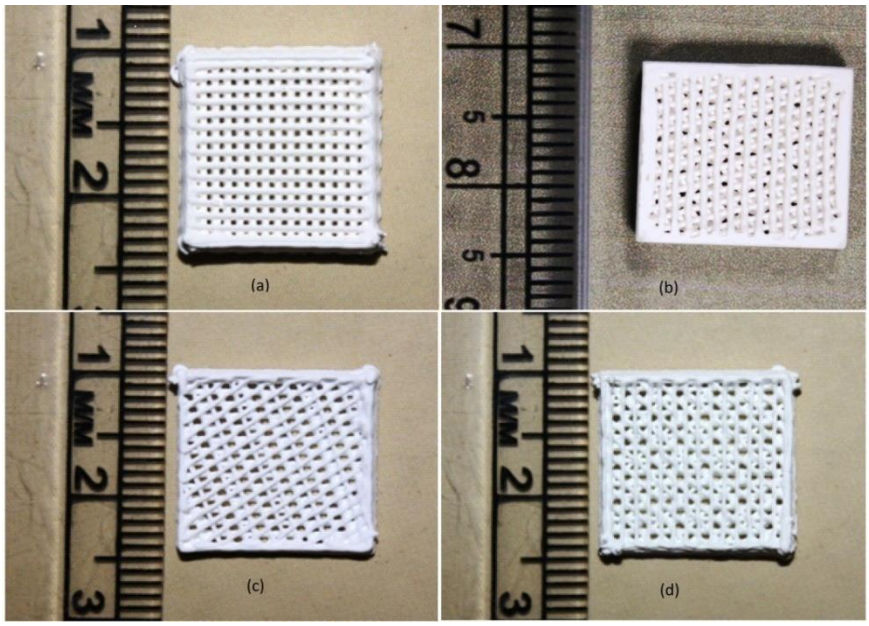


Figure 2: Sintered β-TCP/Zirconia scaffolds with different lay down pattern (a) 0^o - 90^o (b) 0^o - 72^o - 144^o - 36^o - 108^o (c) 0^o -108^o -216^o (d) 0^o- 45^o -90^o - 135^o.

PE plastic nozzle (Nordson, USA) having length 32 mm and an inner diameter of 400 μm was used to print scaffolds. The pore size of around 500 to 600 μm was generated by setting the distance between strands to 1mm for all architectures. 15 mm/s was set as the printing speed with a pressure of 3.6 bars at 30^o C as the printing head temperature. The fabricated scaffolds were kept at room temperature for 24 hours to allow them to dry. The afterward sintering process is carried out in silicon carbide furnace as follows:

RT → 600^oC in 480 min → 600^oC Temp Hold for 120 mi → 1500^oC in 590 min → 1500^oC Temp Hold for 120 min → furnace cooling → RT.

2.3 Porosity analysis

Liquid displacement method was used to calculate porosity of all four sintered scaffolds with different strand orientations. Ethanol was used as the displacing liquid because after getting absorbed it does not induce bulging or contraction in the sample. A 15 ml Eppendorf tube containing a known volume of ethanol (V1) was used to immerse scaffold sample. To fill the micro and macro pores within the sample completely, the tube was sealed and the sample was kept in ethanol for 2 hours, and the final reading was recorded as (V2). After taking out the sample soaked with ethanol from the tube, the remaining ethanol volume was measured as (V3). The formulae, p = (V1-V3) / (V2 - V3) x 100 was used to calculate percent porosity of individual scaffold [Wang, Wang and Wan (2011); Xiong (2002); Guan, Fujimoto and Sacks (2005); Sun, Meng and Li et al. (2015)].

2.4 Mechanical testing of HA/β-TCP scaffolds

Uniaxial compression tests were performed in order to find the mechanical strength

property of composite scaffold for different strand orientations. Compressive strength and compressive modulus properties of the samples were found using Instron 4467 mechanical tester with a load cell of 30 kN. In order to smoothen the contact surface for compression, samples were slightly filed before testing. The load was applied at normal room temperature conditions with no preloading at a crosshead velocity of 1mm/min [Serra, Planell and Navarro (2013); Ramay and Zhang (2004)].

2.5 Fourier transform infrared spectroscopy (FTIR)

To find the components of scaffold samples, Fourier transform infrared spectroscopy was performed. Nicolet is5 ranging from 4000/cm to 400/cm from Thermo Scientific (Waltham, MA, USA) was used to record infrared absorbance spectra of sintered β-TCP/Zirconia scaffolds. To get the final plot, a spectrum of atmospheric moisture was taken out without the sample and was further subtracted from the actual sample spectra as an open system of measurement was used [Sarikaya and Aydin (2015)].

2.6 Scanning electron microscopy (SEM)

Scanning electron microscope was used to analyze the macro pore distribution, strand size and distance between adjacent strands in manufactured β-TCP/Zirconia scaffolds after sintering. The double adhesive tape was used to place the samples on aluminum studs and JEOL JFC 1600 Auto fine coater from Japan was used to coat the samples with gold-palladium. Pictures at different magnifications were taken using JOEL-6380A scanning electron microscope [Haberstroh, Ritter and Kuschnierz et al. (2010)].

2.7 X-ray diffraction analysis (XRD)

X-ray diffractometer (XRD) examined the crystalline phases of TCP/Zirconia scaffolds. X'Pert PRO, PANalytical scanned the samples from 10^o – 70^o (2θ) where θ is the diffraction angle, at a scan rate of 1^o 2θ min^{-1}. The samples rested on aluminum supports using double adhesive tape and the copper tube was operated at 45 kV and 40 mA.

2.8 Cell culture and cytotoxicity assay

2.8.1 Materials

National Centre for Cell Science (NCCS) (Pune, Maharashtra, India) helped us in acquiring human Osteosarcoma cell line (MG-63 cell line). Himedia, India provided us with heat-inactivated fetal bovine serum, antibiotic solution, phosphate buffered saline and Dulbecco's modified eagle medium (DMEM). N-methyl sulfate (PMS), 2,3-bis-[2-methoxy-4-nitro-5sulfophenyl]-2H-tetrazolium-6-caroxanilide inner salt (XTT), and other chemicals were procured from Sigma Co. (St. Louis, MO, USA). Axiva Sichem Pvt. Ltd. (New Delhi, India) provided plastic wares used for cell culture.

2.8.2 Culture of MG-63 cells

DMEM medium was supplemented with 10 µl/ml penicillin, 10% heat-inactivated fetal bovine serum, 25 µg/ml streptomycin and 25 µg/ml amphotericin B (complete DMEM medium) to routinely maintain MG-63 cells. After every 4-5 days, cells were sub-cultured.

2.8.3 Trypsinization protocol for sub-culturing

PBS was used to wash confluent cells in order to remove excess medium. Further, 2 ml of 0.05% trypsin and (0.04%) 5 mM EDTA was added to the flask to trypsinize the cells. The flask was kept in the incubator for 2 to 3 min to bring the cells in the suspension form. The culture medium of the equal volume was added to the suspended free cells to stop trypsinization. 15 ml centrifuge tube was used to collect suspended cells and centrifugation was done at 1000 RPM for 10 min. The supernatant was discarded and fresh DMEM culture medium was used to re-suspend the cells after centrifugation. WBS counting chamber (Neuber's chamber) was used to count the cells as per standard protocol.

2.8.4 Cell cytotoxicity assay

XTT assay was used to evaluate in-vitro cell proliferation ability of each architecture type. XTT was used to assess cell proliferation after 18, 36 and 54 hours for human Osteosarcoma cells which were seeded in the presence and absence of scaffolds in a 24 well plate (4 × 104 cells/ml). 40 µl of XTT containing phenazine metrosulphate (PMS) (1 mg/ml XTT solution) was added to each sample and then kept in an incubator at 37° C in 5% CO2 humidified atmosphere for 4 hours. Robonic Readwell ELISA well plate analyzer was then used to measure the absorbance at 450 nm. In culture medium, 100% viability was considered for well-containing cells without scaffolds and the absorbance of these cells was used as a control for comparison.

3 Results and Discussion

3.1 Physical and mechanical analysis of scaffolds

Physical analysis demonstrated light-yellow shade to the scaffolds after sintering and also continuous interconnected compact structures were exposed by the scaffolds. Volumetric shrinkage was the only deformation due to high-temperature sintering. No other deformations like the geometry of the pores, variation in the architecture or breakage of the strands were observed. After sintering the scaffolds, 14 mm x 14 mm x 4.66 mm dimensions were obtained which measured 15 mm x15 mm x 5 mm before sintering. There are two main reasons for the shrinkage: First, diffusion of HA/TCP particles and second, evaporation of binder. Mechanical and porosity testing revealed higher strength and porosity values of 12.025 Mpa and 69.27% respectively for the scaffold with 0° - 72° -144° -36° -108° architecture. Scaffold with 0° - 108° -216° architecture though has highest porosity value but demonstrates low mechanical strength. Scaffolds with (0° - 90°) and (0° - 45° - 90°-135°) showed comparable values for strength and porosity. Further, in order to find the best architecture which has optimum mechanical strength, porosity and cell culture capability, all the samples were tested for its cell proliferation abilities.

Table 1: Mechanical results obtained for composite scaffolds sintered at 1500° C

Strand Orientation	Strand Size (μm)	Dist. b/w Strands (μm)	Total Porosity (*p*) (%)	Compressive Modulus (Mpa)	Compressive Strength (Mpa)
0° – 90°	400	1000	60.06	197.4383	9.403
0° – 45° – 90° – 135°	400	1000	66.56	226.456	11.785
0° – 108° – 216°	400	1000	76.46	160.398	7.639
0° - 72° - 144° - 36° - 108°	400	1000	69.27	204.199	12.025

3.2 FTIR & XRD analysis

To find the phases present in the sintered scaffolds, FTIR analysis was used to examine the functional groups after sintering in β-TCP / Zirconia scaffolds. Due to absorbed moisture, bands around 3426.18 cm^{-1} and 1633 cm^{-1} is visible and assigned to the stretching vibration of O-H bond. Absorption of non-bridging OH group is evident and is shown by the peaks around 1384cm^{-1}. Also, the monoclinic structure of ZrO_2 has been assigned the sharp bends around 745 cm^{-1} and the tetragonal structure of ZrO_2 has been assigned the wide bands of 550 cm^{-1} and 610 cm^{-1}. The presence of three single P-O bonds was indicated by bands from 1029.65 cm^{-1} to 1051.31cm^{-1} and that of the P-O single double bond was indicated by bands around 970 cm^{-1} in β-TCP. Different FTIR spectra showed no major variation. For all the samples, the presence of β-TCP, ZrO_2 along with $CaZrO_3$ peaks with 30% ZrO2 content was revealed by XRD profile of the sample sintered at 1500° C. $CaZrO_3$ was formed due to the reaction between ZrO_2 and CaO, where CaO is produced due to the decomposition of β-TCP at high temperature. When CaO gets dissolved in ZrO_2, the Cubic phase of ZrO_2 is formed from the tetragonal phase. Among the different samples, no major variations were observed in XRD profile.

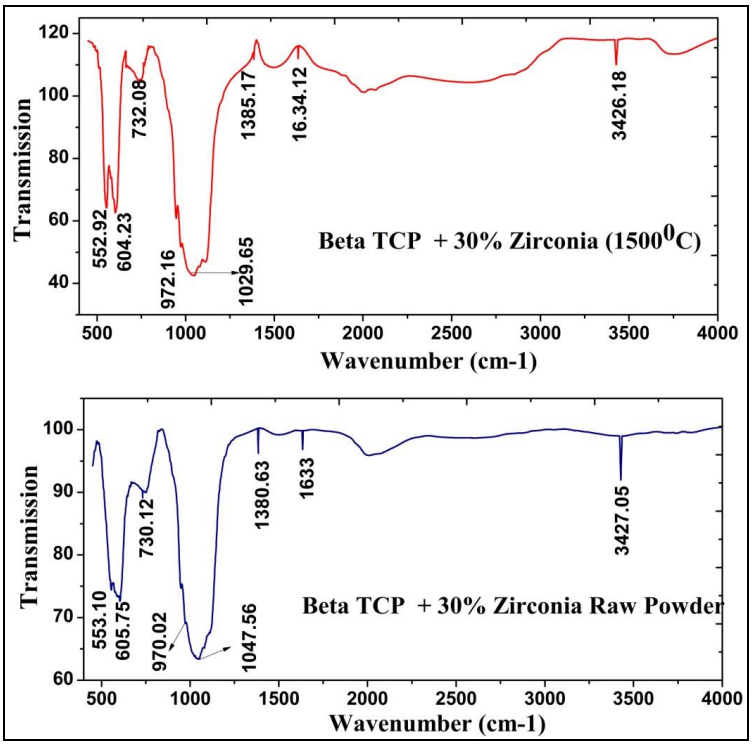


Figure 3: FTIR profile for β-TCP/Zirconia scaffold sintered at 1500° C

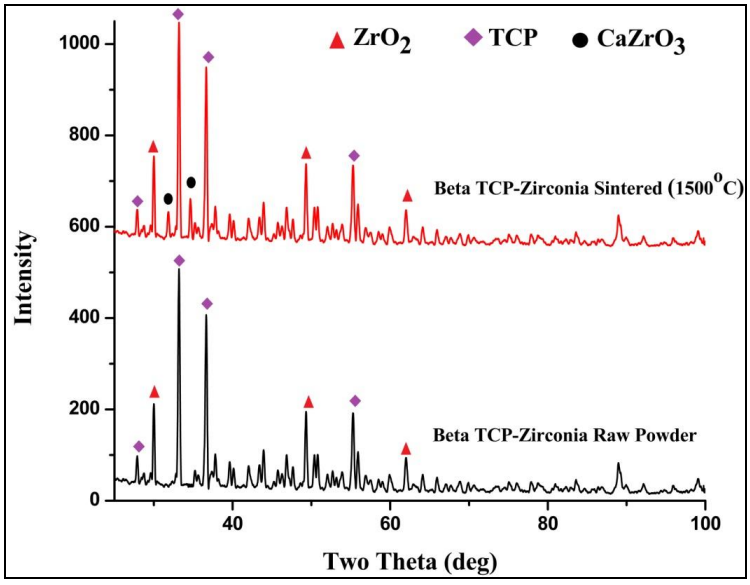


Figure 4: XRD pattern for β-TCP/Zirconia scaffold sintered at 1500° C

3.3 Scanning electron microscopy analysis

Uneven distribution of macro porosity is caused due to powder material processing. For efficient transport of nutrients and oxygen, macropores ranging from 666 µm to 705 µm are present in all samples and is shown by SEM micrograph. Also, on the scaffold surface, micropores were observed (3 to 32 µm) which is required for the initial cell adhesion. For efficient cell growth and nutrient transport, high level of interconnected porosity is required as stated previously in the text. It was observed that the overall porosity and pore connectivity network of the scaffold was affected with the type of architecture and reduction in the proportional volume of the sample was also observed. Removal of water vapor and binder from the scaffold surface and conversion of tetragonal to the cubic structure of ZrO_2 are the reasons that may be attributed to the decrease in porosity of the scaffold samples. Conversely, an increase in compressive strength was observed for sample with staggered architecture. Sample with (0^o - 72^o - 144^o - 36^o - 108^o) structure exposed more strength and porosity value, this may be attributed to the increase in the micro porosity associated with it. Without any perceptible distortion, the surface of the scaffolds maintained their respective structure after sintering.

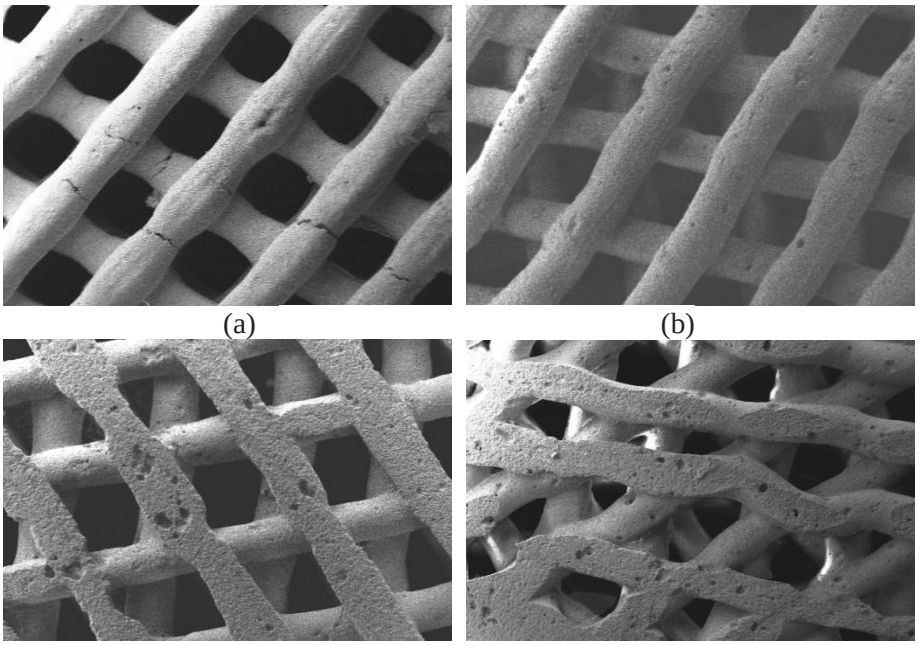


Figure 5: SEM micrograph of HA/TCP/Collagen scaffolds with different lay down pattern (a) 0^o - 90^o (b) 0^o - 72^o - 144^o - 36^o - 108^o (c) 0^o - 108^o - 216^o (d) 0^o - 45^o - 90^o - 135^o.

3.4 Cell cytotoxicity

XTT assay was performed to evaluate cell proliferation of β-TCP/Zirconia composite scaffold for 18, 36 and 54 hours. For scaffolds with 0^o -90^o, 0^o - 45^o - 90^o -135^o and 0^o -

72º - 144º - 36º - 108º architecture, there was an increase in optical density values with culture time. On the other hand, optical density values for the scaffold with 0º -108º-216º decreased. Further after 54 hours of incubation scaffold with 0º - 72º - 144º - 36º - 108º structure displayed the maximum OD value. This proves that scaffold with 0º-72º - 144º - 36º - 108º structure has morphologically well spread inside architecture which further helps cells to perfectly adhere, migrate and proliferate.

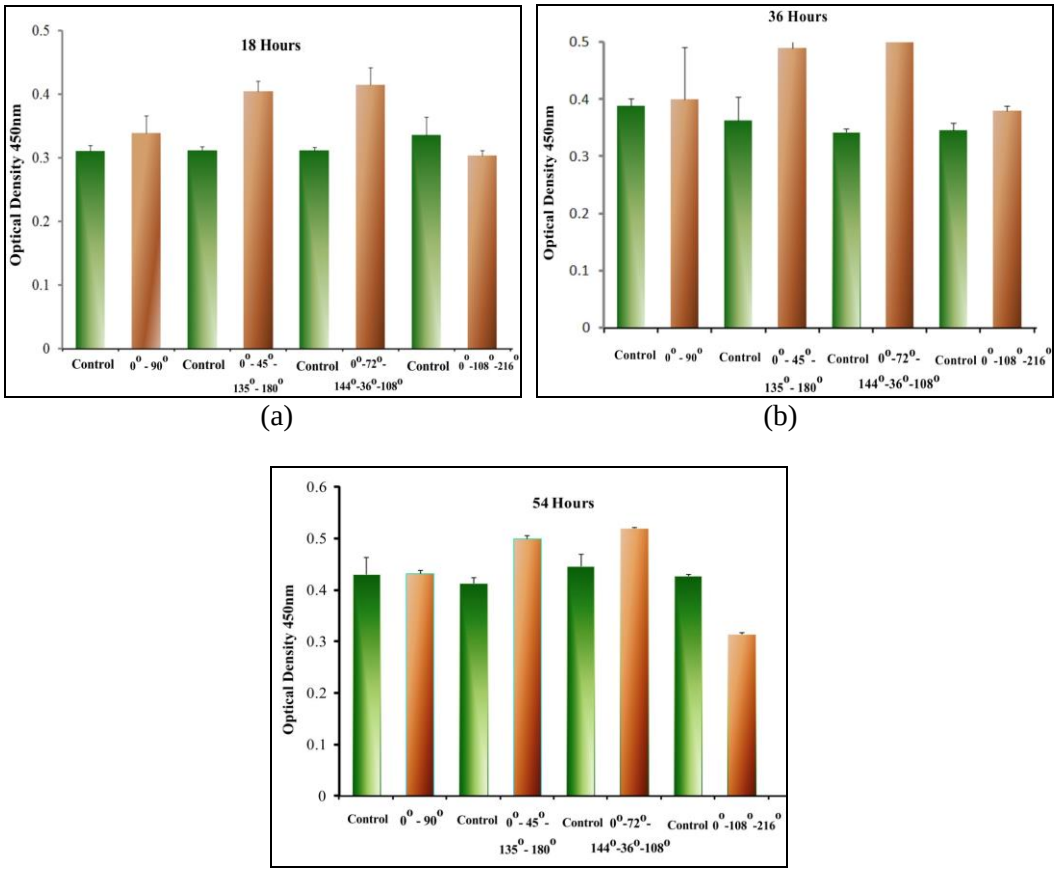


Figure 6: The depiction of optical density values for β-TCP/Zirconia scaffolds sintered at 1500º C for 18 h (a), 36 h (b) and 54 (c).

4 Conclusion

In the present study, Beta TCP-Zirconia composite scaffolds were fabricated using the 3D-Bioplotting system. Four different architectures were fabricated and were further evaluated for its mechanical strength and Cell proliferation capabilities. After high-temperature sintering, it was revealed that scaffold with 0º-72º-144º-36º-108º architecture presented excellent mechanical and cell proliferation ability. It is concluded that the present architecture with 70/30 Beta TCP-Zirconia ratio is very promising for application where bone grafting is essential to heal large size Bone defects. Future studies will include

fabrication of Haversian canals inside the scaffold architecture in order to facilitate the formation of blood vessels and promote vascularization.

5 Future Scope

Future studies will include the development of a computer programme for the fabrication of patient-specific scaffold directly from the CT data of the patient. The CT data collected in DICOM format can be used to find the point cloud to get the 3D volumetric model of the defective part. Further, the model can be saved in. STL file format which can be directly uploaded to the 3D printing machine. Also, computed assisted modeling of different scaffold architectures in relation to their mechanical strength, interconnectivity, porosity and pore size will be addressed in future studies. Furthermore, a computer programme for modeling of Volkmann's canals and Haversian canals which are responsible for efficient nutrients transport will be part of future studies.

Conflict of interest statement: The authors declare no conflict of interest.

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