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Synthesis and characterization of bioscaffolds using freeze drying technique for bone regeneration

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ABSTRACT

Bioscaffolds composed of polymeric biomaterials must be bio-compatible, absorbable and biodegradable upon implantation into humans. The aim of the current study is to synthesize highly porous bioscaffold that provides the appropriate environment for the regeneration of bone tissue. To accomplish this, bioscaffold was prepared by Freeze drying method using Beta-tricalcium phosphate, chitin and gelatin. The Scaffold was characterized by X-ray diffractometry (XRD), Fourier transform infrared spectroscopy (FTIR), and Field emission scanning electron microscopy (FESEM). The in-vitro bioactivity was tested by calcium phosphate rich layer on surface of soaked scaffold in different pH. FESEM demonstrated that the prepared scaffold had interconnected pores with a pore size of 50–60 nm. The XRD showed the composite structure and FTIR revealed the incorporation of Beta-tricalcium phosphate into chitin and gelatin. Porosity calculated by Archimedes principles was 76%. The degradation of scaffold biomaterial was pH dependent. The acidic pH had high weight loss initially ranging from 35% to 40% compared to neutral pH. However, the degradation rate was slowed down after 10 h. Thus, our study preliminarily concludes that Beta-tricalcium phosphate, chitin and gelatin incorporated bio-scaffolds could be an ideal scaffold for bone regeneration. However, further research is needed to confirm the use of synthesized scaffold for bone tissue engineering or bone defects.

1. Introduction

Fractures are usually caused by a fall, blow or other traumatic event. Pathological fractures are caused by disease that weakens the bones, which occur with little or no trauma. Osteoporosis is a disorder in which the bones thin and lose strength as they age, causing fractures each year, especially in the hip, wrist and spine (Philadelphia, 1974). Worldwide, it was estimated that 1 in 3 women and 1 in 5 men above the age of 50 will be experience osteoporotic fractures (Kanis et al., 2000). India with a population of 1.2 billion people is the second most populated country in the world with approximately 10% of population over 50 years of age affected with osteoporotic disorder (Mithal et al., 2014). Bone is a dynamic, highly vascularized hard tissue with a unique capacity to heal and remodel without leaving a scar. Bone healing process can be initiated by many methods like grafting, bone replacement etc., but now-a-days tissue engineering is being extensively researched for the benefit of society.

Tissue engineering is “an interdisciplinary field of research that applies the principles of engineering and the life science towards the development of biological substitutes that restore maintain or improve tissue formation”. The classic biomaterial approach is based on the understanding of tissue formation, regeneration and aims to induce new functional tissue. Tissue engineering is the combination of cells, scaffolds and bioactive factors and also a promising field for tissue regeneration and repair. There are currently thousands of research papers and reviews available on bone tissue engineering, but there is still a major discrepancy between scientific research efforts on bone tissue engineering and the clinical application of such strategies. Today major reconstructive surgeries (due to trauma or tumor removal) are still limited by the autologous materials available and donor site morbidity (Jan Henkel et al., 2013). Recent advances in the development of scaffold-based Tissue Engineering (TE) have given the new options for restoring form and function.

The 3D scaffolds are adopted to provide the appropriate environment for the regeneration of fractured bone and tissue (Anh-Vu Do, 2015). The requirements that must be satisfied by such scaffolds include providing a space with the proper size, shape and porosity for tissue development from the surrounding cells to migrate into the matrix. Osteoblast cells migrate towards the site of fracture by several cellular signaling pathways and involves in formation of bones. These

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cells migrate through the pores into the scaffold, take the nutrients and heal the defective area. An ideal scaffold has biocompatible, biodegradable and mechanical properties. Researchers hope to reach this goal by combining knowledge from physics, chemistry, engineering materials, biology and medicine in an integrated manner. The matrix is *in vivo* 3D scaffold for cells and provides them with a tissue specific environment and architecture. Furthermore it serves as reservoir of water, nutrients, cytokines and growth factor for tissue regeneration. Besides the choice of adequate materials that are addressed later, the macro and micro structural properties of the material are of utmost importance. Such properties affect not only cell survival, signaling, growth, propagation and reorganization but also their gene expression by preservation of their phenotype. Biopolymers which comprise of polysaccharides including cellulose, chitin, chitosan and gelatin are suitable materials for bone scaffolding but Chitosan scaffold was reported to collapse after implementation (Thein-Han et al., 2008).

Recently, combinations of bioceramic and biopolymer scaffolds reported to be more beneficial due to their strength, stiffness and osteoconductivity with cell compatibility and reabsorbability. (Kane et al., 2015; Basha et al., 2015). Biopolymers such as chitosan, gelatin is commonly used as natural biopolymer reinforced with hydroxyapatite and β-tricalcium phosphate (Dorozhkin, 2012). Chitin has been used in bone tissue engineering due to its structural similarity with glycosaminoglycan in extra cellular matrix of bone (Suh and Matthew, 2000). Gelatin is useful for bone tissue engineering and has many desirable properties such as excellent osteoconductivity, lack of antigenicity etc. (Kim et al., 2005). According to literature survey no study has been reported previously on the combination of Beta-tricalcium phosphate with chitin and gelatin as scaffolds for bone regeneration. The objective of the current study is to develop a Beta-tricalcium phosphate scaffold with chitin and gelatin for bone healing mechanism and bone cells migration simultaneously. Several standard analyses such as porosity, pore size and uniformity of the scaffold for bone regeneration capacity of beta tricalcium phosphate with chitin and gelatin gel scaffolds were investigated.

2. Materials and methods

2.1. Chemicals

All chemicals of analytical grade used in this research were procured from Sigma Aldrich.

2.2. SCAFFOLD preparation

The scaffold was fabricated using freeze drying technique**.** The standard procedure for the preparation of scaffold by freeze drying technique was as described by Giovanni et al. (2015). The scaffold material was prepared with the following components of gelatin, chitin and beta tri-calcium phosphate with the ratio of 1:0.5:3.5**.** Briefly 1.3 g of gelatin dissolved in 99% of double distilled water and 1% of glacial acetic acid at an optimum temperature of 60 °C. Later, 0.2 g of chitin was added into the gelatin solution at 60 °C to obtain gelatin and chitin mixture. Then 3.5 g of Beta-tricalcium phosphate was added and stirred well for 2 h till a white dense solution was formed and was stored in deep freezing temperature (-3 °C) for 12 h. Later, the sample was dried by lyophilization. The bioscaffold preparation was performed three times using same method under same laboratory condition. The scaffolds were stored under frozen state in glass bottles for further studies.

2.3. CHARACTERIZATION of scaffold

The morphology of bioscaffolds was examined under field emission scanning electron microscopy (model: FESEM-SUPRA 55 - CARL ZEISS, GERMANY). The sample was placed under tungsten grid and the grid was used to allow ambient temperature for drying the sample for FESEM analysis. The structure was examined under X-ray diffractometry. The X-ray diffractometry (Model: XRD-SMART lab - Rikagu, JAPAN) used Cu K-alpha with a scanning step 2θ range of 3–70 °C. Fourier transform infrared spectroscopy (Spectrum one: FT-IR Spectrometer) was used to identify the functional group of the bioscaffolds within the wavelength of 4000 cm^{-1}-300cm^{-1} using KBr as standard.

2.4. Invitro biodegradation analysis

The degradation testing was performed according to the ISO 10993–14 “Biological evaluation of medical devices – Part 14: Identification and quantification of degradation products from ceramics”. It was performed in duplicates at different pH values. The degradation analysis of the scaffold was performed in Phosphate Buffer solution (pH 7.4) which was referred to as simulation solution testing; and also at pH 3.0 using citric acid that was referred as extreme solution testing. The tests were done at different time intervals (from 1 to 12 h) every 1 h. The sample weight was taken before degradation (W_0) and after degradation (Wt) washed with distilled water and calculated by the following formula (Tao et al., 2017).

Weight loss (%) = [(Wo –Wt) / Wo] X 100

2.5. Porosity analysis

The porosity and pore size of scaffold was calculated by Archimedes principles. The sample was placed in a beaker containing double distilled water for 1 h to calculate the porosity of scaffold according to the following formula (Figueiredo et al., 2009).

P = Wc-Wb/Wc-Wa X 100

Where, Wa - air dried sample tied with nylon string, Wb-sample submerged in water and Wc-is dry weight.

3. Results and discussion

Tissue engineering has continued to develop as an emerging and multidisciplinary field focusing to develop biological substitutes to restore, replace or regenerate defective tissues (Lanza et al., 2013). The scaffolds typically composed of polymeric biomaterials must have biocompatible and biodegradable properties upon implantation in to the human body. They should be absorbable by the human tissue and also be porous with interconnected pores of adequate size to allow cell adhesion and cell proliferation. They must also have the capacity to bind to surrounding tissues with proper interfacing (Langer and Vacanti, 1993). Different techniques have been adopted by the researchers for the development of scaffold for bone regeneration. Both natural and synthetic biomaterials have been used for making porous scaffolds for tissue engineering (Meyer et al., 2009).

3.1. Scaffold preparation

Bioscaffold is an artificial structure, implanted in the body to repair or regenerate tissue growth in an injured or damaged organ. Numerous techniques have been developed to fabricate highly interconnected, porous scaffold for bone tissue engineering applications with the help of biomolecules such as chitosan, collagen, gelatin, silk, etc. with different combinations (Preethi Soundarya et al., 2018).

As shown in Table 1, five samples (S1, S2, S3, S4, S5) with different ratios of Beta-tricalcium phosphate, gelatin, and chitin were prepared to standardize the scaffold biomaterial preparation using freeze drying technique. Among the samples used to prepare the scaffold, sample S1 and S2 exhibited a perfect porous structure than the other combinations. Hence, S1 and S2 sample ratio was considered as the ideal

Table 1
Standardization ratio of bioscaffolds preparation.

S.No	Sample	β-Tricalcium Phosphate (ratio)	Chitin (ratio)	Gelatin (ratio)	Results
1	S1	3.5	0.2	1.3	Solid form
2	S2	3.5	0.3	1.2	Solid form
3	S3	3.5	0.1	1.4	Solid deformed
4	S4	3.0	0.4	1.6	Very flexible
5	S5	4.0	0.5	0.5	Powered

Each ratio of bioscaffold preparation was performed three times.

combination for the preparation of scaffold with good and perfect scaffold structure for biomedical application. Therefore, S1 sample was subjected to further characterization studies. The bio-scaffolds with a diameter of 50–60 nm were obtained by freeze drying technique. Scott Stratton et al. (2016) fabricated collagen scaffolds by freeze drying technique in order to develop the porous surface structure. The porosity with mean pore size of about 80 nm also highlights the rough surface morphology often associated with collagen used for scaffold fabrication. The gelatin gel gives a potential strength to the bio-scaffolds to support the re-growth of the bone. The porous nature in scaffold gives its unique support to the proliferating cells in the bone. The standardized ratio of bioscaffolds preparation is given in Table 1.

3.2. Microstructural charaterization

FESEM image of bioscaffolds showed a mixed homogeneous structure at different magnification as depicted in Fig. 1. The FESEM images (1 & 2) depicted a homogenous structure and images (3 & 4) showed that 80% of the bioscaffold was porous. The side or open pores (surface pore of bioscaffolds) were shown in 5 and 6. The grain size particles were less than 200 nm which were evenly distributed with inter connected structure. Several recurrences of pores were recorded throughout the scaffold. The Bioscaffold was slightly flexible and strong enough to handle in dry conditions. Porosity in a scaffold is very important for a cell to proliferate through the scaffold. The presence of pores as well as inter structure binding gives the scaffold its strength to suit its application. Garg et al. (2012) reported that 3D microstructures of scaffolds prepared using chitosan were heterogeneous with well interconnected porous structure. But the combination of beta-tricalcium phosphate with gelatin and chitin with standardized ratio is more efficient than other combinations. The beta-tricalcium phosphate scaffold gives slight flexibility and is strong enough with high interconnected pores. Zahra Mohammadi and Rasouli-Disfani (2016) suggested that multi-phasic calcium fiber used for scaffold preparation exhibit increased pore size with increasing fiber contents. However, our study confirmed that increasing the temperature to 60 °C increased the pore size of scaffold structure with high interconnectivity of pores. The important factor of freeze-drying technique is to maintain a freezing temperature and drying phase (vacuum) which effect the formation of scaffold. The pores are widely formed on the layer of scaffold so that cell can proliferate and differentiate easily. These studies inferred the structural formation and bonding reaction of scaffold efficiency, cell proliferation rates, and biodegradable rate. Beta-tricalcium phosphate scaffold examined in FESEM showed 3D pore microstructure and the pores are interconnected where the diameters of pores range from 10 to 20 μm. Murphy et al. (2010) reported that collagen scaffold having the optimum pore size of 325 μm promote cell migration and proliferation in bone tissue engineering. The pores having an oval shape diameter between the range of 100 and 500 μm were ideal for use as cell scaffold. Our study demonstrated that the composite scaffold material with surface roughness and porous formation is efficiently interconnected.

3.3. Functional charaterization

FTIR spectral analysis of bioscaffolds was represented in Fig. 2. Beta-tricalcium phosphate exhibits characteristic peak bands of phosphate group (PO_4), chitin, gelatin and water. The peaks at 1041cm-1 and 603.96cm-1 are assigned to vibration of the phosphate group, $PO4^{3-}$(Senthilarasan et al., 2014).The phosphates having double bonded group PO_4 form intensive IR absorption bands at 560cm-1 to 600 cm-1 and at 1000cm-1 to 1100 cm-1. The FTIR absorption band of beta-tricalcium phosphate peaked at 563cm-1 to −1006.97cm-1.The Beta-tricalcium phosphate ($Ca_3(PO_4)_2$) contain calcium, phosphorous and oxygen groups. Zahra Mohammadi et al. (2016) reported that absorption band of phosphate group is 563cm-1 to $-602cm^{-1}$

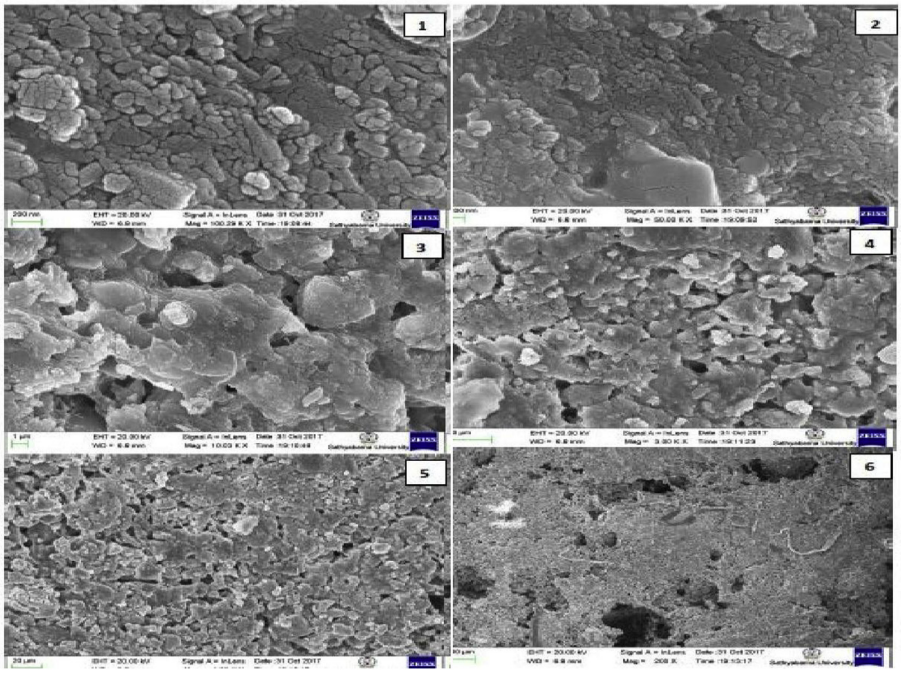

Fig. 1. FESEM image of bioscaffolds with the magnification 1.(100kx), 2.(50kx), 3.(10kx), 4.(3kx), 5.(1kx), 6.(200x).

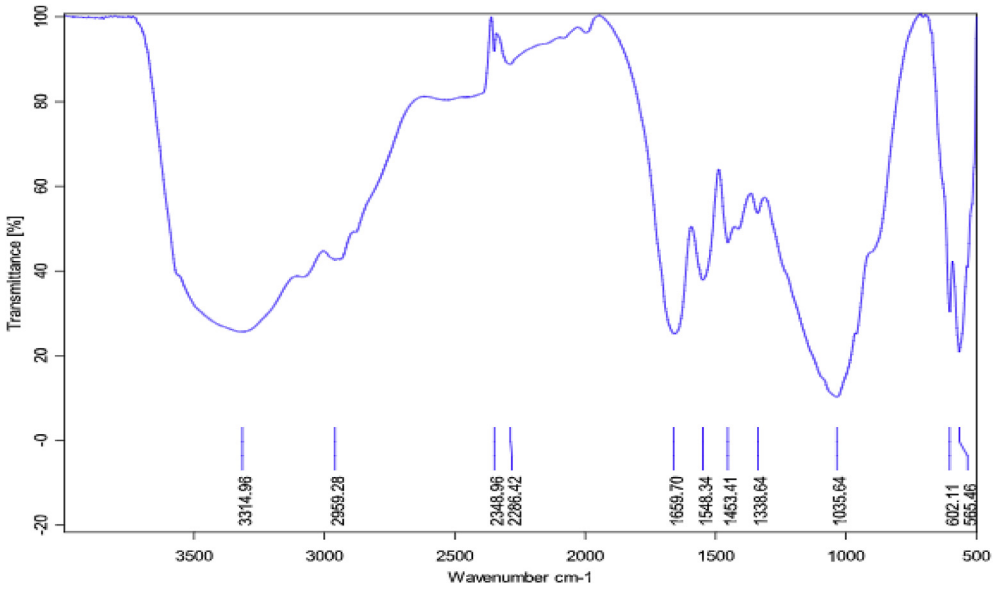

Fig. 2. FTIR spectra Beta-tricalcium phosphate with chitin/gelatin scaffold.

corresponding to the bending mode of O–P–O bonds. This result corroborated with the present investigation.

The absorption bands of amide I and amide II related to the chitosan can be observed in spectra of composite scaffold. Leon-Mancilla et al. (2016) observed that IR spectral study of collagen scaffold band peaked at 1650–1665 cm − 1 C = O stretch corresponding to Amide I. In the present study the absorption band of amide I related to gelatin can be observed in the FTIR spectra of scaffold. The gelatin standard peak value is 1700 cm-1to1600 cm-1. Nipu et al. (2018) reported that FTIR analysis of scaffold composite of Carboxymethyl chitosan and gelatin biomaterials give strong peak at 1650 cm-1 owing to N–H bending of amine group. These results indicated that the carboxymethylation process had occurred at the C6 position of chitosan. The most sensitive spectral region to the protein secondary structural and the gelatin spectra peaked at 1548cm-1 to1666.19cm-1. Gelatin is a type of protein that contains many amino groups. The acetic acid can be characterized as a strong peak and it falls on the spectra of 1669cm-1. The water contains elastic bands and the wavelength falls on 3000cm-1 to 3500cm-1. But the present study showed that gelatin contain amide group of C = O stretching hydrogen bond in the IR spectra band. Chitin has biocompatibility, biodegradability and is cationic. The chitin was used for several applications in the field of tissue engineering and was absorbed by the Fourier spectra peaked on 2285cm-1 to 2348cm-1. The bands are generally large due to the macromolecular structure of the compound containing numerous intermolecular bindings.

3.4. XRD study of bioscaffolds

The XRD examination showed the amorphous structure of the scaffold biomaterials. The XRD of Chitin/Beta tricalcium phosphate and gelatin composite revealed the typical XRD pattern of amorphous Hap (Hap standard ICDD-PDF-00-024-0022) with diffraction peaks at 20° values of 23.6504, 32.5893, 40.9785, 47.7673, 50.0772, 55.4628, 64.9482, 88.4892, which are indexed to (002), (211), (300), (310), (222), (213) and (004) planes. The degree of amorphous structure is increased along with the original Hap content in the composites. The amorphous size measured from the XRD data was lower than 25 nm in the bioscaffolds samples. The XRD pattern of chitosan/TiO2 scaffold is slightly amorphous showed peaks at 25.7°, 35.8°, 36.9°, 40.2°, 46.6° and broad phase from 18 to 21° corresponding to chitosan (Pawan Kumar, 2018). The X-ray diffraction pattern of the composite is depicted in Fig. 3.The XRD patterns of bioscaffolds shows a peak around 20°, which is related to the amorphous nature of beta-tricalcium phosphate with gelatin/chitin matrix. An increase of fiber content was associated with widening the characteristic of chitosan pattern (Mohammad and Rasouli-Disfani, 2016). But here the widening is done with the help of heating effect (ie., increasing the temperature of the sample instead of fiber content associated with widening of beta-tricalcium phosphate pattern).

3.5. Physicochemical properties

3.5.1. Invitro biodegradation analysis

Scaffold biomaterials are not considered as permanent implants. Therefore, the biomaterials should be reabsorbed with an appropriate rate of degradation and also colonizing the adjacent cells to regenerate the tissue. The choice of a biodegradable biomaterial is thus of great importance with regard to tissue engineering and must be accurately done bulk material (Ludovica Parisi et al., 2018). Degradability of scaffold material is a concerned property for implanting biomaterials because it is crucial for bone induction, conduction, metabolism and longevity on implants. (Hench and Polak, 2002). The degradation testing was performed according to the ISO 10993–14 “Biological evaluation of medical devices – Part 14: Identification and quantification of degradation products from ceramics”. Fig. 4 shows the swelling ratio of Beta-tricalcium phosphate scaffold in citric acid and phosphate buffer saline in different time intervals. Gruskin et al. (2012) had used HCl for demineralization of hard tissue for allograft implantation using tissue engineering. Here the samples were subjected to treat two different pH solution of citric acid with the pH of 3.0 and phosphate buffer saline with the pH of 7.3. The acidic pH (A) had high weight loss percent during the first 10 h (ranging from 35% to 40%) compared to the neutral pH of samples (B) treated with phosphate buffer saline. The present study showed that degradation of scaffold biomaterial is pH dependent. The graph shows the degradation percent of scaffold for both A & B samples. After 1 h the scaffolds absorbed more water from the initial weight followed by gradual increase up to 10 h under experimental condition. Reduction in the weight of the bioscaffold significantly increased the degradation rate throughout the experimental time. However, the degradation rate of the bioscaffolds was slowed down in the next 38 h. Gelatin is a denatured protein derived by the partial hydrolysis of collagen. The amount of collagen renovation into gelatin is related to the temperature, pH, and time of the hydrolysis process. Gelatin based scaffold is biodegradable and biocompatible

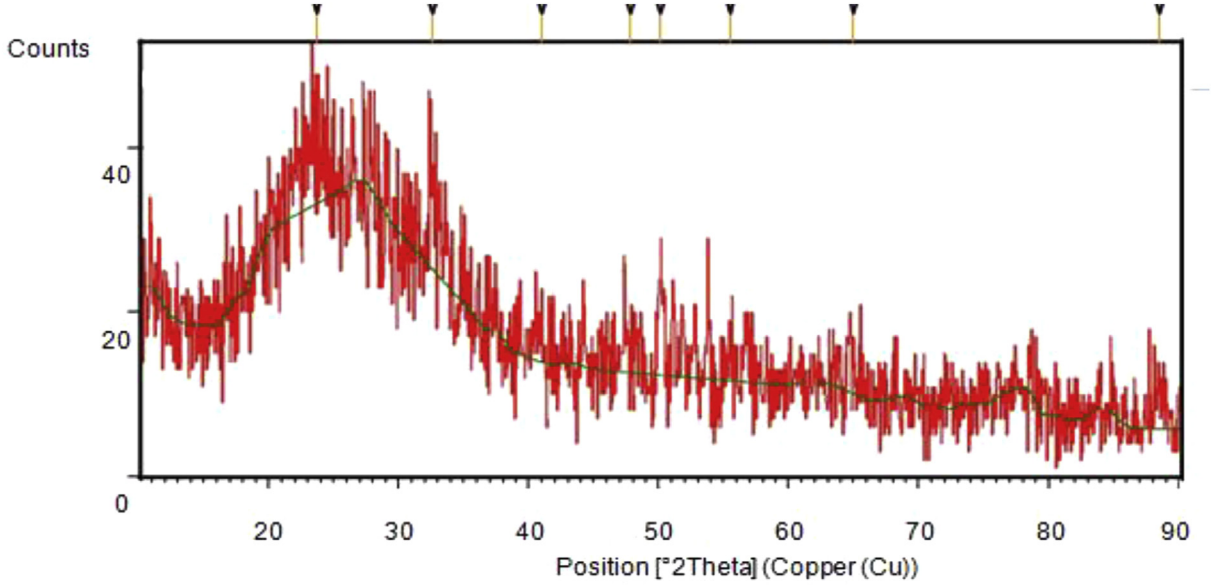

Fig. 3. XRD patterns of Beta-tricalcium phosphate with gelatin/chitin scaffold.

biomaterials both *in vitro* and *in vivo* conditions. Gelatin with a solid to gel transition under the human body temperature (37 °C) must be chemically cross linked to stabilize its structure (Chang et al., 2017). Leon Mancilla et. al., (2016) studied thermogravimetric analysis of collagen scaffold using different pH and temperature represented the 6.07%wt of the bone matrix occurred at 79 °C by the loss of the physisorbed and chemical water in the scaffold material. At the same time for collagen scaffold the loss of water corresponded to 9.6%wt and occurred at 114.74 °C. These results are agreed to those reported by Lozano et al. (2003). Ahmed et al., (2011) reported significant increase in the mean weight loss percent throughout the study period. They observed high weight loss percent during the first week (ranging from 35% to 40%) in all tested scaffold samples.

3.5.2. Porosity analysis

Porosity is the fraction of void space within the scaffold materials and it plays a significant role in bioscaffold design *in vitro* and *in vivo* bone formation (Athanasou, 1996). The total porosity is related to the amount of pore space present in the scaffold. Biomaterial with high specific surface area and porosity may assist cell migration and proliferation. The biomimetic microenvironment of scaffold materials can influence the interactions between scaffold and cells to regulate the biological behavior of the cells for bone regeneration (Ma and Zhang, 2014). In this study, the scaffold biomaterial exhibited 76% of porosity (approx.) as presented in Table 2. The increased porosity of scaffold may induce cell migration rapidly for faster bone regeneration. This is agreed with the study of Ahmed et al. (2011) reported that porosity of scaffold was in a range above 55% at surface material with the combination of nano-hydroxyapatite and b-tricalcium phosphate induce faster cell migration. Ghassemi et al. (2018) reported that porous composite materials implant have been introduced in next generation of orthopedic medicine for tissue engineering.

4. Conclusion

Bioscaffold prepared using gelatin and chitin based macroporous composite containing beta tricalcium phosphate exhibited better *in vivo* osteoconductivity and biodegradability. It is hereby concluded that beta tricalcium phosphate can increase alkaline phosphatase activities of mesoblasts thus enhancing cell proliferation and promoting cell

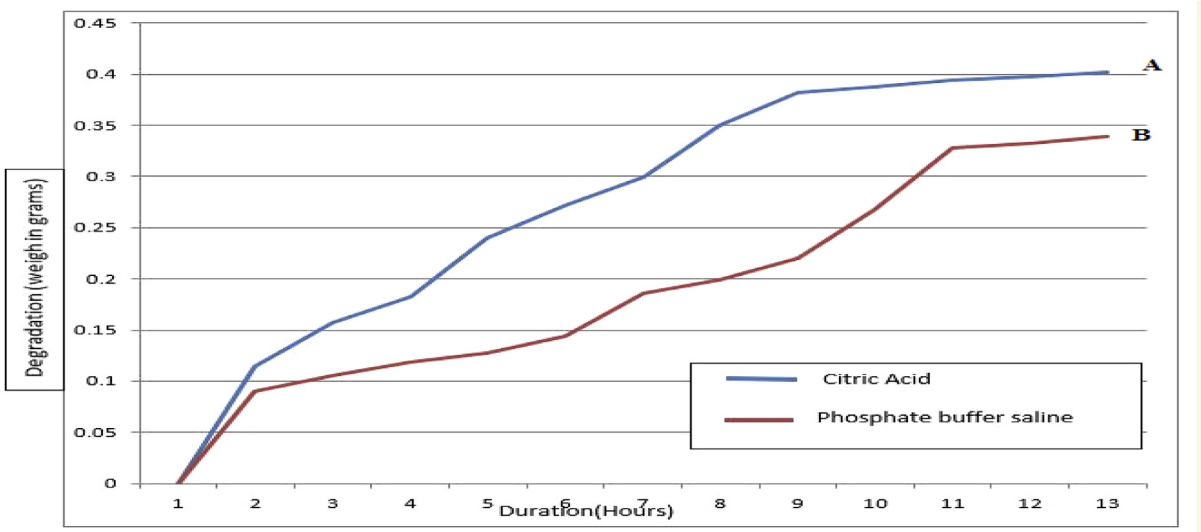

Fig. 4. Degradation of bioscaffolds A. Citric acid B. Phosphate Buffer saline.

Table 2
Porosity of scaffold biomaterial.

Sample	(Wc-Wb)	(Wc-Wa)	(Wc-Wb)/(Wc-Wa)	Porosity (%)
Scaffold	4.3 gm	5.8 gm	0.76 gm	76% (approx)

Values are average in triplicates.
Where.
Wa - air dried sample tied with nylon string.
Wb-sample submerged in water.
Wc-is dry weight.

adhesion which mimicked the native architecture of bone trabecular tissue. The characterization studies of bioscaffolds showed augmented porosity as well as increased surface volume ratio with its equal grain distribution throughout the structure. This composite structure becomes a quite promising material for tissue engineering and bone regeneration. Thus bioscaffold prepared by freeze drying technique using beta tricalcium phosphate, chitin and gelatin unfolds a new arena in orthopedic medicine for tissue engineering.

Conflict of interests

The authors declare that there is no conflict of interests to publish this research article in your esteemed journal.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101184.

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