

SYNTHESIS AND CHARACTERIZATION OF NANO CALCIUM PHOSPHATES EXTRACTED FROM BIOGENIC CANCELLOUS BOVINE BONE IN POLYMERIC SCAFFOLDS FOR BONE TISSUE REGENERATION: IN VITRO STUDY

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ABSTRACT

Introduction: Tissue damage and organ failure are serious problems that affect many individuals worldwide. Bioactive calcium phosphate ceramics can be used to repair or replace damaged bone. The scientific community is very interested in developing new approaches to enhance healthcare using alternative materials of biogenic origin. Utilizing these wastes will help the economy, the environment, and society in a number of ways. **Aim:** To synthesize 3D PCL/zein polymeric scaffolds loaded with calcium phosphate ceramic fillers as hydroxyapatite (HAp), chemically synthesized from pure chemical reagents, and biphasic HAp/ β -TCP prepared from biogenic cancellous bovine bone to obtain bioactive materials for BTE applications. **Material and Methods:** The calcium phosphate ceramics are blended individually with PCL/Zein polymer scaffold matrices using the solvent casting/particulate leaching method. The scaffolds, pore sizes, compressive strength, porosity percentage, bioactivity, and rate of biodegradation were also evaluated. **Results:** The SEM measurements showed the fabricated scaffolds had pore sizes varying from about 279 to 305 μ m. The composite scaffold containing HAp synthesized from pure chemical reagents showed the highest compressive strength; the mechanical strengths of the other groups containing ceramics from CBB ranged in the mechanical strength of cancellous bone (2–12 MPa). Furthermore, an insignificant difference existed among the different groups concerning the weight loss percentage after 6 months of immersion in PBS. All the composite scaffold groups were bioactive; this was confirmed by SEM and EDX results that showed the formation of new apatite crystals on their surfaces. **Conclusion:** According to our findings, the meager cost of manufacturing nano calcium phosphates derived from the biogenic waste of cancellous bovine bone can be used to develop biocompatible composite scaffolds suitable for bone tissue engineering applications.

INTRODUCTION

Many clinical approaches are annually undertaken to replace damaged body parts, including autografts, allografts, and xenografts. However, these approaches suffer from many drawbacks, such as donor scarcity, disease transmission, graft rejection, and morbidity at the donor site. The field of tissue engineering was introduced to overcome these drawbacks by rebuilding the damaged tissues instead of replacing them^(1–3). A porous 3D scaffold is required to support initial

cell attachment and subsequent tissue formation. An ideal scaffold should have appropriate porosity and pore size, enhance cell ingrowth, have enough mechanical strength, have adequate degradation properties, and be biocompatible^(4,5).

The two major classes of biomaterials used in scaffold synthesis are polymers and ceramics. Because no single material can satisfy all the ideal scaffold's requirements, composite scaffolds, which blend the benefits of multiple materials, are more promising. To improve physical, mechanical, and biological performance, polymer/bioceramic composite scaffolds can be used to combine the adaptability and simplicity of shaping polymers with the strength, rigidity, and bioactivity of bioceramic fillers⁽⁶⁻⁸⁾.

Natural polymers, known for their exceptional biocompatibility, superior biodegradability, and non-toxic nature, play a significant role in the polymer domain. Widely recognized in bone tissue engineering, substances such as collagen, chitosan, gelatin, zein, alginate, cellulose, and starch are commonly employed. However, their application is constrained by factors like inadequate mechanical strength, elevated production expenses, limited availability, and variability between production batches⁽⁹⁻¹¹⁾. In contrast, synthetic polymers stand out due to their customizable attributes, including porosity, degradation period, and mechanical characteristics, in addition to their consistent and reproducible mechanical and physical qualities. Polyesters like polycaprolactone (PCL), polylactic acid (PLA), and polyglycolic acid (PGA) are among the synthetic polymers frequently utilized in tissue engineering.

Polycaprolactone (PCL), aliphatic polyester with biodegradable properties, is extensively utilized in tissue engineering owing to its ready availability and cost-effectiveness. Also, its chemical, biological, and mechanical properties can

be greatly modified⁽¹²⁾. PCL's surface, on the other hand, has intrinsic hydrophobic properties that slow its degradation, making it unsuitable for cell growth, restricting bone apposition or bonding on the polymer surface⁽¹³⁻¹⁶⁾, limiting its use in tissue engineering. Therefore, PCL is more frequently used as a copolymer with other natural or synthetic polymers, as well as bio-ceramics, to overcome the problems of using a single polymer.

Zein biopolymer is abundant in maize, accounting for about 44% -79% of the endosperm's protein content. Despite having a hydrophobic nature, it is soluble in alkaline aqueous solutions with a pH of 11 or in ethyl alcohol solutions with a concentration of between 60 - 95 percent. Polycaprolactone (PCL) exhibits significant potential for drug delivery and tissue engineering applications, attributed to its renewable natural origin, biodegradability, non-toxic nature, and biocompatibility⁽¹⁷⁻¹⁸⁾.

Calcium phosphate ceramic materials are widely used in the preparation of dental and orthopedic applications such as implants and scaffolds. The key physical and chemical characteristics of these bioceramics, including solubility, biodegradability, and mechanical properties, are primarily governed by their structural composition, which is largely dictated by the calcium to phosphate (Ca/P) ratio present in their empirical formula. Bioceramic materials, such as hydroxyapatite (HAp), beta-tricalcium phosphate (β -TCP), and biphasic (HAp/ β -TCP), act as bone substitutes and tissue regeneration matrices by reacting with the physiological fluids, also forming strong bonds to hard tissues as a result of their cellular activity. Typically, these ceramic substances are produced through pure chemical reactions that use different chemical reagents. More recently, some bioceramic materials were prepared from natural sources of biogenic waste materials, such as bovine bone, eggshells, cuttlefish bones, and marine shells.

Utilizing these wastes will have a variety of positive societal, economic, and environmental effects^(18,19).

In the present study, two sources are used for nano-HAp synthesis: first, pure chemical reagents, and second, biogenic cancellous bovine bone. In addition, biphasic bioceramics (HAp/ β -TCP) derived from biogenic cancellous bovine bone were prepared. Each type of these bioceramics was separately blended with polycaprolactone / zein to form 3D composite scaffolds for bone tissue engineering. To study the effects of the different sources of bioceramics on the properties of the composite scaffolds, these scaffolds were physically, mechanically, and biologically evaluated.

MATERIALS AND METHODS

Prior to commencing the study, the research received approval from the Ethics Committee for Scientific Research at the Faculty of Dentistry, Suez Canal University, Protocol No. 123/2018.

Synthesis of nano-hydroxyapatite powder from pure chemical reagents

The sol-gel technique described by Padmanabhan et al.⁽²⁰⁾ was used to prepare the nano-hydroxyapatite (n-HAp) powder. The chemical substances employed in the preparation were acquired from Sigma-Aldrich Company. The preparation involved a reaction between calcium nitrate tetrahydrate [$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$] and di-ammonium hydrogen phosphate [$(\text{NH}_4)_2\text{HPO}_4$], maintaining a stoichiometric Ca/P ratio of 1.67 at a pH level of 10.

Synthesis and Characterization of Nano-HAp and Biphasic β -TCP/HAp from Cancellous Bovine Bone (CBB):

The synthesis mechanisms of nano-HAp and biphasic β -TCP/HAp from cancellous bovine bone

are illustrated in **Figure 1**. The cancellous bovine bone was obtained from calf femoral condyles, properly washed using distilled water, and cut into small parts (1-2 cm) before being boiled in water for 4 hours to facilitate the removal of macroscopic adhering tissues. Spongy bone (cancellous) in the present study was used instead of compact bone because spongy bone has a larger surface area and is therefore more active. This is in addition to the ease of grinding. Afterwards, the bones were soaked in a 5% sodium hypochlorite (NaOCl) solution for two hours, followed by a 10% H_2O_2 solution for 3 hours to remove any blood remnants. Again, they were washed with distilled water before soaking in 70% ethyl alcohol for one hour. Finally, the bone material was dried at 80°C for 24 hours, and then calcined in an electric furnace at 700 °C for two hours, using a temperature rate of 10 °C per minute. A white and clear HAp was obtained free from any organic substance. The samples were cooled to room temperature (25 °C) and then divided into two parts, the first for getting n-HAp and the second for preparing biphasic β -TCP/HAp. The first part was crushed in a porcelain mortar until reaching a size of 500–1000 microns, and then milled in a zirconia ball mill for six hours to obtain n-HAp. To prepare the biphasic β -TCP/HAp, a procedure described by Lin F.H. et al.⁽²¹⁾ was used. The second part of the calcined CBB cubes was immersed in 0.6 M of ammonium dihydrogen phosphate solution ($\text{NH}_4\text{H}_2\text{PO}_4$) for 24 hours at room temperature. Then, it was dried at 80 °C for 48 hours. Afterwards, they were heated at 900°C for three hours in air using a Stuart Scientific Temperature Control Electrical Furnace, employing a heating rate of 10°C/min. Finally, the CBB cubes were cooled slowly at ambient temperature, crashed, and milled in a zirconia ball mill for three hours to obtain nano-biphasic (HAp/ β -TCP).

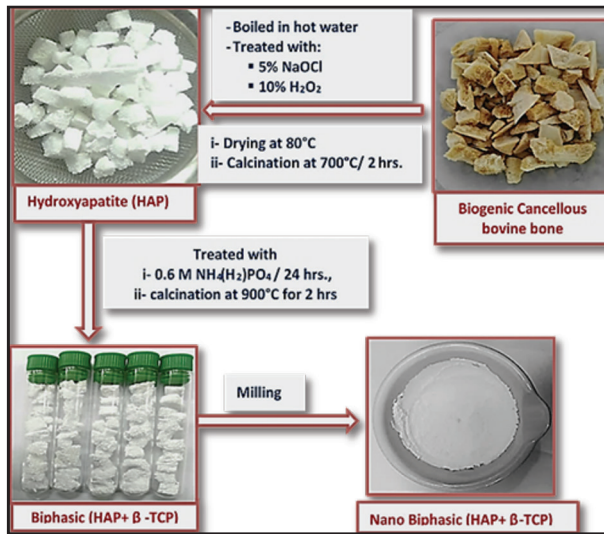


Fig. (1) Schematic diagram of HAp and HAp/ β -TCP synthesized from bovine bone bio-waste. The bioactivities of the nano ceramic powders were evaluated by soaking one gm of each ceramic powder in 100 ml of PBS for 14 days, and then examined using XRD, FTIR and SEM.

Preparation and characterization of the composite scaffolds

In this study, three distinct scaffold groups (as detailed in **Table 1**) were fabricated utilizing the solvent casting/particulate leaching method ^(1, 22). This method is easy to remove NaCl salt from the polymeric scaffold and is fast and inexpensive to form different diameters of pores.

Table (1) Composition of the synthesized scaffold groups in the present study

Group No.	Composition (Wt. %)
Group (I)	PCL + Zein + HAp* (60: 20: 20)
Group (II)	PCL + Zein + HAp** (60: 20: 20)
Group (III)	PCL + Zein + Biphasic (HAp+TCP)** (60:20:20)

*: Chemical synthesized

** : Synthesized from Biogenic Cancellous Bovine Bone

A 10% w/v solution of PCL was created by dissolving six grams of PCL pellets in 60 ml of dry chloroform solvent, utilizing a mechanical stirrer. Subsequently, predetermined ratios of Zein protein and bioceramic powders were incorporated. To this mixture, sodium chloride (NaCl) crystals, acting as pore-forming particles and ranging in size from 250 to 500 μ m, were added in a 1:5 ratio to the PCL solution. The mixture was then stirred until homogeneous blends were achieved.

Immediately after preparation, the composite mixtures were poured into uniquely designed disc-shaped Teflon molds of varying dimensions. The first mold size, with a diameter and length of 8 x 3 mm as illustrated in (**Figure 2a**), was employed to assess the porosity of the fabricated scaffolds. Meanwhile, the second mold size, featuring a diameter and length of 10 x 20 mm shown in (**Figure 2b**), was utilized for evaluating the mechanical strength of the scaffolds.

On the other hand, another portion of each composite mixture (50-mm diameter and 10mm thickness) was poured into a petri dish (**Figure 2c**) for biodegradation tests. The prepared samples were then kept at room temperature for 48 hours to ensure the complete evaporation of chloroform solvents, as well as hardening of the composite scaffolds. Following their extraction from the molds, the scaffolds were submerged in distilled water. This water was replaced every four hours over the initial 48-hour period to facilitate the removal of the NaCl crystals (porogen) from the scaffolds. To ensure the complete dissolution of the porogen (NaCl), a few drops of a 0.1M AgNO₃ solution were added to the water used for immersing the scaffolds. The complete removal of chloride ions was verified by the absence of a white precipitate, indicative of AgCl. Once free of NaCl, the scaffolds were dried in a vacuum oven at 40 °C for 12 hours

and subsequently stored under vacuum in a glass desiccator. For the biodegradation tests, scaffold samples were prepared and placed in Petri dishes.

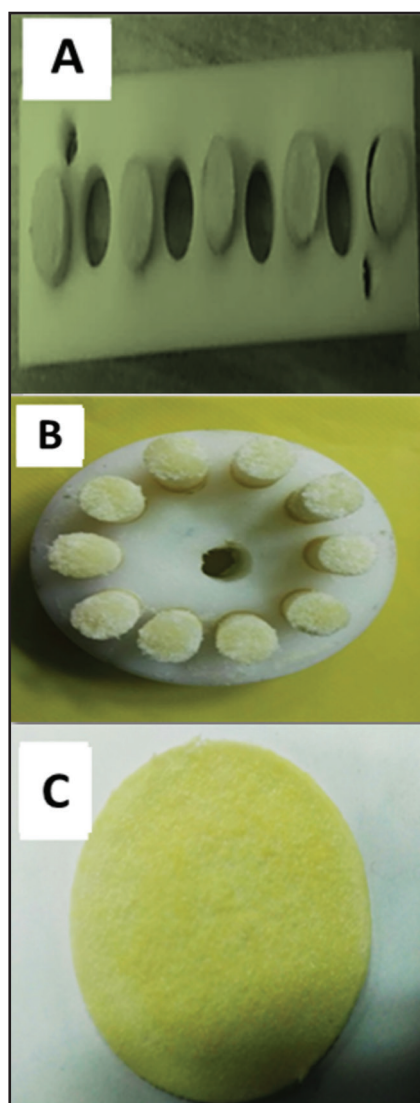


Fig. (2) (A) The scaffold samples and the Teflon mold were used to prepare the samples for the porosity tests. (B) Scaffold samples and the Teflon mold used to prepare the samples for mechanical testing. (C) The petri dish used to prepare the samples for the biodegradation tests.

Scaffolds Characterization

Quantitative assessment of scaffold porosity:

The porosity of the scaffolds was measured employing the Archimedes' liquid displacement technique^(1,22). Each scaffold sample was submerged in a graduated cylinder containing a predetermined volume (V_1) of 100% dry ethyl alcohol. Because it is non-solvent to the polymers, ethanol alcohol easily permeates the pores without producing shrinkage or swelling of the scaffolds.

To facilitate the infiltration of ethyl alcohol into the pores of the scaffold, the cylinder was positioned within a vacuum pump system. The vacuum cycle was repeated until air bubbles ceased to emerge from the scaffolds. The liquid level in the graduated cylinder, representing the combined volume of residual ethanol and the ethanol-saturated scaffold, was recorded as V_2 . Subsequently, the scaffold infiltrated with ethyl alcohol was removed from the cylinder, and the volume of the remaining ethanol was measured (V_3).

$$\text{Porosity (\%)} = (V_1 - V_3) / (V_2 - V_3) \times 100\% \quad (1)$$

Scanning electron microscopy (SEM) qualitative assessment of scaffold porosity

The pore morphology, microstructure, and dimensions of the composite scaffolds were analyzed using a Scanning Electron Microscope (SEM) and Energy Dispersive X-ray (EDX) analysis, specifically employing the Quanta 250 FEG (Field Emission Gun) model.

Compressive strength evaluation

Each sample, with six samples per group, was individually and vertically mounted on a computer-controlled Instron Universal Testing Machine (England), specifically the Frame Model: 3345.

The machine was equipped with a Load Cell: 500 N, N Model: 2519-104, Serial No. 2710-113, to measure its compressive strength. Later, the samples were statically exposed to compression at a speed of one mm/min and compressed to 25% of their original height ⁽²³⁾. The compressive strength, measured in mega Pascal (MPa), was calculated using the equation: Compressive strength = $4P/\pi d^2$. In this formula, P represents the load at the failure point, and d is the diameter of the cylindrical specimen.

Biodegradation test of the scaffolds

To evaluate the degradation rate of the synthesized scaffolds, six samples from each group were accurately weighed individually to determine their initial dry weight (W_i) using a Fisher Scientific electric balance (minimum display: 0.1mg, USA) prior to starting the biodegradation tests. Each sample was individually placed in a plastic vial, which contained 10 ml of Phosphate Buffered Saline (PBS). These vials were then incubated at a temperature of $37\pm 1^\circ\text{C}$ for duration of six months. To prevent bacterial growth, 0.02 wt.% sodium azide (NaN_3) was incorporated into the Phosphate Buffered Saline (PBS). This solution was refreshed every 15 days to maintain consistent ion activity.

After the completion of the predetermined immersion periods, the samples were extracted from the immersion solution and thoroughly rinsed with distilled water. They were then dried for 48 hours at a temperature of 40°C in a drying oven. Upon drying, the samples were reweighed to ascertain their final weight (W_f). The percentage of weight loss for each sample (W_{loss}) was calculated using the following equation:

$$W_{\text{loss}} \% = W_i - W_f / W_i \times 100 \quad (2)$$

Bioactivity of the composite scaffolds

The evaluation of the bioactivity of the composite scaffolds was based on their capacity to facilitate the formation of apatite crystals on their surfaces. Each scaffold sample was immersed individually in 50 ml of Phosphate Buffered Saline (PBS) with a pH of 7.4 ⁽²⁴⁾ for a duration of 14 days. These samples were stored in an incubator maintained at a temperature of $37\pm 1^\circ\text{C}$, with the PBS solution being replaced every three days. Post-immersion, the samples were gently rinsed first with 100% ethyl alcohol and then with distilled water. Following this, they were dried at a temperature of 40°C for 24 hours. The surface morphology of the samples was then analyzed using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) techniques.

Statistical Analysis

The statistical analysis of all the scaffold samples tested was conducted using IBM SPSS for Windows, version 25, developed by IBM Corp. in Armonk, NY. To compare the means, a One-way ANOVA was utilized, and in instances where the ANOVA test indicated significance, Bonferroni's post-hoc test was employed for pairwise comparisons. The threshold for statistical significance was established at $p \leq 0.05$.

RESULTS

Characterization of the synthesized bioceramic powders: before and after bioactivity tests in PBS for 14-days

X-Ray Diffraction (XRD):

The peaks of the hydroxyapatite ceramic powders matched the ICSD standard for synthetic hydroxyapatite (Reference code: 01-086-1199) and

beta-tricalcium phosphate (Reference code: 04-006-2291) as a secondary phase in the case of biphasic powder (synthesized from bovine bone). No secondary peaks were detected in the investigated ceramic powders (**Figure 3**). The maximum relative intensity (I/I₀) peak of 100% was found at 2θ = 31.62 and dÅ (value) = 2.82 for hydroxyapatite powders and their average of crystallite sizes of 19 and 77 nm for HAp (synthetically prepared and from CBB respectively) (**Table 2**). On the other hand, the biphasic ceramic powder prepared from

CBB at 900°C was found to consist of a mixture of HAp and β-TCP having crystal sizes of 56 and 38 nm respectively with a phase ratio of 75.3 and 24.7% respectively (**Table 2**). Relatively small changes in the values of crystal sizes for HAp and/or β-TCP phases after soaking in PBS for 14-days were detected. After soaking in PBS, the ratios between the HAp and β-TCP phases of biphasic ceramic powder differed, with an increased β-TCP content and a decreased HAp content.

Table (2) XRD phase specifications of the synthesized ceramic powders before and after soaking in PBS for 14-days

Powder No.	Specification of the powder	Crystal Size (nm)	2θ at I/I ₀ (100%)	dÅ- Spacing at I/I ₀ (100%)
1	Synth. Hap	19	31.629	2.826
2	Synth. HAp soaked in PBS	18	31.62	2.827
3	Synth. HAp from cancellous bovine bone	77	31.60	2.826
4	Synth. HAp. from cancellous bovine bone soaked in PBS	46	31.69	2.821
5	Synth. Biphasic from cancellous bovine bone	56 for HAp* phase 38 for β-TCP** phase	31.73 (for HAp)	2.817 (for HAp)
6	Synth. Biphasic from cancellous bovine bone soaked in PBS	70 for HAp+ phase 30 for β-TCP++ phase	31.73 (for HAp)	2.817 (for HAp)

Abbreviations:

* HAp content in Biphasic= 75.3% ** β-TCP content in Biphasic= 24.7%
 +HAp content in Biphasic= 70.6% ++ β-TCP content in Biphasic= 29.4%

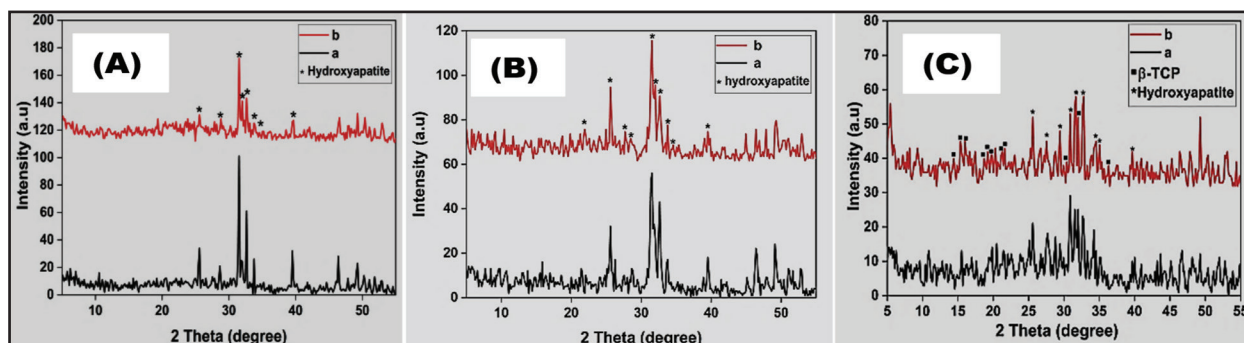


Fig. (3) XRD patterns of chemically synthesized HAp (A), biogenic HAp (B), and (C) biogenic HAp/β-TCP: (a) before soaking (a) and after soaking (b) in PBS for 14 days

Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR)

The IR spectra of both types of HAp ceramic powders before and after 14 days in PBS revealed a large congruence (Fig. 4). They showed typical absorption bands related to the bending vibration modes of O-P-O bonds in phosphate group $(\text{PO}_4)^{3-}$ at: $473 \text{ cm}^{-1} (\nu_2)$, $570 \text{ cm}^{-1} (\nu_4)$ and $602 \text{ cm}^{-1} (\nu_4)$. Moreover, the vibration mode of asymmetric stretching vibration P-O bonds in the phosphate group appeared at $962 (\nu_1)$, $1044-45 (\nu_{3,a})$ and $1090 (\nu_{3,c}) \text{ cm}^{-1}$ respectively. Additionally, two absorption bands were detected at 3571 cm^{-1} and 1455 cm^{-1} . The first band is attributable to the stretching vibrational

mode (ν_s) of the hydroxyl (-OH) group in hydroxyapatite (HAp), while the latter is indicative of the presence of surface carbonation. The soaked powder samples in PBS showed a relative increase in the intensity of absorption bands in respect to the HAp phase ^(25,26).

The infrared spectrum and wave numbers of the synthesized biphasic powders (with and without soaking in PBS) (Fig 4) have a great similarity in peak positions, with little differences in their relative intensities, also revealing spectra of phase formation for HAp and β -TCP ^(26, 27). The presence of HAp and β -TCP phases confirmed the formation of a biphasic system.

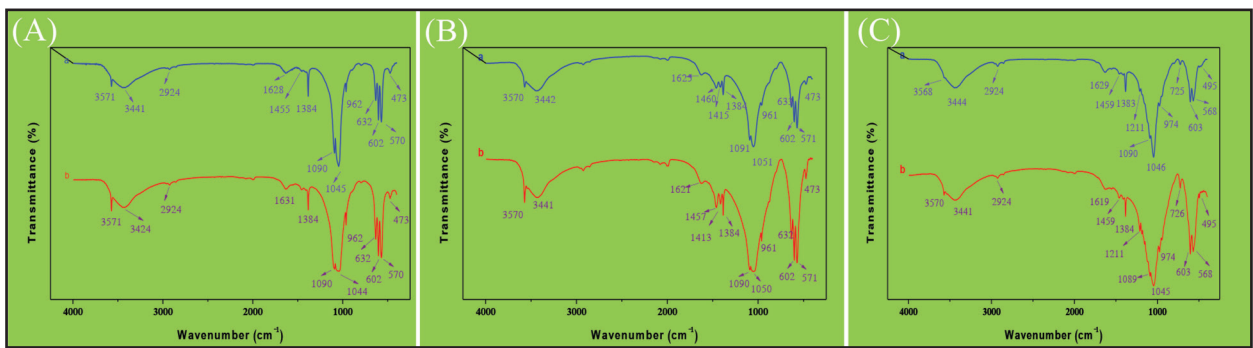


Fig. (4) FTIR patterns of chemically synthesized HAp (A), biogenic HAp (B), and biogenic HAp/ β -TCP: (a) before soaking (a) and after soaking (b) in PBS for 14 days

SEM of the prepared biphasic (HAp/ β -TCP) synthesized from the bovine bone after soaking in PBS for 14-days

The obtained scanning electron micrograph (SEM) of the biphasic (HAp/ β -TCP) synthesized from bovine bone after soaking in PBS for 14-days **Figure (5)** showed the presence of agglomeration of mostly irregular globular shaped particles of varying crystal sizes and the sedimentation of small hydroxyapatite crystals on the surface of the original ceramic powders.

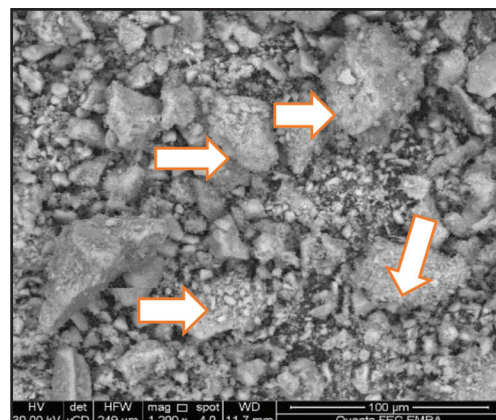


Fig. (5) SEM of the biphasic (HAp/ β -TCP) synthesized from the bovine bone after soaking in PBS for 14 days (the arrows refer to the new formation of HAp crystals)

Physico-mechanical characterization of the prepared scaffolds

Porosity:

Scanning Electron Microscope (SEM) measurements

Scanning Electron Microscopy (SEM) images of the prepared scaffolds (as depicted in **Figure 6**) showed that the scaffolds possessed a highly porous architecture, characterized by well-defined, interconnected open pores. According to SEM measurements (**Table 3**), the average pore size ranged from 279 to 305 μm , with the scaffold containing the chemically synthesized hydroxyapatite (Group I) having the largest average pore size. While the scaffolds containing hydroxyapatite and biphasic hydroxyapatite/ β -TCP from CBB (Groups II and III) do not show a difference between their average pore sizes. The interior walls of the pores contain a

large number of ceramic particles that consolidate them. Furthermore, the SEM analysis revealed that the ceramic particles were thoroughly integrated within the porous Polycaprolactone (PCL)/Zein matrix, and instances of particle agglomeration were also observable.

Table (3) Illustrates the mean pore diameters (μm) measured by SEM for the scaffold groups

Scaffold Group NO.	(I)	(II)	(III)
Average Pore Size (μm)	305	279	280

The calculated porosity (%):

The results of the calculated porosity percent (**Figure 7**) showed that group III had the highest mean porosity percent (79%) while group I had the lowest mean porosity percent (73.667%).

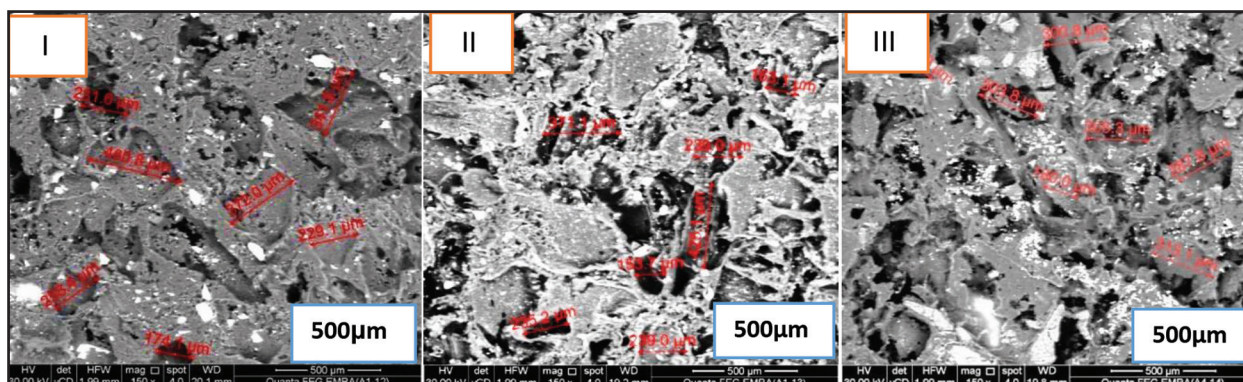


Fig. (6) SEM micrograph of the scaffolds

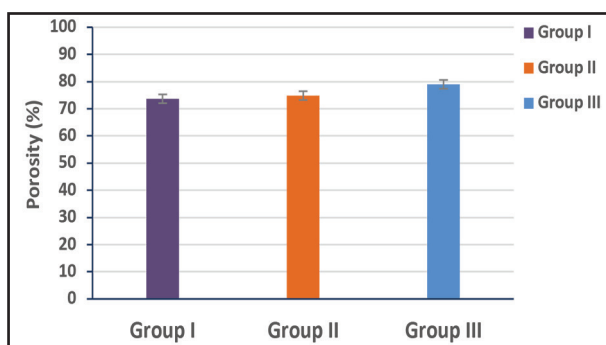


Fig. (7) Bar chart representing the porosity (%) of the different groups

The results of a one-way ANOVA test indicated that there was no statistically significant difference in porosity (%) among the different groups, as evidenced by a *P*-value of 0.08 and an effect size of 0.285.

Mechanical strength:

Compressive strength (MPa):

The results of the compressive strength test, which are shown in **Figure 8**, showed that group I had the greatest mean compressive strength value (5.452 MPa), whereas group II had the lowest value (2.616 MPa). The results of a one-way analysis of variance (ANOVA) test indicated that there was a statistically significant difference in compressive strength (MPa) across the various groups investigated (P -value < 0.001, effect size = 0.712). The results of the pair-wise comparisons between the groups showed that there was a statistically significant difference between groups I and other groups, but there was no statistically significant difference between groups II and III.

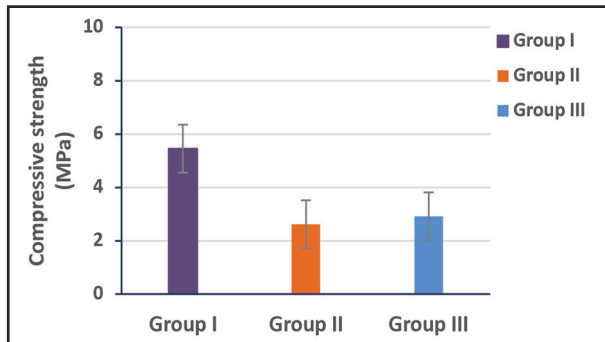


Fig. (8) Bar chart representing the mean values for compressive strength (MPa) of the different groups.

Bio-degradation (weight loss %):

The results of the weight loss percentages of the various groups were shown in **Figure 9**. The results revealed that group III had the greatest mean weight loss percentage value (24.625%), while group I had the lowest value (21.763%). The results of the one-way analysis of variance (ANOVA) test indicated that there was no statistically significant difference in the percentages of weight reduction across the various groups (P -value < 0.198, effect size = 0.194).

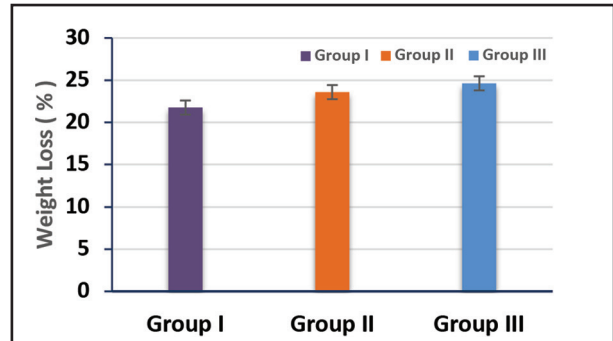


Fig. (9) Bar chart representing the mean values for weight loss % of the different groups

Imaging of the composite scaffolds before and after degradation in PBS after six months at $37 \pm 1^\circ\text{C}$ (**Figure 10**) demonstrates degradation from the scaffold's external edges as well as a damaged interior structural framework.

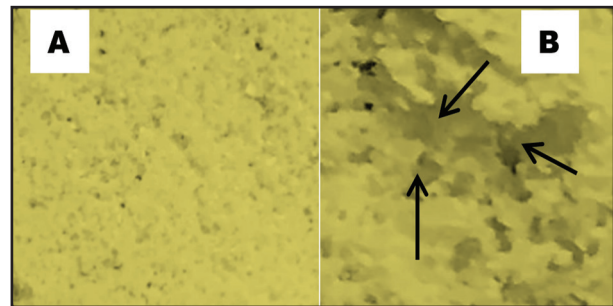


Fig. (10) Composite scaffolds Before (A) and after (B) degradation in PBS for 6 months (The arrows point to the locations where the scaffold has degraded)

The scaffolds in vitro bioactivity

SEM and EDX of the scaffolds bioactivity

After 14 days in PBS, scanning electron micrographs (SEM) of the composite scaffolds revealed the presence of freshly formed apatite crystals in various crystal sizes (**Figure 11**). These apatite crystals precipitated as tiny, atypically shaped crystals on the polymer surfaces and inside the interior walls of the scaffolds.

In the instance of scaffold group III, the EDX imaging of the different scaffold groups allowed for the detection of the $K\alpha$ radiation of Ca, P, and O elements for HAp ceramic powders. Additionally, the TCP phase was determined for scaffold group III. In addition to the deposits of hydroxyapatite formed due to soaking in the PBS solution. On the other hand, the appearance of $K\alpha$ radiation of Na and Cl arises from the phosphate buffer solution.

Additionally, the zein protein structure is responsible for the emergence of the $K\alpha$ radiation of nitrogen in all scaffold groups. While the emergence of the $K\alpha$ radiation of carbon (C) in high proportions in all of the groups, varying from 41.05 to 68.1 weight percent, resulted from the $(CH_2)_n$ -chains forming the PCL polymer structure and the amino acids present in the zein protein structure.

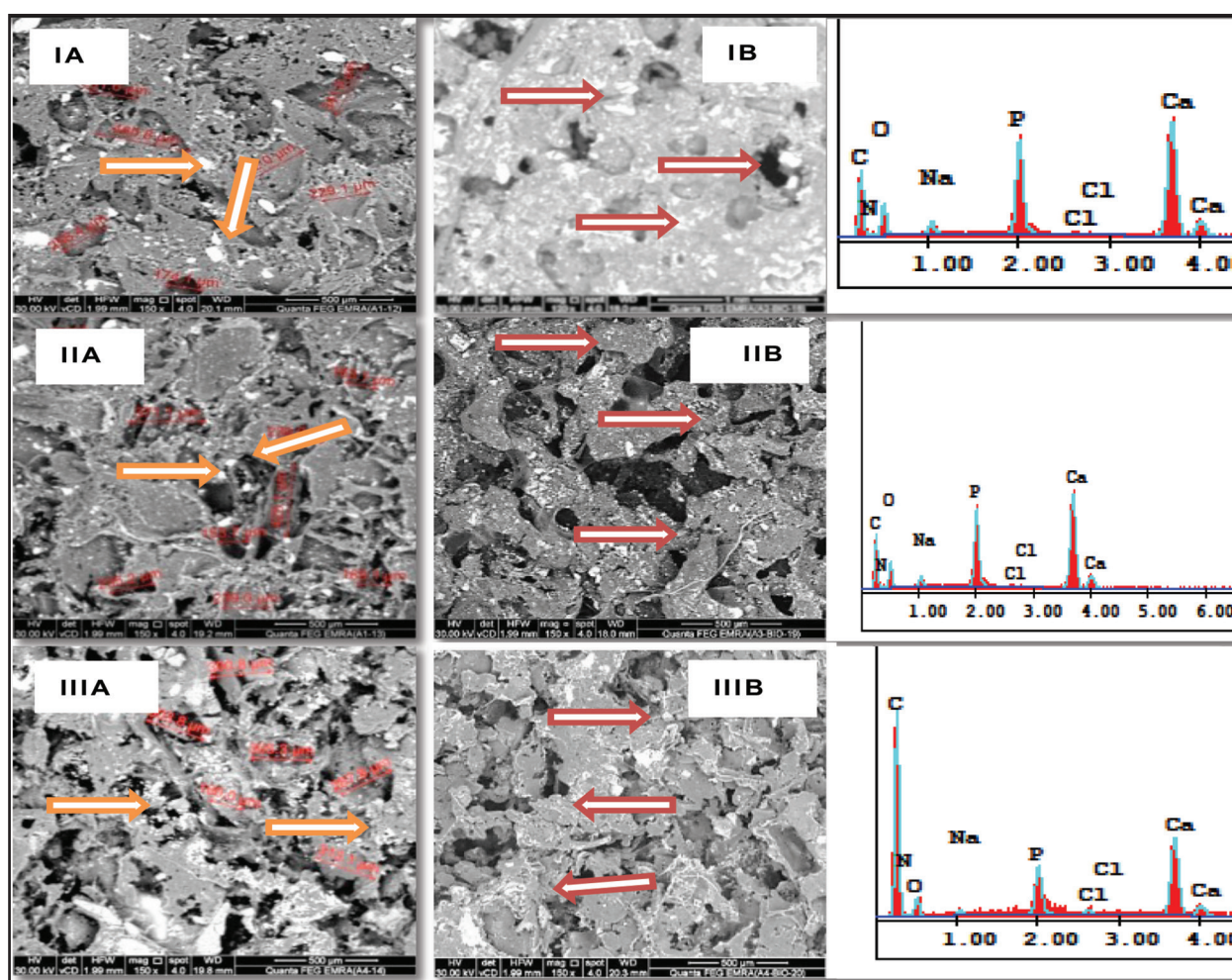


Fig. (11) SEM and EDX of the bioactivity of scaffold groups I, II & III before (A) and after (B) immersion in PBS for 14 days at 37°C (the yellow arrows refer to zein particles, while the red arrows refer to the formation of HAp crystals).

DISCUSSION

In the field of bone tissue engineering, composite scaffolds, which are made by mixing bioceramics and polymers, are becoming more promising. By combining the advantages of polymer flexibility and simple shape with the increased strength, stiffness, and bioactivity of bioceramics, they are able to build scaffolds that have superior performance in terms of their physical, mechanical, and biological properties. Polycaprolactone and zein protein polymers are subjects of attention at the moment due to the fact that they are biocompatible, possess favorable mechanical characteristics, and are readily available. Among the most promising bioceramic materials are calcium phosphates, either chemically prepared or extracted from biogenic sources such as hydroxyapatite, biphasic HAp/ β -TCP, and bioglass. This is because of their characteristics of biocompatibility, bioactivity, and osteoconductivity, which indicates that they have a natural affinity for bone tissues and a predisposition to link directly with them⁽²⁸⁾.

The findings of the XRD, FTIR, and SEM studies of the ceramic powders that were created indicated the creation of new apatite crystals on the surfaces of the powders, which is an indication of the powders' propensity to perform biological functions. This was confirmed through the increased main relative intensity XRD peak $I/I_0=100$ (at $2\theta = 31.8$) for the HAp phase (Fig. 3). Infrared analyses supported the X-ray results by increasing the relative intensities of the absorption bands at 609 and 561 cm^{-1} due to the triply degenerate mode of PO_4^{3-} in the HAp phase (Fig. 4)^(29,30). On the other hand, SEM supports the formation of newly formed apatite crystals on the biphasic ceramic surface (Fig. 5).

Solvent casting is one of the techniques used for composite scaffold fabrication. It is capable of producing scaffolds with an interconnected pore

structure and controlled pore size and distribution⁽³¹⁾. The polymer is dissolved in a suitable organic solvent and mixed with a porogen. NaCl was used as a porogen in this work as it remains the gold standard due to its ease of use, very cheap price, and high solubility, which ensures complete porogen removal.

The SEM images (**Figure 6**) revealed highly porous well inter-connected pore structure of the composite scaffolds of all groups, with pore sizes ranging from approximately 279 to 305 μm (**Table 3**). Pore size variation, including macro- and microporosity, is an important factor in vascularization, cell colonization, and survival, as well as the supply of oxygen and nutrients to transplanted cells⁽³²⁾. Furthermore, macroporous scaffolds with pore sizes ranging from 100 μm to 600 μm allow for better integration, vascularization, and bone distribution with host bone tissue. Increasing the size of the pores increases the permeability, which in turn increases the growth of the bone; however, small pores are more appropriate for the growth of soft tissue⁽³³⁾. The variation in the average pore sizes among the different groups may be attributed to the variation in the grain sizes of the NaCl porogen (250 to 500 microns and vary from batch to batch). The internal walls of the composite scaffolds appeared rough; this may be attributed to the exposure of the nano bioceramics after the complete extraction of the NaCl porogen. Such rough surfaces would function as favorable sites for osteoblastic attachment, facilitating quicker tissue ingrowth⁽³⁴⁾. The microstructure of the synthetic composite scaffolds prepared in this research is anticipated to be very promising, due to their high porosity and a well-developed network of interconnected pores with sizes greater than 100 microns. These results generally agree with the statistically computed porosity data. According to the statistical findings, the porosity of all the composite scaffolds used

in this research ranged from 73.67 to 79 percent, which was a satisfactory high percentage and would be highly beneficial for bone ingrowth.

The results of the compressive strength in this study showed that scaffolds containing chemically synthesized HAp (scaffold I) revealed the highest compressive strength (5.45 MPa), followed by scaffolds III (2.92 MPa) and II (2.62 MPa), which contained biphasic HAp/ β -TCP and HAp derived from CBB in their matrices, respectively.

Concerning the mechanical properties of the scaffold, smaller pore sizes and a lower porosity percentage are strongly correlated with a higher compressive strength. However, this disagreed with our data, since scaffold group I, containing chemically synthesized HAp, had the highest compressive strength value and the largest average pore sizes with an intermediate porosity percent at grain boundaries and manufacturing processes⁽³⁵⁾. While scaffold group II containing HAp from CBB had the least compressive strength, average pore size, and porosity percentage. This may be attributed to the polycrystalline nature of the calcium phosphate materials. As a result, their mechanical properties are affected not only by the porosity but also by the degree of crystallinity, composition, presence of impurities, grain sizes, grain boundaries, and manufacturing processes⁽³⁵⁾.

Also, it was reported that composite scaffolds prepared by incorporating the ceramic powder into a polymer matrix would encounter several potential difficulties and/or challenges, such as agglomeration of the ceramic particles and their distribution through the matrix and insufficient advantages of the composite structure, which may result in significantly inferior mechanical properties⁽³⁶⁾.

The compressive strength values for the scaffold groups under study, as depicted in **Figure 8**, fell

within the mechanical strength range of cancellous bone, which lies between 2 and 12 MPa⁽³⁷⁾. This suggests that these scaffolds are appropriately suited for use in bone tissue engineering applications in areas that are not subjected to significant stress. Additionally, the square pores that were created when the NaCl porogen crystals disintegrated would add steady mechanical strength⁽³³⁾.

After six months of scaffold immersion in PBS, the results of the weight loss percentage (**Figure 9**) due to hydrolytic degradation did not reveal statistically significant differences between the various groups. The scaffold group I, containing chemically synthesized HAp, had the lowest weight loss percentage (21.76%), while the scaffold containing HAp from CBB had a weight loss of 23.58 percent. Scaffold group III, containing the biphasic (HAp/ β -TCP) ceramic powder from CBB, showed the highest weight loss percentage (24.62%). As the resorption rate of TCP is higher than that of HAp, porous biphasic HAp/ β -TCP implants typically degrade more quickly than HAp implants do; agrees with our results. Different types of HAp have been used in earlier experiments, but there have been questions about how well this material degrades. The chemistry of the crystalline HAp and its small surface area makes it less likely to resorb⁽³⁸⁾. Incorporating calcium phosphate fillers like Hydroxyapatite (HAp) and/or biphasic HAp/ β -TCP into the Polycaprolactone (PCL)/zein matrix significantly influences the acceleration of the degradation rate of the hydrophobic composite comprising PCL and zein polymers. At the onset of the degradation process, these calcium phosphate fillers enhance the water absorption rate (hydrophilicity) of the PCL/zein matrix⁽³⁹⁾.

Furthermore, these fillers, being entrapped within the PCL/Zein matrix, interfere with the formation of PCL crystallites in the composite

mixture. Consequently, this leads to a reduction in the crystallinity of the PCL/zein matrix. Such a decrease in crystallinity results in an enhanced hydrolysis rate, attributable to the increased exposure of the ester linkage present in the amorphous phase of the matrix⁽⁴⁰⁾. The different scaffold groups were examined in vitro to confirm their apatite-forming ability. The formation of apatite layers on the material surface is necessary to bond the biomaterials to the living bones⁽³⁸⁾. In this study, the apatite-forming behavior of the different synthesized composite scaffolds was examined after immersion in PBS at 37°C for 14 days. The post-immersion SEM (**Figure 11**) showed the formation of new apatite crystals on the surfaces of the composite scaffolds of all groups and some other crystals precipitated within their inner walls. This is confirmed by the increase in the concentration values of the K α radiation for both Ca and P elements measured by the EDX technique following immersion in PBS for 14 days. Thus, the ability of the synthesized composite scaffolds in this study to form new apatite crystals indicates them being beneficial in bone tissue engineering. Furthermore, it has been demonstrated that the presence of β -TCP in nano sized biphasic enhances mesenchymal cell growth, with a 50:50 HAp: β -TCP ratio being optimal⁽⁴¹⁾.

CONCLUSION

Promising hydroxyapatite (HAp) and biphasic calcium phosphate were derived from biogenic cancellous bovine bone and incorporated as fillers in polycaprolactone (PCL) and zein scaffold matrices. This approach aimed to create bioactive materials for bone tissue engineering, with a focus on recycling bio-waste materials to provide low-cost precursor alternatives. The study processed PCL/zein/HAp (chemically synthesized), PCL/zein/HAp (CBB), and PCL/zein/biphasic (HAp/ β -TCP)

(CBB) composite scaffolds using the solvent casting/particulate leaching method. The objective was to evaluate their physical and mechanical properties, as well as their in vitro bioactivity. Results showed that all composite scaffold groups exhibited a highly porous, interconnected structure with pore sizes ranging from 279 to 305 μ m, based on SEM measurements. These pore sizes are conducive to the growth of blood vessels and bone tissue. Furthermore, there was no significant difference in porosity percentages among these groups. The highest compressive strength was recorded in the composite scaffold containing chemically synthesized HAp (5.452 MPa). However, the mechanical strengths of the other groups with ceramics from CBB fell within the typical range of cancellous bone strength (2–12 MPa). Additionally, there was no significant difference among the groups in terms of weight loss percentage after six months of PBS immersion. The bioactivity of all composite scaffold groups showed new apatite crystal growth on their surfaces after 14 days of PBS soaking. It was demonstrated that biogenic wastes of cancellous bovine bone are a viable source of calcium phosphate for developing composite scaffolds suitable for bone tissue engineering applications.

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