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EGGSHELL DERIVED NANOHYDROXYAPATITE REINFORCED CHITOSAN CRYOGEL BIOCOMPOSITES FOR TISSUE ENGINEERING APPLICATIONS

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Abstract: Hydroxyapatite has a biocompatible, biodegradable and natural apatite characteristic to be used in biomedical applications such as bone tissue engineering. The objectives of this study were to synthesize hydroxyapatite from domestic waste eggshells which is utilized as pure calcium source; compare the properties of biosynthesized hydroxyapatite with commercially purchased hydroxyapatite; and produce biosynthesized hydroxyapatite reinforced chitosan cryogels for possible tissue engineering applications. Calcium oxide powders obtained after calcination of waste eggshells showed different particle sizes depending on calcination temperature. It was found that increased temperature of calcination led to the powders of smaller particle sizes. Structural changes at carbonate groups of calcined eggshell were determined by FTIR analysis. The effect of the biosynthesized hydroxyapatite on the morphology of chitosan cryogel biocomposites were determined. The changes in the chemical bond structure of the cryogels were analysed by FTIR and swelling behavior of produced chitosan cryogels was determined by swelling ratio tests.

Keywords: Biosynthesis, eggshell, hydroxyapatite, chitosan, biocomposite scaffold.

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INTRODUCTION

Hydroxyapatite (HAp, $Ca_{10}(PO_4)6(OH)_2$) exhibits excellent biocompatibility, biodegradability, osteoconductive and bioactive properties due to its chemically similar composition to the inorganic component of natural bone minerals (1). HAp can be produced by chemical synthesis such as sol-gel method, hydrothermal method, sono-chemical synthesis, co-precipitation and mechanochemical method or by extraction from natural sources such as corals, sea shells, animal bones and eggshells (2). The worldwide availability, unlimited supply, low cost, simple, inexpensive, economical, and efficient production are the advantages of obtaining HAp from natural biological sources (3). Eggshell is one of the major waste product of food industry, and it becomes useless after the use of egg contents and its derivatives (4). Eggshell is composed of calcium carbonate (94%), organic matter (4%), calcium phosphate (1%) and magnesium carbonate (1%) (5). In recent years, the combination of a polymeric matrice (especially made of a natural polymer) with a biocompatible, reinforcing and bioactive component like HAp has shown significant improvements as biomaterials for clinical applications (6). In this study, we aimed to obtain biosynthesized HAp (bio-HAp) from waste egg shells and combine it with chitosan cryogel scaffolds. The chitosan used in this study was extracted from the blue crap, as demonstrated in our previous study (7). The present study firstly demonstrates producing calcium oxide (CaO) powder from domestic waste eggshells (a cheap and widely available biological source in worldwide) by using a simple heat treatment process at different calcination temperatures. Then, describes the synthesis and characterization of nanometer scale bio-HAp from this CaO powder by co-precipitation method. The properties of synthesized CaO powders and bio-HAp was characterized by fourier transform infrared spectroscopy (FTIR) and dynamic light scattering (DLS) analysis. Finally, we combined bio-Hap with chitosan cryogels. The chemical structure and swelling behaviour of chitosan-HAp cryogels were demonstrated for possible tissue engineering applications.

MATERIAL AND METHODS

Material

In this study, eggshells which were collected from domestic wastes were used as starting material for the synthesis of biosynthesized HAp (bio-HAp). Phosphoric acid was obtained from Merck, Germany. The commercial HAp (com-HAp) used for the comparison of the properties of bio-HAp was purchased from Sigma Aldrich, USA. Chitosan from blue crap shells were used for the production of cryogel scaffolds. Glutaraldehyde (25%, v/v) as the crosslinker was obtained from Merck, Germany. All solution preparations and washing steps were performed by using distilled water.

Preparation of Waste Eggshells and Synthesis of CaO

Waste eggshells were first washed with tap water to remove contaminants. The cleaned eggshells were washed with distilled water and dried at 105 °C for 4 hours. The pretreated eggshells were crushed, milled and sieved to a standart powder (500 µm sieve). For CaO synthesis, powder of eggshells were calcined in an ash furnace (Protherm, Plf 130/45, Turkey) at different temperatures (400, 600, 800 and 1000 °C) for 2 hours with a heating rate of 10 °C/min. During calcination, the eggshells were converted to calcium oxide by releasing carbon dioxide (CO_2) according to following reaction:

$$CaCO_3 \rightarrow CaO + CO_2 \quad \text{(Eq. 1)}$$

The CaO samples obtained from the eggshells were stored in an oven at 40 °C for further analysis.

Synthesis of Bio-Hap

Bio-HAp were prepared from CaO powders by co-precipitation method according to Kunjalukkal et al, 2015 (6). The calcination temperature of 800 °C was selected in order to prepare CaO powders for certainty of the complete transformation of $CaCO_3$ to CaO based on TGA results. A stoichiometric amount of CaO powder (2 grams) were weighed and hydrolysed in 250 mL of distilled water to obtain calcium hydoxide ($Ca(OH)_2$) solution as follows:

$$CaO + H_2O \rightarrow Ca(OH)_2 \quad \text{(Eq. 2)}$$

Under continuous stirring at 1400 rpm, 1350 µL phosphoric acid solution was added drop by drop to the $Ca(OH)_2$ solution at 100 °C by using a magnetic stirrer. The expected reaction is as follow:

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca_{10}(PO_4)6(OH)_2 + 18H_2O \quad \text{(Eq. 3)}$$

After the bubbling was finished, the mixed solution was kept on the same conditions for 2 h. The resulting solution was allowed to cool down to room temperature and the resulting precipitate was filtered out using filter paper. Wet sample was put in an oven at 40 °C for 24 h. The dried precipitate was then collected and stored.

Characterization of Synthesized CaO AND Bio-HAp

Thermogravimetric analysis, TGA/DTG of waste eggshell was carried out using Perkin Elmer Pyris 1 TGA, ABD. A total of 7.5 mg of the sample was used and TGA curve was obtained from 40 °C to 1000 °C in nitrogen atmosphere with a heating rate of 10 °C/min. FTIR (Perkin Elmer, FTIR Spectrometer Frontier ATR, USA) was used to determine the functional groups and chemical compositions of the CaO powders synthesized by the calcination of waste eggshells at different temperatures and synthesized bio-HAp at selected calcination temperature. FTIR analysis were performed at a resolution of 4 cm^{-1} in the wavelength range of 450-4000 cm^{-1}. For the characterization of particle size, the Dynamic Light Scattering (DLS) (Malvern, Nano ZS90, England) was used to measure powder particle size distribution of synthesized CaO and bio-HAp samples. The samples were dispersed in acetic acid (100% purity, Glacial).

Production of Chitosan-HAp Cryogel Biocomposites

The chitosan-HAp cryogels were synthesized at different amounts of synthesized HAp by cryogelation method. Chitosan solution (3%, w/v) was prepared in acetic acid solution (6%, v/v) and mixed on a magnetic stirrer until solution was homogenous and clear. At the end of mixing, three different ratio of synthesized HAp (1:1, 1:2, 1:3, ratio of chitosan to HAp) was added to prepared chitosan solutions. 1 mL of GA solution (3%, v/v) was added to 2 mL of prepared chitosan-HAp solution. The whole solution was immediately poured into a 2 mL plastic syringe and transferred into the cryostat. The reaction mixture was incubated in the cryostat at -16 °C for 2 h. After this period the cryogels were stored in the fridge at same conditions for 24 h. After the reaction was completed, the frozen samples in the syringe molds were thawed to room temperature and washed several times to remove the unreacted reagents. The samples were lyophilized before characterization.

Characterization of Produced Chitosan-Hap Cryogel Biocomposites

The obtained cryogels' chemical structure was analysed by FTIR in the range of 450-4000 cm^{-1}, with automatic signal gain collected in 20 scans at a resolution of 4 cm^{-1}. To determine the swelling behavior of chitosan-HAp cryogels, the samples were dried at room temperature to a constant weight (W_D). Then, dried samples were immersed in distilled water to obtain swollen cryogels. The excess water on the surface of the cryogels was removed and the samples were weighed (W_S). Swelling ratio was calculated by the following equation:

$$SR\% = ((W_S - W_D) / (W_D))*100 \quad \text{(Eq. 4)}$$

RESULTS AND DISCUSSION

Characterization of Synthesized CaO and Bio-Hap

FTIR analysis was carried out to determine the chemical composition of the waste eggshell and CaO powders synthesized after calcination of eggshells at different temperatures. Figure 1 shows the FTIR spectra of the waste eggshell and CaO powders. The bands at between 873 and 1413 cm^{-1} were attributed to C-O bond of carbonate (CO_3) groups of eggshell. However, the CO_3 ions disappeared from the structure at high temperatures (800 and 1000 °C) during heat treatment. The intensity of the O-H stretching band observed at 3642 cm^{-1} wavelength is due to the O-H bond in $Ca(OH)_2$, which is formed during the adsorption of water by CaO (8). The peak at 2981 cm^{-1} is characteristics of free CO_2 due to the background of the measurement system or reaction process (9). Presence of these characteristic bands is a proof of the CaO powder formed.

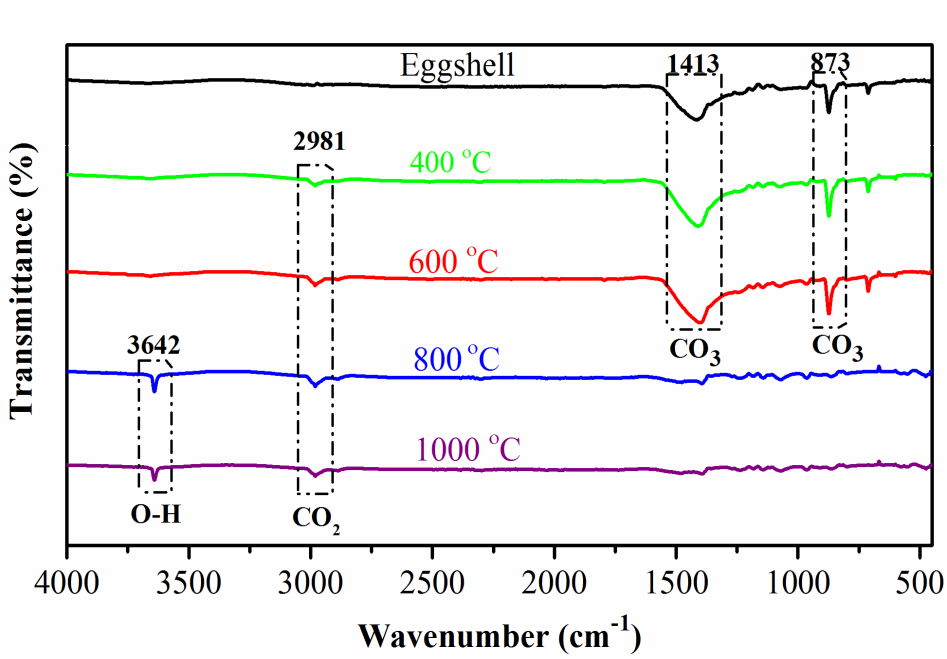

Figure 1. FTIR analysis of waste eggshell and CaO powders synthesized at 400, 600, 800 and 1000 °C.

$CaCO_3$, the main component of the eggshell, can completely decompose in CaO and CO_2 with the increase in calcination temperature (10). In this study, eggshells were calcined in an ash furnace at 400, 600, 800 and 1000 °C, respectively to evaluate the effect of calcination temperature on formation of CaO. Thermal analysis of the waste eggshell was performed to determine the optimum calcination temperature (Figure 2). The weight decrement during the heating process was determined by TGA. The CaO content in the eggshells were determined as about 49% (w/w). With the increase in temperature from 600 to 800 °C, a significant weight loss has occurred because of the CO_2 molecules moving up from the structure of eggshell. The $CaCO_3$ decomposes completely into CaO at a maximum temperature of 760 °C.

Beside the thermal analysis of waste eggshell, DLS analyser measurements were made to observe the effect of the calcination temperature on the particle size of the synthesized CaO. Figure 3 shows the variation of CaO powder size as a function of calcination temperature of eggshells. It reveals that increasing the calcination temperature results in smaller size of CaO powder which was in close agreement with the (11). At the calcination temperature of 800 the particle size was 103.5496 nm. The calcination temperature was chosen as 800 °C for the synthesis of bio-HAp.

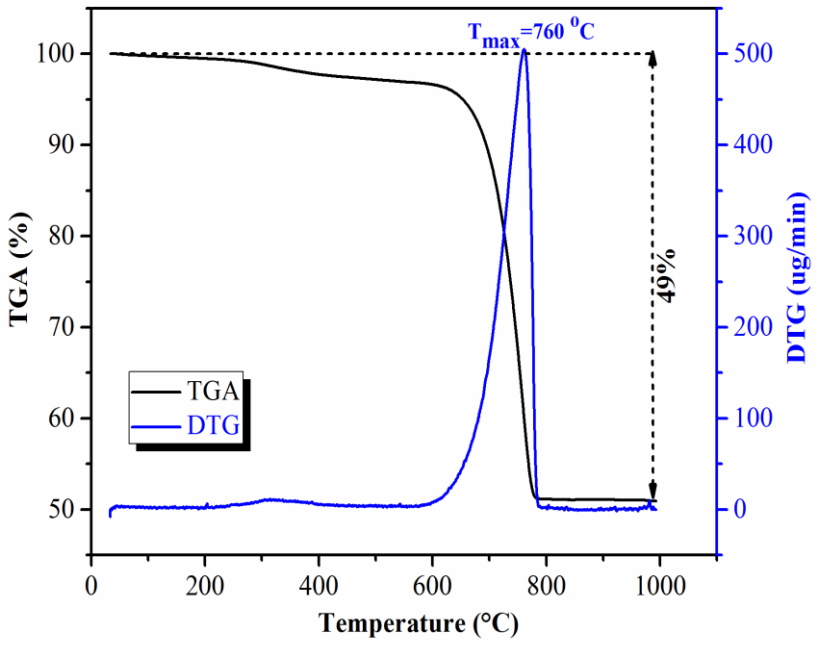

Figure 2. TGA and DTG curves of waste eggshell.

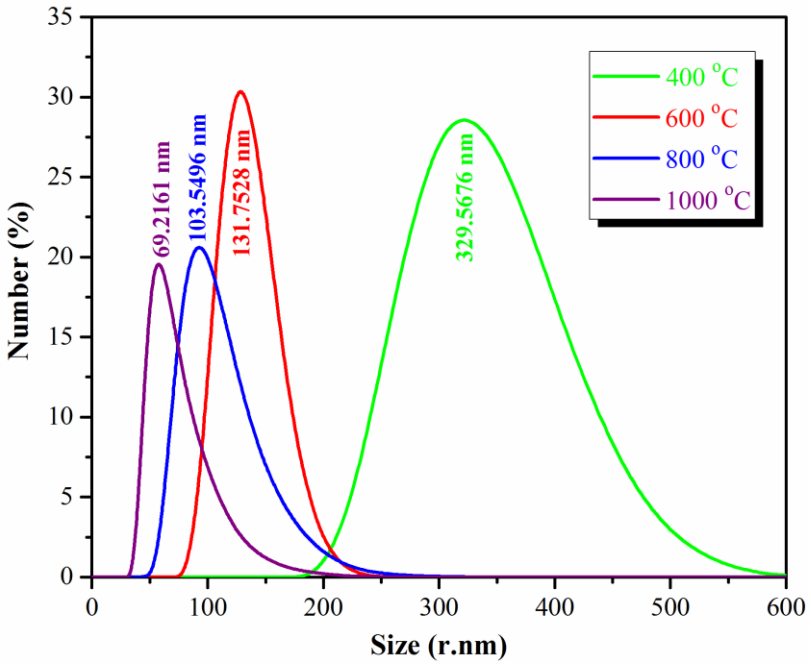

Figure 3. Particle size distribution of CaO powders synthesized at 400, 600, 800 and 1000 °C.

Characterization of Bio-Hap

FTIR analysis was performed to compare the functional groups of bio-HAp and commercially purchased hydroxyapatite (com-HAp). The FTIR spectrum of the bio-HAp was chemically in good agreement with the spectrum of com-HAp and the FTIR spectrum reported by another study that demostrated HAp powder synthesized from hen eggshells (12). The band at 1027 cm^{-1} is the characteristic band of phosphate (PO_4) stretching vibration whereas the bands at 562 and 501 cm^{-1} are due to phosphate bending vibration (13). According to spectral data, the absorption

peak at 1412 cm^{-1} corresponds to the asymmetric stretching of carbonate ion substitution. FTIR spectra indicated that bio-HAp was successfully derived from CaO calcined at 800 °C.

The particle size distribution of bio-HAp and com-HAp samples was analysed (Figure 5). The average size of bio-HAp was 33.08 nm while the size of com-HAp was 115.98 nm. The polydispersive index (PDI) of samples were 0.462 and 0.722, respectively. This PDI value showed that the synthesized sample was homogeneous and uniform in size (14). Moreover, DLS analysis showed that the synthesized bio-HAp was in the nanometer size.

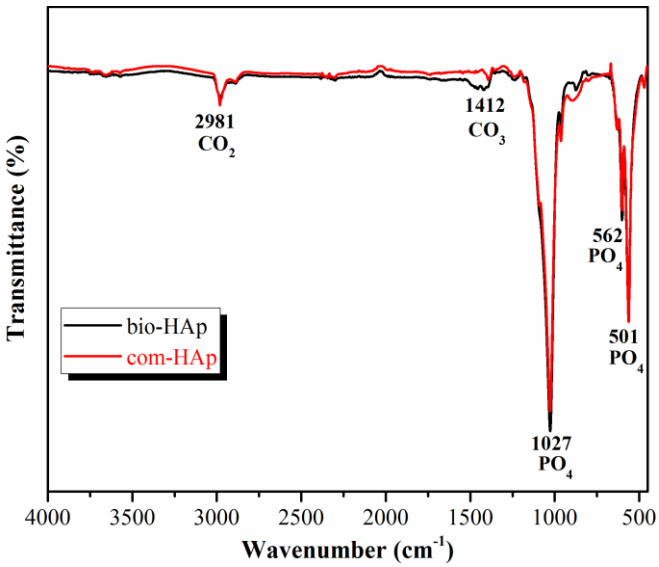

Figure 4. FTIR analysis of com-HAp and bio-Hap.

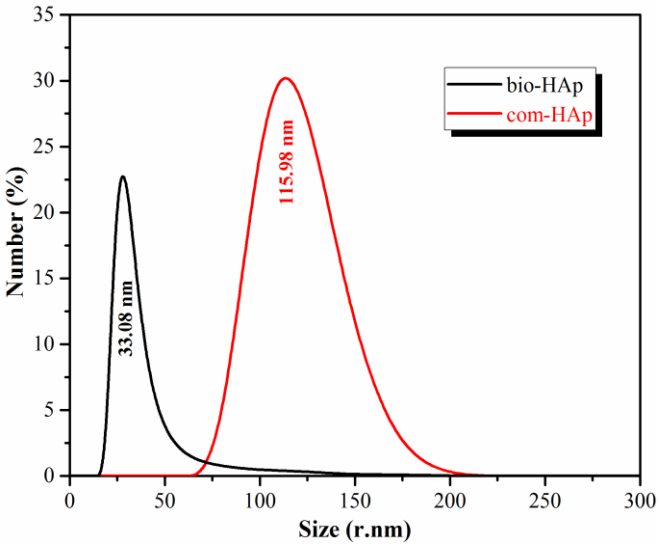

Figure 5. Particle size distribution of com-HAp and bio-Hap.

Characterization of Chitosan-Hap Cryogels

The ratio of bio-HAp to chitosan solution was varied in this study. The amount of bio-HAp affected the chemical, physical, mechanical, morphological and porous structure of cryogels. Figure 6

shows the photograph of chitosan-HAp cryogels after cryogelation reaction is completed (in wet form) and after lyophilization (in dry form). The colour of cryogels including bio-Hap were more opaque than the plain chitosan cryogel. Also with increasing the amount of bio-HAp in the chitosan cryogels, a more smooth and elastic surface was reached.

Furthermore, FTIR analysis demonstrated the functional groups of bio-HAp, interactions between bio-HAp and chitosan, crosslinking bonds between glutaraldehyde and chitosan. The FTIR spectra of the cryogels, as shown in Figure 7, demonstrated bands corresponding to hydroxyl, phosphate and amine groups. The major absorbance bands of the spectra correspond to hydroxyapatite. Width of bands decreases with increasing bio-HAp content of cryogels (15). With the increase in the amount of bio-HAp a sharp peak was observed at near by 1050 cm^{-1}. This peak shows the interaction of chitosan with the phosphate groups of bio-HAp (16). The bands between 1550-1700 cm^{-1} are attributed to mode superposition of the hydroxyl group of bio-HAp and amide groups of chitosan (15). The hydroxyapatite phosphate bending bands are at 562 cm^{-1}. The broad peak started at 3480 cm^{-1}, gradually decreased and became narrower with the increase in the amount of bio-HAp.

At the cryogelation step, chitosan-HAp cryogels with interconnected pores were obtained. Swelling ability of a cryogel is related with the highly porous and spongy morphology of the cryogels (7). The swelling ratio results of the plain chitosan and chitosan-HAp cryogels are demonstrated in Figure 8. Plain chitosan and all chitosan-HAp cryogels showed a swelling ratio higher than 3000% in the first 5 min. It was observed that as the amount of bio-HAp in the cryogels increased the swelling ratio decreased. The decrease may be due to the decrease in the pore size of the cryogels. As the amount of bio-HAp increased the pore walls of the cryogels were filled with more bio-Hap. Plain chitosan cryogel showed the highest swelling ratio (8660.43%) after 60 minutes of swelling time.

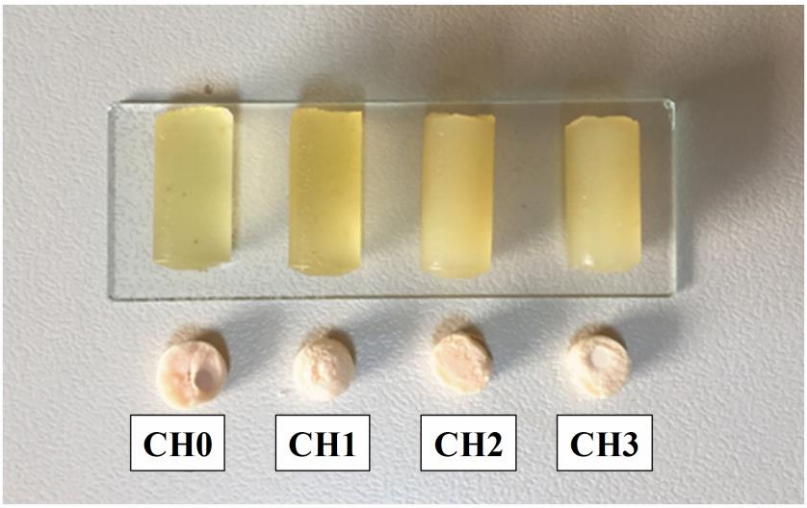

Figure 6. Photograph of blank chitosan and chitosan-HAp cryogels in wet and dry form (CH0= plain chitosan, CH1=1:1, CH2= 1:2, CH3= 1:3 (chitosan:HAp))

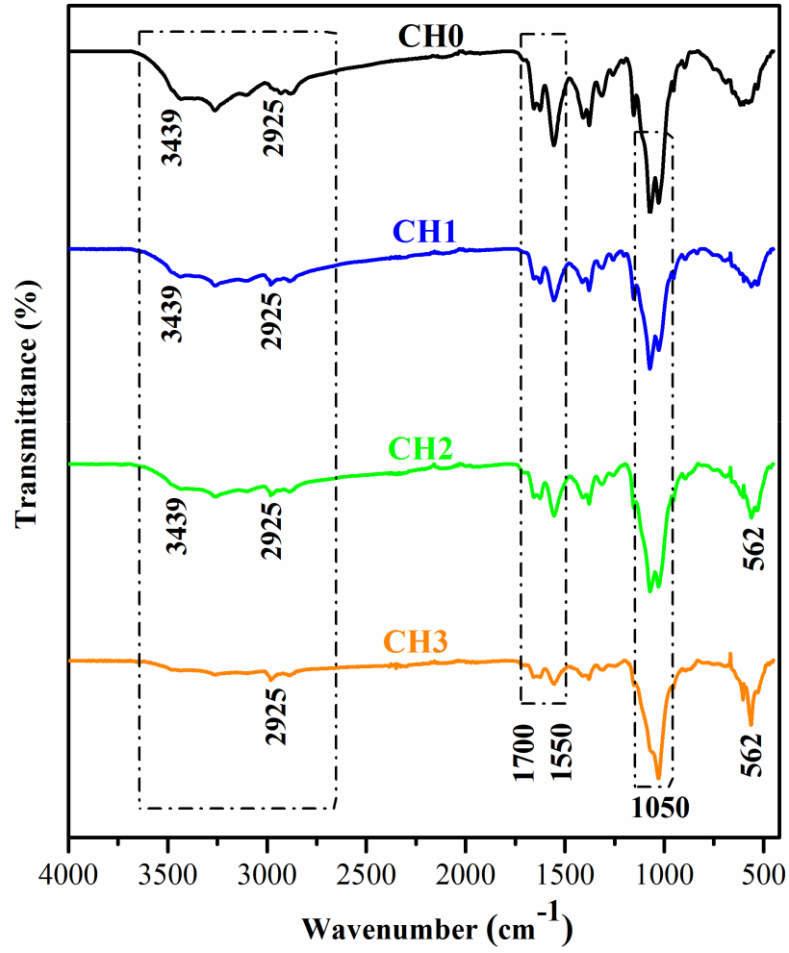

Figure 7. FTIR analysis of plain chitosan and chitosan-HAp cryogels.

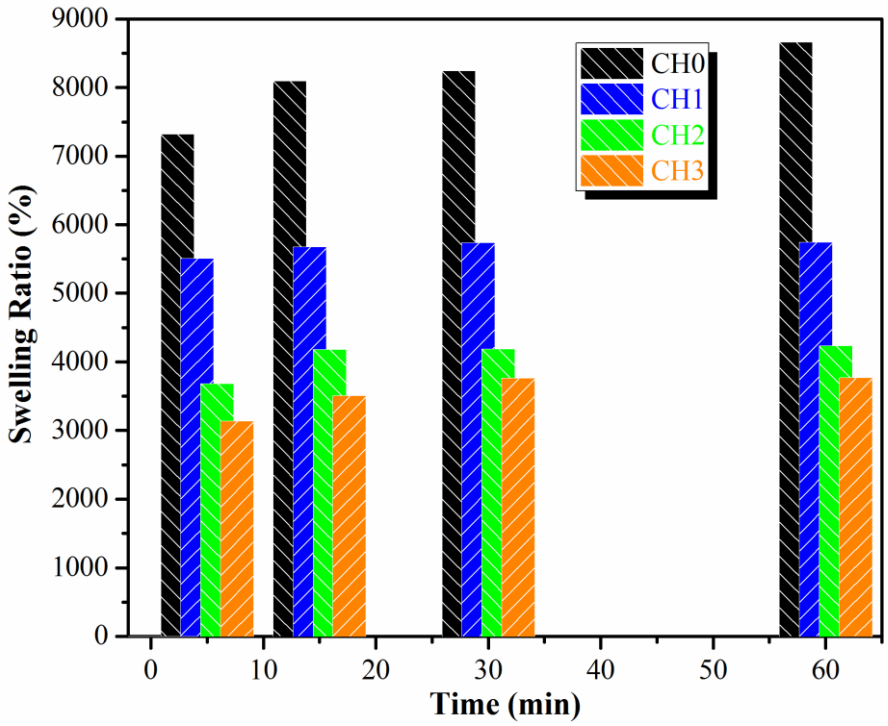

Figure 8. Swelling ratio analysis of plain chitosan and chitosan-HAp cryogels.

CONCLUSION

CaO powder was produced by using domestic waste eggshells through a calcination process at different temperatures. Pure bio-HAp was synthesized by co-precipitation method using the CaO powder which was calcined at 800 °C. The particle size of the synthesized bio-HAp was measured as in nano scale. FTIR analysis showed the purity of bio-HAp. This process can lead to the development of a cost effective biomaterial and can improve waste management in future. Chitosan cryogel scaffolds reinforced with bio-Hap were successfully produced for possible tissue engineering applications. Hence, from the results, it can be concluded that the synthesized bio-HAp can be economically produced from waste eggshells by a simple calcination and co-precipitation method for wide range of biomedical applications especially for tissue engineering. The bio-HAp reinforced chitosan cryogel biocomposites can be potential scaffold candidates to be used in possible tissue regeneration.

CONFLICT OF INTEREST

The authors declare that no conflict of interest occurred in this work.

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