

## Preparation of Nanocomposite Via Calcium Phosphate Formation in Chitosan Matrix Using in Situ Precipitation Approach

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**Abstract:** Calcium phosphate-based biomaterials are of special interest in bone regeneration due to their biocompatibility and biodegradability; on the other hand, chitosan has novel properties such as antibacterial, biocompatibility and biodegradability. In the present study a new chitosan (CS)/ calcium phosphate (CaP) nanocomposite biomaterial is introduced which is prepared using the in situ precipitation approach. The fabricated nanocomposites were characterized by Fourier transformer infrared spectra (FT-IR), X-ray diffraction (XRD), transmission electron microscopy (TEM) and scanning electron microscope (SEM). The crystals were rod like with the average size of about 100 nm in length and 20 nm in diameter. Calcium phosphates such as hydroxyapatite (HA), brushite (DCPD) and  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) were found to crystallize simultaneously in the chitosan matrix. The results showed that the crystalline calcium phosphate nanoparticles were mineralized in chitosan and the interaction between  $\text{Ca}^{2+}$  in CaP and negative-charged functional groups in chitosan molecular chains was formed. The effect of chitosan on the crystallization of calcium phosphate particles was studied and it was found that polymer has controlling role in the growth of calcium phosphate crystals.

**Key words:** Nanocomposites • Calcium phosphate • Interaction • Polymeric phase • Brushite

### INTRODUCTION

Recently a great deal of attention has been focused towards creating bioactive ceramic/polymer composites to be used as a bone substitute whereas the natural bone is made of calcium phosphate nanoparticles scattered in the organic matrix [1]. Calcium phosphates which constitute the major inorganic phase of human hard tissues like the bone and teeth are bioactive and can be rapidly integrated into the human body. There are many bioceramic materials based on calcium such as hydroxyapatite (HA), tricalcium phosphate (TCP), calcium aluminate (CA) and dicalcium phosphate dehydrate (brushite) which have been used as fillers to repair bone defects [2]. Three calcium phosphate cements, brushite (BR),  $\beta$ -tricalcium phosphate and hydroxyapatite, were recently developed as bone substitution materials in orthopaedic surgery [3-4]. Hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ) (HA) is a naturally occurring inorganic material that its chemical and crystallographic are similar to the inorganic component

found in natural bone [5]. Moreover, HA has been widely investigated because it is an excellent biocompatible and bioactive material for a number of clinical demands in the areas of orthopedics and dentistry for the past several decades [6]. Compared to HA, tricalcium phosphate (TCP:  $\text{Ca}_3(\text{PO}_4)_2$ ) is generally considered as a resorbable bio-ceramic.  $\beta$ -TCP is preferred as a bio-ceramic among all TCPs because of its mechanical strength, good tissue compatibility and ability to bond directly to tissue to regenerate bone without any intermediate connective tissue. In addition, fast bone regeneration and proper bio-resorption rate are other additional attributes of  $\beta$ -TCP [7-8]. Brushite (dicalcium phosphate dihydrate, DCPD,  $(\text{CaHPO}_4)_2 \cdot 2\text{H}_2\text{O}$ ) is another calcium phosphate which has a higher solubility than HA at physiological pH and an ideal in vivo resorption rate that can match the rate of new bone formation [9]. Besides having bioresorbability characteristic, brushite-based orthopedic cements are biocompatible, osteoconductive and bioresorbable and have mechanical properties similar to those of cancellous

bone [10]. Nevertheless both compounds are considered biocompatible and bioactive in the sense of osteoconduction and resorbability [11], their application as a monolithic in artificial bone materials is limited due to some disadvantages such as their migration from the implanted site into surrounding tissues by causing damage to health tissue, their low flexural strength and poor formability [12].

Therefore, there is a growing interest in composite materials because no single existing material possesses all the necessary properties required in an ideal bone graft. The addition of biodegradable polymers to calcium phosphate ceramics would allow for better manipulation and control over both the macro-and micro-structure in shaping composites to fit bone defects [13]. Moreover, biodegradable polymers can be used as binders for ceramic to reduce their brittleness [14]. Some polymers are studied as useful modifiers and binding agents with hydroxyapatite such as fibrin, collagen, alginates, gelatin and chitosan [15-16]. Recently much attention has been given to utilize chitosan in biomedical applications such as space filling implants. Chitosan (CS), which is generally obtained by deacetylation of chitin which is a natural cationic polysaccharide [17]. Due to its numerous desirable properties, e.g. no antigenicity, good biocompatibility and biodegradability, it has been widely used in the biomedical field [18]. In addition, it has been reported that chitosan can promote adhesion and functional expression of osteoblasts because of its similarity to glycosaminoglycan in structure [19-20].

Hence, the present work is concerned with the preparation of chitosan/ calcium phosphate nanocomposites by in situ precipitation approach to be used as bone substitute. Three types of CS/CaP composites with weight ratios of 40/60, 50/50 and 60/40 were prepared. The crystallization and morphology of calcium phosphate in polymeric matrix were examined by X-ray diffraction (XRD), transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Ionic interaction between  $\text{Ca}^{2+}$  in CaP particles and negatively charged functional groups in molecular chains of chitosan was also analyzed by Fourier transform infrared spectroscopy (FTIR). Moreover, the effects of the chitosan on the properties of calcium phosphate crystals were evaluated.

## MATERIALS AND METHODS

**Materials:** Chitosan powder was purchased from the Sigma Chemical. Calcium nitrate tetrahydrate ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ) and diammonium hydrogen phosphate ( $(\text{NH}_4)_2\text{HPO}_4$ )

were prepared from Sigma-Aldrich Inc.. Glutaraldehyde was of analytical grade. And all other chemicals were used as received without any further purification. Deionized water was used throughout the experiment.

### Synthesis of Chitosan-Calcium Phosphate Composites:

Composites of Chitosan / Calcium Phosphate were synthesized by in situ precipitation. The amount of ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ), ( $(\text{NH}_4)_2\text{HPO}_4$ ), chitosan was calculated to make 2 g of composites with different ratio of organic/inorganic phase. The first step is to prepare CS solution by dissolving CS in 40 ml of acetic acid solution (2 vol.%) with stirring at room temperature to get a perfectly transparent solution. Then  $(\text{NH}_4)_2\text{HPO}_4$  was added to the CS solution under agitation until the salt was entirely dissolved. Subsequently,  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  was solved in deionized water and was added dropwise into CS solution. Next, 150  $\mu\text{l}$  glutaraldehyde (25 wt.%) was added to the previous mixed solution as a crosslinker. The solution was continually stirred until an opaque hydrogel was produced. The synthesized hydrogel was stored under ambient conditions for 24 h to achieve complete crosslinking. It was then kept in ammonia solution for 48 h at 25°C. Under this alkaline condition, CaP crystals precipitated within the hydrogel matrix gradually. Then the composite was rinsed with distilled water to neutral with distilled water. Finally, the composites were lyophilized with a freeze dryer and the CS/CaP composites were obtained. Different samples were synthesized with organic/inorganic phase weight ratios of 40/60, 50/50, 60/40 which are named CCP 46, CCP 55 and CCP 64, respectively.

**Characterization:** Crystallinity of the composites was determined using a X-Ray Diffractometry (JEOL, model JDX- 8030, Japan) under the operating conditions of 30 kV and 20mA was performed on the CS/CaP composites matrix. Measurements were taken from  $2\theta = 10$  to  $50^\circ$ . The size and morphology of the powders were observed using a Phillips EM208S, Netherlands Transmission Electron Microscopy (TEM) with a 100 kV accelerating voltage. And the morphology of the composites also was observed using a Phillips XL30, Netherlands Scanning Electron Microscopy (SEM) equipped. The composites were vacuum coated with gold in a Polaron SC500 sputter coater. Chemical bonding between CaP crystalline and functional groups of chitosan matrix was estimated using a Fourier Transform Infrared (FTIR) spectroscopy (Lexus, 670EST, USA) and the spectra was collected over the range of  $4000\text{-}400\text{ cm}^{-1}$ .

## RESULTS

**XRD:** X-ray diffraction was performed on the composites to determine the phases in presence. The indexed X-ray spectrum is represented in Fig. 1 CS/CaP composites with different ratio of organic/inorganic phase. Three major characteristic diffraction peaks could be observed for all composites: one at  $2\theta$  of  $\sim 10$  to  $11$  is belonging to Brushite, second at  $2\theta = 21$  which might be attributed to  $\beta$ -TCP and the other group is ranging from  $2\theta = 30$  to  $2\theta = 31$  can be used to monitor the HA formation in composites. Figure 1 shows that the peaks intensity of brushite gradually increased by increasing of organic/inorganic (Cs/CaP) ratio. This may be the result of the harder penetration of the NaOH solution into the matrix which is due to increased organic phase. Therefore, much more brushite is produced due to low pH within the matrix that is because of the possible presence of residual acid. On the other hand, the amount of HA formed increases with decreasing organic phase because of enhancing the permeability solution. In CS/CaP composites by increasing of CS, less HA and  $\beta$ -TCP were formed. Moreover, the XRD pattern of the CS/CaP nanocomposite showed sharp peaks with high crystallinity around the characteristic diffraction for brushite, which suggested high crystallinity of Calcium Phosphate (BR) in the CS/CaP nanocomposite.

**FTIR:** Fourier Transform Infrared Spectroscopy (FTIR) identifies chemical bonds in a molecule by producing an infrared absorption spectrum. FT-IR spectra of chitosan and CS/CaP composites are shown in Fig. 2. The bands at  $1031$ ,  $602$  and  $562$   $\text{cm}^{-1}$  correspond to different modes of  $\text{PO}_4^{3-}$  groups which implied the existence of the organic-inorganic interaction between calcium phosphate (HA and Brushite) crystals and polymer macromolecules, while the  $\text{PO}_4^{3-}$  bands at  $1130$ - $1140$   $\text{cm}^{-1}$  were assigned to BR specifically [21]. The OH band at  $3568$   $\text{cm}^{-1}$  indicates the surface-free water on calcium phosphate crystallites and the intensity decreased with the increase of polymer content. The spectrums of chitosan exhibited an apparent absorption band at  $1658$   $\text{cm}^{-1}$  assigned to amide I, C=O stretching mode, shift to  $1648$   $\text{cm}^{-1}$  in all composites. The significantly weakened C=O stretch vibration peak may result from formation of the bonding between Ca ions and C=O bonds. Amino II ( $-\text{NH}_2$ ) peaks at  $1552$   $\text{cm}^{-1}$  in chitosan move to lower wavenumber at  $1541$   $\text{cm}^{-1}$  for composites which indicate existence a kind of chemical interaction between  $\text{PO}_4^{3-}$  and amino groups in chitosan, or between amino groups of polymer and calcium ions in

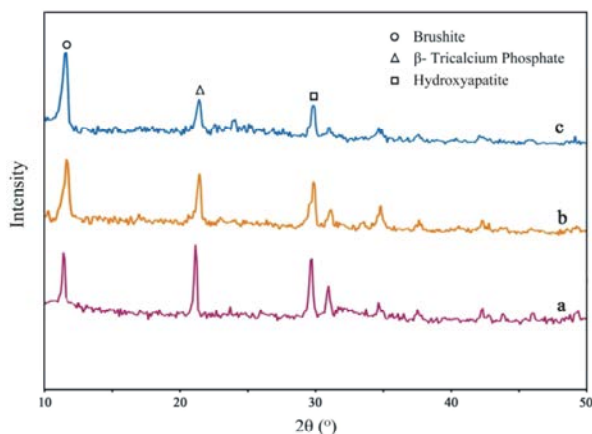


Fig. 1: XRD pattern of the CS/CaP composite: (a) CCP 46; (b) CCP 55; (c) CCP 64

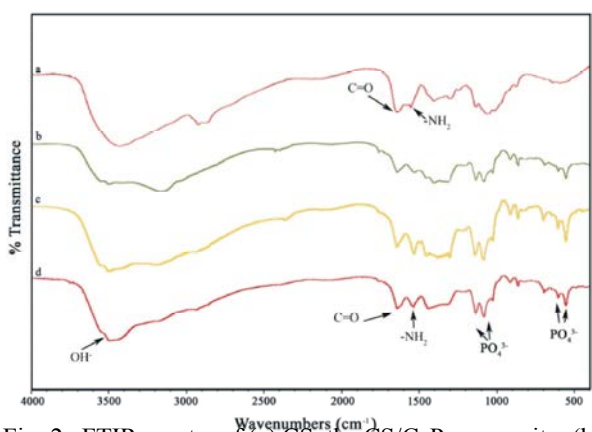


Fig. 2: FTIR spectra of (a) CS; the CS/CaP composite: (b) CCP 46; (c) CCP 55; (d) CCP 64

formed crystals. Results offered that the charged functions groups that existed on the surfaces of polymeric phase, e.g. Positively charged amino groups of chitosan or negatively charged groups including C=O groups of chitosan may begin to act as heterogeneous nucleation centers for calcium phosphate particles. At first, a layer of  $\text{Ca}^{2+}$  ions was formed on the surface of chitosan via strongly bound to carbonyl groups and amino groups of chitosan. Then another layer of  $\text{PO}_4^{3-}$  ions was attracted on the  $\text{Ca}^{2+}$  layer through electrostatic interaction.

**SEM and TEM Observation:** The structure morphology of calcium phosphate in the CS/CaP composites with weight ratios of 40/60, 50/50 and 60/40 are shown in Fig. 3 which are named GH 46, GH 55 and GH 64, respectively. SEM observations revealed the presence of calcium phosphate particles into polymeric matrix and typical images of all CS/CaP composites show that the inorganic crystals have a high affinity with the polymeric matrix, because the

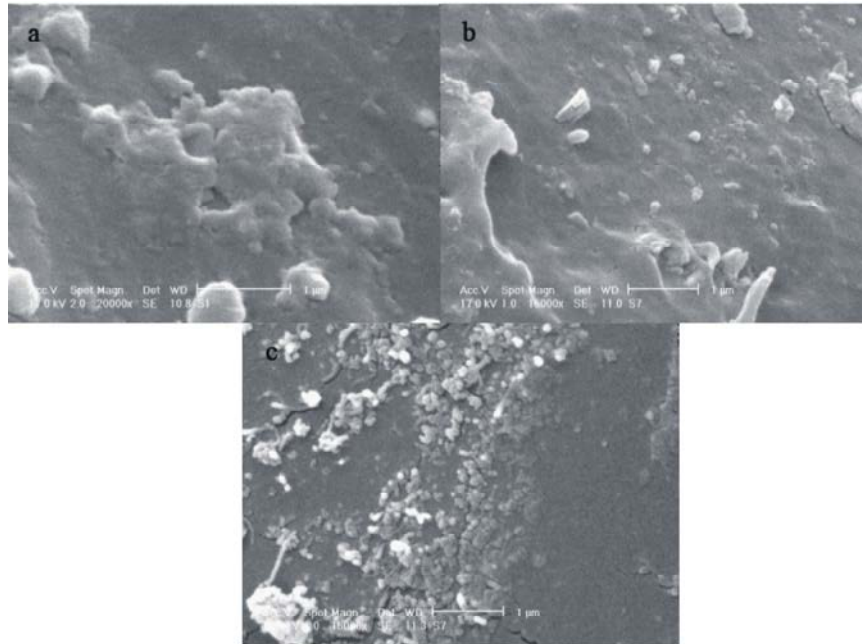


Fig. 3: SEM images of CS/CaP of different organic/ inorganic ratio: (a) CCP 46; (b) CCP 55; (c) CCP 64

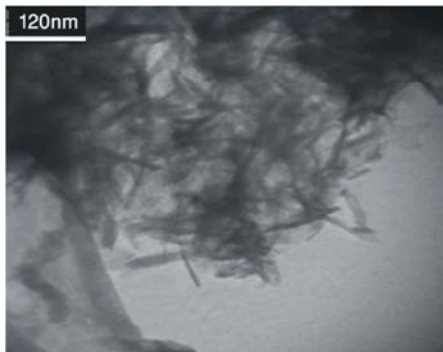


Fig. 4: Transmission Electron Microscopy (TEM) micrographs of chitosan/calcium phosphate composites

interface between the inorganic and organic phases was indistinguishable. These figures proved that the inorganic particles formed in CS/CaP composites were agglomerated more in smaller amount polymer and the dispersion was less. Moreover, SEM result of CS/CaP and composites demonstrated particle size of inorganic crystal in composites was about nanoscale and suggest that formation of calcium phosphate in polymeric matrix was controlled by density polymer network. The greater amount of polymer is used, the smaller crystal of inorganic phase become. That may be due to increasing interaction between polymer and calcium phosphate crystals which can limit the growth of calcium phosphate crystals within the matrix.

Figure 4 shows the TEM images of CS/CaP composite that revealed some interesting information. For example, calcium phosphate particles in the composites prepared by the in-situ precipitation method displayed crystalline morphology of rod like with a typical size of nanoscale. It can be seen that calcium phosphate crystals in composites have a good dispersive property and display a short rod crystals with an average size of about 100 nm in length and 20 nm in diameter which proved SEM results.

## CONCLUSION

The present consideration offers a simple approach for the preparation of a family of novel biocomposite materials for tissue regeneration. The chitosan/calcium phosphate composites composed of different weight ratios of organic/inorganic phase. IR, XRD, SEM and, TEM were applied to study the properties of composite. The results of XRD indicated that the formed particles into the polymeric phase are hydroxyapatite, Brushite and  $\beta$ -tricalciumphosphate. FT-IR confirmed that the chemical interactions between the inorganic and organic constituents in the composite, probably take place via the chemical bonding between  $\text{Ca}^{2+}$  and the amino group or the amide bands of chitosan. SEM result suggested that the amount of polymer greatly influenced the nucleation and the development of calcium phosphate crystalline. In

addition, TEM observation proved that particles formed in organic matrix in nano size for chitosan/calcium phosphat composites.

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