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Formation of calcium deficient HAp/collagen composites by hydrolysis of α-TCP

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Abstract Bone-like composites containing calcium deficient hydroxyapatite (CDHAp) were formed by the hydrolysis of alpha-tricalcium phosphate (α-TCP) in the presence of type I collagen. CDHAp-collagen composites were synthesized using two techniques. In one technique α-TCP was mixed with non-milled (as-received) collagen prior to the addition of the aqueous solution. In the second, the collagen was milled with α-TCP in heptane at room temperature prior to its conversion to CDHAp. The effect of milling strongly facilitates the formation of CDHAp at physiological temperature. The proportion of milled collagen between 5 and 20 wt% present in the α-TCP/collagen composites has no significant effect on the rate of CDHAp formation. Variations in pH and in calcium and phosphate concentrations were determined as a function of collagen processing and variations specific to the presence of collagen were discerned. Compared to CDHAp or to composites containing non-milled collagen, diametrical and compressive strengths of CDHAp increased in the presence of milled collagen. Lack of collagen dispersion and incomplete formation of CDHAp during 48 h were the bases for reduced strengths of composites containing non-milled collagen.

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1 Introduction

Formation of synthetic hydroxyapatite (HAp) at physiological temperature from calcium phosphate precursors is significant in biomedical applications. Calcium phosphate bone cements based on HAp formation from α-TCP have attracted continuous attention since the 1980s [1]. Compared to the β polymorph (β-TCP), α-TCP does not exhibit a true solubility and is therefore expected to be more reactive. It is also recognized that resorbable bioceramics containing α-TCP exhibit higher resorption rates than those constituted to contain HAp [2]. Hydrolysis of α-TCP to calcium deficient HAp (CDHAp) between 60 and 100°C was described by Monma and Kanazawa [3]. More recently, the mechanisms of conversion at physiological temperature were investigated [4, 5]. Prior work showed that α-TCP slurries can hydrolyze to HAp within 15 h at physiological temperature [6]. In order to achieve rapid hydrolysis, priority has been given to the preparation of α-TCP in a pure state and with a small particle size. Preparing α-TCP by different methods has shown that its reactivity depends strongly on the origin of the precursors, impurity levels, and thermal history. Durucan et al. [7] extensively studied different procedures of preparation, reaction kinetics and properties of α-TCP. During α-TCP hydration to produce CDHAp, the pH values changed from 9 to 6, consequently, α-TCP would not be expected to have a toxic effect on the human tissue when it is implanted as a bone substitute. Although employing α-TCP may offer the significant advantage of direct hydrolysis to CDHAp, its rate of reaction may be slow. Consequently, there is interest in identifying means to accelerate its reaction. One such means is to produce composites which provide templating surfaces on which HAp can nucleate.

It is well known that HAp and related calcium phosphates are brittle and their mechanical properties do not match those of natural bone tissue. This limits the clinical applications of calcium phosphate cements. As a consequence, it is desirable to synthesize materials having mechanical properties comparable to those of native tissue. Combining a mineral constituent with a polymer to produce a composite is one method for obtaining mechanical properties more closely related to those of the bone. The presence of a polymer is anticipated to improve the toughness of a composite.

Collagen is an abundant protein in body tissue and has been developed as material for use in biomedical applications [8–10]. In 1977 Mittelmeier proposed the concept of dispersing small particles of hydroxyapatite in a purified, non-immunogenic and lyophilized collagen sponge. This collagen sponge is obtained from porcine skin and has been used successfully as a hemostatic agent for many years [11]. HAp-collagen composites may show great promise in clinical applications because of their compositional and structural similarity to natural bone [12–14]). For this reason many techniques have been used to prepare HAp/collagen composites. Rhee et al. [15, 16] and Lickorish et al. [17] prepared HAp/collagen composites using a soaking technique. A collagen membrane was soaked in supersaturated simulated body fluid (1.5 SBF) solutions for 4 weeks and HAp crystals were formed on the surface of the collagen membrane. Kikuchi et al. [18] and Sena et al. [19] used the simultaneous titration method for the preparation of HAp/collagen composite. In this method the HAp precipitated when calcium hydroxide mixed with phosphoric acid in presence of collagen. Tenhusein et al. prepared HAp/collagen composite using an acid–base reaction using tetracalcium and dicalcium phosphate as precursors [20, 21].

Many studies have demonstrated the ability of collagen to nucleate and grow HAp crystals [22–24]. This ability is attributed to the presence of the anionic groups (carboxylic groups) of the collagen fibers [25–27]. Koutsoukos et al. [28] reported that a poly-nucleation mechanism is the basis for the growth of hydroxyapatite on the collagen surfaces. Zhang et al. [29] used Fourier transform infrared spectroscopy to investigate the nucleation sites of calcium phosphate crystals during collagen mineralization. It was found that there are two types of sites on collagen surfaces where nucleation preferentially occurs, the carbonyl groups and carboxylic groups. Infrared spectra indicated that the calcium ions bond to carboxylic groups on the collagen surfaces. The structure of the collagen/HAp composite was also investigated by Zhang et al. [30]. HAp crystals grew on the fibril surfaces in such a way that their C-axes were oriented along the longitudinal axes of the fibrils. The mineralized collagen fibrils aligned parallel to each other to form mineralized collagen fibers. Sugihara et al. [31] appear to have been the first to investigate collagen/α-TCP as a type of bioactive bone cement.

It is well understood that intimacy of mixing can be important in the expeditious formation of HAp and its composites by cement-type reactions. If the objective is to confer bioactivity, it is desirable to form a calcium deficient HAp. While this can be achieved using a variety of precursors, the simplest is α-TCP because it hydrolyzes directly to CDHAp. This attribute, coupled with the fact that the influence of collagen on α-TCP hydrolysis seems not to have been systematically investigated, motivated the present study.

It had been reported that ball milling may increase the active surface area of collagen by more than 200 times without chemically altering or denaturing it [32]. Enhancing the interaction between the collagen and HAp was anticipated to provide a mechanism for improving the mechanical properties of the composites. Thus, our goal was to investigate the effect of the comminution of collagen with α-TCP to evaluate the effects of milled vs. non-milled collagen on the kinetics, pH variations, microstructure and mechanical properties of the CDHAp composites formed.

2 Experimental approach

2.1 Preparation of α-TCP

α-TCP was prepared as previously described [33] by a solid-state reaction between reagent grade calcium carbonate ($CaCO_3$) (Osram Sylvania, Towanda, PA) and thermally synthesized pyrophosphate ($Ca_2P_2O_7$). $CaCO_3$ was fired at 1,010°C for 2 h to produce calcium oxide that was then hydrolyzed to calcium hydroxide. This calcium hydroxide was mixed with phosphoric acid (Fisher Inc.) at a molar ratio of 1:1 with stirring at 65°C and at a pH of 3.4 ± 0.2 for 1 h to produce monetite ($CaHPO_4$). Monetite was filtered, dried in an oven at 100°C overnight. Monetite was fired at 500°C for 2 h to produce pyrophosphate. The pyrophosphate was mixed and milled with calcium carbonate overnight then ball milled at room temperature in heptane. The slurry of pyrophosphate and calcium carbonate was filtered and air-dried for 24 h in a vacuum. The dry physical mixture of pyrophosphate and calcium carbonate was fired at 1,180°C for 1.5 h to produce α-TCP. α-TCP was directly air quenched to avoid formation of the *β*-polymorph. The α-TCP was ground using a mortar and pestle and milled in a ball mill for 16 h in heptane. α-TCP slurry was filtered and dried at room temperature over night, and re-milled in an attrition mill for 8 h to obtain an average particle size of 3–4 μm. Formation of α-TCP was confirmed by XRD.

CDHAp formation occurred by hydrolysis of α-TCP in deionized water at the required temperature according to the following reaction:

$$3\alpha - Ca_3(PO_4)_2 + H_2O \rightarrow Ca_9HPO_4(PO_4)_5(OH)$$

X-ray diffraction analysis was used to confirm the purity and formation of CDHAp after hydrolysis. Tendon insoluble collagen, type I, (Sigma–Aldrich, Inc.) was used in the as-received condition without any treatment.

2.2 Composite formation

Two techniques were used to prepare α-TCP/collagen composites containing 5 and 20 wt% collagen. The first was the direct mixing of the as-received collagen with α-TCP using a mortar and pestle. The physical mixture was reacted at 37°C to produce CDHAp. The second technique was the mixing and milling of the collagen (Type I) and α-TCP in heptane for 16 h in a ball mill at room temperature. The slurry of collagen/α-TCP was filtered and dried under vacuum at room temperature for 48 h.

2.3 Isothermal calorimetry

The rates of CDHAp formation by the hydrolysis of α-TCP in the presence and absence of milled and non-milled collagen were established at 37°C by isothermal calorimetry. Each pre-composites containing collagen and α-TCP was weighed to attain a sample mass of 1.5 g, and placed in a copper calorimeter cup. The calorimeter cup was sealed with plastic wrap, and a placed in calorimeter chamber. A syringe containing an equivalent mass of de-ionized water was placed in the upper chamber of the calorimeter. The calorimeter cup and syringe were equilibrated for at least 30 min. After thermal equilibrium was established, reaction was initiated by injecting the de-ionized water into the calorimeter cup. The temperature was controlled using a water bath connected to the calorimeter chamber. The thermopiles surrounding the calorimeter cup respond to the changes of heat liberated from the reaction of the α-TCP. The thermopiles convert the heat evolved to a voltage output that was processed using a data acquisition card (PCMCIA, model NI4350, National instruments, Inc, TX) and stored on a computer. The data were collected using the LAB VIEW software. The output data were plotted as rates of reaction verses time. The unit of the rate data is watt/s. The accumulated heats of reaction were calculated by integrating the rate data. The accumulated heat curves are presented in kJ/mole of calcium deficient hydroxyapatite (CDHAp). Formation and purity of CDHAp produced in these experiments was confirmed by XRD.

2.4 Solution chemistry

Variations in pH during the α-TCP hydrolysis in the presence of collagen at physiological temperatures were monitored using a glass electrode connected to pH meter (Orion 940, Thermal Electron Corporation, MA). The pH measurements were carried out at 37°C by connecting the system to a controlled temperature water bath. 200 ml of deionized water was thermally equilibrated before adding 2 g of α-TCP/20 wt% collagen. Nitrogen gas was bubbled through the solution throughout the experiments to minimize the presence of the carbon dioxide. A datum point was collected every 30 s using a computer equipped with Orion software. After pH changes appeared to reach completion, the slurry was filtered and rinsed with acetone to stop any extraneous reactions and the precipitate was air-dried for 24 h. The formation of calcium deficient hydroxyapatite was confirmed using X-ray diffraction.

The calcium and phosphate concentrations were determined during the formation of CDHAp at physiological temperature for 24 h in the presence and absence of collagen as a function of time. At each measurement, 20 ml of the slurry were drawn by a syringe, and filtered. The filtrate was analyzed as follows: Phosphate was determined by ion chromatography using a Dionex Model 2010i. Calcium was determined by flame atomic absorption spectroscopy using a Perkin-Elmer Model 5100ZL. All standards were prepared by serial dilution of NIST traceable stock solutions. All samples were diluted with ASTM Type I deionized water.

2.5 Composite characterization

Formation of CDHAp in the composites produced by the reaction of α-TCP in the presence of 20 wt% of milled or as-received collagen at different temperatures was characterized by XRD. Other compounds, if present, were also determined using X-ray diffraction. XRD analysis was performed using a Scintag automated diffractometer (Scintag, 2) with scan rate 4°/min, over 2θ range of 20°–40°. Scanning electron microscopy was performed using a Hitachi S-3000H SEM. Fracture surfaces were mounted on conducting carbon tape, coated with gold and visualized using an accelerating voltage of 20 keV.

2.6 Mechanical measurements

The composites were subjected to two mechanical tests: diametrical and compressive. α-TCP/20 wt% milled or nonmilled collagen was mixed with water at room temperature in a mortar to produce a paste. The ratio of powder-to-liquid is 3:1. The sample paste was then introduced into Teflon molds and allowed to set to harden in an

incubator that has controlled temperature and 100% relative humidity for 48 h.

Tensile strengths of the composites were established using a diametrical compression test (Brazilian test) [34, 35]. The dimensions of the composite samples prepared for tensile strength were 6.35 mm diameter and 6.35 mm thickness. The prepared composite sample dimensions for compressive test were 6.35 mm diameter and 12.7 mm thickness. The specimens were tested using an automated Instron machine with 1 KN load cell and the cross-head speed was set at 0.3 mm/min. The force required to attain failure was collected using Lab view software. The failure load of the specimen characterizes the tensile and compressive strength for the tension and compressive tests, respectively, of the composite. The tensile strength was calculated according to Eq. 1:

$$(\sigma_t) = 2P/\pi DT \quad (1)$$

where σ_t the tensile strength at failure, P is the load required for failure, D is the diameter of the specimen and T is the thickness of the specimen. The compressive strength was calculated according to Eq. 2:

$$(\sigma_p) = P/\pi r^2 \quad (2)$$

where σ_p is the compressive strength at failure, P is the load required for failure and πr^2 is the area of the sample.

3 Results and discussion

3.1 Reaction kinetics at physiological temperature

Two preparative routes were used to produce CDHAp-collagen composites. The first route is the dry mixing of collagen and α-TCP without milling. The second route is the milling of collagen with α-TCP in ball mill in heptane at room temperature for 16 h before hydration. The reasons for using the milling process were to enhance the homogeneity of the collagen and α-TCP in the mixture, to decrease the particle size of the collagen fibers and to provide a basis for comparing between the effects of the two routes on the kinetics, structure and mechanical properties of CDHAp.

Calorimetric rate curves for the formation of CDHAp by the hydrolysis of α-TCP at physiological temperature in the presence of milled or non-milled collagen are shown in Fig. 1. The curves were normalized according to the weight of α-TCP. Three calorimetric peaks occur in all curves, showing wetting (A), nucleation (B), and growth (C) of HAp. The intensity of the wetting peak is higher in the presence of collagen than in the absence of collagen regardless of whether the collagen is milled or non-milled. Non-milled collagen was found to induce the nucleation of

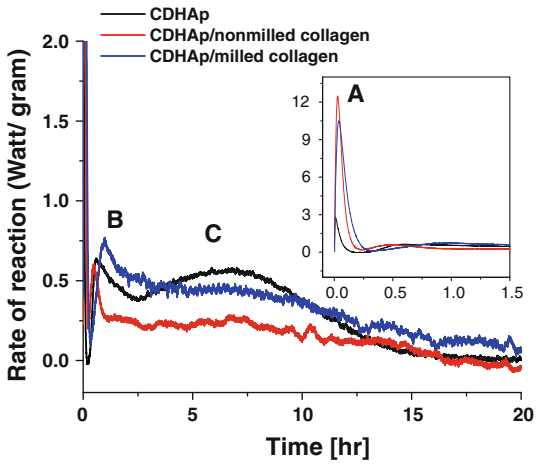

Fig. 1 Rate of heat evolution curves during the formation of CDHAp by hydrolysis of α-TCP at 37°C depending on the presence of collagen and the manner of its processing: The three peaks are (*a*) wetting, (*b*) nucleation and (*c*) growth

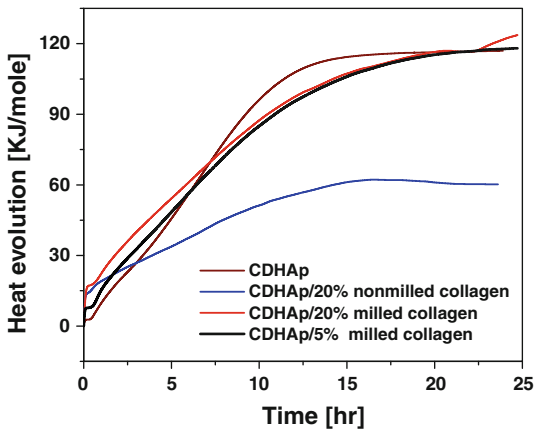

Fig. 2 Heat evolution curves during the formation CDHAp by α-TCP hydrolysis at 37°C depending on the presence of collagen content and the manner of its processing

CDHAp more effectively than the milled collagen. The peak for CDHAp nucleating in the presence of non-milled collagen reached to a maximum value after ~30 min while the peak for CDHAp nucleating in the presence of milled collagen reaches its maximum after 1 h. Milling of collagen has a strong influence on the growth of CDHAp. The intensity of the CDHAp growth peak in the presence of milled collagen is higher than that of CDHAp formed in presence of non-milled collagen indicating more extensive conversion. These data indicate that milled collagen provides more nucleation sites. Consequently competition for calcium and phosphate from α-TCP dissolution requires longer for the nuclei to reach critical size. However, once critical size is reached, the extent of α-TCP conversion via the growth of CDHAp crystallites is more rapid.

The effect of collagen processing on the total heat evolved of CDHAp formed by α-TCP hydrolysis at

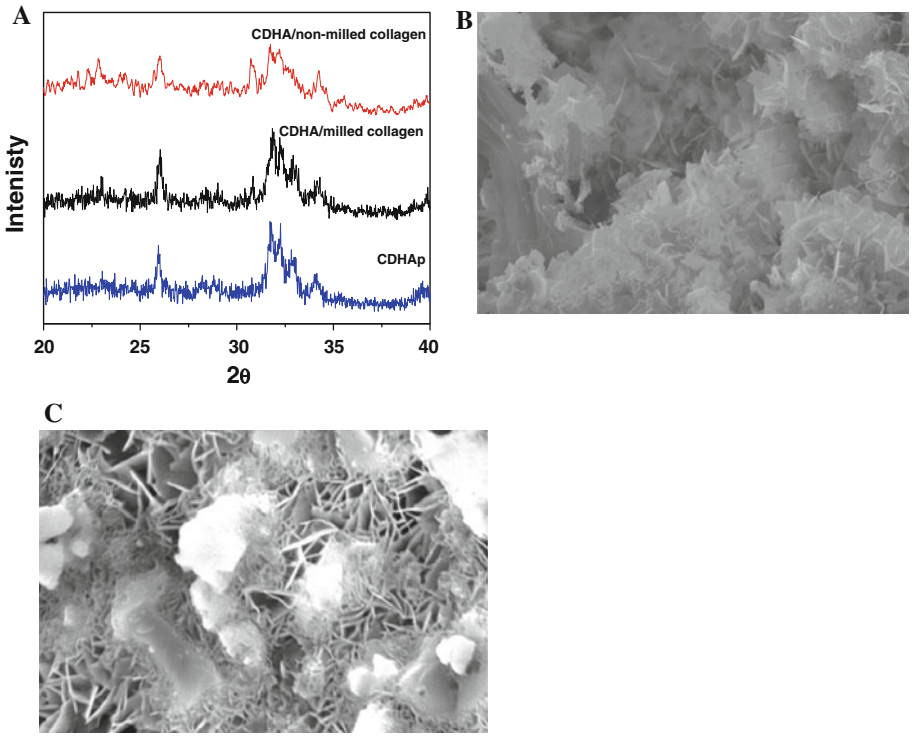

Fig. 3 X-ray diffraction analysis of CDHAp formed from the hydration of α-TCP at 37°C as a function of collagen (**a**). Microstructure of CDHAp formed in the presence of **b** milled collagen and **c** non-milled collagen

physiological temperature is shown in Fig. 2. The total evolved heat during CDHAp formation is not strongly affected by the presence of milled collagen. Figure 2 shows that the total heat evolved during the formation of the composite containing milled collagen is approximately similar to that of CDHAp formed in the absence of collagen. Heat evolved in 24 h in the presence of non-milled collagen is approximately half of this value. X-ray diffraction and scanning electron microscopy revealed that big portion of the α-TCP remained unreacted after 24 h of hydrolysis in the composite containing non-milled collagen (Fig. 3). Reaction of α-TCP was substantially complete in the composite containing milled collagen by 24 h of hydrolysis at physiological temperature.

The effects of the proportion of milled collagen on the total evolved heat at physiological temperature were also evaluated by hydrolyzing α-TCP with 5 and 20 wt% milled collagen. There is no significant difference in the value of the total heat evolved at 37°C depending on the proportions of milled collagen.

3.2 Variations in solution chemistry

The effects of the presence 20 wt% of milled or non-milled collagen on the pH variations during the hydrolysis reaction of α-TCP for 24 h at physiological temperature are shown in Fig. 4. Rapid increases in pH occur immediately after mixing regardless of the presence or absence of collagen. In the presence of collagen, the pH values slowly descend from about 8.7; in the absence of collagen the pH values are consistently higher and slowly descend from a maximum value of 9.1. Collagen milling has no significant effect on pH at the onset of the hydrolysis reaction and the pH behavior during the first 8 h in the presence of milled and non-milled collagen is similar. However, after 8 h a decrease the pH of the solution in contact with milled collagen occurs until it reached a value near 6 after 24 h. A similar trend occurs when TCP hydrolyzes in the absence of collagen. The pH of the solution in contact with non-milled collagen does not undergo such an accelerated decrease within 24 h but descend to a value near 8. These results also indicate that dispersing small fibers of the milled collagen facilitates the formation CDHAp.

It has been reported that, the initial period during which the pH variations were very slow is associated with the dissolution of α-TCP and the nucleation of CDHAp crystallites while a decrease in pH was related to further conversion to CDHAp resulting in the uptake of OH ions from the solution [7]. Based on the data shown in Fig. 4 the presence of milled collagen induces a faster dissolution of α-TCP and enhances more rapid conversion to CDHAp than does non-milled collagen.

In the calorimetric experiments the liquid-to-powder ratio was 1:1, while in the pH experiments the ratio was 200:1. The similarity between calorimetric results and pH variations indicates that the liquid-to-solid weight ratio does not greatly affect the kinetics of CDHAp formation at

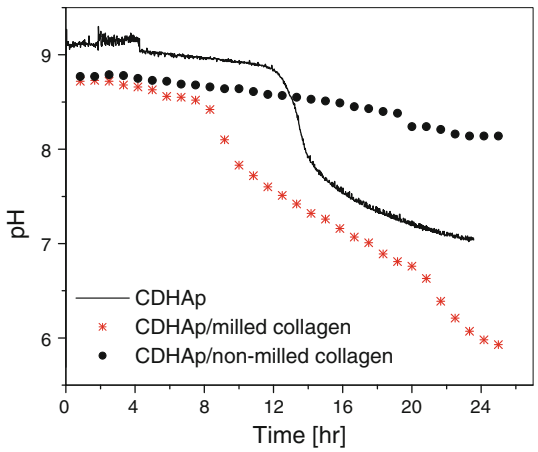

Fig. 4 pH variations during CDHAp formation from α-TCP at 37°C depending on the presence of 20 wt% milled or non-milled collagen: CDHAp, CDHAp/milled collagen and CDHAp/non-milled collagen

physiological temperature, provided water can contact the TCP surfaces.

Figure 5a–c shows the variations in calcium and phosphate concentrations during hydrolysis of α-TCP at 37°C for 24 h in the presence and absence of collagen. While the presence of collagen reduces the pH during the first 8 h of hydrolysis, it elevates the calcium and phosphate concentrations. In the absence of collagen, the variations in [Ca] and [P_i] occur in two stages. During the first 4 h of reaction, they increase showing distinct maxima before subsequently decreasing. However, in the presence of the collagen the variations in [Ca] and [P_i] are more complex. Regardless of collagen milling [Ca] and [P_i] slowly decease after the concentration spikes associated with initial dissolution, show local minima after several hours, increase again to local maxima, and then decrease. These trends are most apparent in the presence of non-milled collagen in that the data points show less scatter. Generally this behavior is consistent with initial dissolution, [Ca] and [P_i], mild depletion due to CDHAp nucleation, followed by greater reductions in ionic strength during growth of CDHAp. The persistently higher [Ca] and [P_i] values in the presence of milled collagen accord with the more rapid dissolution of α-TCP in the more well-dispersed system.

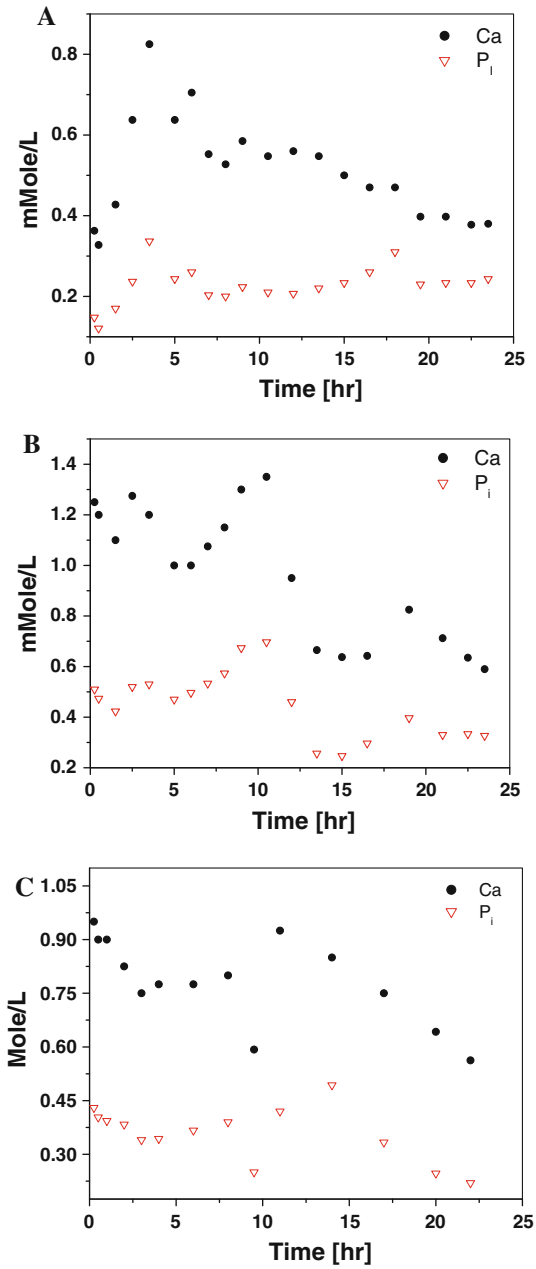

Fig. 5 Variations in calcium and phosphate concentrations during CDHAp formation at 37°C in the presence or absence of 20 wt% collagen: **a** CDHAp, **b** CDHAp/milled collagen, and **c** CDHAp/non-milled collagen

3.3 Mechanical properties

The effect of collagen on the diametrical tensile strengths, σ_t, of the composites after aging for 48 h at physiological temperature is shown in Fig. 6. The average value of the tensile strength for CDHAp formed in the absence of collagen is 2.5 ± 0.5 MPa. This is consistent with a previously reported value of 3 MPa [36]. Non-milled collagen was found to decrease the strength of the composites to an average tensile strength value of 1.0 ± 0.2 MPa. The tensile strength of the composite formed in the presence of non-milled collagen was reduced by the agglomeration of collagen fibers and by the incomplete reaction of α-TCP. Agglomeration of collagen fibers affects the extent of the movement of deionized water, thus affecting the hydration of α-TCP. X-ray diffraction analysis and microstructural examination revealed that un-reacted α-TCP was still remained there after 48 h of the hydration reaction. The presence of un-reacted α-TCP will be a major factor decreasing composite tensile strength. The average strength

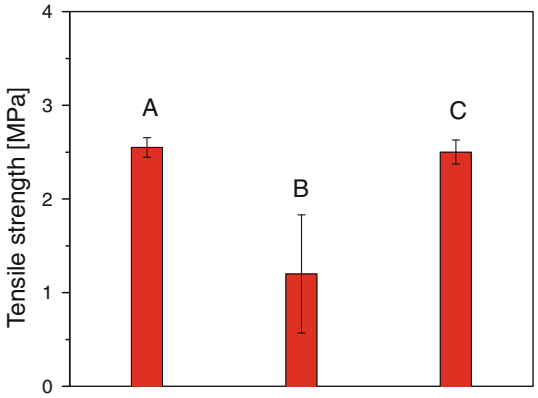

Fig. 6 Strengths as determined by diametrical compression of CDHAp formed at 37°C (A) from α-TCP, (B) CDHAp formed from α-TCP in the presence of 20 wt% milled collagen, and (C) CDHAp formed from α-TCP in the presence of 20 wt% non-milled collagen

of composites containing milled collagen is equivalent to that in the absence of collagen.

Figure 7 shows the compressive strength values of CDHAp and composites formed in the presence of milled and non-milled collagen at physiological temperature for 48 h. The average value of the compressive strength of the CDHAp formed in the absence of collagen was 8 ± 0.2 MPa. The average value for the compressive strength of the composites containing the non-milled collagen was 4 ± 0.2 MPa.

The factors responsible for the low value of the compressive strength are the same as for the low tensile strength. It has been reported that compressive strengths of hydroxyapatite tripled when 3% of apatite seeds were added to the calcium phosphate precursors [37]. Seeding promotes CDHAp formation and improves strength by providing homogeneously distributed nucleation sites. Figure 7 shows the average value of the compressive strength of composites of CDHAp and non-milled collagen

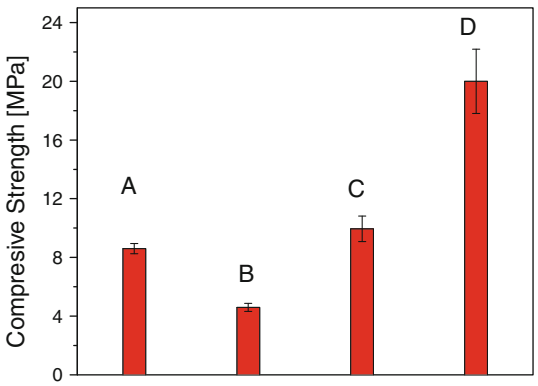

Fig. 7 Compressive strengths of CDHAp formed at 37°C (A) by the hydrolysis of α-TCP, (B) in the presence of 20 wt% non-milled collagen, (C) in the presence of 20 wt% non milled collagen +5% apatite seed, and (D) in the presence of 20 wt% milled collagen

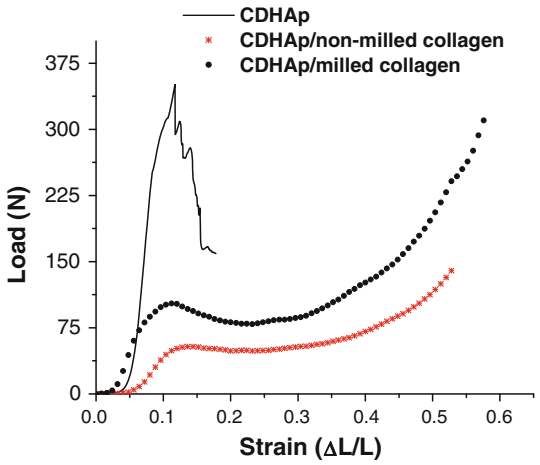

Fig. 8 Stress-strain behavior of CDHAp formed at 37°C by the hydrolysis of α-TCP, CDHAp formed in the presence of 20 wt% non-milled collagen, and CDHAp formed in the presence of 20 wt% milled collagen

increased to about 10 MPa when 5 wt% of apatite seeds, produced by hydrolysis of α-TCP, were present.

Composites constituted using milled collagen had an average compressive strength of 20 ± 0.5 MPa. These results indicate that the improvement in the compressive strength is due to improved dispersion of the milled collagen, the increase in homogeneity between the collagen and α-TCP and the increase of the activity of the surface area of the collagen fibers as a result of the milling process. Thus, the effect of milling collagen improved the compressive strength of seeded CDHAp/non-milled collagen composites by a factor of 2.

The dependence of the work of fracture of the CDHAp formed in the absence and presence of collagen is shown in Fig. 8 which shows the behavior of CDHAp and the composites determined at a constant rate of strain. The work of fracture values were obtained by integration of the areas under the stress-stain curves. CDHAp formed in the absence of collagen shows little strain capacity and has a value of work of fracture been 28.15 N. In the presence of non-milled collagen the value of work of fracture was 30.5 N; the corresponding value in the presence of milled collagen was 67.7 N. These values indicate the importance of composite behavior in improving toughness while also demonstrating the importance of the distribution of the collagen.

4 Conclusions

The formation of CDHAp by the hydration of α-TCP in the presence of collagen was investigated. CDHAp-collagen composites were synthesized either by direct mixing of α-TCP with collagen or milling of collagen with α-TCP

before hydration. The calorimetric rate curves show that the intensity of the wetting peak of CDHAp is higher in the presence of collagen. Heat evolution curves show the milled collagen has no significant effect on the total heat of CDHAp, whereas the total evolved heat of CDHAp formed in the presence of non-milled collagen is approximately half of the value for CDHAp formed alone. Although non-milled collagen was found to enhance the nucleation of CDHAp, X-ray diffraction and scanning electron microscope revealed that about 40% of un-reacted α-TCP remained after 24 h in the composite containing non-milled collagen.

The proportion of milled collagen has no significant effect on the rate of CDHAp formation at physiological temperature, pH, and calcium and phosphate concentration decreased faster in the presence of milled collagen than in the presence of non-milled collagen indicating that the dispersion of smaller fibers facilitated the formation of CDHAp.

The presence of milled collagen has no significant effect on the diametrical tensile strengths of CDHAp while the presence of non-milled collagen reduced tensile strength. The average compressive strengths of CDHAp formed in the presence of milled collagen were 20 MPa while the corresponding values of CDHAp formed in the presence of non-milled collagen was 4 MPa.

The dispersion of small fibers of milled collagen in the composites appears to be a significant factor in improving the mechanical properties of CDHAp-collagen composites.

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