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# Calcium concentration dependent collagen mineralization

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ABSTRACT

Mineralization of collagen fibrils is a regular combination process of organic and mineral components mainly involving calcium, phosphate and collagen. We report the influence of calcium to the self-assembly of collagen by changing the concentration of calcium ion in the process of mineralization. Low concentration of calcium results in the well collagen self-assembly while poor mineral crystallization. Relatively, high concentration of calcium can hinder collagen self-assembly, whereas it is benefited to mineral crystallization. We also reveal that collagen self-assembly happens in advance of the formation of better mineral crystals. These results interpret the mechanism of collagen mineralization further.

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## 1. Introduction

Changes of extracellular calcium ion concentration modulate diverse biological activities, such as secretion, neurotransmission, and muscle contraction [1–4]. Recently, some studies also show that fluctuation in extracellular free ionized calcium accompanies bone formation and remodeling [5–7]. At cellular and molecular levels, different calcium ion levels affect bone cells bioactivities such as the formation of osteoclast-like cells, osteoblastic proliferation and type I collagen synthesis [1,5,8]. Moreover, many researches are reported that the elevated extracellular calcium ion levels induce matrix mineralization of smooth muscle cells *in vitro*, which indicate that extracellular calcium plays an important role in the process of biomineralization [6,9,10].

Bone is the major biomineralized tissue in human body, which contains 60–70% (w/w) of calcium phosphate minerals, 20–30% (w/w) of organic matrix and 10% (w/w) of water [11]. Biomineralization is an important process for bone formation and remodeling to have a hierarchically ordered organization, comprised of organic and mineral components [12–15]. Moreover, proper mineralization is benefit to develop well-structured artificial bone materials. Mineralized collagen fibril is an essential building block of bone tissue with unique structural property [16]. It was inferred that collagen fibril could be the template to chelate calcium ion and serve as nucleation site for calcium phosphate [12,17]. Calcium and phosphate were mineralized initially in hole zones of collagen fibril [18,19]. Noncollagenous matrix macromolecules contributed to mineralization by stabilizing amorphous calcium phosphate (ACP) and initiating nucleation and hierarchical assembly of apatite within the collagen scaffold [20]. There were many researches aiming to explore how different factors influence collagen self-assembly, growth of calcium phosphate crystals and their interaction [21–24]. Since extracellular free ionized calcium is a crucial factor, it was broadly investigated [20,25–28]. Some researchers used low calcium ion concentrations in their experiments. For example, Liu et al. [20] floated carbon-coated grids containing cross-linked collagen over polyacrylic acid-containing simulated body fluid (SBF) with 2.5 mM $Ca^{2+}$. Deshpande et al. [27] also placed collagen fibril in mineralization solution with 1.67 mM $Ca^{2+}$. Other researchers used high calcium ion concentrations in their experiments. For example, Zhang et al. [25] and Zhai et al. [26] prepared mineralization solution with over 10 mM $Ca^{2+}$. Despite all these progresses, effects of various calcium concentrations towards the mineralized hierarchical structure of collagen is still not systematically investigated [29]. Researching the influence of its concentrations on collagen mineralization is benefited not only to getting in-depth knowledge of interaction between calcium phosphate crystals and collagen but also developing the artificial bone substitute with fine properties.

Many researchers reported the process of collagen mineralization *in vivo* follows that collagen fibril was formed by self-assembly of collagen triple helices, and then calcium phosphate crystals were formed initially in the gap zones between collagens [30]. The crystals then grew along the surface of collagen fibril, which resulted in a cross-striation periodicity of nearly 60–70 nm of each mineralized collagen fibril [12,15,31]. Scientists attempted to mimic the collagen mineralization process *in vitro* in order to achieve a better understanding of the hierarchical

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structure in naturally occurring tissues in which the major organic matrix is collagen [32,33]. Without the complicated *in vivo* biological environment the mineralization can be regulated easily and the interaction between collagen and calcium become more evident *in vitro*. Here, the influence of calcium on collagen self-assembly is investigated and the results present that increasing the concentration of calcium can hinder collagen self-assembly whereas it has positive effect on mineral crystallization. These finding is benefit to the future development of mineralized collagen composites as bone substitute materials.

## 2. Experimental methods

### 2.1. Collagen mineralization

Concentration of 4.01 mg/mL acid soluble collagen type I (Rat Tail, BD Biocoat) was used as the source of collagen. It was diluted in a certain quantity of double distilled water and mixed with 0.1 M $Ca(NO_3)_2$ and 0.1 M $(NH_4)_2HPO_4$ to keep *Ca*: P in the mole ratio of 1.67 and the collagen concentration in 0.6 mg/mL. There were 3 experimental groups which had 3 different calcium ion concentrations through adding different volume of 0.1 M $Ca(NO_3)_2$ and 0.1 M $(NH_4)_2HPO_4$ while keeping the same mole ratio of calcium to phosphorus. Specifically, group 1 to 3 had 1, 7, and 14 mM calcium, respectively. The control sample had the same component with experimental groups except calcium and followed the same experimental steps with others. The pH value was adjusted to 7.0 by addition of 0.1 M NaOH solution primarily and 0.1 M ammonium hydroxide was used to adjust the pH slightly. The mixture was kept in 25 °C for a period of times (0.5, 1, 2, 3, 6, 9 h, respectively) [34–37]. At the designated time points, the mixture was centrifuged (1500 rpm, 1 min) and washed 3 times with double distilled water. At last, the homogenously mineralized collagen fibrils were collected by centrifugation as a wax-like, colorless material. The samples were freeze dried for the following characterizations.

### 2.2. Fourier transform-infrared spectroscopy (FTIR)

The chemical compositions and crystalline phases of the mineralized collagen composites were investigated by FTIR. The samples were ground with 1% KBr in an agate mortar, compressed to tablets and analyzed under nitrogen atmosphere from 4000 to 400 $cm^{-1}$ using a Nicolet 6700 FTIR (USA).

### 2.3. X-ray diffraction (XRD)

XRD analysis of the mineralized collagen composites was performed on Rigaku D/Max X-ray diffractometer (Japan) with Cu Kα radiation source (wavelength = 1.54 Å). The supplied voltage and current were set to 40 kV and 120 mA, respectively. The samples were exposed at a scan rate of 10°/min from 5 to 60°.

### 2.4. Scanning electron microscope (SEM)

The freeze dried mineralized collagen composites were stuck onto conducting tape and gold coated for 5 min. The samples were examined in a JSM-6460LV Field Emission SEM (Japan) using an accelerating voltage of 5 kV.

### 2.5. Atomic force microscope (AFM)

For topological assessment by AFM, the samples were prepared by deposition of a 200 μL droplet of the mixture solution onto mica sheet. The mica sheet was washed by $ddH_2O$ for 2–3 times and dried under room temperature for 12 h. The surface topography of the mineralized collagen composites was investigated *via* tapping mode by Tap300-G (40 N/m) to record error signal (deflection) images using Dimension Icon (USA).

### 2.6. Transmission electron microscopy (TEM)

The freeze-dried samples were cut into $1 \times 1 \times 1$ $cm^3$ bulk and embedded in Spurr resin at 65 °C for 18 h. Ultrathin sections were no more than 100 nm cut by diamond knife using LEICA UC7 (USA) and then transferred onto carbon coated copper grid. The samples were investigated by FEI F20 instrument (USA) operated at 80 kV for conventional TEM examination.

### 2.7. Differential thermogravimetry (DTG) and differential scanning calorimetry (DSC)

The thermal stability of the samples was assessed in the STA 449 C Jupiter Thermogravimetric analyzer (Germany) using aluminium pans, in nitrogen flow. The heating ramp rate was 10 °C/min from 30 to 800 °C. In the case of DSC testing, an empty aluminium pan was used as reference. Each group sample was carried out in triplicate, and all data were presented as the mean ± standard deviations (SD). From the DTG and DSC curves obtained by means of the Universal Analysis software, the following parameters, which describe the thermal behavior of these samples, were calculated: temperatures of thermal denaturation, the degradation onset temperature, final temperature, percentage of the corresponding mass loss. The ratio of collagen loss weight to residue content weight was the mass ratio of collagen and apatite.

## 3. Results and discussions

### 3.1. Collagen self-assembly and mineralization under various $Ca^{2+}$ concentrations

FTIR of the control group and mineralized collagen composites (1, 7 and 14 mM) between 4000 and 400 $cm^{-1}$ at 3, 6, and 9 h is shown in Fig. 1. Typical bands could be observed such as N—H stretching at 3310 $cm^{-1}$ for the amide A, C—H stretching at 3063 $cm^{-1}$ for the

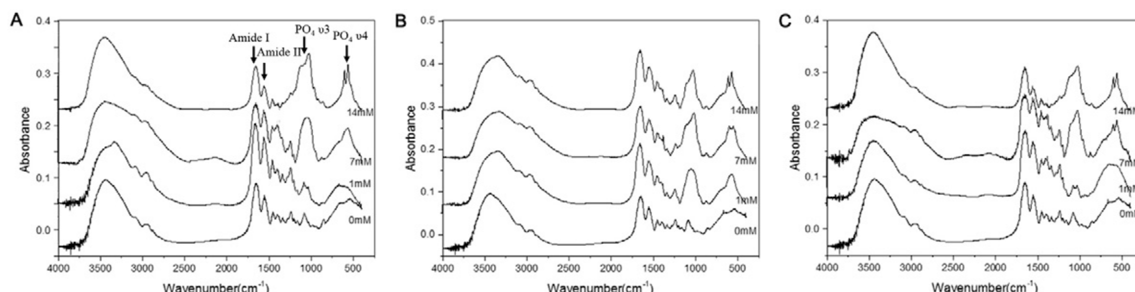


**Fig. 1.** FTIR of the control group and mineralized collagen composites with 1, 7 and 14 mM $Ca^{2+}$ at 3 (A), 6 (B) and 9 h (C).

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