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Morphology Development of HAp Crystallites in GEL Matrix

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ABSTRACT

The crystal morphology of hydroxyapatite [HAp] phase in gelatin [GEL] matrices was investigated with the condition of a GEL precursor treatment in an aqueous solution of H_3PO_4 at 37-80°C. Needle-shaped nanocomposite particles were prepared through a dynamic reaction during a coprecipitation process using a phosphoric GEL solution. Various types of mineralized morphology appeared with a phosphorylated condition of the GEL solution. HAp/GEL nanocomposite slurries showed the existence of an octacalcium phosphate [OCP] phase during the process.

Key words : Hydroxyapatite, Gelatin, Morphology, Mineralization

1. Introduction

Biomimetic synthesis of inorganic compounds such as calcium phosphate and apatite has received an increasing amount of attention over the last few years. The major goal is to understand the specific interactions and processes that enable nature to form such sophisticated objects as bone.[1,2] The formulation of such a composite would be especially advantageous for application as a bone substitute, perhaps incorporating knowledge of hydroxyapatite, one of the major constituents of hard tissue. Calcified tissue, such as bone and teeth, is considered a biologically and chemically bonded composite between HAp nanocrystals and type-I collagen.[1] Recently, Chang *et al.*[3-6] developed a HAp/COL nanocomposite through a coprecipitation reaction of HAp nanocrystals in soluble collagen. The characteristic feature of this process is a dynamic reaction using an active $Ca(OH)_2$ precursor[3] as a free Ca^{2+} source instead of $CaCl_2$ or $Ca(NO_3)_2$ in a double diffusion process.[7] Since 2001 the development of a HAp/GEL nanocomposite[8-10] has been researched using commercially available GEL materials rather than expensive type-I COL. GEL materials have been well studied over several decades,[11] but most of this research has been focused on the physico-chemical properties of GEL itself. Information on the subject of chemical reactions between GEL and calcium phosphate minerals is less well known, despite the fact that a large percentage of commercial GEL is produced from the calcified tissue such as cow bone or pigskin.

The development of apatite phase in GEL matrices is very complicated,[7-9] as commercial GEL contains variety of protein species and different stages of degraded products. In the development of a HAp/GEL nanocomposite, most of the interest is on the formation reaction of apatite nanocrystals in the GEL matrix and the chemical coordination between the formed apatite crystals and the GEL matrix. The morphology development of apatite phase in GEL matrices is reported with further degradation of GEL molecules.

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2. Materials and Methods

2.1. Sample Preparation and Characterization

The preparation details of the HAp/GEL nanocomposite were comprehensively described by Chang.[8] The precursors used in the present study were $CaCO_3$ (Alkaline analysis grade, Aldrich, USA), H_3PO_4 (AP grade, Aldrich, USA), and Gelatin (Unflavored, Canada). The preparation details of $Ca(OH)_2$ powders from $CaCO_3$ precursors has been reported elsewhere.[3] The reactivity of an aqueous solution of $Ca(OH)_2$ powders depends on the formed [Ca^{2+}] in H_2O. The solvated status of the Ca-OH complex in H_2O is presumably related to the precipitation of apatite phase and to the organic-inorganic interaction between the GEL and apatite nuclei. Normally, the reactivity of powders was reduced with days. The amount of $Ca(OH)_2$ and H_3PO_4 was calculated to make 10 g of HAp. The amount of GEL was typically set as 1 g while in some cases 3-5 g GEL batches were prepared. GEL powders were dissolved in an aqueous solution of H_3PO_4 and kept at 37°C for a designated amount of time before coprecipitation. The coprecipitation reactions were conducted between 37°C and 80°C. After the reaction, the obtained slurry in the solution was aged at 37°C for 24 h. A sample of the slurry was collected after aging and microstructures were characterized by transmission electron microscopy TEM (JEM-1210, JEOL, Japan).

2.2. Animal Test

A surgical operation was performed on a rat while the rat

was under anesthesia. When the rat was clearly sedated, the surgical area of its two back legs and its back, was shaved and disinfected with a betadine solution. After the rat was induced into deep sedation, the dorsa of both legs were shaved with a shaving machine and scrubbed with the betadine disinfectant solution. The surgeries were performed according to the surgical protocol, which had indicated a randomized selection for the experimental site and a control site on both legs. After the material was implanted into the femur the incision was sutured using chromic gut for muscle repair and ethilon nylon for skin repair.

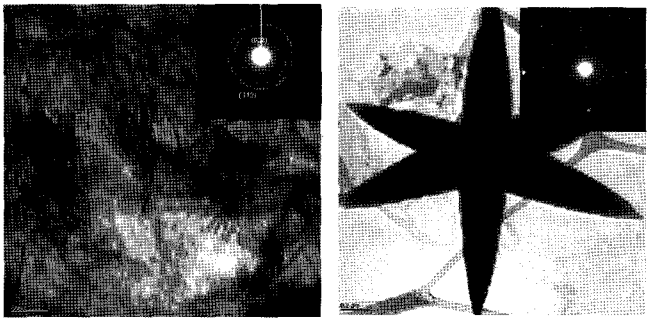
Euthanasia was performed after a designated time to observe the progress of the material. Ketamine (0.45 mL) was used for anesthesia and administered intraperitonealy. The rats were enthanized after 2, 4, 6, and 8 weeks. Once it was clear that the rat was sedated, Ketamine (0.5 mL) was used for euthanasia by injecting it pericardially. When the rat had died, the femurs were amputated and placed into labeled containers for further observations. Both femurs were taken out following disarticulation of the knee and hip joints. The gross findings on the experimental and control sites of the femurs were observed in order to determine whether the wound was stable or fractured. The specimens were immersed into a Formalin solution for tissue fixation.

3. Results and Discussion

3.1. TEM and ED Pattern

3.1.1. Needle-Shaped Particles

Versatile morphologies such as needle-shaped particles, rectangular-type and others were observed. Fig. 1 shows an example of the typical TEM morphology of the HAp nanocrystallites in GEL matrices prepared at 37°C. The needle-shaped particles were observed as often as in previous reports.[8-10] The crystals are several nm thick and HAp nanocrystals are preferentially aligned along the c-axis, from the direction of arc-like (002) diffraction spots. During the coprecipitation reaction at 37°C, the HAp/GEL composite showed the preferred orientation of the c-axis during HAp crystal development.



(a) scale 50 nm (b) scale 0.2 μm

Fig. 1. TEM morphology and ED pattern for the samples synthesized at 37°C (a) and 80°C (b). The ED pattern for HG1-37 shows the preferred orientation of the HAp crystals along the c-axis of the GEL molecule. The scale bars are 50 nm for (a) and 0.2 μm for (b).

Normally, the molecular structure of type-I COL[11] is denatured above 40°C and the commercial GEL partially maintains its self-assembling activity in spite of the denaturation during the production process. Thus, GEL shows limited activity for self-assembling, and the activity mostly disappears above ~40°C, while the organic-inorganic interaction continues to work at temperatures up to and above 80°C.[10] Below 50°C, the HAp phase is heterogeneously nucleated; it lightly grows on the GEL molecules matrices[7]. Above 50°C,[9] the growth of the HAp crystal becomes dynamic, and the pattern of crystal growth is close to the hydrothermal behavior, as shown in Fig. 1(b). In the hydrothermal reaction, the crystal growth is kinetically controlled depending on the reaction temperature.

3.1.2. Variously Shaped Particles

Fig. 2 shows the various TEM morphologies of the HAp nanocrystallites in GEL matrices prepared at 37°C. These types of morphology are related with the degradation of the gelatin precursors in the H_3PO_4 solution with DI water. GEL powders were dissolved and degraded in an aqueous solution of H_3PO_4 before the beginning of the coprecipitation process. The long term degradation of the GEL in the H_3PO_4 solution contributed to the formation of needle-shaped particles of HAp phase in the GEL matrix. The formation of needle-shaped apatite crystals was caused by the c-axial alignment in the type-I COL molecular structure.[3,11,12] The molecular structure of commercial GEL is further degraded by using phosphoric acid and the individual molecular structure of GEL fibrils becomes homogeneous. The microstructure of commercial GEL maintains the partial morphology of the original collagen precursor; thus, mineralization of the partially degraded GEL molecules occurs. The morphology shown in Fig. 2(a) is the stria patterns[12] of the HAp phase, which can be well observed in bone tissue.[11,12] In biological bone type-I collagen [COL] a long helical texture exists that builds an architectural nanocomposite between COL molecules and the HAp phase. It can be shown as the stria patterns of HAp crystals in COL under TEM observation.[12] The ED pattern shows clear (002) diffraction spots indicating the HAp phase. The stria of a GEL molecule is roughly estimated to be one thousand nm in length and 30 nm width. Fig. 2(b) also shows the stria patterns of the HAp phase in the GEL matrix, which can be critically confirmed from the ED pattern. Although fine mineralization of the apatite phases do not clearly appear in this figure, the presence of loosely dispersed fine particles was confirmed through higher magnification observation with the ED pattern.

Fig. 2(c) also shows a clear image of HAp mineralization in the GEL molecules matrix. It shows the clearly mineralized region in the GEL macromolecules. HAp crystallites were confirmed from the ED pattern spots.

For the cases of Fig. 2(a), (b), and (c), the black contour of the mineralized region in the GEL matrix revealed clear HAp crystallites from the ED ring pattern. A diluted region

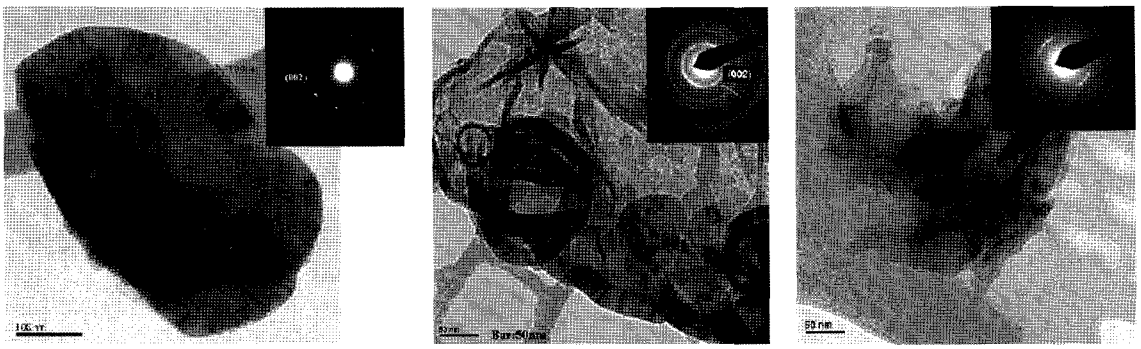


Fig. 2. TEM morphology for HG1-37(a), (b), and (c). The scale bars are 100 nm for (a) and 50 nm for both (b) and (c).

of the GEL matrices was observed through higher magnification of the ED pattern. The region was loosely mineralized rather than amorphous. It was occasionally observed to resemble an amorphous phase, but it is believed that the crystallites are too small to clarify the crystallinity.

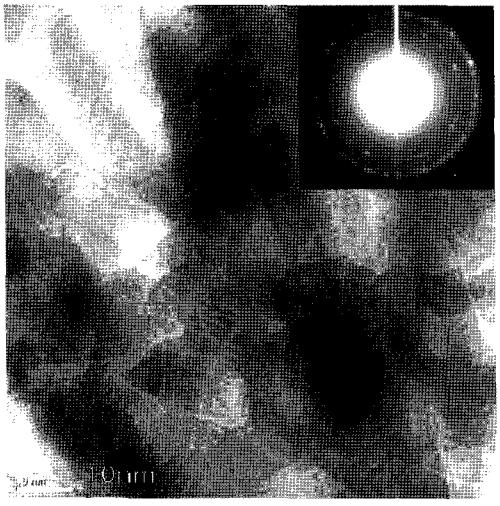


Fig. 3. (a) TEM morphology and ED pattern for the sample synthesized at 37°C (a). The lattice image shows the existence of OCP phase. (b) The theoretical schematic model for the lattice structure for the sample shown in Fig. 3. Apatite grows epitaxially on the (100) plane of OCP.

Fig. 3(a) shows the lattice patterns as a TEM and a pattern. A part of the lattices corresponds to the image of the OCP phase. The OCP phase is easily transferred to the HAp phase under vacuum during TEM observation.[12] Essentially the lattice change to HAp lattices from OCP lattices could be observed as imaged once for the same region. Thus, the actual phase status of the prepared HAp/GEL nanocomposite slurries may be schematically modeled as shown in Fig. 3(b).

The organic-inorganic phase of the HAp/GEL slurries was chemically processed according to a similar biological process of mineralization similar to that of in-vivo bone. The difference in the process is mostly based on the chemical kinetics. As shown in Fig. 4 the bone regeneration of these samples in a big mouse proved the evidence of this biomimetic process.[13] The biological chemistry of this artificial bone will be normalized through an analysis of the bone regeneration and organic-inorganic interaction between the GEL and HAp phase.

3.2. Crystal-Protein Interaction

The length scales defining the structure and organization

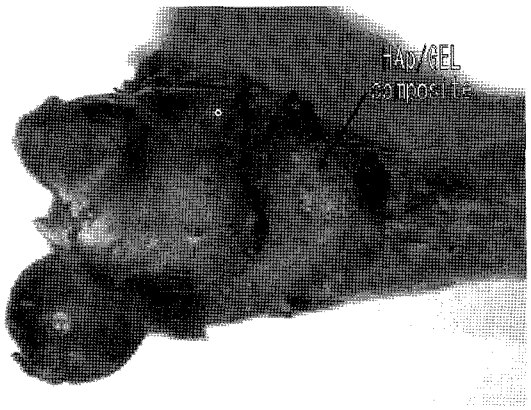


Fig. 4. Bone regeneration of a HAp/GEL nanocomposite sample in a rat femur. Six weeks after the material was implanted into the femur the sample was well bonded into the femur bone.

determine the fundamental characteristics of a material and nanostructural design is accomplished through the self-assembly of organics.[2] Organic materials are organized on length scales of 1-100 nm and used as frameworks for specifically oriented and shaped inorganic crystals. The obtained HAp/GEL nanocomposite materials showed a strong chemical bond between HAp and GEL macromolecules. This low costs process can have an economic impact. The experimental approach can be extended to incorporate bone growth factors due to low temperature procedures.

The nucleation of isotropic or anisotropic crystals in the protein matrix induces the hierarchically modulated crystal morphology. This can be used for the organization of a microcircuit, with the organization controlled at the atomic or molecular level. There are two concepts to be considered; "the preparation of an organic template for the coming nucleation of crystals", and "the control of the heterogeneous nucleation along the designed pattern". If the organic template has a singular pattern corresponding to a single phase and morphology, heterogeneous nucleation is controlled by the concentration of the template organics.

In the HAp/GEL nano-composite, it was possible to observe several types of formed morphology, which resulted from both the coprecipitation variables (pH, temperature, and the input of dynamic energy such as the stirring speed during the reaction) and the morphology of the protein precursor. Sheet type morphology results from the self-organization of the fully disintegrated GEL precursor in water. The fully disintegrated GEL glands are preferentially mineralized by calcium phosphate and easily organized to form the uniform organic sheet.

4. Conclusions

The morphologies of HAp/GEL nanocomposite slurries were analyzed through TEM observations of the ED patterns. Various types of morphology were revealed according to the denaturing condition of the GEL precursors in an aqueous solution of H_3PO_4. The organic-inorganic interaction and the resulting morphology can serve as a guide to develop a microcircuit design in a biomass.

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