

# Synthesis of Hydroxyapatite/Collagen Bone-Like Nanocomposite and Its Biological Reactions

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## 1. Introduction

Bone is a typical nanocomposite of inorganic and organic substances mainly composed of nanocrystals of hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , HAp) with nonstoichiometry 20-40 nm in length and type-I collagen molecules 300 nm in length. In microscopic observation of bone, collagen molecules are covered with HAp nanocrystals, and they form nanocomposite fibers in which HAp *c*-axes are approximately aligned along with collagen molecules (Bacon et al., 1979, Sasaki & Sudoh, 1997). Bone has two important roles in vertebrates; one is as a structural material to maintain vertebrates body structure and to guard important internal organs, such as brain, heart and lung, and another is as an organ to control calcium ion homeostasis by resorbing bone mineral according to deficiency of calcium ion. These two roles are kind of antinomial properties as industrial materials, *i.e.*, TOUGH to endure to external forces for whole life and BREAKABLE to allow calcium release by bone cell functions on demand. However, change our viewpoint about toughness of material, solution of the antinomy is concluded to one property. That is, every materials fatigue by long time use; thus, periodic renewal of material is necessary to maintain enough toughness of bone for whole life. Thus, bone has to be renewed easily by cell functions. That is almost the same meaning as a breakable property of bone. Accordingly, requirement of bone can be translated as follows; bone has to be decomposed by cell functions but be stable without them.

Fortunately, our ancestors needed to preserve calcium and phosphate ions in their body when they left from sea. Calcium and phosphate easily form insoluble compound in aqueous solution, brushite ( $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ ), octacalcium phosphate ( $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$ ) and HAp, and all calcium phosphates are changed into HAp in aqueous or moistured condition, because it is most stable compound in these conditions. Further, HAp formed in regular

biological condition is a nanocrystal. Our ancestors, as a result, preserve HAp in endoskelton as a calcium and phosphate reservoir. Collagen had been developed in evolutionary process as a structural organic molecule and chosen as main organic component of endoskelton. Accumulation of HAp in endoskelton and following well-aligned nanostructure of HAp and collagen produced highly hardness and elasticity of bone to barely endure to external force in short period. In addition, HAp is very stable in neutral pH condition but unstable in acidic and basic conditions, and collagen is a protein and can be decomposed by appropriate enzymes. Thus, bone is stable in our body and become unstable when cells release proton and enzyme.

Some readers wondering why HAp ceramics, sold as artificial bones and fillers, are considered as a non-resorbable material even with the same chemical properties as bone HAp. One reason is difference in chemical composition. Bone HAp contains large amount of carbonates due to high amount of carbonate ion concentration in our body fluid. Carbonate ion increases HAp solubility and inhibits HAp crystal growth. Another and much important reason is size of HAp. Generally, HAp sintered bodies including pillar and wall of porous HAp ceramics is dense, and crystal size of HAp in them is approximately 1  $\mu\text{m}$ . In this dense and large crystal size, HAp needs significantly longer period to be decomposed by cell functions in comparison to HAp nanocrystals dispersed in collagen molecules because of difference in surface area.

According to these considerations, mimicking of bone nanostructure can be a solution of problems existing in present artificial bone filler materials.

## 2. Synthesis of HAp/Col bone-like nanocomposite

### 2.1 Bone remodeling process

Bone remodeling process is a regular bone metabolism regulated by cells aiming to maintain mechanical strength of bone and Ca ion homeostasis in body fluid and constantly repeated in our bone to achieve the former aim. This regular bone remodeling is carried out by coupling of an osteoclast and an osteoblast. The osteoclast is one of a multinucleated giant cell forms through the fusion of mononuclear precursors, hemopoietic progenitors and resorbs bone by controlling biochemical condition of target site, lowering pH to 3-4 to dissolve HAp and releasing enzyme to decompose collagen. This bone-resorbed site is called "Howship's lacunae." The osteoblasts are accumulated to the Howship's lacunae to form new bone. The formation of bone is simplified as follows:

1. The osteoblasts release collagen molecules and they form fibers.
2. Calcium phosphate nanocrystals, generally considered as brushite, are supplied from matrix vesicle synthesized by the osteoblasts.
3. Hydroxyapatite nanocrystals epitaxially grow on collagen molecules (with some support of calcium phosphates released from matrix vesicles.)

In fact, bone nanostructure formation mechanism have not completely understood yet; however, orientation between HAp nanocrystals and collagen fibers cannot be well explained without some kinds of epitaxy because of its extremely small scale for cells. Thus, functions of cells to form the nanostructure is assumed to be supply of raw materials and adjustment of surrounding condition, at least initial stage, i.e., formation of collagen fibers and formation of HAp nuclei on collagen. These cell functions can be technologically mimicked.

## 2.2 Processes for mimicking bone formation

Prior to explain our method, several approaches to prepare composite of HAp and collagen are introduced.

First trial to prepare the composite is just mixing HAp or related calcium phosphates in collagen sol and freeze-dry it after gellation of collagen sol (e.g., TenHisen et al, 1995). This method is very simple but difficult to prepare homogeneous composite in which HAp amount is similar to bone (70mass%). Further, this method does not form bone-like nanostructure. So, biological reaction to the composite obtained from this method is just a combination of implantation of collagen sponges and HAp particles.

Second trial is precipitation of HAp nanocrystals on collagen fibers in its sponge or membrane (e.g., Tampieri et al., 2003). This method allows orientation of HAp and collagen fibers at only low HAp amount. Further, HAp nanocrystals completely cover collagen fibers. This is microcomposite in a sense, i.e., primary components, HAp and collagen, are nano-size but their secondary components are HAp microsized layer and collagen microsized fibers. Accordingly, initial biological reaction is similar to HAp and suddenly changed into reaction to collagen sponge.

Third trial is to mimic bone nanostructure. Polymer induced liquid precursor method allows to synthesis bone-like nanocomposite with almost similar process of bone formation (Olszta et al., 2007). Coprecipitation of collagen fibers and HAp from thin mixture solution of collagen and calcium phosphate allows nano to millimeter level bone-mimicking structure (Nassif et al., 2010). Both methods assumed to need long period to form HAp on collagen fibers ( $\geq 1$  weeks) and not suit for mass production. Further, the latter paper have not confirm the orientation of HAp and collagen in bone-like HAp content.

Our simultaneous titration method is very simple coprecipitation method to allow self-organization of HAp and collagen (Kikuchi et al., 2001). This method can easily synthesis bone-like oriented HAp/collagen (HAp/Col) composite with high HAp amount even greater than bone ( $\geq 80$ mass%). Further, length of the bone-like composite is up to 75 mm at HAp/collagen mass ratio of 3/2 (Kikuchi et al., 2003). Accordingly, this method is considerably better for mass production which make the HAp/Col composite available to use in practical medicine. The method in details is explained in the followings section.

## 2.3 Simultaneous titration method

Calcium hydroxide is synthesized by hydration of CaO obtained by burning of  $\text{CaCO}_3$  (alkaline analysis grade, Wako Pure Chemical, Japan) to ignore influence of minor elements, Mg and Sr. Schematic drawing of synthesis apparatus is shown in Fig. 1. Aqueous suspension of  $\text{Ca(OH)}_2$  and aqueous solution of  $\text{H}_3\text{PO}_4$  (Regent grade, Wako Pure Chemical, Japan) which contains porcine dermal type-I atelocollagen (Biomedical grade, Nitta Gelatin, Japan) are simultaneously added into a reaction vessel through tube pumps. The HAp/Col forms almost ideally, thus HAp/Col mass ratio and amount obtained are expected by amounts and concentrations of starting materials. composite Pure water is added in the reaction vessel beforehand to allow measuring pH. The pumps are turned on and off by pH meter with power control unit to maintain pH at 9 in the reaction vessel. The reaction vessel is placed in water bath to maintain reaction temperature to 40 °C. The pH 9 is very important because isoelectric point of the collagen exists in pH 7 to 9, and HAp is stably formed by simultaneous titration of  $\text{Ca(OH)}_2$  and  $\text{H}_3\text{PO}_4$  around pH 9. Temperature is also important to allow collagen fiber formation. Temperature up to 40 °C allows competitive reaction of collagen fiber formation and collagen degeneration. At more than 40 °C, degeneration of

collagen become dominant reaction and at lower temperature allows to degenerate collagen to gelatin due to delaying of collagen fibril formation at low ionic concentration as in the present reaction. Figure 2 shows transmission electron micrographs of the HAp/Col composite at HAp/Col mass ratio of 4/1. The HAp/Col composite in this photo is approximately 30  $\mu\text{m}$  in length, and selected area electron diffraction from the HAp/Col composite shows orientation of HAp nanocrystals along with collagen fiber.

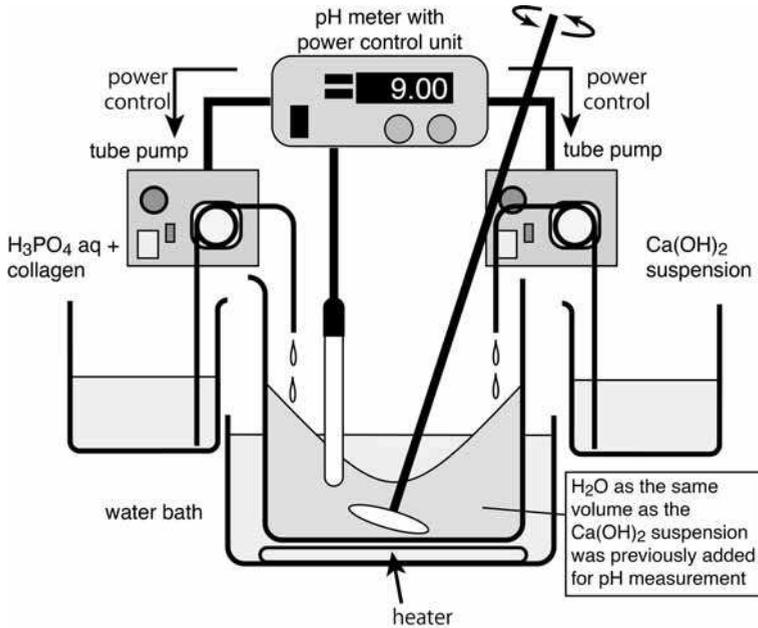


Fig. 1. Schematic drawing of synthesis apparatus for HAp/Col bone-like nanocomposite.

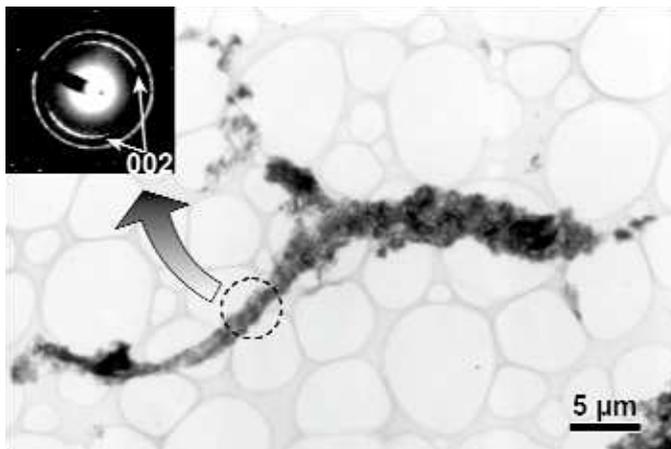


Fig. 2. Transmission electron micrograph and selected area electron diffraction of HAp/Col bone-like nanocomposite.

In addition, HAp formation on collagen molecules is supposed to promote collagen fiber formation (Kikuchi et al., 2003). Therefore, concentrations of starting materials in the reaction vessel control fiber length of the HAp/Col composite. Longer fibers are obtained from thinner solution and low HAp/Col ratio. To control the HAp/Col fiber length, starting materials concentrations, their amounts and amount of H<sub>2</sub>O previously added in the reaction vessel are controlled as shown in Table 1. Figure 3 shows that the HAp/Col fibers are grown with decreasing of starting material concentration. In fact, most important concentration is Ca, P and collagen concentrations in the reaction vessel. Calcium concentration in the reaction vessel measured with Ca ion electrode shown in Fig. 4 demonstrates that changes in Ca ion concentration in the reaction vessel for low starting material concentrations is more steady than high starting material concentration. Further, presence of collagen molecules also stabilize Ca concentration change at the same Ca and PO<sub>4</sub> concentration. These results suggest that stable and slow HAp heterogeneous and epitaxial nucleation on the collagen molecules occurs under low starting material concentration in the reaction vessel. On the contrary, higher starting material concentration rapidly increases Ca and PO<sub>4</sub> ion concentrations and allows homogeneous nucleation of HAp in the solution like HAp formation in non-collagen system as well as a heterogeneous nucleation on the collagen.

Ca(OH) <sub>2</sub>	Concentration / mM	50	100	200	400
	Amount / ml	1600	800	400	200
H <sub>3</sub> PO <sub>4</sub>	Concentration / mM	15	30	60	120
	Amount / ml	3200	1600	800	400
Total Ca/P ratio		1.67 (stoichiometric for HAp)			
Collagen / g		2.01			
H <sub>2</sub> O in reaction vessel / ml		1600	800	400	200
HAp/Col mass ratio		4/1			

Table 1. Concentration and amount of starting materials for controlling fiber length of HAp/Col bone-like nanocomposite.

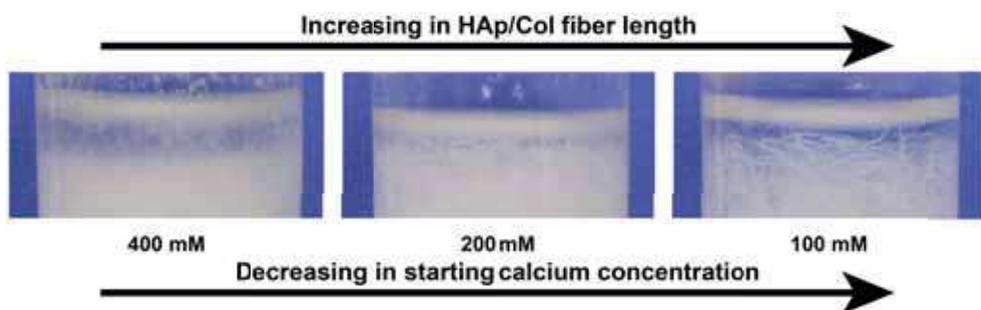


Fig. 3. Photographs of HAp/Col bone-like nanocomposite fibers increasing their length with decreasing in starting material concentrations.

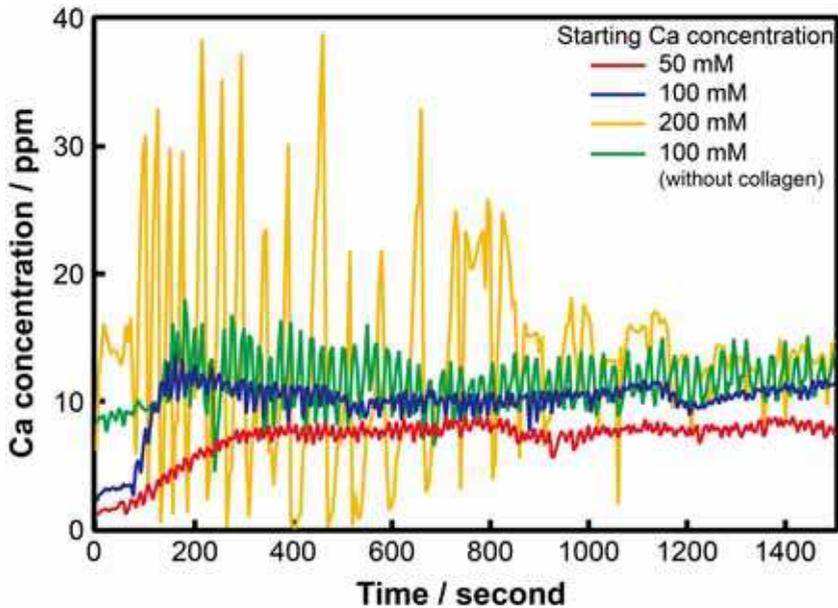


Fig. 4. Changes in calcium concentration in reaction vessel. Fluctuation of concentration decreases with decreasing in concentration or presence of collagen.

#### 2.4 Preparation of artificial bone

Several forms of artificial bone can be prepared from the HAp/Col nanocomposite fibers.

A dense body is prepared by compaction of the HAp/Col with uniaxial and/or cold isostatic pressing methods. The HAp/Col dense body demonstrates maximum 3-point bending strength of  $39.5 \pm 0.9$  MPa and maximum Young's modulus of  $2.54 \pm 0.38$  GPa. These values are 1/5 of cortical bone but higher than those of cancellous bone (Kikuchi et al., 2001).

A paper-like membrane is prepared by filtration of the HAp/Col fibers followed by pressing as shown in Fig. 5 (Kikuchi, 2007). The membrane is easily deform as a wavy membrane as in Fig. 6 (Kikuchi, 2008).

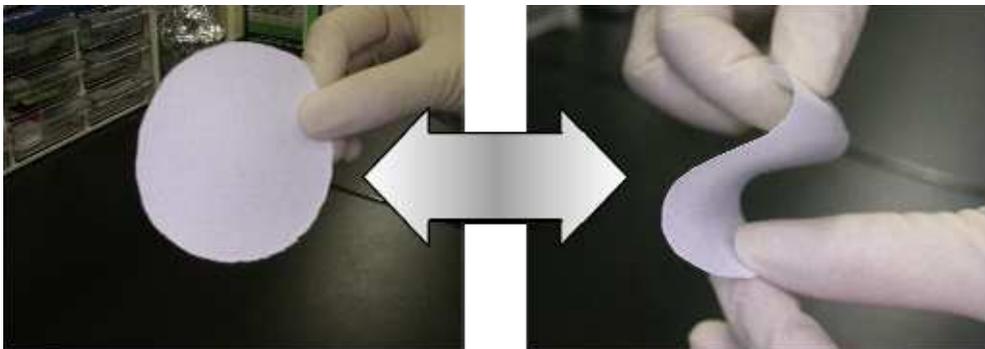


Fig. 5. Flexible membrane prepared from HAp/Col bone-like nanocomposite fibers.



Fig. 6. Wavy membrane prepared from HAp/Col flexible membrane.

A porous body is prepared by lyophilization of the frozen HAp/Col suspension. The HAp/Col fibers, collagen solution, phosphate buffered saline are mixed homogeneously and frozen to grow ices in the mixture. After the freezing, the mixture is lyophilized to remove ices as pores. Collagen molecules in the porous HAp/Col obtained are crosslinked to decrease resorption rate (Kikuchi et al., 2004a). The porous HAp/Col demonstrates sponge-like visco-elasticity as shown in Fig. 7 (Kikuchi et al, 2004b). When the HAp/Col mixture is frozen from one side, unidirectional ice growth create unidirectional porous structure instead of random pores (Yunoki et al, 2006). Another way to prepare unidirectional porous body is rolling-up the wavy membrane as shown in Fig. 8(Kikuchi, 2008).

### 3. Biological reactions

#### 3.1 Regeneration of bone defect

All animal tests described in this section are accepted by Tokyo Medical and Dental University Institutional Animal Care and Use Committee. The cylindrical dense HAp/Col with central hole and satellite holes as shown in Fig. 9 were implanted into segmental defect of beagle's tibia 20 mm in length. This size of defect never regenerate without support of

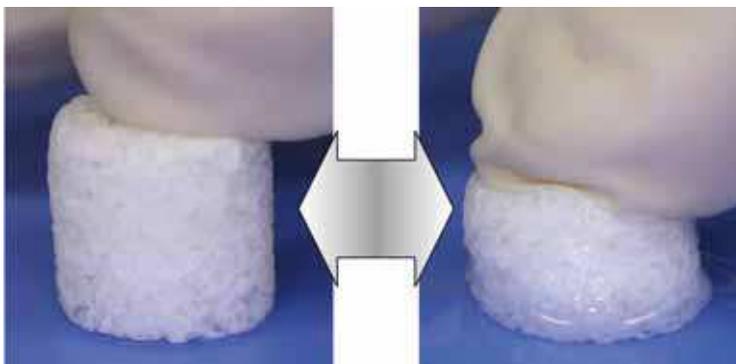


Fig. 7. Viscoelastic porous body prepared from HAp/Col bone-like nanocomposite.



Fig. 8. Unidirectional porous HAp/Col bone-like nanocomposite.

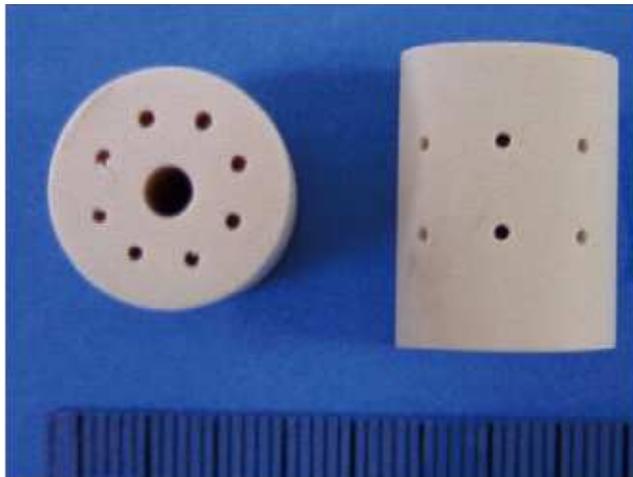


Fig. 9. Dense HAp/Col bone-like nanocomposite with central and satellite holes.

autologous bone transplantation or other material implantation. After 12 weeks of implantation of the HAp/Col, the HAp/Col cannot be detected with X-ray photo. Naked-eye observation of the host site shows no HAp/Col presence and complete regeneration of bone (Fig. 10a). From the histological observation shown in Fig. 10b, regenerated bone is still young but completely regular bone. Multinucleated giant cells are attached on the surface of the HAp/Col debris found in the new bone tissue. The cells are tartarate-resistant acid phosphatase positive. Thus, the cells are identified as osteoclasts. Spindle-shaped cells are attached on the opposite side of the HAp/Col with non matured tissue between. These cells are alkaline phosphatase (AlkP) positive and identified as osteoblasts. These results mean that the HAp/Col is resorbed by the osteoclasts followed by new bone formation by the osteoblasts. This osteoclast-osteoblast coupling is completely the same as the bone remodeling process, especially as the same reaction when autologous bone is transplanted. That is, the HAp/Col is incorporated into bone remodeling process (Kikuchi et al., 2001). In fact, this is the first evidence of incorporation of the HAp/Col into bone remodeling process

and the world first reported material that completely incorporated into bone remodeling process. The HAp/Col porous body also revealed the same biological reaction when it is implanted into a hole of rabbit's tibia (Tsuchiya et al., 2008). According to the results, enough pore size and well interconnected porous structure to allow cell and tissue migration was important for good bone regeneration. Recently, importance of pore structure in critical bone defect regeneration has been being investigated using series of unidirectional porous HAp/Cols (Aoki et al., 2009). Further, effects of combination of cytokine and the HAp/Col were investigated. The HAp/Col is a good carrier of recombinant human bone morphogenetic protein-2 (Itoh et al., 2001) and regenerates large osteochondral defect with small amount of basic fibroblast growth factor (Maehara et al., 2010).

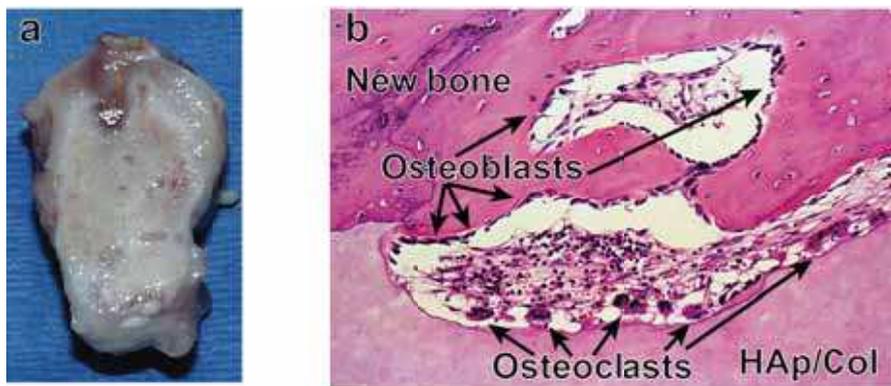


Fig. 10. Naked-eye (a) and histological (b) observations of dense HAp/Col bone-like nanocomposite after 12 weeks' implanted into segmental defect of beagle's tibia.

### 3.2 Cell reactions in vitro

Influence of osteogenic activity of the HAp/Col was examined by three-dimensional culture with pressure/perfusion on the porous HAp/Col (Yoshida et al., 2010). Human osteoblast-like cells, MG-63, were seeded to the HAp/Col sponge and cultured for 24 hour in static condition to allow secure attachment of cells on the wall of HAp/Col sponge. Then, the cell-seeded HAp/Col sponge was transferred into pressure-proof culture column. The cells were cultured in the column in growth medium (Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin) for 6 day with medium perfusion at 1.3 ml/min to allow cell proliferation into the sponge. After 6 days' incubation, the medium was changed into osteogenic medium (growth medium supplemented with 50 µg/ml L-ascorbic acid phosphate magnesium salt and 10 mM β-glycerophosphate). In addition, static pressure at 3.2 MPa was applied with a back-pressure regulator with 1.3 ml/min perfusion. The medium was stocked in the reservoir and changed every 3 days. Conventional collagen sponge was used as a control.

In the first 10 days, no significant difference in cell proliferation estimated from total DNA amount is found between cells on the HAp/Col and collagen sponges; however, the cells on the HAp/Col sponge demonstrates significant higher proliferation against the cells on the collagen sponge. On the contrary, AlkP gene expressions, one of osteogenic markers, of the cells on the collagen sponge at day 7 and 10 are significantly greater than those of the cells

on the HAp/Col sponges. Other osteogenic marker, osteocalcin, gene expression shows no significant difference between them. The reason of these phenomena is explained by histological and scanning electron microscopic observations. Histological sections at day 21 shown in Fig. 11 demonstrate that both sponge are covered with cell layers (Fig. 11a and 11b); however, many cells migrate into the center of HAp/Col sponge (Fig. 11c) and very few cells migrate into the center of collagen sponge (Fig. 11d). The AlkP gene expression of osteoblastic cells requires certain numbers of cells and their junction by their accumulation. On the collagen sponge, this accumulation is easily achieved by cell proliferation only on its surface and continued up to the end of experimental period. Contrarily, cell proliferation on the HAp/Col sponge promotes not only on the surface but in the HAp/Col sponge along with its pore walls. These differences in cell proliferation manners explain that higher AlkP activity on the collagen sponge at early stages without significant difference of cell proliferation on both sponges as well as significant higher cell numbers (total DNA amounts) for the HAp/Col sponge. Another significant difference is an extracellular matrix (ECM). The ECM from cells on the collagen sponge has no calcium phosphates but that from the cells on the HAp/Col sponge contains calcium phosphates as shown in Fig. 12. These results suggest the HAp/Col sponge is expected to be a good scaffold for bone tissue regeneration as well as a bone filler material.

Further, enhancement of osteogenic activity of MG63 cells is confirmed under two-dimensional culture on the HAp/Col membrane (Kikuchi, 2007). Cells grow very well on the membrane and become confluent in 7 days as the same as the cells on conventional tissue culture polystyrene (TCPS) well as shown in Fig. 13. The cells on the HAp/Col

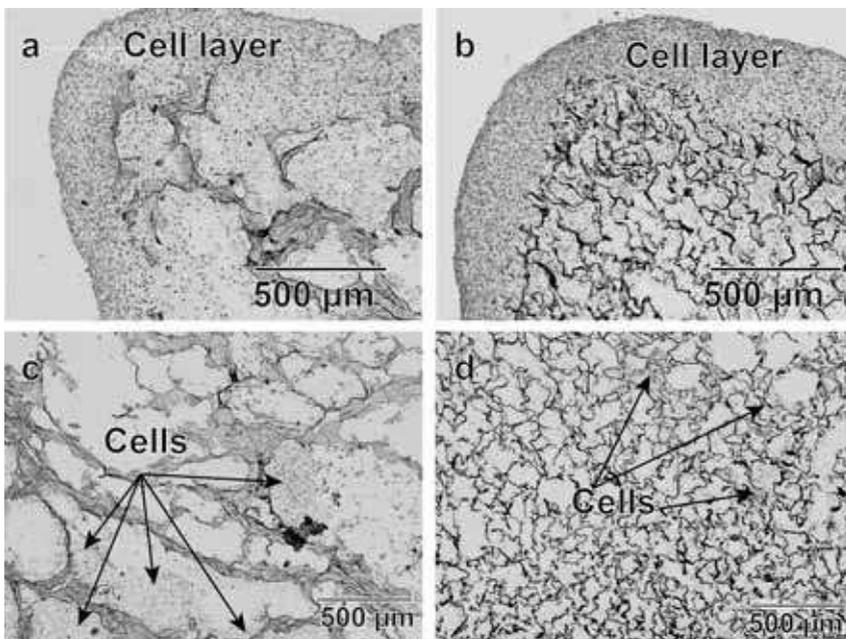


Fig. 11. Hematoxylin and eosin stained histological section of HAp/Col (a: periphery, c: center) and collagen (b: periphery, d: center) sponge at day 21.

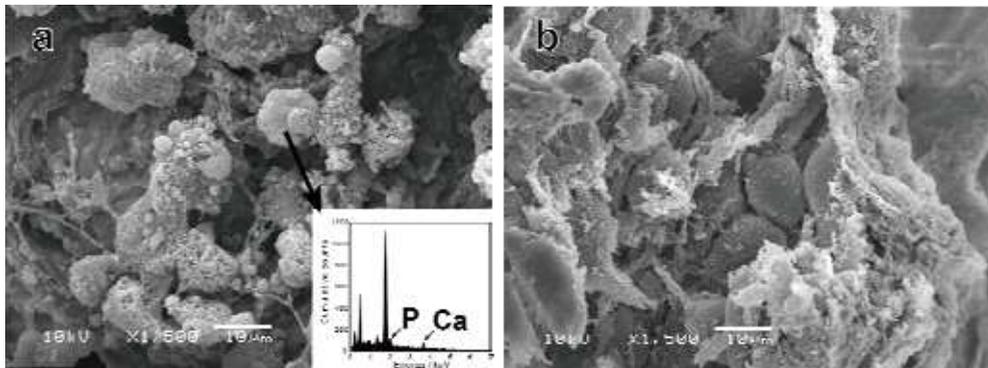


Fig. 12. Scanning electron micrographs of cells on HAp/Col sponge (a) and collagen sponge (b). Both cells secreted extracellular matrices (ECM); however, calcium phosphates only formed on ECM secreted by cells on HAp/Col as shown in inset chart.

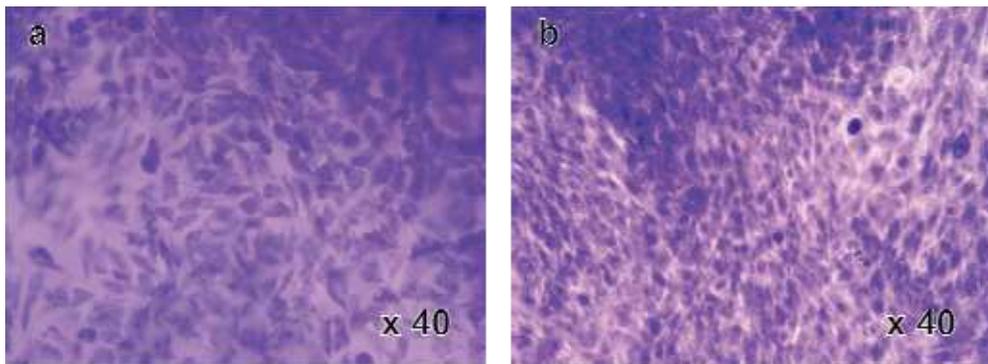


Fig. 13. Phase contrast micrographs of MG63 cells on HAp/Col membrane (a) and tissue culture polystyrene (b) at 7 days.

membrane were observed with a conventional translucent phase contrast microscope, thus image is not clear as the TCPS but enough to observe cell morphology. Alkaline phosphatase gene expression of the cells on the HAp/Col membrane is 3.5 times greater than that on TCPS when cells culture without the osteogenic supplements. When the cells culture with the supplements, AlkP gene expression on the HAp/Col is 3.2 times greater than that on the TCPS. That is, the HAp/Col is expected to be a good scaffold for osteoblast culture for bone regenerative medicine.

In addition, mouse primary bone marrow cells cocultured with mouse primary osteoblasts differentiate into osteoclast without adding of any differentiation promoting supplements as shown in Fig. 14 (Kikuchi & Irie, 2009). The mechanism of differentiation led by the HAp/Col is still under investigation, but the HAp/Col may affect osteoblast's activities to release some signals for osteoclast differentiation as well as to activate osteogenic activity.

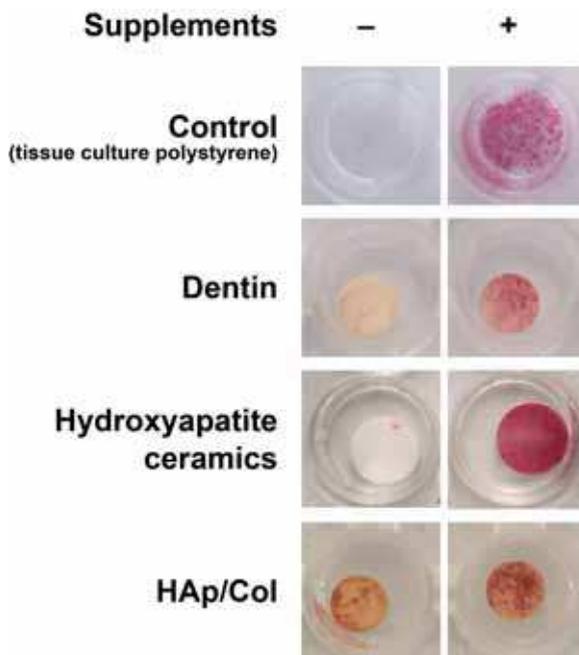


Fig. 14. Photographs of tartrate-resistant acid phosphatase stained specimens after coculture of mouse osteoblasts and bone marrow cells. Osteoclasts differentiated from bone marrow cells exist at red-stained parts.

#### 4. Conclusion

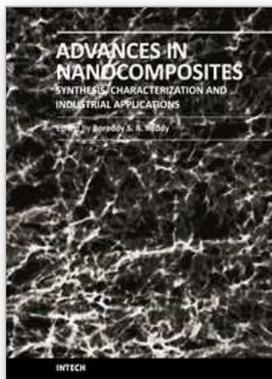
Bone nanostructure is reproduced by simple simultaneous titration method with maintaining pH and temperature suit for self-organization by controlled HAp nanocrystal formation on collagen molecules. This result is supportive for reproduction of biominerals with control of starting materials, their concentration and surrounding conditions for biomineral formation. As an artificial bone material, the HAp/Col bone-like nanocomposite is a promising material due to its excellent bioactivity, biocompatibility, formability, cytocompatibility and viscoelastic property. In addition, the HAp/Col is also beneficial as a cell scaffold.

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Advances in Nanocomposites - Synthesis, Characterization and Industrial Applications was conceived as a comprehensive reference volume on various aspects of functional nanocomposites for engineering technologies. The term functional nanocomposites signifies a wide area of polymer/material science and engineering, involving the design, synthesis and study of nanocomposites of increasing structural sophistication and complexity useful for a wide range of chemical, physicochemical and biological/biomedical processes. "Emerging technologies" are also broadly understood to include new technological developments, beginning at the forefront of conventional industrial practices and extending into anticipated and speculative industries of the future. The scope of the present book on nanocomposites and applications extends far beyond emerging technologies. This book presents 40 chapters organized in four parts systematically providing a wealth of new ideas in design, synthesis and study of sophisticated nanocomposite structures.

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