Influence of Concentration of Starting Materials on Fibrillogenesis of HAp/Col Self-Organized Nanocomposites

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Abstract

Fibrillogenesis of hydroxyapatite/collagen (HAp/Col) self-organized composites was examined at several synthesis conditions. The fibrillogenesis of HAp/Col composites at low Ca, P and Col concentrations was continuous growing of composite fibrils via interaction between hydroxyapatite nanocrystals and collagen molecules. An asepsis test after sterilized by several methods showed no bacterial colonies. The porous sponge, net and paper-like sheet of composites were formed with simple procedures; they could be incorporated into bone remodeling process *in vivo*.

Introduction

Hydroxyapatite is well known as a main inorganic component of vertebrates' bone. The *c*-axes of Hydroxyapatite nanocrystals in bone were as a whole aligned to a specific direction parallel to molecular axes of collagen. The authors assumed that such organized nanostructure of hydroxyapatite and collagen could affect bioactivity through cell attachment *etc.*, and then could be incorporated in normal bone metabolism. The self-organization mechanism and biological reaction of HAp/Col composite have been reported [1-5]; however, very long fibrils of the HAp/Col composite could not obtained. A control of ionic strength in the reaction vessel could control the fibrillogenesis of collagen because collagen fibrillogenesis is influenced by ionic strength in the solution[6]. In the present paper, HAp/Col composite fibrils up to 75mm in length were synthesized with a closely

controlled self-organization condition. Further, an asepsis test after several sterilizations and the biological reaction of the resultant composite were examined.

Materials and Method

Calcium hydroxide, orthophosphoric acid (Reagent grade, Wako Pure Chemicals Ind.) and atelocollagen were used as starting materials. The concentration and amount of the starting materials (**Table 1**) were determined for HAp/Col mass ratio to be 80/20; the total mass was 10g. Pure water, the same amount as the $Ca(OH)_2$ suspension, was previously added in a reaction vessel to measure pH of reaction solution from a starting point of synthesis. The HAp/Col composite was synthesized at pH9 and 40°C by a simultaneous titration method [1] at an adding rate of $15cm^3$/min of both titrants. Calcium concentration in the reaction solution was measured by Ca ion electrode (DKK-TOA Co.). The length of HAp/Col fibrils synthesized was measured with a Rapid-View® system up to 100μm and with a rule over 1mm. The composite compact prepared by uniaxial pressing was cut into $5\times3\times20mm^3$. Three-point bending strength of them before and after sterilizing by a vacuum-heat, a plasma, a γ-ray (10 and 25 kGy) and an electron beam (15, 25 and 50 kGy) was measured by a universal testing machine (AGS-1kN, Shimadzu) at a crosshead speed of 500μm/min with a span of 15mm. Further, under a condition of 100mM of Ca and 30mM of P starting concentration, HAp/Col composite fibrils were also synthesized at HAp/Col mass ratios of 70/30 and 60/40. The composite fibrils were formed into porous sponge by a lyophilization and Japanese-paper-like sheet by a filtration. Animal test was carried out using Wistar rats and Japanese white rabbits.

Table 1. Concentration and amount of starting materials.

$Ca(OH)_2$ suspension	Conc. [mM]	50	100	200	300	400
	Amount [cm^3]	1600	800	400	166.7	200
H_3PO_4 solution	Conc. [mM]	15	30	60	90	120
	Amount [cm^3]	3200	1600	800	333.3	400
Collagen	Amount [g]			2		

Results and Discussion

The HAp/Col fibers became longer and thicker with decreasing in concentration of the reaction solutions. The maximal length of the fibers was about 20mm at a Ca concentration of 100mM, while it was 20μm or less at a higher concentration [1]. Figure

1 indicated that the Ca concentrations of a titrant and a reaction solution was correlative and each Ca concentration of all reaction solutions was higher than that of hydroxyapatite saturated solution but about a quarter of that of human blood plasma. The variation and mean Ca concentration for 100mM without collagen and for 200mM with collagen was greater than that for the other conditions. The process of the nucleation of hydroxyapatite crystal and the fibrillogenesis of collagen molecule explained the reason of these differences. Under the existence of collagen hydroxyapatite crystals can form on carboxyl groups of collagen that act as nucleation centers to decrease the activation energy [1,7]. In addition, the hydroxyapatite formation on the carboxyl groups promoted the fibrillogenesis of collagen molecules, though Ca ions were generally regarded as an inhibitor for the fibrillogenesis [6]. Therefore, heterogeneous hydroxyapatite formation on collagen molecules and collagen fibrillogenesis occurred simultaneously and continuously at low concentration of starting materials. However, in the case of 200mM of Ca titrant concentration, addition of high concentration of starting materials led both homogeneous in solution and heterogeneous on collagen functional group nucleation of hydroxyapatite crystals due to acute raise of Ca (and P) concentrations, although collagen was added in the reaction solution. The fibril growth led higher 3-point bending strength to the composite about 20MPa in comparison to 10MPa with short fibrils. At a Ca starting concentration of 50mM, the self-organized fibrils grown up to

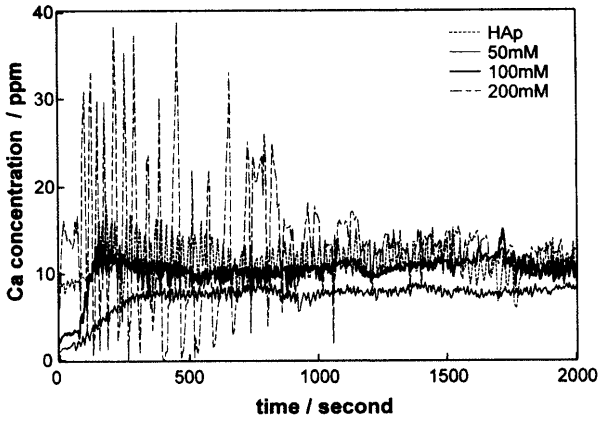

Fig. 1. Ca concentration in a reaction solution as a function of time.

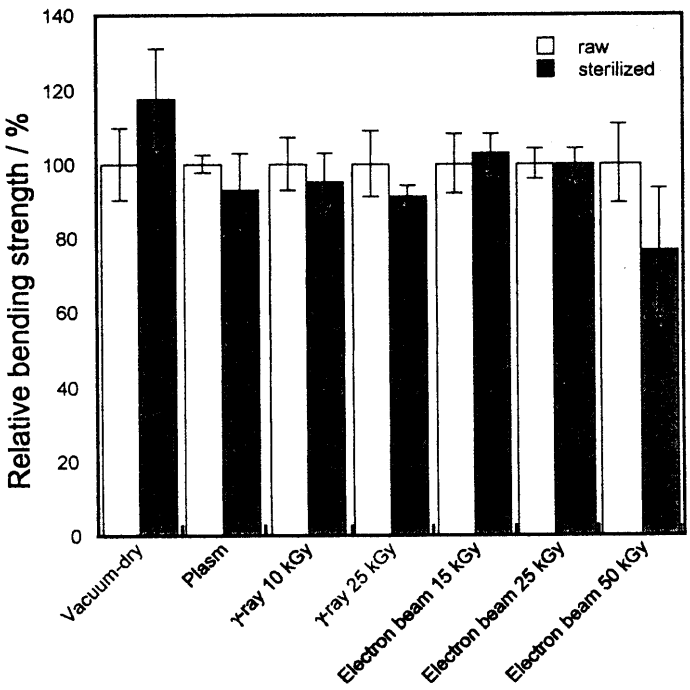

Figure 2. Relative bending strength of the HAp/Col composites before and after sterilization.

75mm in maximum length but the bending strength and Young's modulus because the fibrils packed into the mold to dehydration could not be compacted dense by a spring-like function of fibrils and sizes.

Relative bending strength of the HAp/Col composites before and after sterilization is shown in Fig. 2. The vacuum-dry sterilization induced obvious increasing of the bending strength due to dehydration crosslinkage of collagen. On contrary, the electron beam sterilization at 50 kGy induced drastic decreasing of it due to denaturation of collagen. However, other sterilization methods showed no significant changes in bending strength. Further, SDS-PAGE of collagen extracted from the composites indicated that no significant changes in collagen were detected for collagen after plasma sterilization.

The porous HAp/Col composites at a porosity of 90-97% prepared by a lyophilization of water, the fibrous composite and collagen mixture indicated sponge-like elasticity at wet condition. Japanese-paper-like sheets were prepared by a method conformable to manufacture paper using the composite fibrils.

The results of animal tests demonstrated that the composite was incorporated into a bone remodeling process, *i.e.*, the composite was resorbed by osteoclastic cells in the first 7days followed be new bone formation by osteoblasts in the lacunae on the composites. The coupling of the osteoclastic resorption and osteogenesis continued for at least 4 weeks. This process was considered to be quite similar to that observed for autogenous transplantation of bone. This "bio-integrated materials" could be useful for implant materials and expected to good materials for tissue engineering.

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