Calcium Phosphate Formation on Highly-oriented Collagen Fibrils

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Calcium phosphate formation on collagen fibrils was studied. Collagen fibrils deposited from a collagen solution were insolubilized by UV-irradiation. The collagen fibrils looked to be precipitated and arranged in one direction in a narrow space due to convection stream from bottom to top of test tube. Highly-oriented collagen fibrils precipitated were soaked in simulated body fluid (SBF) solution at 36.5 °C. After immersion of SBF solution at 36.5 °C for 3 days, calcium phosphate was formed on insolubilized collagen fibrils. In contrast, no calcium phosphate was formed on collagen fibrils without insolubilization treatment.

Human bone is a composite biologically produced, constituted with apatite and collagen fibrils, which is classified into two types; cortical bone and spongy bone. The cortical bone possesses periodical architecture with nano-size dimensions, and consists of cylindrical osteons or Haversian systems which are held together by a hard tissue stroma. In a hard tissue stroma, fine apatite crystals are with the long axis parallel to the collagen fibrils. The spongy bone is formed through intra-membranous ossification, and collagen fibrils are randomly arranged. The spongy bone is changing into the cortical bone with remodeling. The remodeling goes like concentric circle around blood vessel with dilation of primary spongiosa, which leads haversian systems. So the ordered structure of collagen fibrils is believed to be obtained by making hierarchically structured matrix grow in a narrow space. Considering the above findings, in this letter, highly-oriented type I collagen fibrils precipitated in a narrow space were prepared. Furthermore, calcium phosphate was formed on highly-oriented collagen fibrils from simulated body fluid (SBF) solution at 36.5 °C and the calcium phosphate-collagen composite were prepared. Given that human bone is the material combines strength, elasticity and bioactivity due to its structure, the materials closely resemble the human bone in structure are to be sought as a hard tissue replacement, and such study can make towards the understanding of biomineralization process in the body.

All chemicals used in this study were supplied by Wako Pure Chemical Industries, Ltd. and used without further purification. 10 ml of collagen solution (3 mg/ml in HCl, pH 3) were placed in a 30 ml test tube immersed in ice-gel water. 15 ml of "TRIS" ($(CH_2OH)_3CNH_2$) and HCl was added to the test tube. The TRIS/HCl acted as a buffering agent keeping the pH of the solution within the range 7.2-7.4. The solution was warmed calmly up to a temperature of 36.5 °C. Then the test tube was placed 10 cm away from the UV lamp (10 W, 254 nm) for 4 h at room temperature. The precipitated collagen fibrils were placed in a 30 ml test tube to which 20 ml of SBF solution was added. The test tube was placed in a water bath thermostatically controlled at 36.5 ℃ for periods of 3 days. A precise description of SBF solution is published in earlier reports.[1] The ion concentration of SBF solution are almost the same as those of body plasma solution. Scanning electron microscopy and EDX analyses were performed using a Hitachi S-530 scanning electron microscope and a Horiba EMAX-2200 X-ray microanalyzer. The determination of ions in 1.5xSBF solution before and after immersion of collagen fibrils was carried out using a Nippon Jarrell-Ash ICAP-1000S ICP-AES instrument. The turbidity of the solution was measured using Shimadzu UV Spectrometer at 660 nm.

Figure 1 shows the turbidity of collagen solution in "TRIS"

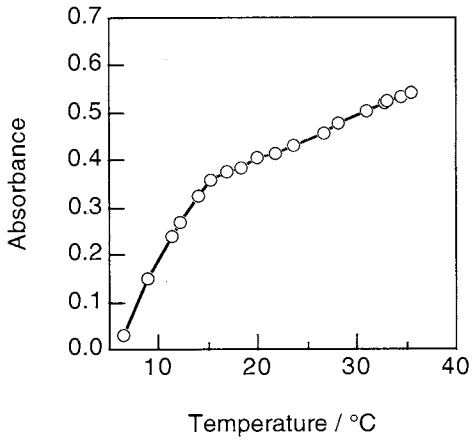

Figure 1. Absorption change of collagen solution with "TRIS" buffer as a function of a temperature of collagen solution.

buffer as a function of a collagen solution temperature. With an increase of temperature, the absorption (at 660 nm) of collagen solution increased. The increase in turbidity is due to the precipitation of collagen fibrils from the solution. The precipitated collagen fibrils were insolubilized by UV-irradiation according to the method of Hino,[2,3] in UV

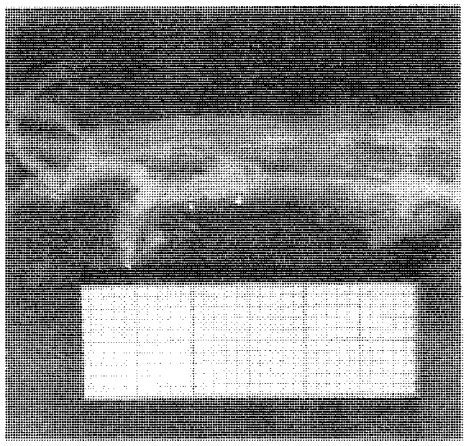

Figure 2. Photo of precipitated collagen fibrils. (1 grid is 1 mm).

sterilization box. UV-irradiation gives a cross-linking to collagen fibrils, and collagen fibrils became stable after irradiation with UV for more than 4 h. In case of collagen solution without UV-irradiation, the absorption (at 660 nm) of solution decreased, but the absorption of collagen solution UV-irradiated was kept constant for at least several days.

When collagen solution was neutralized and its temperature was increased to body temperature from ice temperature, collagen fibrils were precipitated randomly in flat bottomed flask. 20-30 capillaries of 2 mm diameter were placed in a 30 ml test tube, where collagen fibrils looked to be precipitated and arranged in one direction several centi-meters long as shown in Figure 2. The fibrils might grow along glass capillaries due to convection stream from bottom to top of test tube.

Figure 3 shows the EDX profile of products on collagen

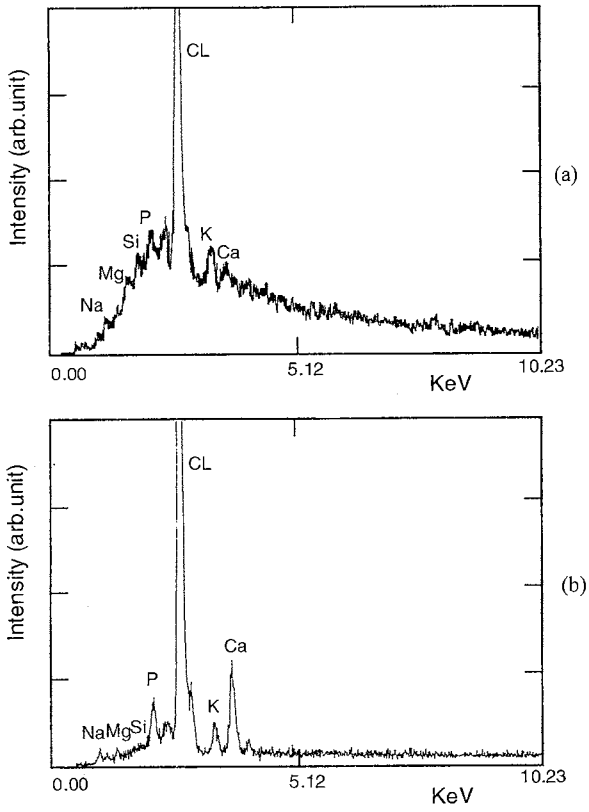

Figure 3. EDX profile of products on collagen fibrils (a) without UV irradiation treatment, (b)UV irradiated, after immersion in SBF solution at 36.5°C for 3 days.

fibril not insolubilized (a), and insolubilized (b) by UV-irradiation for 4 h after 3 day's immersion in SBF solution at 36.5 °C. The line intensities corresponding to Ca and P of the products on collagen fibrils UV-irradiated (Figure 3b) were apparently strong compared to those of the products on collagen fibrils without UV-irradiation treatment (Figure 3a) This suggests that insoluble collagen fibrils are stable in surface structure and may give certain nucleation sites of calcium phosphate, but the collagen fibrils without UV-irradiation treatment shows a soluble character in aqueous solution and does not give nucleation sites of calcium phosphate. ICP-measured calcium or phosphorous content in SBF solution slightly fell after immersion using collagen fibrils UV-irradiated, but these contents kept almost constant using collagen fibrils without UV irradiation treatment. The EDX-derived Ca/P ratio of the products on collagen fibrils UV-irradiated was around 1.56, which indicates the Ca-deficient apatite formation.

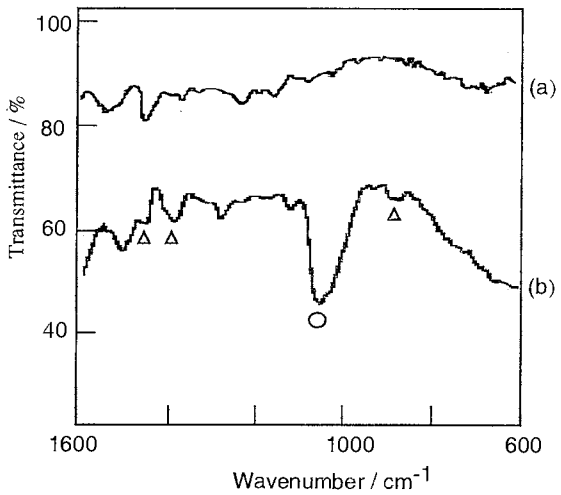

Figure 4. FT-IR spectroscopy of the products on collagen fibrils (a) without UV irradiation treatment, (b)UV irradiated, after immersion in SBF solution at 36.5 °C for 3 days.

Figure 4 shows the results of FT-IR spectroscopy measurement of the products on the collagen fibrils without UV-irradiation treatment (a), and the collagen fibrils UV-irradiated (b). For the products on the collagen fibrils UV-irradiated (b), the round shows the peaks denoted the stretching mode of PO_4 ion and the triangle shows the peaks assigned with the stretching mode of CO_2 ion, which shows the formation of carbonate apatite on the collagen fibrils UV-irradiated. However, those peaks were not found for the products on the collagen fibrils without UV-irradiation treatment. Small amount of calcium phosphate was found to be formed on collagen fibrils. Collagen was shown to be less effective in promoting the calcium phosphate deposition, and the addition of O-phosphoserine[4] or phosvitin[5,6] on to collagen fibrils should be required for the induction of calcium phosphate formation.

This study describes the formation of calcium phosphate on collagen fibril insolubilized by UV irradiation. The collagen fibrils looked to be precipitated and arranged in one direction in a narrow space due to convection stream from bottom to top of test tube. After immersion in SBF solution for 3 days, calcium phosphate was formed on insolubilized collagen fibrils. In contrast, no calcium phosphate was formed on collagen fibrils without insolubilization treatment.

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