Bone-Like Apatite Formation On Collagen Fibrils By Biomimetic Method

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Formation of collagen-apatite composite by biomimetic methods without any previous treatment of the collagen fibrils has been investigated. Reconstituted collagen fibrils obtained from Type I collagen solution were incubated in conventional and revised simulated body fluid (C and R-SBF) solutions to form calcium phosphate. Spherical deposits of B-type carbonate apatite started precipitating along the fibrils from day 1 of incubation in both C and R-SBF. But the large number of carbonate ions in R-SBF has changed the microstructure of the apatite precipitates. The calcium rich apatite deposits are more in R-SBF than in C-SBF. Percentage of Cl^- ions in the precipitates of R-SBF were less than in C-SBF.

Natural bone is a three dimensional composite of the inorganic crystalline hydroxyapatite (HAP) reinforced within an organic collagen fiber matrix. Hence attempts are being made to develop an artificial bone and is expected to have similar properties to those of human bone.[1,2] Apatite coating obtained by biomimetic process are referred as bone-like apatite which render a favorable environment for bone cell seeding and proliferation than sintered HAP. Apatite formation by such biomimetic methods has been attempted by various investigators on polymeric substrates like collagen, chitin, cellulose, poly L-lactic acid and poly (lactide-co-glycolide).[3–6] Collagen is less effective in promoting calcium phosphate precipitation. Pretreatment of collagen fibrils with O-phosphoserine or phosphovitin or acetic acid is needed for faster nucleation and growth of calcium phosphate crystals.[7–9] In the present study, collagen-HAP composite has been prepared from both C-SBF and R-SBF mimicking the *in vivo* conditions without any previous treatments of collagen fibrils.

Wako chemicals, Tokyo, Japan, supplied all the chemicals used in this experiment. Type I collagen solution (porcine skin, 3 mg/ml in 1 N HCl, pH 3) was adjusted to pH 7.2 by adding appropriate amounts of 1.5 M NaCl, 0.1 M Na_2HPO_4 and 0.1 M NaOH. Concentration of collagen after pH adjustment was about 1 mg/ml and was allowed to polymerize at 36.5 °C for one day. Polymerized gels were cross-linked in glutaraldehyde vapour atmosphere. The C-SBF solution was prepared by dissolving NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, K_2HPO_4 and Na_2SO_4 in distilled water together with TRIS ($(CH_2OH)_3CNH_2$) and HCl which acted as a buffering agent keeping the pH of the solution to be within a range 7.10–7.50 during the soaking experiments.[10] While in R-SBF, TRIS and its counter agent HCl was replaced by 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid $C_8H_{18}N_2O_4S$ (HEPES) and NaOH and Na_2CO_3 is added additionally.[11] The gels were immersed in 15 ml of SBF contained in plastic bottles with airtight lids and maintained at 37 °C. SBF solution was renewed once in two days. Experiments were conducted for the soaking periods 1, 3, 5, 7 and 14 days. After incubation, the gels were washed with water and dried by critical point drying. Thin film X-ray diffraction (TF-XRD) pattern of the gels were recorded on a MAC Science MXP diffractometer using monochromated CuKα radiation at 40 kV and 20 mA. Micro FT-IR spectra was recorded on a Jasco Micro-FTIR Jansen Fourier transform infrared spectrometer by encasing the sample in a transparent KBr matrix. Scanning electron microscopy (SEM) and EDX analyses were performed using a Hitachi S-3000N scanning electron microscope and Horiba EMAX-7000 X-ray micro analyzer.

Microstructure analysis of the gels obtained from the above experiments showed bundles of lengthy collagen fibrils. Gels soaked in C and R-SBF has shown spherulites precipitated along the collagen fibrils. The SEM observation of the gels soaked in C and R-SBF for 1, 3, 5 and 7 days has shown the gradual formation of spherical deposits along the fibrils. The spherical deposits begin to form from day 1 of soaking both in C and R-SBF. Three days soaked samples showed developing of more spherical deposits along the fibrils. With five and seven days of soaking the spherical deposit formation is almost complete. At high magnification, these spherulites were found to contain large

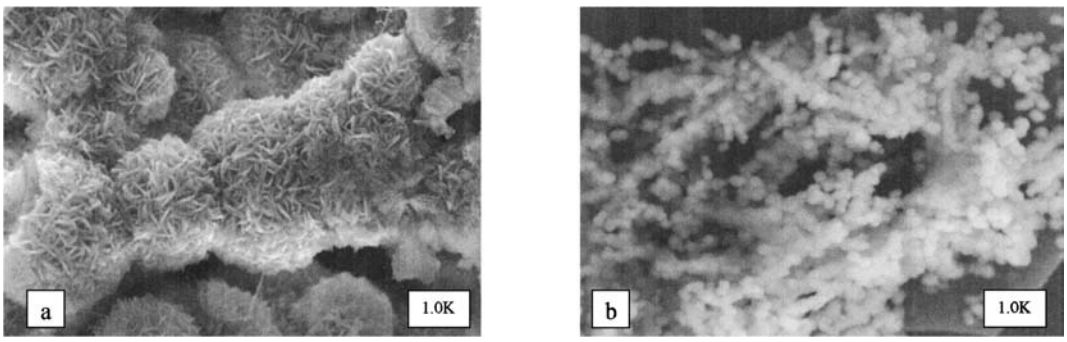

Figure 1. SEM micrograph showing (a) C-SBF soaked sample and (b) R-SBF soaked sample.

number of tiny flakes when soaked in C-SBF but the flakes are not distinct in R-SBF soaked samples which may be due to the larger number of carbonate ions in it (Figure 1).

Figure 2 shows the TF-XRD patterns of the deposits obtained on the soaked gels for two weeks. The broad peak in the range 31 to 34 degrees is due to the 211, 112 and 300 diffractions of apatite[12] and is broader in the case of R-SBF than in C-SBF sample. A non-apatite peak at 27.38 degrees has also appeared. The broadness of the peaks may be due to the low crystallinity or the small crystallite size of the apatite, which may be due to the larger number of $(CO3)^{2-}$ ions in R-SBF.

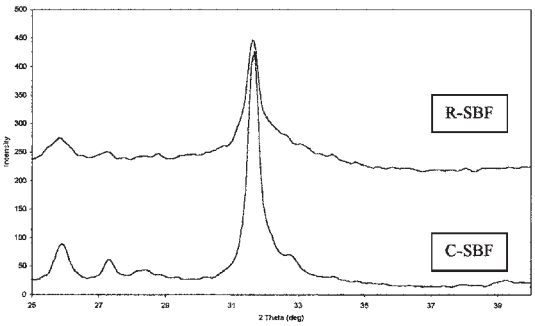

Figure 2. XRD patterns of the deposits obtained on the gels.

Figure 3 shows the FT-IR spectra obtained for the samples soaked in C and R-SBF. The bands at 601 and 564 cm^{-1} are the characteristic of the γ_4 vibrations of the PO_4 group in apatitic structure precipitated from solution.[13] The out of plane mode of $(CO_3)^{2-}$ ion is observed at 875 cm^{-1}. Bands in the region 968–1200 cm^{-1} in C-SBF are due to the γ_3 vibrations of the PO_4 group[14] and it splits into three peaks in R-SBF. The stretching mode of $(CO_3)^{2-}$ is observed around 1428 and 1470 cm^{-1}. The peak around 1670 cm^{-1} arises from the C=O of the peptide bond of the collagen. The carbonate peaks at 875, 1428 and 1470 cm^{-1} suggests that the carbonate substitution has occurred in the B site.[15] Hence the apatite formed on the collagen fibrils is confirmed to be B-type carbonate apatite.

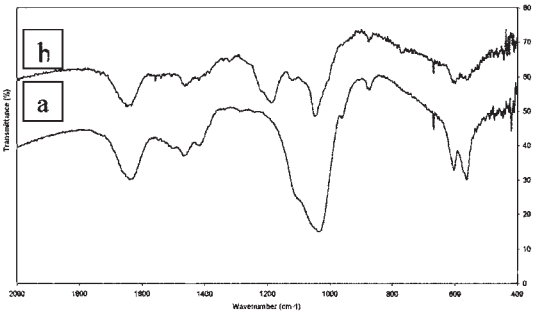

Figure 3. FT-IR spectra of deposits in collagen fibrils when soaked in (a) C-SBF (b) R-SBF.

The Ca/P ratios of the spherical deposits along the collagen fibrils have shown values from 1.68 to 2.38 in the case of C-SBF and 1.68 to 3.41 in the case of R-SBF. The deposits are close to apatite in composition and are heavily enriched in calcium. The excess calcium ions in the deposits can be attributed to calcium carbonate.[16] Analysis of the Cl^- content of the spherical deposits reveals that it is comparatively less in R-SBF samples than in C-SBF samples.

Collagen is known to be less effective in promoting calcium phosphate precipitation. Generally biomimetic investigations are being done using 1.5 × SBF (with ionic concentration equal to 1.5 times than normal SBF). Early investigation of apatite formation on collagen has also been done using 1.5 × SBF. Rhee and Tanaka[17] have reported that presence of 1 mM citric acid in 1.5 × SBF promoted the apatite formation on collagen membranes.

Collagen solutions at 37 °C, neutral pH and at optimum conditions results in the reconstitution of collagen fibrils with a structure identical to that of the collagen fibrils observed in tendons.[18,19] In the present study the collagen fibrils thus formed has precipitated apatite along it from both C-SBF and R-SBF with ionic concentrations equal to that human blood plasma without citric acid. From this it is evident that the collagen fibrils obtained by this gel method has the potential to induce apatite formation by itself when soaked in SBF solution.

From the XRD and FT-IR analyses the precipitates obtained on the collagen fibrils is confirmed to be bone-like carbonated apatite. The low crystallinity of the precipitates may be due to the carbonate substitution in the B site. The microscopic and EDX examination confirmed that the large number of carbonate ions in R-SBF affects the microstructure of the spherical deposits and increases the Ca/P ratio.

In the present study, reconstituted collagen fibrils obtained from collagen gel formed at pH 7.2, temperature 36.5 °C and cross-linked has formed calcium phosphate precipitate from C and R-SBF solutions. Analyses of the precipitates from both the solutions have shown that the precipitates are calcium rich B-type carbonate apatite. The higher carbonate content in R-SBF has influenced the microstructure, crystallinity and calcium content of the precipitates.

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