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# Deposition of bone-like hydroxyapatite on the surface of silk cloth with the aid of immobilized urease

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**Abstract** Continuous layers of hydroxyapatite were deposited on silk cloth from aqueous solutions by using urease as the precipitant supplier. Silk cloth was surface-modified with urease and was immersed in an aqueous solution containing $Ca^{2+}$, $PO_4^{3-}$, and urea. As urea was hydrolyzed to form ammonia with the aid of the immobilized urease, hydroxyapatite precipitated predominantly on the surface of the silk cloth. It took only a few hours to form continuous layers of hydroxyapatite on the silk cloth. The resultant hydroxyapatite was found to be bone-like apatite because it had low crystallinity, contained carbonate ion in the lattice, and had a calcium-deficient composition.

## 1 Introduction

Three-dimensional, porous biocompatible polymers coated with hydroxyapatite are promising materials for orthopedic implants for bone repair or scaffolds for tissue engineering, as the unique biological properties of hydroxyapatite including bone-bonding ability and protein adsorption capability may be utilized. A number of methods to prepare hydroxyapatite coatings have been known. In the so called biomimetic method [1–5], substrates are immersed in the simulated body fluid (SBF) whose ion concentrations are very similar to those of human extracellular fluid or 1.5SBF which has 1.5 times higher ion concentrations than those of the SBF. Although the biomimetic method is well-known and has been used by many researchers, one drawback is that it usually takes quite a long time, such as several days, for hydroxyapatite to precipitate. The processing time may be shortened by ‘‘alternate soaking method’’ in which substrates are alternately soaked in two solutions containing $Ca^{2+}$ and $PO_4^{3-}$ ions, respectively [6, 7]. However, this method may be laboring because it requires continual soaking operation every few hours.

Banks and coworkers [8] deposited hydroxyapatite coatings on collagen tapes by alternately soaking the tapes in the solutions containing calcium *β*-glycerophosphate and alkaline phosphatase, respectively. In this process, as soon as inorganic phosphate ion was produced with the aid of alkaline phosphatase immobilized on the collagen tapes, it reacted with calcium ion to form hydroxyapatite. As the result, hydroxyapatite was precipitated predominantly on the surface of the tapes. In this case, the enzyme determined the rate and location of the supply of the precipitant of hydroxyapatite. Therefore, such enzymes that may promote the formation of the precipitants of certain inorganic precipitates may be regarded as ‘‘precipitant suppliers’’. Making use of enzymes as precipitant suppliers in ceramic processing enables us to control morphology of ceramics and ceramic precursors. Various types of enzymes including urease [9, 10], lipase [11], and glucose oxidase [12] as well as alkaline phosphatase [13, 14] have been proved to be effective for the purpose.

Alkaline phosphatase is active only in a weakly basic media (pH 8.0–9.0). In contrast, urease is active in a wider pH range and more readily available than

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alkaline phosphatase. In the present work, as an attempt to establish a method to form continuous layers of hydroxyapatite on polymer substrates in a short time period, urease was immobilized on silk cloth substrate and hydroxyapatite was precipitated with the enzymatically derived ammonia as the precipitant. The morphology and the composition of the resultant hydroxyapatite were characterized. Silk cloth was used as the substrate because it has been proved that the surface protein of raw silk fiber, sericin, has an ability to induce nucleation of hydroxyapatite in 1.5SBF [15]. Without any pretreatment, it took approximately 7 days for hydroxyapatite to start deposition on silk in 1.5SBF. The present method is compared with the above observation.

## 2 Materials and methods

Silk cloth made from raw silk fiber was supplied by the Kyoto Prefectural Institute for Northern Industry. Specimens were cut from the cloth into 15 × 15 $mm^2$ sheets, and were used without any pretreatments. Water-soluble carbodiimide (WSC, 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride) was purchased from Dojindo Laboratories Co. Urease, 5000 U/g, originated from jack bean and all the other inorganic reagents of analytical grade purities were purchased from Kanto Reagents, Chemicals & Biologicals Inc (Tokyo, Japan).

The immobilization of urease onto the surface of the cloth was conducted in the following manner. The specimens were immersed in a phosphate buffer solution (PBS, pH 5.8) containing 0.052 $mol/dm^3$ of WSC and were kept at 310 K for 2 h to modify the silk surface with carbodiimide. After being rinsed with fresh PBS solutions three times, the specimens were transferred to another PBS solution containing 1.0 $g/dm^3$ of urease and were kept at 310 K for 18 h to immobilize urease.

The urease-modified specimens were attached to glass slides with polyimide tape as schematically shown in Fig. 1 and were immersed in a 100 $cm^3$ of aqueous solution having 0.10 $mol/dm^3$ of $Ca(NO_3)_2$, 0.06 $mol/dm^3$ of $H_2NH_4PO_4$, and 0.11 $mol/dm^3$ of urea, whose pH had been adjusted at 6.0 with an appropriate amount of ammonia, at 310 K for 1, 3, and 6 h. The glass slides were tilted in the beakers so that the surfaces of the specimens face downwards to prevent piling up of powdery precipitate generated in the bulk of the solutions. During the immersion, the hydrolysis of urea was promoted by the urease (Eq. 1) and the resultant ammonia precipitated hydroxyapatite (Eq. 2). It should be noted that the precipitation of hydroxyapatite took place predominantly on the surfaces of the specimens, especially in the beginning stage of the immersion. When the cloth specimens were immersed for longer periods than 6 h, the precipitation occurred not only on the surfaces of the cloth but in the bulk of the solutions.

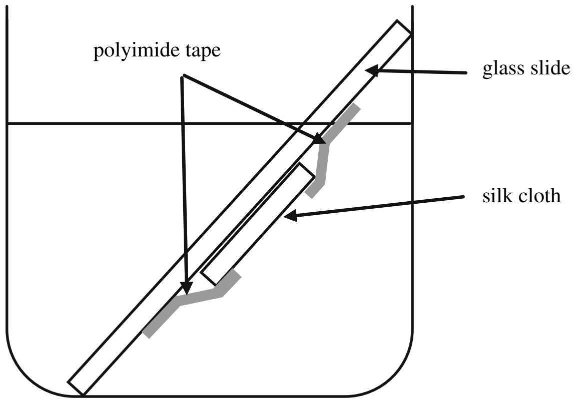


**Fig. 1** Schematic arrangement of the silk cloth substrate in the apatite precipitation experiment

$$H_2N\text{-}CO\text{-}NH_2 + 3H_2O \rightarrow 2NH_4OH + CO_2 \tag{1}$$

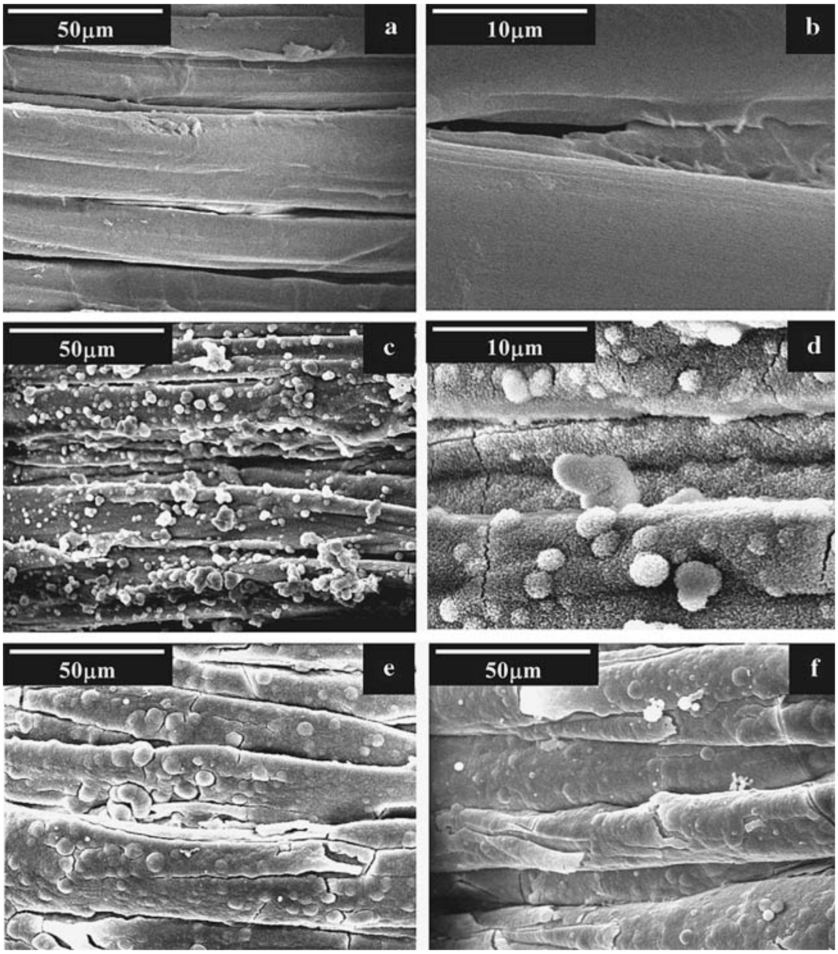
$$10Ca^{2+} + 6PO_4^{3-} + 2OH^- \rightarrow Ca_{10}(PO_4)_6(OH)_2 \tag{2}$$

Specimens were taken out of the solution after immersing for the predetermined time periods, rinsed with deionized water, dried at room temperature, and were subjected to scanning electron microscopic (SEM, JSM6330F, JEOL, Tokyo, Japan) observation. The precipitate was scraped off the substrates and subjected to X-ray diffraction (XRD, Mini Flex, Rigaku Co., Tokyo Japan), Fourier transform infra red (FT-IR, FT-720, Horiba, Kyoto, Japan) spectroscopy, and inductively coupled plasma (ICP, ICPS-7000, Shimadzu, Kyoto, Japan) analysis for Ca/P ratio.

## 3 Results and discussion

Figure 2 shows the SEM photographs of the surfaces of the silk substrates. Within the first one hour of the soaking, spherical particles of precipitate appeared on the surface of the substrate (Fig. 2c). At the same time, the surface had been covered with a very thin layer of precipitate (Fig. 2d). As will be described later, the precipitate was identified to be hydroxyapatite. The thickness of the layer increased with increasing soaking time (Fig. 2e, f). It should be noted that it took quite a

**Fig. 2** Scanning electron micrographs of the surfaces of the silk substrates immersed in the solution for 0 h (**a, b**), 1 h (**c, d**), 3 h (**e**), and 6 h (**f**)



short period of time for hydroxyapatite layers to be formed compared with the biomimetic method. As the layer became too thick, the cloth lost flexibility and the hydroxyapatite layer became brittle and prone to crack upon drying or handling.

Figure 3 shows the XRD profile of the precipitate. Broad diffraction peaks characteristic to hydroxyapatite were observed around 25.6, 31.9, 39.6, 47.6, 49.3, 52.9 degrees. Other phases than hydroxyapatite were not detected. In our preliminary experiments, $CaHPO_4 \cdot 2H_2O$ (DCPD) precipitated from solutions having higher concentrations than the present solution. As long as the initial concentrations of $Ca^{2+}$ and $PO_4^{3-}$ are 0.10 and 0.06 mol/dm$^3$ or lower, respectively, impurity-free hydroxyapatite was obtained. The broadening of the diffraction peaks is commonly observed for hydroxyapatite precipitated from aqueous solutions near room temperature where an increase in the crystallinity may be kinetically hindered.

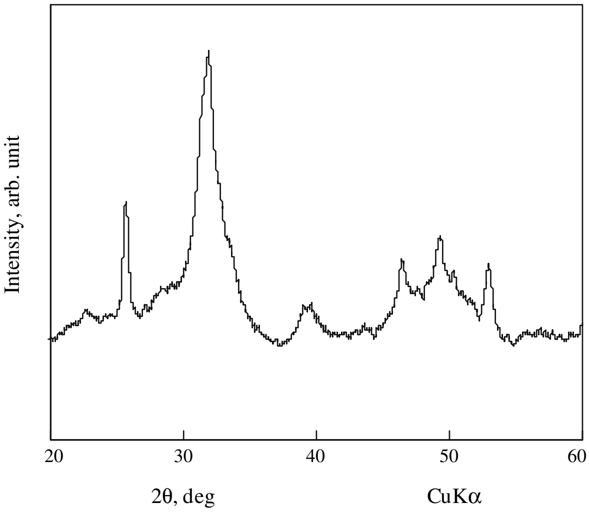


**Fig. 3** X-ray diffraction profile of the precipitate on silk substrate

Figure 4 shows the FT-IR spectra of the present hydroxyapatite powder and commercially available hydroxyapatite powder (HAP-100, Taihei Chemical Industrial, Osaka, Japan). Absorption bands characteristic to $CO_3^{2-}$ were recognized around 1450 cm$^{-1}$ and 880 cm$^{-1}$, respectively. The former is attributed to

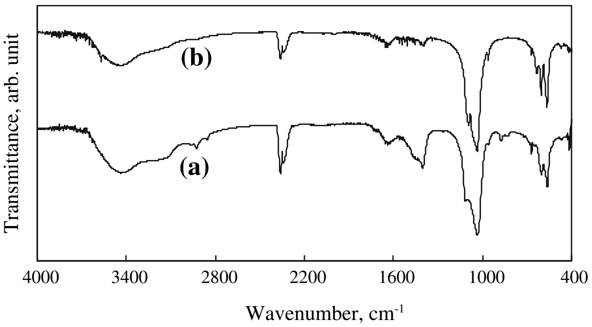


**Fig. 4** FT-IR spectra of (**a**) the present hydroxyapatite powder and (**b**) commercial powder

carbonate ion occupying the phosphate ion site and the latter that in the hydroxide ion site [16]. Therefore, in the present hydroxyapatite, carbonate ion was incorporated in both the phosphate and hydroxide sites. The carbonate ion should have been generated in the enzymatic hydrolysis of urea (Eq. 1).

ICP analysis revealed that the Ca/P ratio in the present hydroxyapatite was 1.59, being slightly lower than that in stoichiometric composition. It is generally accepted that calcium-deficient, carbonate-containing hydroxyapatite with low crystallinity is called ‘‘bone-like’’ apatite and has higher solubility to water than stoichiometric, highly crystalline hydroxyapatite.

The use of urease to prepare continuous layers of bone-like apatite on polymer substrates is advantageous for the following reasons; (1) precipitation of hydroxyapatite takes place near room temperature where the increase in the crystallinity can be kinetically avoided (2) precipitation of hydroxyapatite takes place under carbonate-containing environment because the generation of carbonate ion is accompanied upon hydrolyzing urea (3) precipitation of bone-like apatite occurs predominantly near the surface of the substrates where urease is immobilized, and (4) the present technique is able to form continuous layers of bone-like apatite in much shorter time period than the conventional biomimetic method or alternate soaking method. The present method, therefore, may be applicable to the quick preparation of biomedical implants or ion absorbents. The adherence of the apatite layer to substrates shall be the point to be improved for potential applications in the future.

## 4 Summary

A method has been proposed to precipitate continuous layers of bone-like apatite on the surface of silk cloth in much shorter time period than the conventional methods. Silk cloth made of raw silk fiber was surface-modified with urease and was immersed in an aqueous solution containing $Ca^{2+}$, $PO_4^{3-}$, and urea. As urea was enzymatically hydrolyzed to form ammonia, hydroxyapatite precipitated predominantly on the surface of the silk cloth. It took less than 3 h for continuous layers of hydroxyapatite to be formed. The resultant hydroxyapatite had low crystallinity, calcium-deficient composition and carbonate ion incorporated in both phosphate and hydroxide sites. The advantages of the use of urease as a precipitant supplier in the preparation of hydroxyapatite coatings are that hydroxyapatite is precipitated at room temperature, at a considerably high rate, in carbonate-containing environment, and predominantly in the vicinity of urease.

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