Mechanism of Biomineralization of Apatite on a Sodium Silicate Glass: TEM−EDX Study In Vitro

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The mechanism of biomineralization of apatite on a Na_2O-SiO_2 glass was investigated in vitro, in which the glass surface was surveyed by transmission electron microscopy and energy-dispersive X-ray spectrometry as a function of soaking time in simulated body fluid (SBF), complemented with Fourier transform infrared reflection spectroscopy of the glass surface and atomic emission spectroscopy of the fluid. The glass was found to exchange Na^+ ions with H_3O^+ ions in the SBF to form silanol (Si−OH) groups on its surface at an early stage of soaking. Immediately after they were formed, the Si−OH groups incorporated calcium ions in the fluid to form an amorphous calcium silicate. After a long soaking time, the calcium silicate incorporated phosphate ions and further calcium ions in the fluid to form an amorphous calcium phosphate with a low Ca/P atomic ratio of 1.43. The amorphous calcium phosphate was eventually converted into crystalline apatite, which contained small amounts of Na, Mg, and Cl and had a Ca/P ratio of 1.65, similar to bone mineral. The bonelike apatite grew spontaneously by consuming the remaining calcium and phosphate ions in the fluid. We propose that the initial formation of the calcium silicate is a consequence of an electrostatic interaction of negatively charged $\equiv Si-O^-$ units, formed by dissociation of the Si−OH groups, with the positively charged calcium ions in the fluid. The calcium silicate is postulated to gain a positive charge and interact with the negatively charged phosphate ions in the fluid to form an amorphous calcium phosphate, which then stabilizes into the crystalline apatite.

Introduction

Apatite is the major mineral phase in the human body, which constitutes hard tissue such as bone and dentin. The biomineralization of apatite, which is usually in normal hard tissue, is known to occur also on the surfaces of synthetic calcium phosphates, silicate glasses, and glass ceramics, so-termed bioactive ceramics.[1−6] In fact, the bioactive ceramics spontaneously bond to and integrate with living bone through the apatite layer mineralized on their surfaces.[3−6] This phenomenon has been very useful for developing clinical bone-repairing materials, e.g., Bioglass,[7] sintered hydroxyapatite,[8] and glass-ceramic A-W,[9] but a detailed insight into its mechanism is still unclear. Particularly, the surface chemistry of bioactive silicate glasses involved in the biomineralization of apatite has long been a subject of much scientific and technical interest.

Addressing inorganic surface events on the bioactive ceramics that occur on exposure to body fluid is considered to provide a fundamental way to explain the mechanism of biomineralization of apatite on their surfaces. In this context, a protein-free and acellular simulated body fluid (SBF) with ion concentrations nearly equal to those in human blood plasma has been proposed and extensively confirmed to reproduce apatite formation on bioactive ceramics in vivo.[10−13] In vitro studies have shown that in the SBF the bioactive glasses use surface functional sites composed of silanol (Si−OH)

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groups in inducing mineralization of apatite.[14−17] Much attention has been paid thereafter to the process of apatite formation by the Si−OH groups, which has been explained by nonexperimental models such as molecular orbital calculations.[18−20]

The present authors recently reported that even a simple Na_2O-SiO_2 glass is able to form the Si−OH groups, thereby inducing mineralization of apatite in the SBF.[21] While this glass has a simple composition free of calcium and phosphate, apatite formation on its surface was observed in vitro to be very sluggish,[21,22] which may be highly beneficial to trace out the surface changes involved in apatite formation. On immersion in the SBF, this glass is likely to present a prime in vitro model, giving an experimental background to develop a detailed mechanism of biomineralization of apatite on bioactive glasses. This study presents the pathway of apatite formation on the Na_2O-SiO_2 glass, which was investigated by surveying the glass surface as a function of soaking time in the SBF by transmission electron microscopy (TEM) and energy-dispersive X-ray spectrometry (EDX), complemented with Fourier transform infrared reflection spectroscopy (FT-IRRS) of the glass surface and inductively coupled plasma (ICP) atomic emission spectroscopy of the fluid. The mechanism of biomineralization of apatite on the glass is discussed in terms of electrostatic interaction of the glass surface with ionic species in the SBF.

Experimental Procedure

1. Preparation of the Na_2O-SiO_2 Glass Specimen. A glass with a nominal composition of 20 mol % Na_2O and 80 mol % SiO_2 was prepared by a conventional melting−quenching technique: a powder mixture of reagent-grade chemicals of Na_2CO_3 and SiO_2 (Nacalai Tesque, Inc., Kyoto, Japan) was placed in a platinum crucible, melted in a $MoSi_2$ furnace at 1600 °C for 2 h, poured onto a stainless steel plate, formed into a 1 mm thick glass plate, and then annealed at 500 °C for 2 h. The glass plate was pulverized into powder about 30 μm in size or cut into rectangular specimens $10 \times 10 \times 1$ mm^3 in size and polished with 3−4 μm diameter diamond paste.

2. Immersion of the Glass Specimen in SBF. Either 0.25 g of the glass powder or a single glass substrate was immersed at 36.5 °C in 20 mL of SBF[10] of pH 7.40 and ionic concentrations (142.0 mM Na^+, 5.0 mM K^+, 1.5 mM Mg^{2+}, 2.5 mM Ca^{2+}, 147.8 mM Cl^-, 4.2 mM HCO_3^-, 1.0 mM HPO_4^{2-}, and 0.5 mM SO_4^{2-}) nearly equal to those in human blood plasma. The SBF was prepared by dissolving reagent-grade chemicals of NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$, and Na_2SO_4 (Nacalai Tesque, Inc., Kyoto, Japan) in distilled water and buffering at pH 7.40 at 36.5 °C, using tris(hydroxymethyl)aminomethane ($(CH_2OH)_3CNH_2$) and 1.0 M hydrochloric acid (Nacalai Tesque, Inc., Kyoto, Japan). After soaking for given periods, the glass powder and substrate were removed from the fluid, washed with distilled water, and dried in a clean bench.

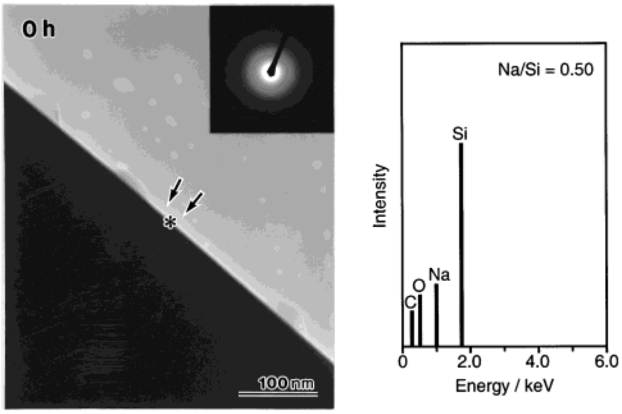

Figure 1. TEM photograph and EDX spectrum of the surface of the Na_2O-SiO_2 glass before soaking in the SBF.

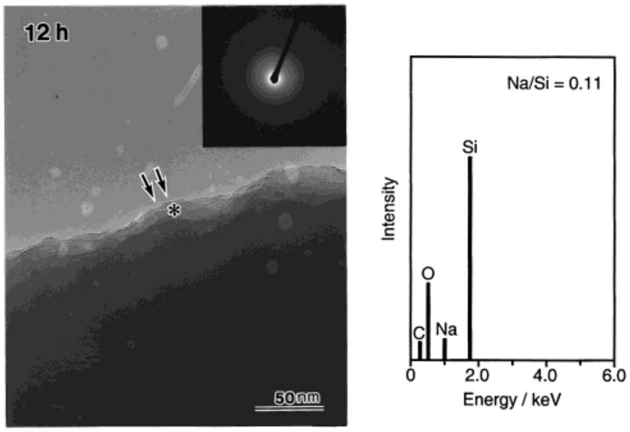

Figure 2. TEM photograph and EDX spectrum of the surface of the Na_2O-SiO_2 glass after soaking in the SBF for 12 h.

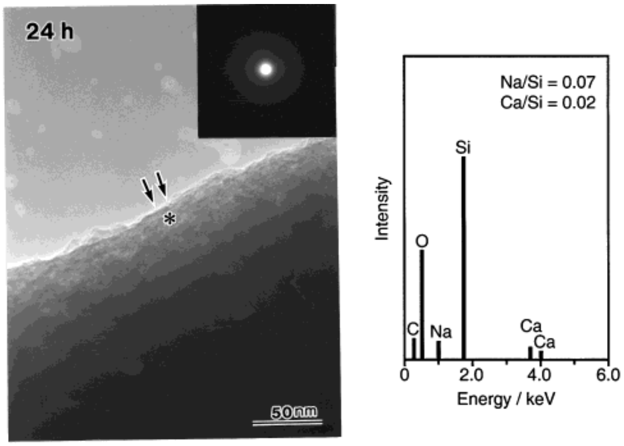

Figure 3. TEM photograph and EDX spectrum of the surface of the Na_2O-SiO_2 glass after soaking in the SBF for 24 h.

3. TEM−EDX and FT-IRRS Analyses of the Glass Specimen and SBF. The surfaces of the glass powder and substrate were subjected respectively to TEM (JEM-2000FX III, JEOL Co., Tokyo, Japan) with an EDX (VOYAGER III, Noran Instruments, Inc., Middletown WI) attachment and FT-IRRS (Magna IR860, Nicolet Instrument Co., Madison, WI). In the TEM−EDX analyses, the glass powder was dispersed in ethanol and deposited onto a poly(vinylformal) film supported by a 200 mesh, 3 mm diameter nylon grid. The EDX results were calibrated using tricalcium phosphate ($Ca_3(PO_4)_2$;

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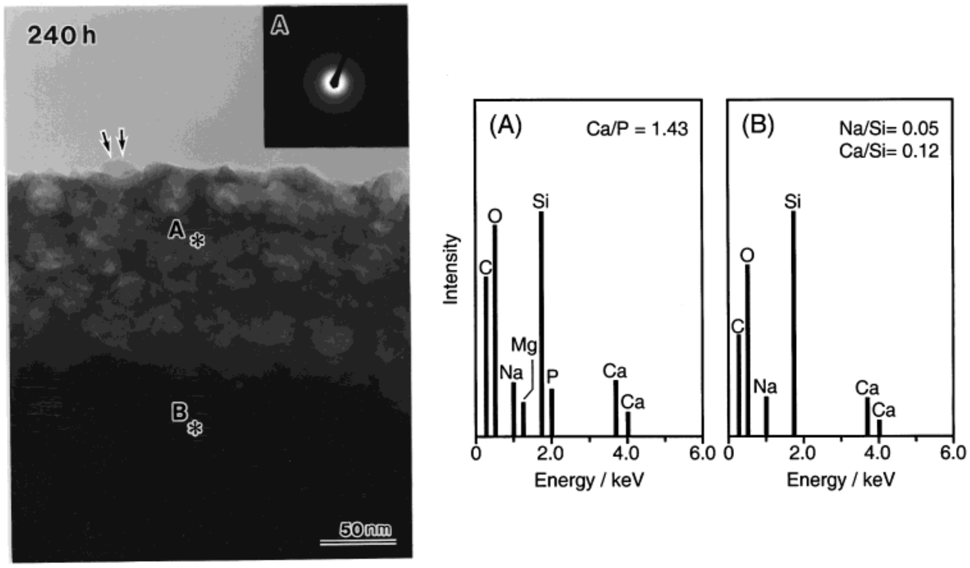

Figure 4. TEM photograph and EDX spectrum of the surface of the Na_2O-SiO_2 glass after soaking in the SBF for 240 h.

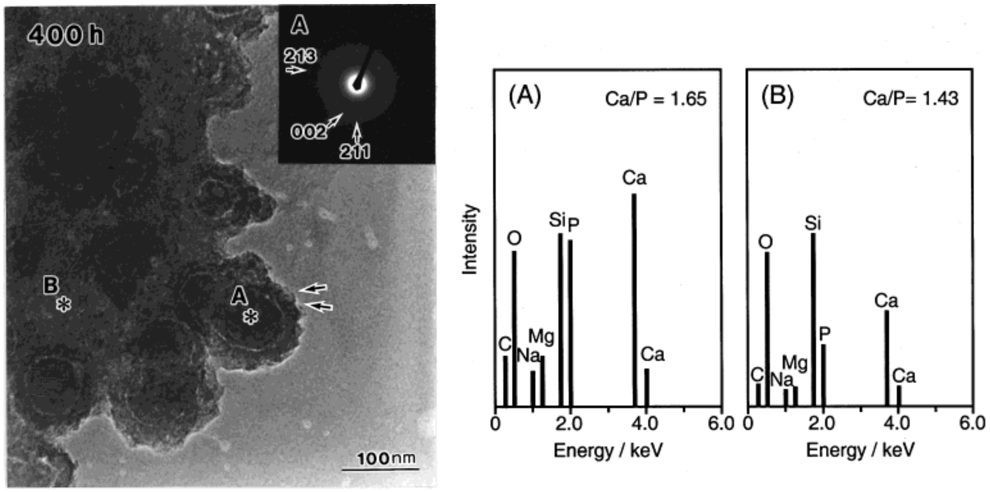

Figure 5. TEM photograph and EDX spectrum of the surface of the Na_2O-SiO_2 glass after soaking in the SBF for 400 h.

Taihei Chemicals, Osaka, Japan) and hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; Taihei Chemicals, Osaka, Japan) as reference samples.

The pH and element concentrations of the SBF before and after soaking the glass substrate were measured using ICP atomic emission spectroscopy (model SPS1500, Seiko Instruments Co., Chiba, Japan) and an electrolyte-type pH meter (model D-14, Horiba, Ltd., Kyoto, Japan).

Results

Figures 1–5 show TEM photographs and EDX spectra of the surface of the Na_2O-SiO_2 glass powder soaked in the SBF for various periods. The TEM observation was performed to present roughly two-dimensional images around the glass surface denoted with arrows, finding sharp edge sides of the glass particles. The asterisks on the TEM images indicate the centers of the electron diffraction and EDX analysis areas, which range over less than 100 nm in diameter. The glass surface before soaking in the SBF (0 h; see Figure 1) showed no specific TEM image nor a diffraction pattern. The EDX detected Na, Si, C, and O, which were attributed to the glass and the polymer film and grid. The Na/Si atomic ratio in the EDX area was 0.50, which was exactly equal to that of the nominal composition of the glass.

After soaking in the SBF for 12 h (see Figure 2), the glass revealed a continuous transparent layer of about 20 nm thick on its surface. Including this transparent product, the glass showed no electron diffraction pattern. The EDX detected the same elements as those before soaking (see Figure 1), where the Na/Si atomic ratio had decreased to 0.11 after this soaking time.

After 24 h (see Figure 3), fine particle-like precipitates were observed on the surface and inside the transparent layer on the glass surface. The electron diffraction around these new products showed no resolvable pattern, where the EDX detected Ca in addition to Na, Si, C, and O. The Na/Si and Ca/Si atomic ratios were 0.07 and 0.02, respectively, showing that the Na/Si atomic ratio had decreased further during this soaking time. After 120 h, the TEM image and EDX elements remained unchanged at the glass surface, where the electron diffraction still showed no resolvable pattern. However, the Na/Si atomic ratio had decreased to 0.04, while the Ca/Si ratio had increased to 0.04.

After 240 h (see Figure 4), a new product with network morphology (A) was observed on the glass

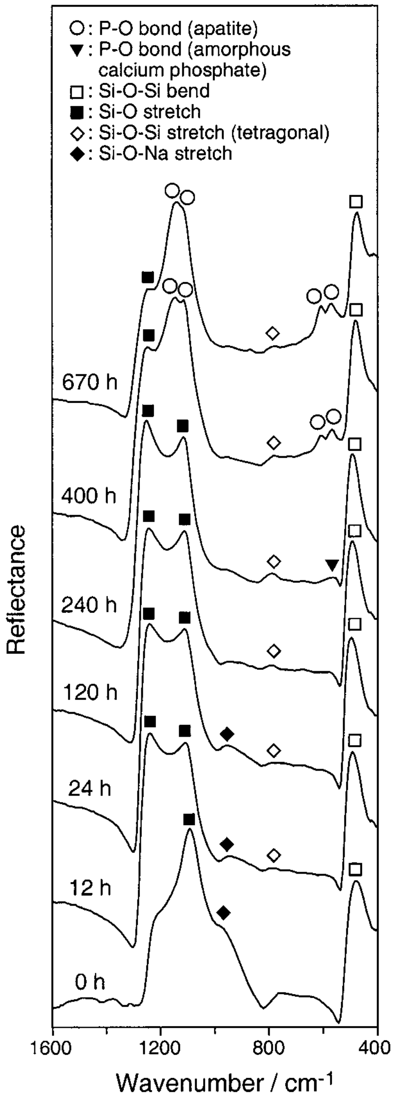

Figure 6. FT-IRRS patterns of the surface of the Na_2O-SiO_2 glass after soaking in the SBF for various periods.

surface (B). The electron diffraction around this new product showed no resolvable pattern, where the EDX detected P and Mg in addition to Ca, Na, Si, C, and O. The Ca/P atomic ratio of this product was 1.43. The EDX detected the same elements in the bulk side (B) as those after 120 h, while the Na/Si and Ca/Si ratios were 0.05 and 0.12, respectively.

After 400 h (see Figure 5), large particle-like products (A) were precipitated inside the network-like products (B) that were observed after 240 h (see Figure 4). This particle-like product (A) showed electron diffraction patterns ascribed to (211), (213), and (002) planes of crystalline apatite. The EDX detected the same elements both in the apatite (A) and in the network-like product (B) as those after 240 h (see Figure 4A). The Ca/P atomic ratio of the apatite (A) was 1.65. After 670 h, these particle-like apatite products increased appreciably in number and size, giving more distinct electron diffraction patterns. The EDX detected newly appearing Cl around the apatite, where the Ca/P atomic ratio was apparently unchanged.

Figure 6 shows the FT-IRRS spectra of the surface of the Na_2O-SiO_2 glass after soaking in the SBF for various periods, in which the IR peaks were assigned in accordance with previous studies.[13-17,21] Up to a soaking time of 12 h, the glass was appreciably deprived of the Si—O—Na stretching peaks around 930 cm^{-1} and revealed new Si—O—Si and Si—O stretching peaks around 800 and 1250 cm^{-1} ascribed to a silica hydrogel abundant in Si—OH groups on the glass surface. After 240 h, the glass revealed new IR peaks for the P—O bond, which may be attributable to an amorphous calcium phosphate. After 400 h, the glass exhibited IR peaks for the P—O bond at 570, 610, 1050, and 1120 cm^{-1} ascribed to the crystalline apatite, whose intensities increased after 670 h.

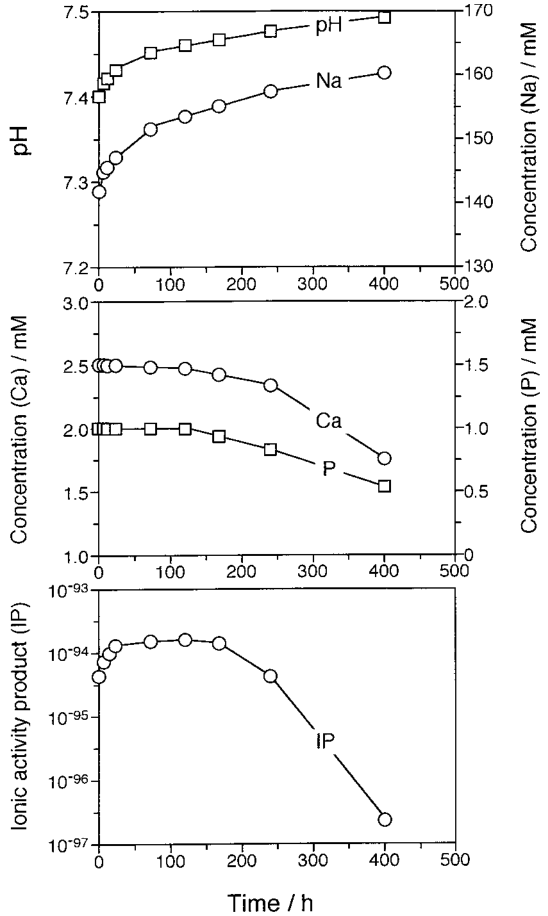

Figure 7. pH, Na, Ca, and P concentrations, and ionic activity product of apatite (IP) of the SBF as a function of soaking time of the Na_2O-SiO_2 glass.

Figure 7 shows the pH, element concentrations, and ionic activity product of apatite (IP) of the fluid as a function of the soaking time of the Na_2O-SiO_2 glass. The IP was calculated from the concentration and pH values at each soaking time point by the method described elsewhere.[14] Immersion of the glass resulted in an appreciable increase in the Na ionic concentration and simultaneously in the pH of the SBF, whereby the IP of the apatite was raised. This effect was pronounced during the early soaking period of up to 120 h. The observed decreases in Ca and P ionic concentrations and the IP in the SBF are because of the formation of apatite on the glass and were viable after 120 h.

Discussion

In a dimensional assumption, the surface area to solution volume ratio of powder glass in TEM—EDX analyses was estimated to be over 80 times higher than that of plate glass in FT-IRRS and ICP analyses, which could affect the rate of apatite formation on a bioactive glass surface. In the current glass model, the immersion time required for the apatite formation appeared to be approximately the same between the powder and plate glasses. Complementing the TEM−EDX results with the FT-IRRS spectra, the transparent phase observed on the Na_2O-SiO_2 glass surface after soaking in the SBF for 12 h is the silica hydrogel abundant in Si−OH groups (see Figures 2 and 6). In this soaking period, the glass released a large amount of Na^+ ions into the SBF and gave rise to an appreciable pH increase in the fluid (see Figures 1, 2, and 7). The TEM−EDX results indicate that the fine particle-like product observed after 24 h (see Figure 3) and 120 h is an amorphous calcium silicate. The FT-IRRS spectra showed that the product with network morphology observed after 240 h is an amorphous calcium phosphate (see Figures 4 and 6). Therefore, it is apparent from the above results that the Na_2O-SiO_2 glass forms apatite on its surface in the SBF via the process described below.

When the glass was immersed in the SBF, it released Na^+ ions into the surrounding fluid via exchange with H_3O^+ ions in the fluid (see Figures 1, 2, and 7). As a result of this ion exchange, the glass formed Si−OH groups on its surface within 12 h (see Figure 6). The Si−OH groups, immediately after they were formed, incorporated the calcium ions in the fluid to form an amorphous calcium silicate within 24 h (see Figure 3). The calcium silicate thereafter incorporated the phosphate ions in the fluid to form an amorphous calcium phosphate with a low Ca/P atomic ratio on its surface as late as 240 h after the immersion (see Figures 4 and 6). The amorphous calcium phosphate increased its Ca/P atomic ratio and crystallized into apatite within 400 h (see Figures 5 and 6). These observations imply that the Si−OH groups formed on the glass surface induce apatite formation not directly but by formation of an amorphous calcium silicate and an amorphous calcium phosphate.

Concerning the induction of apatite formation by the Si−OH groups on the bioactive glasses, Hench et al. in an early study proposed on the basis of a molecular orbital calculation that the Si−OH groups may chelate a calcium phosphate cluster present in the solution to form a Ca−P unit on the glass surface.[18,19] The Ca−P unit was assumed to provide favorable sites for nucleation of hydrated calcium phosphate, which would be the precursor of apatite.[18,19] Hayakawa et al. supported this proposition by a magic-angle-spinning nuclear magnetic resonance study of the Na_2O-SiO_2 glass immersed in the SBF and proposed that the Si−OH groups would be condensed into a $Si(OSi)_3O^-$ unit to combine with a calcium phosphate cluster $(Ca-O-PO_3-Ca)^{\delta+}$.[22] Contrary to these speculations, the present results show that the Si−OH groups do not combine with the calcium and phosphate ions simultaneously.

On the other hand, Karlsson et al. predicted in a computer-aided simulation of bioactive glass surface that the Si−OH groups would interact not with a calcium phosphate cluster but with the calcium ions directly to form calcium silicate chelates which mediate formation of calcium phosphate.[20,23] This speculation is consistent with the present experimental results.

We suppose that formation of an amorphous calcium silicate and an amorphous calcium phosphate is the result of electrostatic interactions between the Si−OH groups on the glass surface and the calcium and phosphate ions in the SBF. The formation of the Si−OH groups via ion exchange is expressed as follows:[1,7,13,21,22,24]

$$\equiv\text{Si}-\text{ONa(glass)} + \text{H}_3\text{O}^+ + \text{OH}^- \rightarrow \equiv\text{Si}-\text{OH(glass)} + \text{H}_2\text{O(glass)} + \text{Na}^+ + \text{OH}^- \tag{1}$$

$$\equiv\text{Si}-\text{O}-\text{Si}\equiv\text{(glass)} + \text{H}_2\text{O(glass)} \rightarrow 2\equiv\text{Si}-\text{OH(glass)} \tag{2}$$

Namely, the Si−OH groups are formed by (1) ion exchange and by (2) hydration of the glass network (≡Si−O−Si≡). The ion exchange (1) accompanies a pH increase, and thereby the IP of apatite, in the fluid (see Figure 7). The pH of the SBF (7.40) is much greater than the isoelectric point of the silica (•2.0)[25] even before the immersion of the glass, which induces a pH increase. The Si−OH groups are therefore postulated to dissociate into negatively charged units.[24,25]

$$\equiv\text{Si}-\text{OH} + \text{OH}^- \bullet \equiv\text{Si}-\text{O}^- + \text{H}_2\text{O} \tag{3}$$

The glass thereby acquires a negative charge that enhances electrostatic interaction with the positively charged calcium ions in the fluid. This reaction results in the formation of an amorphous calcium silicate comprising a neutral or positive charge as follows:[20,23]

$$2(\equiv\text{Si}-\text{O}^-) + \text{Ca}^{2+} \rightarrow (\equiv\text{Si}-\text{O})_2\text{Ca} \tag{4}$$

$$(\equiv\text{Si}-\text{O}^-) + \text{Ca}^{2+} \rightarrow \equiv\text{Si}-\text{OCa}^+ \tag{5}$$

The glass surface is thereby postulated to acquire a positive charge. Interestingly, the glass surface was shown to incubate the calcium silicate for a fairly long period (see Figure 3), during which the EDX results showed that the Ca/Si atomic ratio of the glass surface continued to increase. This implies that the calcium silicate may slowly grow on the glass surface for a long period. In view of the fact that the calcium silicate underlays the calcium phosphate (see Figure 4), it is the calcium silicate that triggered the formation of the amorphous calcium phosphate. The formation of amorphous calcium phosphate is attributed to an electrostatic interaction between the positively charged calcium silicate and the negatively charged phosphate ions in the SBF. The amorphous calcium phosphate was shown thereafter to grow toward the outside of the glass surface by incorporating the calcium and phosphate ions in the SBF (see Figure 4). It contained minor components of Na and Mg and revealed a Ca/P atomic ratio of 1.43 (see Figures 4 and 5). This Ca/P atomic ratio is much lower than that of stoichiometric hydroxyapatite

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($Ca_{10}(PO_4)_6(OH)_2$; 1.67)[26] but in the range (1.31−1.48) of those of other amorphous calcium phosphates produced in aqueous solution.[27]

The amorphous calcium phosphate was shown to eventually crystallize into crystalline apatite (see Figures 5 and 6), which can be explained by the stability of calcium phosphates in aqueous media. Theoretically, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) has a lower solubility in water than any other calcium phosphates; i.e., it is the most stable calcium phosphate in aqueous media.[27] Namely, the amorphous calcium phosphate is a precursor of hydroxyapatite and can be stabilized by being crystallized into hydroxyapatite. The EDX results indicate that the crystallized apatite on the glass contained minor components such as Na, Cl, and Mg and showed the Ca/P atomic ratio to be 1.65 (see Figure 5). These compositional characteristics are the same as those of bone mineral.[26] The crystalline apatite, once formed, spontaneously grows by consuming the calcium and phosphate ions in the SBF, because the fluid is highly supersaturated with respect to the apatite (see Figure 6).[28]

Conclusions

The Na_2O-SiO_2 glass induces mineralization of apatite on its surface in the SBF by forming surface functional groups of Si−OH groups by exchanging the Na^+ ions with the H_3O^+ ions in the fluid. The Si−OH groups on the glass were shown to induce apatite formation not directly but by formation of an amorphous calcium silicate and an amorphous calcium phosphate with a low Ca/P atomic ratio. The amorphous calcium phosphate was thereafter crystallized into apatite, which contained small amounts of Na, Mg, and Cl, had a Ca/P ratio of 1.65, similar to bone mineral, and grew spontaneously by consuming the calcium and phosphate ions in the fluid. The initial formation of the calcium silicate is a consequence of electrostatic interaction of negatively charged $\equiv Si-O^-$ units, which are formed by dissociation of the Si−OH groups, with the positively charged calcium ions in the fluid. The calcium silicate is postulated to gain a positive charge and interact with the negatively charged phosphate ions in the fluid to form an amorphous calcium phosphate.

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