Interfacial Serum Protein Effect on Biological Apatite Growth

Motohiro Tagaya,*‡ Masaki Takeguchi,§ Nobutaka Hanagata, ‡ and Junzo Tanaka†

‡Department of Metallurgy and Ceramics Science, Tokyo Institute of Technology, Tokyo 152-8550, Japan
§Biomaterials Center, National Institute for Materials Science, Ibaraki 305-0047, Japan
†Advanced Nano-Characterization Center, National Institute for Materials Science, Ibaraki 305-0003, Japan

ABSTRACT: Biological apatite (BAp) growth from a simulated body fluid on various substrate surfaces (gold (Au), titanium (Ti), and hydroxyapatite (HAp)) with and without preadsorption of fetal bovine serum protein (FBS) at the initial nucleation stage was investigated using a quartz crystal microbalance with the dissipation (QCM-D) technique. The protein composition in the FBS adlayer and viscoelastic property was elucidated by the Voigt-based viscoelastic model and antibody-binding analysis, respectively. The bare HAp effectively induced the BAp growth to increase the mass to 25.9 ± 3.2 μg cm⁻² in 40 h; however, a slight increased mass of less than 1 μg cm⁻² was detected on the Au and Ti, indicating the superior biocompatibility of the HAp surface. The FBS adsorption on the bare Au and Ti rapidly reached a plateau as compared to that on the HAp, and the adsorbed amount of the FBS on the HAp was approximately 2 times greater than those on the Au and Ti. The FBS adlayer on HAp showed the higher saturated ΔD/Δf values in the ΔD−Δf plots, and the higher elasticity and viscosity were evaluated by the Voigt-based model. These viscoelasticities of the FBS adlayer depending on the surfaces were predominantly attributed to the adsorbed protein species (immunoglobulin (IgG) and albumin) based on the antibody-binding results. Particularly, the FBS adsorbed on HAp significantly inhibited the BAp growth, while that on Au and Ti showed a slight BAp growth. The growth rate on the FBS adlayers depended on the substrate surface, and the rate was in the order of HAp > Ti > Au. The rate on the FBS adsorbed on HAp was 10 times less than that on the bare surface. Although the FBS adlayer clearly inhibits the BAp growth, the relationship between the adsorbed IgG amount and the BAp growth was significantly correlated, indicating that the IgG molecules would promote the growth. It was suggested that the interfacial viscoelastic changes with the BAp growth depended on the substrate surface as well as the adsorbed protein properties. Therefore, the FBS adlayer significantly affected the BAp growth at the interface, indicating the importance of the interfacial protein effect.

1. INTRODUCTION

When biomaterials are implanted in the body, ion and protein adsorption initially occurs on the surfaces whose physicochemical property including the amount and conformation of proteins is of great importance for biocompatibility.1,2 The protein adlayers on the surfaces are subsequently recognized by cells, which affects the attachment, migration, proliferation, and differentiation.3–7 The other important event that occurs in hard tissues is mineralization/calciﬁcation with a complex biological phenomenon.8–10 The direct bone bonding ability is one of the necessary properties for bioactive ceramics.

Biological apatite (BAp) growth from a simulated body fluid (SBF) with similar inorganic components of plasma serum is one of the evaluation methods for bioactive materials.11,12 The growth dependence on the surface hydrophilicity of the hydroxyl and carboxyl groups has been reported; the negatively charged surface can accelerate the formation of the BAp by complexation of the calcium ions.13–16 The roles of the monocomponent proteins, such as fibrinogen (Fng), albumin (Ab), and ﬁbronectin (Fn), against the BAp growth have been investigated on titanium (Ti) and hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂; HAp).17–24 The proteins dispersed in a solution or adsorbed on the surfaces affect the nucleation and subsequent BAp growth by coexistence with calcium phosphate. However, the techniques for detection of the in situ interfacial BAp growth behavior (growth rate, deposition mass, viscoelasticity, etc.) are still controversial, and the effect of adsorbed proteins on the surface activation of bioactive ceramics has not been clarified. Thus, the BAp growth using protein-modified bioactive ceramics should be investigated.

A quartz crystal microbalance (QCM) technique in a liquid has been developed for in situ mass changes as a frequency shift in the nanogram range25,26 and has been applied for monitoring the BAp growth on crushed silica powder,27 a self-assembled monolayer,28 and TiO₂/Ti surfaces. These results suggested...
that the bare gold (Au) and Ti surfaces could not induce the direct BAp growth. The QCM with the dissipation (QCM-D) technique can simultaneously detect the mass changes and viscoelastic property as a dissipation shift, which has been developed by Kasemo et al.28−30 The technique clarified the protein adsorption/desorption,31,38−42,44,46 antigen−antibody reaction,32 lipid bilayer reactions in membrane proteins,33 and cell attachment.34,55 However, the QCM-D technique has not been employed for the BAp growth on bioactive ceramics with/without serum proteins. To comprehensively understand and control the BAp growth mechanism and protein−BAp interactions, in situ monitoring of the interfacial phenomena in a liquid is needed.

HAp is a bioactive and biocompatible ceramic whose protein adsorption property has been widely investigated.35−37 The HAp sensor applicable for the QCM was fabricated in our group by an electrophoretic deposition (EPD) method.38−42 The benefit of the EPD is to apply HAp nanocrystals with less than a 20 nm size by homogeneous deposition on any conductive substrates as a nanolayer. The sensor constructed of HAp nanocrystals has a thickness of approximately 20 nm, a surface roughness of 6.3 ± 1.5 nm, and a contact angle for water of 44.4 ± 2.3°.38,42 The mild protein adsorption on HAp as compared to Ti and Au and the reuse of the surface have been described on the basis of QCM-D results.38,41,56,72 The interfacial phenomena with the cell adhesion on protein-adsorbed HAp nanocrystals have been investigated, indicating a biocompatibility due to the surface properties.44,50,71,73,74 The advantage of the QCM-D technique based on the thin film fabrication is that it allows real-time monitoring of biological apatite growth, which is of interest in the field of bone tissue regeneration.40

In this study, the preadsorption of fetal bovine serum (FBS) and subsequent BAp growth on the substrate surfaces (Au, Ti, and HAp) were investigated by the QCM-D technique using an antibody-binding technique and Voigt-based viscoelastic model. The different BAp growth behavior and interfacial viscoelastic property depending on the preadsorbed proteins were evaluated to clarify and control the interfacial phenomena between the proteins and BAp growth.

2. EXPERIMENTAL SECTION

Materials. Ethanol (99.5 vol%), hydrogen peroxide (H2O2, 30.0 vol%), ammonium (NH4, 28.0 vol%), and hydrochloric acid (HCl) as special grade chemicals were purchased from Wako Chemical Co., Ltd. Sodium chloride (NaCl, 99.5 wt %), sodium hydrogen carbonate (NaHCO3, 99.5 wt %), potassium chloride (KCl, 95.0 wt %), dipotassium hydrogen phosphate (K2HPO4, 3H2O, 99.0 wt %), magnesium chloride (MgCl2·6H2O, 99.0 wt %), calcium chloride (CaCl2, 95.0 wt %), sodium sulfate (Na2SO4, 99.0 wt %), and tris(hydroxymethyl)aminomethane (Tris, 99.9 wt %) as special grade chemicals were supplied by Nacalai Tesque, Inc. FBS (product number, 12603C; lot no., 6D097S) was obtained from SAFEC Bioscience Co., Ltd. The phosphate buffered saline (PBS) tablet was from Dulbecco Co., Ltd. The Au sensor (QXS-301) and Ti sensor (QXS-310) were purchased from Q-Sense, Inc. Serum polyclonal IgG antibody for Ab (Anti-Ab; product number, LLB0002; MW, 150 kDa; origin, bovine; immunized animal, rabbit; purity, 99.9%) were purchased from Life Laboratory, Inc. Serum polyclonal IgG antibody for Fng (Anti-Fng; product number, 3642; MW, 150 kDa; origin, bovine; immunized animal, goat; purity, 99.9%) was purchased from American Diagnostica, Inc.

Preparation of Substrate Surfaces (Au, Ti, and HAp). The Au sensor was cleaned at 70 °C for 10 min by immersing in a 5:1:1 mixture of Milli-Q quality distilled water, H2O2, and NH3, and then dried by a stream of nitrogen gas. The Ti sensor was cleaned at room temperature using 2 wt % of SDS solution, washed with Milli-Q quality distilled water, and then dried by nitrogen gas. The cleaning procedures were completed by UV light (λuv = 185 and 254 nm; UV/OZONE, Bioforce Nanoscience Co., Ltd.) for 10 min in air.

Fabrication of HAp Sensor. The HAp nanocrystals dispersed into ethanol at 1 wt % was prepared as follows. The HAp nanocrystals were synthesized at room temperature (21 °C) by a wet chemical method according to a previous report.38 A dilute H3PO4 solution was added dropwise into a Ca(OH)2 suspension until the pH was 8.0. The obtained suspension was centrifuged at 2000g for 15 min, washed three times with ethanol, and ultrasonically dispersed. The HAp sensor was fabricated by the EPD method on the basis of a previous report.38−42 A direct current voltage at 100 V/cm was applied for 1 min to the cleaned Au sensor as the electrode. The surplus nanocrystals were removed by ultrasonic treatment (28 kHz, 100 W) for 1 min in ethanol. The deposited mass and thicknesses of the HAp layer were measured in air by the QCM-D.

Real-Time Monitoring of FBS Adsorption and BAp Growth by QCM-D. The QCM-D (D300, Q-Sense AB) measurements were performed at 37.5 ± 0.05 °C by monitoring the frequency (Δf (Hz)) and dissipation shifts (ΔD (×10−6)) at 15 MHz. The measured Δf was divided by a harmonic overtone (n = 3) as the fundamental frequency of 5 MHz. The mass change (μg/cm2) by the Δf was calculated using the Sauerbrey equation.25 The viscoelastic property of the adlayer was evaluated by the saturated ΔD/Δf value (ΔDsat/Δf sat) in the ΔD−Δf plots.

Monitoring the adsorption of FBS and the subsequent BAp growth was conducted as follows. The baseline was completely stabilized with PBS for 30−60 min, 10% FBS/PBS was introduced into the sample chamber for 1 h, and the proteins weakly bound to the surface were rinsed with PBS for 10−20 min. The FBS adsorbed on Au, Ti, and HAp were abbreviated FBS-Au, FBS-Ti, and FBS-HAp, respectively. Subsequently, the Tris buffer was applied to the surfaces for stabilizing the baseline for 25 min. 1.5SBF was introduced for 40 h, and the surface was rinsed twice with ultrapure water. 0.5 mL of PBS was introduced into the sensor chamber, and the Δf and ΔD were stabilized for 30−60 min.

Analysis of the antibody bindings to the FBS-modified surfaces was conducted as follows. After the FBS adsorption and PBS rinse, 0.5 mL of the antibody solution in the order of Anti-Ab, Anti-Fn, Anti-Fng, and Anti-IgG was introduced into the chamber.

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The Δf and ΔD curves were measured for 60 min, 0.5 mL of PBS was subsequently introduced into the chamber, and the Δf and ΔD were stabilized for 10 min.

Without the FBS adsorption, the baseline was stabilized using the Tris buffer for 30–60 min, the 1.5SBF was introduced into the sample chamber for 40 h, and the surfaces were rinsed with ultrapure water and dried by flowing nitrogen gas. The experiments were repeated three times.

Evaluation of Interfacial Layer by Voigt-Based Viscoelastic Model. The QCM-D data were simulated using the Q-tools software. The protein adlayer is considered to have some degree of viscoelasticity with the surrounding PBS solution, and the QCM surface—solution interface behaves as a Newtonian fluid. Voinova et al. have reported that the measured Δf and ΔD curves by the QCM-D technique have been fitted to a Voigt-based viscoelastic model to characterize the viscoelastic properties of the adlayers as a Newtonian fluid.57,58 Thus, the viscoelastic property is represented by a complex shear modulus G* given by eq 1.

\[ G^* = G' + iG'' = \mu_{ad} + \frac{i2\pi f \eta_{ad}}{\omega} \]  

where \( G' \) is the real part of \( G^* \), \( G'' \) is the imaginary part of \( G^* \), \( f \) is the oscillation frequency, \( \mu_{ad} \) is the elastic shear modulus, and \( \eta_{ad} \) is the shear viscosity. The Δf and ΔD curves were monitored at 15 MHz and fitted with the Voigt-based viscoelastic model; the changes are fitted by the β function as follows:

\[ \Delta f = \frac{1}{2\pi \epsilon_0 \rho_{q}} \]  

\[ \Delta D = -\frac{1}{2\pi \epsilon_0 \rho_{q}} \]  

where \( \beta \) is represented by eq 4 with \( \alpha, \xi_1, \mu_{ad}, \eta_{ad}, d_{ad}, \) and \( \xi_2 \) is represented by eq 5 with \( \mu_{ad}, \eta_{ad}, \) and \( \rho_{ad} \) as follows:

\[ \beta = \frac{2\pi f \eta_{ad} - \mu_{ad}}{2\pi f} \frac{1 - \alpha \exp(2\xi_1 d_{ad})}{1 + \alpha \exp(2\xi_2 d_{ad})} \]  

\[ \xi_1 = \frac{1}{\sqrt{\frac{(2\pi f)^2 \rho_{ad}}{\mu_{ad} + i2\pi f \eta_{ad}}}} \]  

where \( \alpha \) is represented by eq 6 with \( \xi_1, \xi_2, \mu_{ad}, \eta_{ad}, \) and \( \eta_{f} \). \( \alpha \) is represented by eq 7 with \( \rho_{f} \) and \( \eta_{f} \) as follows:

\[ \alpha = \frac{2\pi f \eta_{ad} - \mu_{ad}}{2\pi f \eta_{f}} \]  

\[ \xi_2 = \frac{1}{\sqrt{\frac{2\pi f \rho_{ad}}{\mu_{ad} + i2\pi f \eta_{ad}}}} \]  

where the viscoelastic parameters, such as \( \mu_{ad}, \eta_{ad}, \) density \( \rho_{ad} \), and thickness \( d_{ad} \), were determined using the density \( \rho_{f} \) and viscosity \( \eta_{f} \) of the bulk liquid. In the case of the FBS adlayers, the values of \( \rho_{f} \) and \( \eta_{f} \) were fixed at 1.000 g cm\(^{-3}\) and 1.000 mPa s, respectively, and \( \rho_{ad} \) was maintained constant at 1.010–1.030 g cm\(^{-3}\). The other parameters were optimized within the following boundaries: \( \mu_{ad} \) of 1 × 10\(^{-10}\) to 1 × 10\(^{-9}\) Pa, \( \eta_{ad} \) of 0.692–100 mPa s, and \( d_{ad} \) of 1 × 10\(^{-9}\) to 1 × 10\(^{-3}\) m. The ratio of \( G' \) and \( G'' \) from eq 1 can be calculated as the loss tangent delta \( \tan \delta = G''/G' \) to evaluate the viscoelasticity of the adlayer as shown in eq 8.

\[ \tan \delta = \frac{G''}{G'} \]  

Characterization of Surfaces. The surface morphology and roughness were measured with a scanning electron microscope (SEM; JEOL-5600 LV, JEOL, Japan) and an atomic force microscope (AFM: SPM-9500, Shimazu Inc.) in a 2 × 2 µm area. A silicon probe mounted on a cantilever (OMCL-AC160TS, OLYMPUS, Inc.) was employed for the dynamic tapping mode. The surface roughness was calculated by the root mean squares (rms) in the z-range images, and the surface area was estimated from the three-dimensional images. On the basis of the rms values, the surface area was calculated in this area.

The surface wettability was analyzed in air by the sessile drop method of distilled water using a contact angle meter (CA-W200, Kyowa Interface Science, Inc.). The droplet was controlled at the volume of 1.5 mL by the dripping nozzle, and the initially attached area on the substrate surfaces was 1.6 mm\(^2\) when approaching the nozzle to the surfaces.

The surfaces after the BAp growth were rinsed with 0.5 mL of ultrapure water, dried by following N\(_2\) gas, and also analyzed by FT-IR spectroscopy (Spectrum GX, Perkin-Elmer, Inc.) with an accumulation of 256 times and resolution of 2.0 cm\(^{-1}\). The IR beam entered the chamber through a polarizer and a KBr window, and the once-reflected beam was detected by a mercury—cadmium—telluride (MCT) detector.

All of the measurements were repeatedly conducted five times to obtain the averaged values. The statistical analysis of all of the measurements was evaluated using the Student’s t test, and “***” denotes a significant difference of \( P < 0.01 \).

3. RESULTS AND DISCUSSION

Substrate Surface Properties. We previously reported the XRD pattern of the nanocrystals synthesized at 21°C.43,56 The characteristic peak at approximately 20 = 32° is attributed to the 211 and 112 reflections of HAp. All of the XRD peaks can be assigned to HAp, indicating the absence of other phases, whereas the large peak width reflects the small size of crystalline domains. With the EPD method, the mass change of the HAp nanocrystals deposited on Au was 4.0 ± 0.2 µg cm\(^{-2}\), and the thickness was calculated to be 12.9 ± 0.5 nm based on the density of HAp of 3.14 g cm\(^{-3}\), indicating the formation of an HAp nanocrystal monolayer.

Figure 1 shows the AFM topographic images and the height profiles for an area of 2 × 2 µm on the Au, Ti, and HAp sensors. The rms values from the line analyses (places are denoted by arrows) and the surface areas measured in these areas were 0.8 ± 0.3 nm and 4.012 ± 0.004 µm\(^2\) for Au, 1.3 ± 0.2 nm and 4.044 ± 0.016 µm\(^2\) for Ti, and 4.1 ± 0.4 nm and 4.224 ± 0.048 µm\(^2\) for HAp in the area of 2 × 2 µm. These values in an area of 5 × 5 µm were measured and were almost the same as those of the 2 × 2 µm. The observed particulate sizes were 35–75 nm for Au, 45–75 nm for Ti, and 20–30 nm × 60–80 nm × 10–20 nm for HAp. The contact angles were 82.5 ± 1.4° for Au, 75.9 ± 1.8° for Ti, and 48.7 ± 2.1° for HAp, indicating the hydrophilicity of the HAp surface as compared to the others. These results were consistent with a previous report,39 and the surface properties of the three types of surfaces were significantly different.
The Δf dramatically increased to $-1446 \pm 181$ Hz at 40 h with a critical viscoelastic change at around 4 h, and the saturated ΔD/Δf value at 40 h was $(-0.06 \pm 0.04) \times 10^{-8}$ Hz$^{-1}$. At several hours in the initial stage, the ΔD/Δf value was clearly different depending on the substrate surface, and the absolute value was on the order of HAp > Ti > Au, indicating the interfacial different viscoelasticity with the formation of nucleation.

Figure 3d,e shows the mass changes and growth rates of BAp on Au, Ti, and HAp versus the reaction times. The mass changes on Au and Ti at 40 h were 0.13 ± 0.02 and 0.56 ± 0.06 μg cm$^{-2}$, respectively, and the growth rate showed a maximum at 0.5 h of which the values were 0.19 ± 0.05 0.21 ± 0.06 μg cm$^{-2}$ h$^{-1}$, respectively, and then gradually decreased. The HAp significantly increased the mass change to 25.9 ± 3.2 μg cm$^{-2}$ at 40 h; the growth rate showed the maximum of 3.4 ± 1.9 μg cm$^{-2}$ h$^{-1}$ at 4 h and decreased to 0.65 ± 0.04 μg cm$^{-2}$ h$^{-1}$ at 40 h. Taking into account the viscoelastic changes, the nucleation behavior at the interface would be changed at 4 h. The thickness of the BAp on HAp calculated by the mass changes was 105 ± 13 nm based on the 3.14 g cm$^{-3}$ density of HAp. Thus, the BAp was significantly grown on the HAp surface, indicating the biocompatibility of HAp.

Figure 4 shows the FT-IR spectra of the BAp growth on HAp and the integral absorbance changes of the phosphate groups, and the SEM and AFM images after the BAp growth on HAp at 40 h. With the increase in the reaction time is the increase in the absorbance bands at 875 cm$^{-1}$ (due to stretching of CO$_3^{2-}$ ions$^{60}$), 960, 1030, and 1060 cm$^{-1}$ (due to stretching of PO$_4^{3-}$ groups), 1120 cm$^{-1}$ (due to stretching of HPO$_4^{2-}$ groups), 1420 and 1465 cm$^{-1}$ (due to symmetric and asymmetric stretching of carbonate ions ($ν_{s/as}$ CO$_3^{2-}$)), and 1650 and 3390 cm$^{-1}$ (due to OH group of the BAp and adsorbed water molecules$^{61}$). The increase in the phosphate groups as shown in Figure 4b significantly indicates the BAp growth on the HAp, and the changes correlated with the mass changes. It is known that the peaks of the phosphate groups split into a number of distinct peaks for the case of HAp having a stoichiometric chemical composition and crystallinity$^{62-64}$. Thus, in this study, there was a specific increase in the band at 1030 cm$^{-1}$ with the increasing reaction time, suggesting bone apatite-like mineral characteristics. The SEM images after the BAp growth on HAp at 40 h exhibited a structure covered with spherical nanoparticles, of which the morphology is coincident with the AFM image in Figure 4c. In the SEM and AFM images, the nongrowth parts of BAp were partially observed. Most substrate surfaces were nonhomogeneously fabricated.
covered with the BAp nanoparticles at an rms value of 4.9 ± 1.2 nm, and the nanostructure is quite different from the petal-shape deposited on a bioactive glass or glass ceramics from the SBF solution. Therefore, the surface chemical, morphological, and viscoelastic changes clearly indicate the characteristic BAp growth based on the biocompatibility.

**FBS Adsorption Behavior on the Bare Substrate Surfaces.**

Figure 5a–c shows the typical time course curves of the Δf and ΔD, and ΔD–Δf plots for the FBS adsorption. The Δf curves indicated that the FBS adsorption on Au and Ti rapidly reached a plateau as compared to that on HAp. The Δf and ΔD values at 60 min were −41.3 ± 2.5 Hz and (+3.5 ± 0.7) × 10⁻⁶ on Au, −61.3 ± 6.1 Hz and (+4.7 ± 1.1) × 10⁻⁶ on Ti, and −85.7 ± 2.9 Hz and (+6.7 ± 1.0) × 10⁻⁶ on HAp, respectively. The FBS adsorption amount on HAp was approximately 2 times greater than those on Au and Ti. The absolute value was in the order of HAp > Ti = Au, indicating that the FBS adlayer on HAp shows a higher value than those on Au and Ti. These results correspond to our previous report on the AFM observation of the FBS adsorbed on Au and HAp, indicating the difference in the viscoelastic properties of the adsorbed FBS depending on the substrate surfaces. With the subsequent PBS rinse, the changed Δf and ΔD values on Au were +5.5 ± 1.3 Hz and (−0.5 ± 0.2) × 10⁻⁶ by PBS, whereas those on Ti and HAp showed no change. With the Tris rinse, the changed Δf and ΔD values were −7.6 ± 1.3 Hz and (−0.8 ± 0.2) × 10⁻⁶ on Au, −5.2 ± 1.6 Hz and (+2.4 ± 1.1) × 10⁻⁶ on Ti, whereas those on HAp did not change. These results indicated that the desorption of the adsorbed FBS proteins or change in the hydration structure on Au easily occurred, whereas the proteins were tightly adsorbed on HAp. Thus, the adsorption behavior and adsorbed state of FBS depending on the substrates were successfully monitored by the QCM-D technique.

Table 1 shows the elastic shear modulus (μ_ad), shear viscosity (η_ad), tan δ, and thickness (d_ad) of the FBS adlayer on Au, Ti, and HAp before the rinses. On the basis of the Voigt-based viscoelastic model, the viscoelastic parameters of the FBS adlayers were determined. The μ_ad and η_ad values on HAp were higher than those on Au and Ti, which would be related to the protein adsorption amount on the surfaces, and the tan δ value on Au indicates the elasticity rather than the viscosity of a flexible structure. The viscoelastic parameters in this study were quite different from those of the albumin (Ab) adlayer on Au, which had a μ_ad of 100 kPa and η_ad of 6 mPa s⁻¹, and are higher than those of the laminin adlayer on Au, which had a μ_ad of 7.6 ± 1.9 kPa and η_ad of 1.83 ± 0.11 mPa s⁻¹ indicating a multicomponent. The Voigt-based thicknesses (d_ad) of the FBS adlayer on HAp were greater than those on Au and Ti. Only a few studies of the ΔD_sat/Δf_sat value in the ΔD–Δf plot from the measured
Δf and ΔD curves have been used for evaluating the protein adsorption behavior and the conformation.65–68 The ΔD sat/Δf sat value can be represented by only d sat and tan δ69 indicating that the higher ΔD sat/Δf sat on HAp would be predominantly attributed to the d sat. Therefore, the different viscoelastic properties of the adlayers depending on the substrate surfaces were successfully evaluated. These viscoelastic parameters were found to be important for investigating the protein structures and could affect the BAp growth behavior.

Figure 6a–c shows the typical Δf and ΔD curves of the FBS adsorption and subsequent binding of the Anti-Ab, Anti-Fn, Anti-Fgn, and Anti-IgG to the FBS-Au, FBS-Ti, and FBS-HAp. There was no nonspecific binding of the antibodies to the bare Au, Ti, and HAp. The antibody binding amounts to the surfaces (Δf sat) showed a positive correlation to the adsorption amount of the proteins.70 After rinsing with PBS, the total binding amount (Δf sat total) of the antibodies to the FBS adlayer on the surfaces was −51.8 ± 4.3 Hz on Au, −73.7 ± 6.2 Hz on Ti, and −88.0 ± 7.5 Hz on HAp in the order of HAp > Ti > Au, which corresponds to the adsorbed amount of the FBS proteins. The binding amounts (Δf Anti-Ab, Δf Anti-Fn, Δf Anti-Fgn, Δf Anti-IgG) were determined and are shown in Table 2. The BSA and IgG are the larger mass fractional protein components in the FBS, whereas the ECM proteins of the Fn and Fgn are relatively very minor components when using the QCM-D technique. The adsorbed amounts of the BSA and IgG were higher, and the relative ratio of the Δf sat to Δf sat (Δf IgG/Δf sat) were significantly different depending on the surfaces, which is 0.31 ± 0.13 on Au, 2.38 ± 0.83 on Ti, and 7.32 ± 1.63 on HAp as shown in Figure 6d. The adlayers on the Au and HAp consisted of the highest percentage of BSA and IgG, respectively, and the major component of IgG would predominantly affect the d sat and ΔD sat/Δf sat values. The adsorption amount of FBS on HAp was about 2 times greater than those on Ti and Au, and the adsorption on Ti and Au rapidly reached a plateau as compared to the HAp as shown in Figure 5a,b. On the basis of the results of the antibody binding, the different adsorption kinetics on Au and Ti would be dominated by the BSA adsorption from FBS, and also correspond to the previous report about the BSA adsorption on Ti and HAp.47 Therefore, the different ratio of the IgG to Ab on the surfaces affects the viscoelastic properties and would be one of the dominant factors for the BAp growth.

BAp Growth Behavior on the Protein-Modified Surfaces.

Figure 7a–c shows the typical time course curves of Δf and ΔD, and ΔD–Δf plots for the subsequent BAp growth on FBS-Au, FBS-Ti, and FBS-HAp. The Δf curves showed that the BAp growth on FBS-Au and FBS-Ti rapidly reached a plateau within the first few hours as compared to that on FBS-HAp of which the Δf continuously decreased with the soaking times. The Δf and ΔD values at 40 h on the FBS preadsorbed surfaces were −7.3 ± 1.6 Hz and (+1.1 ± 0.4) × 10−6 on FBS-Au, −29.4 ± 3.9 Hz and (−3.9 ± 0.9) × 10−6 on FBS-Ti, and −49.7 ± 2.1 Hz and (+3.4 ± 0.7) × 10−6 on FBS-HAp, respectively. The changes in the ΔD–Δf plots indicated that the viscoelastic properties with the BAp growth changed through several stages and the behavior was clearly different depending on the surfaces. On FBS-Au, the ΔD increased and subsequently decreased with the decreasing Δf. On FBS-Ti, the ΔD gradually decreased with the decreasing Δf for 40 h, and some parts showed the circulated shapes of the ΔD–Δf curve. On FBS-HAp, the ΔD decreased without changing the ΔD, and, subsequently, the ΔD increased with the decreasing Δf. The BAp growth behavior on the protein-modified surfaces was different from that on the bare surfaces. For the shorter incubation, the little BAp growth on Au with/without the FBS at approximately 7 Hz and similar growth on Ti with/without the FBS at approximately 30 Hz were monitored, of which the behaviors would be attributed to the hydrophobicity of the bare surfaces. On the contrary,
the growth behavior on the hydrophilic HAp with/without the FBS was apparently different, which would be attributed to the adsorbed protein species. Therefore, the behavior on the protein-modified surfaces significantly depended on the substrate surfaces, indicating the difference in the interfacial phenomena involving ion adsorption and reconstruction on the preadsorbed FBS.

Figure 7d,e shows the changes of the mass and growth rate of the BAp on FBS-Au, FBS-Ti, and FBS-HAp versus the reaction time. The mass changes on FBS-Au, FBS-Ti, and FBS-HAp gradually increased to 0.13 ± 0.03, 0.52 ± 0.07, and 0.88 ± 0.12 μg cm⁻² at 40 h, respectively. The rate on FBS-Au, FBS-Ti, and FBS-HAp showed a maximum at 1 h of which the values were 0.02 ± 0.01, 0.25 ± 0.05, and 0.22 ± 0.01 μg cm⁻² h⁻¹, and then gradually decreased. The mass changes on FBS-Au and FBS-Ti were almost the same as those on Au and Ti, and the decreased rates were clearly different from those on the bare surfaces. Therefore, the growth ability depended on the substrate surface, and, particularly, the viscoelastic property as well as the mass change on FBS-HAp were clearly different from those on the bare HAp. The FBS in the body fluid is known to be effective for preventing pathological calcification.21 It is suggested that

<table>
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<th>Surface</th>
<th>μad (kPa)</th>
<th>ηad (mPa s)</th>
<th>tan δ</th>
<th>dad (nm)</th>
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<td>Au</td>
<td>14.9 ± 6.3</td>
<td>0.76 ± 0.25</td>
<td>1.64 ± 0.15</td>
<td>15.7 ± 3.8</td>
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<tr>
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<td>1.18 ± 0.07</td>
<td>1.21 ± 0.55</td>
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<td>HAp</td>
<td>73.3 ± 2.2</td>
<td>2.89 ± 0.15</td>
<td>1.23 ± 0.12</td>
<td>27.9 ± 2.6</td>
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Table 2. Binding Amount of Anti-Ab (Δf/Δf Anti-Ab), Anti-Fn (Δf/Δf Anti-Fn), Anti-Fgn (Δf/Δf Anti-Fgn), and Anti-IgG (Δf/Δf Anti-IgG) on Au, Ti, and HAp

<table>
<thead>
<tr>
<th>Surface</th>
<th>Δf/Δf Anti-Ab (Hz)</th>
<th>Δf/Δf Anti-Fn (Hz)</th>
<th>Δf/Δf Anti-Fgn (Hz)</th>
<th>Δf/Δf Anti-IgG (Hz)</th>
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<tr>
<td>Au</td>
<td>−30.7 ± 3.4</td>
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<td>−4.2 ± 0.5</td>
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<tr>
<td>Ti</td>
<td>−20.6 ± 2.2</td>
<td>−2.4 ± 0.3</td>
<td>−2.9 ± 0.1</td>
<td>−47.8 ± 6.8</td>
</tr>
<tr>
<td>HAp</td>
<td>−9.7 ± 1.2</td>
<td>−1.9 ± 0.6</td>
<td>−6.7 ± 1.0</td>
<td>−69.7 ± 2.4</td>
</tr>
</tbody>
</table>
FBS proteins would strongly bind to the surface ions of the calcified compounds to suppress the surface activation. Therefore, the protein modification on the HAp effectively suppressed the BAp growth.

Figure 8 shows the FT-IR spectra of the BAp grown on FBS-HAp and AFM image after the BAp growth at 40 h. The spectral changes show the absorbance bands at 1660 and 1550 cm$^{-1}$ attributed to the amide I (C=O stretching) and amide II (in-plane N-H bending), respectively, of the adsorbed FBS proteins. With an increase in the reaction time, the BAp grew as the bone apatite-like mineral on the FBS-HAp due to an increase in the absorbance at the bands mentioned above (875, 960, 1030, 1060, 1120, 1420, 1465, 1650, and 3390 cm$^{-1}$), and there was also a relative increase in the band at 1030 cm$^{-1}$, whereas the absorbance of the bands due to the FBS proteins did not change, suggesting the direct BAp growth on the adsorbed FBS surfaces. The increase in the phosphate groups as shown in Figure 8b was significantly related to the mass changes in Figure 7d. The AFM image revealed that the surface was homogeneously covered with the BAp nanocrystals at a rms value of 4.6 ± 0.6 nm, and the nanostructures are quite different from the BAp on the bare HAp surface. Therefore, the surface chemical, morphological, and viscoelastic changes indicate the characteristic BAp growth on the protein-modified HAp surface.

During the BAp growth process, it is well-known that the initial adsorption of Ca$^{2+}$ and PO$_4^{3-}$ on the surface forms BAp nucleation sites. Liu et al. described that the FBS dispersed into SBF is an effective biological regulator to prevent pathological calcification, and that the FBS induced amorphous calcium phosphate nanoparticles by a coprecipitation. During the BAp growth dominated by the interfacial interaction between the ions and substrate surfaces, two processes, which are the heterogeneous nucleation initiating from the protein surfaces and homoepitaxial growth initiating from the already-formed BAp crystals, are competitive. In this study, the interfacial viscoelastic changes with the BAp growth depended on the substrate surfaces; the viscoelastic property and component (IgG/Ab ratio) of the FBS adlayer due to the substrate surface property effectively affected the BAp growth behavior. The interfacial protein effect during the initial crystallization stage would be attributed to the competitive heterogeneous and homoepitaxial ion reactions at the interface.

The FBS contains polypeptides, hormones, amino acids, glucose, and proteins such as Fgn, BSA, Fn, IgG, etc. The major content of BSA and IgG in the FBS protein is 80–90 wt %. The BSA has a globular morphology with an isoelectric point (pI) at 4.7 and molecular weight (66.5 kDa), and has an asymmetric heart-like structure in which the three main domains are divided.
into six subunit domains. According to the Vroman effect, the BSA in the serum initially adsorbs on the material surface and can be replaced with other proteins. The BSA with many carboxyl groups and imidazole groups induces the effective binding to calcium ions and represents a negatively charged surface due to dissociation of the side chains of acidic amino acids, such as glutamic acid, under the experimental pH condition. Thus, the calcium ion-bound BSA surfaces can be a trigger to form nucleation sites of BAp. Thus, the preadsorption of BSA plays an important role in the subsequent reaction particularly during the initial stage. On the other hand, the IgG, which has a wide pI range and molecular weight (150 kDa), is generally basic. The basic amino acid side chains and acidic amino acid side chains would act as nucleation sites in the local environment even when the overall surface charge of the IgG is positive. In this study, the FBS adlayer inhibits the BAp growth, and the relationship between the adsorbed IgG amount and the BAp growth is significantly correlated, indicating that the IgG molecules effectively promote the growth. Therefore, the different nucleation mechanisms of the BSA and IgG would competitively affect the growth behavior on the protein-modified substrate surface. The BAp growth on the surface-modified Au and Ti has been reported. Takanashi et al. reported that the hydrophilic layers fabricated with a self-assembled monolayer or crushed silica on Au effectively promoted the BAp growth. The preadsorption of Fn on Ti suppresses the BAp growth, whereas the effect of the preadsorption of BSA on the growth is still unclear, that is, inhibition or promotion. In the present study, the viscoelastic changes with the BAp growth clearly depended on the surface properties. The viscoelastic properties of the BAp grown on the FBS-Au and FBS-Ti were different from those of the bare Au and Ti, whereas the mass changes were almost the same. The lower $\Delta D_{\text{sat}}/\Delta f_{\text{sat}}$ values of the FBS on Au and Ti in Figure 5c would be attributed to the BSA adsorption. The increase in the $\Delta D$ with the BAp growth on FBS-Au in Figure 7c indicated that the ions would adsorb on the surface and change the interfacial interaction between the BAp and FBS adlayer. The decrease in the $\Delta D$ with the growth on FBS-Ti in Figure 7c indicates that the ions would replace the adsorbed proteins to form the rigid BAp.

In an in vivo situation, the ECM proteins play key roles in cellular behaviors, such as proliferation, migration, differentiation, and survival that affect the mineralization process. Particularly, the ECM proteins of the Fn and Fgn would have characteristic loose and flexible structures on the HAp. The proteins are the structural glycoproteins in the blood plasma with an isoelectric point of 5.5 and are at a lower concentration as compared to the Ab and IgG. Fn shows a high affinity to the HAp and is perpendicularly adsorbed with the cell-binding site up. Fgn has both negative and positive charges and also shows a high affinity to the HAp along with preserving the perpendicular adsorption state. However, the concentration of the Fn and Fgn in the 10% FBS/DMEM is...
very low in this study, and then the QCM-D revealed the minimum detectable quantity of the proteins. On the other hand, the BAp growth on the surface-modified HAp has also been investigated\textsuperscript{17,24,39} The preadsorption of FBS on HAp effectively forms the BAp nucleation.\textsuperscript{17} The relationship between the BSA adsorption and growth has not been clarified; the preadsorption inhibited\textsuperscript{17} or favored\textsuperscript{24} the BAp growth. The acidic and basic proteins adsorb on the positively charged calcium and negatively charged phosphate sites of HAp, respectively.\textsuperscript{39} The neutral protein of IgG preferentially adsorbs on the surface in the present study, and the IgG would affect the higher \( \mu_{ad} \) and \( \eta_{ad} \) values of the adlayer. The viscoelastic properties affected the higher BAp growth on HAp. The preadsorption of FBS proteins on HAp inhibited the BAp growth, indicating that the FBS would suppress the activation of the crystal growth by decreasing the nucleation sites. The ions in the SBF initially adsorb on the proteins along with preserving the structure and show a constant \( \Delta D \) in Figure 7c, and the subsequent BAp growth induces interfacial rearrangement between the BAp and proteins at around 20 h.

4. CONCLUSIONS

The interfacial BAp growth on the Au, Ti, and HAp with/without the preadsorption of FBS was investigated using the QCM-D technique combined with the Voigt-based viscoelastic model and antibody-binding technique. The \( \Delta D - \Delta f \) plots indicated that the viscoelastic changes with the growth behavior depended on the substrate surfaces, and the different growth behavior depending on the preadsorption of the FBS protein was also successfully monitored. The BAp significantly grew on the bare HAp, whereas a small amount of BAp grew on the bare Au and Ti without/with the preadsorption of FBS. These differences were attributed to the interfacial interactions of the near-surfaces with ions, such as Ca\(^{2+}\) and PO\(_4\)\(^{3-}\), indicating the importance of the substrate surface as well as the states of the preadsorbed FBS proteins. An antibody-binding analysis revealed that the different properties of the BAp adlayers were attributed to the adsorbed protein species (IgG and Ab), and the IgG effectively induced the nucleation as compared to the BSA. Thus, it was suggested that the species of the preadsorption of the FBS proteins played key roles in the viscoelastic property and the BAp growth kinetics at the interface. The real time comparative in situ analysis of the FBS adsorption and subsequent BAp growth exhibited significant benefits for investigating the interfacial protein effect and controlling the BAp growth.

**AUTHOR INFORMATION**

Corresponding Author
Tel.: +81-258-47-9345. E-mail: tagaya@mst.nagaokaut.ac.jp.

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