Competitive adsorption of fibronectin and albumin on hydroxyapatite nanocrystals

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Abstract
Competitive adsorption of two-component solutions containing fibronectin (Fn) and albumin (Ab) on hydroxyapatite (HAp) nanocrystals was analyzed in situ using the quartz crystal microbalance with dissipation (QCM-D) technique. Adsorption of the one-component protein (Fn or Ab) and the two-component proteins adjusted to different molar ratios of Fn to Ab at a fixed Fn concentration was investigated. The frequency shift ($\Delta f$, Hz) and the dissipation energy shift ($\Delta D$) were measured with the QCM-D technique, and the viscoelastic changes of adlayers were evaluated by the saturated $\Delta D/\Delta f$ value and the Voigt-based viscoelastic model. For the adsorption of the one-component protein, the Fn adlayer showed a larger mass and higher viscoelasticity than the Ab adlayer, indicating the higher affinity of Fn on HAp. For the adsorption of the two-component proteins, the viscoelastic properties of the adlayers became elastic with increase in Ab concentration, whereas the adsorption mass was similar to that of Fn in the one-component solution regardless of the Ab concentration. The specific binding mass of the Ab antibody to the adlayers increased with increase in Ab concentration, whereas that of the Fn antibody decreased. Therefore, Fn preferentially adsorbs on HAp and Ab subsequently interacts with the adlayers, indicating that the interfacial viscoelasticity of the adlayers was dominated by the interaction between Fn and Ab.

Keywords: QCM-D, competitive adsorption, hydroxyapatite, viscoelastic property, soft interface

1. Introduction

When biomaterials are implanted into the human body, multiple proteins in the body fluid immediately and competitively adsorb on the surface [1–3]. The protein adlayers on the surfaces determine the biocompatibility. Thus, an investigation of the interfacial protein–material interaction is important for designing superior biocompatible materials. While the adsorption of proteins has been widely studied [4–7], the complex behavior during the multiple-protein adsorption at the interface has not yet been elucidated. Substitution of adsorbed fibrinogen on the surface with other abundant proteins in a serum solution is known as the ‘Vroman effect’ [8]. Several studies characterized the multiple-protein adsorption of albumin (Ab) and immunoglobulin G (IgG) labeled with radioactive $^{125}$I and $^{131}$I on poly(ethylene) terephthalate surfaces [9, 10], and Ab, fibrinogen, and IgG on a poly(styrene) surface from a plasma solution [11]. Grainger et al reported the fibronectin (Fn) conformation adsorbed on a poly(tetrafluoroethylene) surface from the Fn and Ab mixtures using an anti-Fn antibody, indicating the Ab masking on the adsorbed Fn [42]. However, the mechanism of the multiple-protein adsorption on the bioceramics has not been investigated.

Hydroxyapatite ($\text{Ca}_{10}$$($$\text{PO}_4$$)_6$($$\text{OH}$$)_2$; HAp) is a biocompatible ceramic that is used as a bone filling material with collagen [12–15] and drug delivery carriers [16–19]. Its biocompatibility is attributed to its
protein adsorption [20–23]. An HAp sensor has been fabricated by our group using electrophoretic deposition, and the adsorption of single and multiple proteins has been studied using the quartz crystal microbalance with dissipation (QCM-D) technique [24–28, 43, 46]. QCM-D is an excellent in situ technique in the liquid phase, which provides information on the mass and viscoelasticity of the adlayers [29, 30]. The interfacial phenomena during the multi-protein adsorption on the HAp surfaces have not been analyzed in situ by the QCM-D technique.

The two major proteins in serum are Ab and globulin. Ab is the most abundant protein in blood and is known to eliminate cell attachment and block nonspecific binding [31, 32]. On the other hand, Fn, collagen and other subtle proteins (osteopontin, laminin, vitronectin, etc) are obligate adhesive proteins for integrin-receptor-based cell adhesion and spreading on the surfaces. Thus, the ratio of the nonadhesive Ab to the adhesive Fn selectively adsorbed on the HAp surface from a multicomponent solution (e.g. serum) is an important parameter for improving the cell adhesion on the surfaces.

The objective of this study is to in situ analyze the competitive adsorption of proteins from a two-component solution containing Fn and Ab on HAp nanocrystals by the QCM-D technique. The adsorption of the one-component protein (Fn or Ab) or two-component proteins dissolved in phosphate-buffered saline (PBS) was investigated versus the concentrations of Ab in order to elucidate the viscoelastic properties of the Ab–Fn compound adlayers using a Voigt-based viscoelastic model.

2. Experimental details

Bovine serum albumin with the isoelectric point (pI) of 4.7 and molecular weight of 66.5 kDa, ethanol (99.5 vol%), a hydrogen peroxide solution (H2O2: 30 vol%), HCl (special grade) and an ammonia solution (NH3: 25 vol%) were supplied by Wako Chemicals Co. Ltd. Bovine plasma fibronectin (Cat. No. 341631) with the pI of 5.6 and molecular weight of 430 kDa was purchased from Calbiochem Co. Ltd, and PBS was obtained from Dulbecco Co. Ltd. A gold sensor (QXS301, thickness: 100 nm) was purchased from Q-Sense Inc. The antibody of the serum polyclonal IgG antibody for Ab (anti-Ab; product number: LLB0002, M.W.: 150 kDa, origin: bovine, purity: 99.9%) and the antibody of the serum polyclonal IgG antibody for Fn (anti-Fn; product number: LLB0004, M.W.: 150 kDa, origin: bovine, purity: 99.9%) were purchased from Life Laboratory Inc.

Table 1. Fn and Ab concentrations in the initial solution, \( \Delta D_{sat}/\Delta f_{sat} \) in the \( \Delta D–\Delta f \) plots of the adlayers, and \( \Delta f_{anti–Fn} \) and \( \Delta f_{anti–Ab} \) with the antibody binding to the adlayers.

| Name   | Initial concentration | Adlayer | Antibody |
|--------|-----------------------|---------|----------|
|        | Fn (nM) Ab (µM)       | \( \Delta D_{sat}/\Delta f_{sat} (\times 10^{-6} \text{Hz}^{-1}) \) | \( \Delta f_{anti–Fn} (\text{Hz}) \) | \( \Delta f_{anti–Ab} (\text{Hz}) \) |
| Fn     | 200 0                | −30.5 ± 1.3 | −44.3 ± 3.2 | – |
| Ab/Fn  = 0.1 | 200 0.02            | −14.5 ± 1.9 | −17.5 ± 2.1 | −1.1 ± 0.3 |
| Ab/Fn  = 1 | 200 0.2             | −9.0 ± 2.1  | −12.4 ± 2.2 | −2.1 ± 0.9 |
| Ab/Fn  = 10 | 200 2               | −6.6 ± 1.4  | −7.2 ± 1.9  | −6.4 ± 2.2 |
| Ab/Fn  = 100 | 200 20              | −6.3 ± 1.6  | −6.3 ± 1.6  | −28.1 ± 3.1 |
| Ab     | 0 20                | −4.5 ± 2.2  | –          | 52.1 ± 2.9 |

The HAp nanocrystals were synthesized at 21 °C by wet chemical method [33, 43]. A dilute H3PO4 solution was added dropwise into a Ca(OH)2 suspension until reaching the pH of 8.0 to precipitate the nanocrystals. The HAp nanocrystal sensor was fabricated by electrophoretic deposition according to previous reports [24, 27]. The HAp suspension was centrifuged at 2000 g for 15 min, washed three times with ethanol, and ultrasonically dispersed in ethanol at 1 wt%. Before the deposition, the gold surface of the sensor was cleaned by immersing it in the APM solution (a 5:1:1 mixture by weight of Milli-Q-quality distilled water, H2O2, and NH3) for 10 min at 70 °C and then dried by blowing with N2 gas. The cleaned surface was irradiated with UV light (\( \lambda_{air} = 254 \) and 185 nm, Bioforce Nanoscience Co. Ltd) for 10 min in air. A dc voltage was applied at 100 V cm\(^{-1}\) for 1 min. According to previous report [27], inhomogeneous deposition with partly exposed gold surfaces was observed at a dc bias of 10 or 50 V cm\(^{-1}\), and the 100 V cm\(^{-1}\) dc bias ensured a homogeneous coverage of the surface with HAp. The surplus nanocrystals were removed by a 1 min of ultrasonic treatment (28 kHz, 100 W) in ethanol. The weight and thickness of the deposited HAp layer were measured by the QCM-D technique in air.

To prepare the one-component solution, Ab at concentrations of 2.0, 7.5, 10 and 20 µM and Fn at concentrations of 50, 100, 200 and 450 nM were dissolved in PBS. The two-component solution was prepared as follows: Ab was dissolved at concentrations of 0.02, 0.2, 2 and 20 µM in a 200 nM Fn solution, and the molar ratios of Ab to Fn were 0.1, 1, 10 and 100, respectively, as shown in table 1. Anti-Ab and anti-Fn antibodies were dissolved in PBS at a concentration of 50 µg ml\(^{-1}\).

QCM-D (D300, Q-Sense Co. Ltd) measurements were performed by monitoring the frequency shift (\( \Delta f \), Hz) and dissipation shift (\( \Delta D \)) at 15 MHz. The temperature was kept at 37.00 ± 0.05 °C. A PBS amount of 0.5 ml was introduced into the sensor chamber and the \( \Delta f \) and \( \Delta D \) readings were stabilized for 30–60 min. Then, 0.5 ml of the one- or two-component protein solution was introduced and the 100 V cm\(^{-1}\) dc bias ensured a homogeneous coverage of the surface with HAp. The surplus nanocrystals were removed by a 1 min of ultrasonic treatment (28 kHz, 100 W) in ethanol. The weight and thickness of the deposited HAp layer were measured by the QCM-D technique in air.
change due to adsorption was calculated with the Sauerbrey equation [34]:

\[ \Delta m = -c \times \Delta f_{n3}/3, \quad (1) \]

where \( c \) is a constant equal to 17.7 ng Hz\(^{-1}\) cm\(^{-2}\). The adsorption amount at the equilibrium state \( (W) \) was calculated with equation (1), and the adsorption isotherms of Ab and Fn were obtained. On the basis of the Langmuir adsorption isotherm formula, the equation of state for the one-component adsorption can be represented as

\[ \frac{C}{W} = \frac{W_{\max}}{K_{eq}} + \frac{C}{W_{\max}}, \quad (2) \]

\( C, K_{eq} \), and \( W_{\max} \) are the protein concentration in the equilibrium state, the adsorption equilibrium constant and the maximum adsorption amount, respectively. The \( W_{\max} \) and \( K_{eq} \) values were determined from the slope of a \( C/W \) versus \( C \) plot based on the Langmuir equation, and the Langmuir-type monolayer adsorption was evaluated using the correlation coefficient.

The viscoelastic properties of the adsorbed proteins were evaluated using the time-saturated \( \Delta D/\Delta f \) value (\( \Delta D_{sat}/\Delta f_{sat} \)) in the \( \Delta D – \Delta f \) plot. The \( \Delta D_{sat}/\Delta f_{sat} \) values from the measured \( \Delta f \) and \( \Delta D \) curves have been used to evaluate the conformational adsorption state [24, 44, 45]. These values correspond to the energy dissipation per unit adsorption mass, characterizing the viscoelasticity of the adlayer.

The QCM-D data were simulated using the Q-tools software. The \( \Delta f \) and \( \Delta D \) curves were fitted with the Voigt-based viscoelastic model to characterize the adlayers as a Newtonian fluid [35, 36]. The viscoelasticity is represented by a complex shear modulus \( G^* \) given as

\[ G^* = G' + iG'' = \mu + i2\pi f \eta, \quad (3) \]

\( G' \) and \( G'' \) are the real and imaginary parts of \( G^* \), respectively, \( f \) is the oscillation frequency, \( \mu \) is the elastic shear modulus and \( \eta \) is the shear viscosity. The viscoelastic parameters \( \mu \) and \( \eta \), as well as density (\( \rho \)) and thickness (\( d \)), were determined using the density (\( \rho_l \)) and viscosity (\( \eta_l \)) of the bulk liquid.

The values of \( \rho_l \) and \( \eta_l \) were fixed at 1 g cm\(^{-3}\) and 1 mPa s, respectively, and \( \rho \) was kept constant at 1.010–1.030 g cm\(^{-3}\) according to previous report [47]. The ratio of \( G'' \) and \( G' \) was calculated as the loss tangent (\( \tan \delta \)).

The crystalline phase of the nanocrystals was identified by powder x-ray diffraction (XRD: Ultima-III, Rigaku, CuK\( \alpha \) radiation, \( \lambda = 1.5418 \) Å, \( V = 40 \) kV). The diffraction patterns were collected at room temperature in the 2\( \theta \) range from 5\(^\circ\) to 60\(^\circ\). The surface morphology and roughness after the treatments were measured by atomic force microscopy (AFM; SPM-9500; Shimadzu Inc.) in an area of 1 \( \times \) 1 \( \mu \)m\(^2\). A silicon cantilever (Olympus, OMCL-AC160TS) was used in the dynamic mode. The roughness was calculated as the root-mean-square value.

3. Results and discussion

The XRD pattern of the HAp nanocrystals synthesized at 21\(^\circ\)C has been reported [33, 43]. The characteristic peak at 2\( \theta = 32^\circ \) is attributed to the 211 and 112 reflections of the synthesized HAp. All the XRD peaks can be assigned to HAp, indicating the absence of other phases, and the large peak width reflects the small size of the crystalline domains. Figure 1 shows the AFM topographic images of the gold and HAp sensors. The weight change in the HAp nanocrystals was measured as 4.0 \( \pm \) 0.2 \( \mu \)g cm\(^{-3}\) by the QCM-D technique, and the thickness was calculated to as 12.9 \( \pm \) 0.5 nm taking the density of HAp as 3.14 g cm\(^{-3}\). In our previous report, the HAp nanocrystals with different crystallinities synthesized at 4, 24 and 80\(^\circ\)C were deposited on gold [27], resulting in HAp nanolayers of 10–20 nm thickness as deduced by ellipsometry. The HAp surface deposited on the gold sensor had the root-mean-square roughness of 4.2 \( \pm \) 0.8 nm, which was slightly larger than that on the gold surface (0.8 \( \pm \) 0.4 nm). The measured surface areas in a 1 \( \times \) 1 \( \mu \)m\(^2\) AFM window were 4.012 \( \pm \) 0.004 \( \mu \)m\(^2\) for gold and 4.224 \( \pm \) 0.048 \( \mu \)m\(^2\) for HAp, and the respective contact angles were 82.5 \( \pm \) 1.4\(^\circ\) and 48.7 \( \pm \) 2.1\(^\circ\), indicating a hydrophilic HAp surface. Thus, a HAp sensor with a nanoscale thickness was successfully fabricated by electrophoretic deposition.
Figures 2(a) and (c) show the $\Delta D - \Delta f$ plots corresponding to the one-component adsorption on HAp at different concentrations. The adsorption amount and $\Delta D_{sat}/\Delta f_{sat}$ value of Ab on the HAp slightly increased with the increase in the initial concentration. The $\Delta D - \Delta f$ plots are linear for Ab and the $\Delta D_{sat}/\Delta f_{sat}$ values match those in our previous report [27]. These values provide information on the viscoelasticity of the adsorption layer [37], suggesting a monomolecular adsorption irrespective of the crystallinity of HAp. In contrast, the adsorption amount and $\Delta D_{sat}/\Delta f_{sat}$ value ($\Delta D_{sat}/\Delta f_{sat} = -30.3 \pm 2.8 \times 10^{-8}$ Hz at a concentration of 450 nM) of Fn were much larger than those of Ab ($\Delta D_{sat}/\Delta f_{sat} = -4.5 \pm 2.2 \times 10^{-8}$ Hz at a concentration of 20 $\mu$M), indicating the higher molecular weight and affinity of Fn. The $\Delta D - \Delta f$ plots of Fn show two-step changes, indicating conformational changes during the Fn adsorption, according to a previous report [38]. Therefore, the adsorption amount and viscoelasticity of Fn were observed to be higher than those of Ab.

The different adsorption behaviors of Ab and Fn with almost the same pI are attributed to their different secondary structures of adsorption models such 'side-on' and 'end-on' [24, 27]. Ab has an asymmetric heart-like structure in which three main domains are divided into six subunit domains and the dissociated carboxyl and imidazole groups; these groups strongly interact with the positively charged calcium ions of the HAp surface. The low adsorption amount of Ab indicates that the adsorption model could be 'side-on' of the tightly adsorbed state [27], which would prevent the protein-specific binding and cell adhesion. The adsorption behavior of Fn on HAp has been described as follows [38]. The monoclonal antibody is more accessible to the cell-binding domain of the Fn adsorbed on HA as compared with that adsorbed on gold. Therefore, Fn was...
adsorbed on HAp with a loose structure, which would accelerate the cell adhesion.

Figures 2(b) and (d) show the adsorption isotherms and linear plots of the one-component adsorption on HAp. The adsorption isotherm types of Ab and Fn are C and L, respectively. The adsorption isotherms were adapted to linear plots using the Langmuir equation, and the correlation coefficients are \( R = 0.992 \) and \( R = 0.999 \) for Ab and Fn, respectively, indicating the monolayer adsorption of Ab and Fn on the HAp. The \( K_{eq} \) in the linear plot are \( 2.3 \times 10^3 \) M\(^{-1}\) for Ab and \( 9.7 \times 10^4 \) M\(^{-1}\) for Fn, indicating the higher affinity of Fn to HAp, and the \( W_{max} \) values are 378 and 1955 ng cm\(^{-2}\) for Ab and Fn, respectively. Sano et al studied the adsorption of ferritin on titanium by the QCM-D technique and deduced the \( K_{eq} \) value as \( 2.6 \times 10^8 \) M\(^{-1}\) [39], which is larger than our values for Ab and Fn on HAp. Therefore, Fn clearly showed a high affinity and viscous adsorption structure on HAp, which would provide the biocompatibility to the HAp surface.

Figure 3 shows the \( \Delta D - \Delta f \) plots of the adsorption from the two-component solution at different molar ratios. Regardless of the Ab concentration, the \( \Delta f \) value at 60 min is similar to that for the one-component solution of the same Fn concentration, which could be attributed to the high affinity of Fn to HAp. With an increase in the Ab concentration, the \( \Delta D_{sat} / \Delta f_{sat} \) value decreased from \(-14.5 \pm 1.9 \times 10^{-8}\) at \( Ab/Fn = 0.1 \) to \(-6.3 \pm 1.6 \times 10^{-8}\) at \( Ab/Fn = 100 \), similar to the one-component Ab solution (table 1), indicating that the viscoelastic behavior of the An–Fn compound adlayer changed to elastic with increasing Ab concentration. Thus, in the adlayer formation process, an increase in the Ab concentration would strengthen the interaction between Ab and Fn, resulting in the elastic behavior.

Figure 4 shows the elastic shear modulus (\( \mu \)), shear viscosity (\( \eta \)), \( \tan \delta \) and thickness (\( d \)) of the two-component adlayers after 60 min of adsorption versus the different Ab/Fn ratios in the solution. The viscoelastic parameters of the Fn adlayer were determined as \( \mu = 15.2 \pm 1.5 \) kPa, \( \eta = 1.8 \pm 0.1 \) mPa s, \( \tan \delta = 4.2 \pm 1.1 \) and \( d = 41.3 \pm 13.0 \) nm. The corresponding values for the Ab adlayer on gold were reported as \( \mu = 100 \) kPa, \( \eta = 6 \) mPa s and \( \tan \delta = 1.8 \) [40], indicating the higher \( \tan \delta \) value for the Fn adlayer on HAp. As to the Ab adlayer, its parameters are \( \mu = 26.1 \pm 3.1 \) kPa, \( \eta = 1.1 \pm 0.2 \) mPa s, \( d = 3.8 \pm 1.0 \) nm and \( \tan \delta = 0.2 \pm 0.1 \). The corresponding values for the laminin adlayer on gold were reported as \( \mu = 7.6 \pm 1.9 \) kPa, \( \eta = 1.83 \pm 0.11 \) mPa s and \( \tan \delta = 7.6 \) [41], indicating the lower \( \tan \delta \) value of the Ab adlayer on HAp. As compared with the gold surface, the HAp surface would induce a rigid structure for Ab and a flexible structure for Fn. The \( \mu \) and \( \eta \) values slightly increased with an increase in the Ab concentration, whereas the \( d \) and \( \tan \delta \) values slightly decreased. It has been reported that \( \Delta D / \Delta f \) can be represented by the \( d \) and \( \tan \delta \) values [48], and thus, the viscoelastic properties can be monitored through \( d \) and \( \tan \delta \) or the \( \Delta D_{sat} / \Delta f_{sat} \) values. It is speculated that Fn interacts...
with Ab during the adsorption to form the elastic structure of the two-component adlayer.

Figure 5 shows the $\Delta f$ and $\Delta D$ curves of the adsorption from the two-component solution and the subsequent binding of anti-Fn and anti-Ab antibodies to the surface. There were no specific bindings of anti-Ab and anti-Fn antibodies to the HAp surface, of anti-Fn to the adsorbed Ab and of anti-Ab to the adsorbed Fn. The binding amount of anti-Fn ($\Delta f_{\text{anti-Fn}}$) to Fn (amount: 1341.2 ± 175.4 ng cm$^{-2}$) was 784.2 ± 56.6 ng cm$^{-2}$, and that of anti-Ab ($\Delta f_{\text{anti-Ab}}$) to Ab (amount: 321.0 ± 34.3 ng cm$^{-2}$) was 922.1 ± 51.3 ng cm$^{-2}$. The relationship between the protein adsorption amount on the HAp surface and the antibody binding amount to the protein showed a significant positive correlation. The $\Delta f_{\text{anti-Fn}}$ value for the two-component adlayer decreased with increasing Ab concentration, whereas $\Delta f_{\text{anti-Ab}}$ increased, as shown in Table 1. The molar ratios of $\Delta f_{\text{anti-Ab}}$ to $\Delta f_{\text{anti-Fn}}$ for the two-component adlayer are 0.03 ± 0.05, 0.20 ± 0.11, 1.14 ± 0.09 and 4.49 ± 0.06 for Ab/Fn = 0.1, Ab/Fn = 1, Ab/Fn = 10 and Ab/Fn = 100, respectively, which would correspond to the Ab/Fn molar ratio in the antibody-accessible surface. It is speculated that the binding of anti-Fn to the Fn adlayers was progressively hindered by the Fn–Ab interaction with increasing amount of adsorbed Ab.

Grainger et al. investigated the conformation of the Fn adsorbed on a poly(tetrafluoroethylene) surface from a mixture of Ab and Fn, and found that the binding of anti-Fn to the two-component adlayer was suppressed by the Ab presence, indicating the Ab-masking on the adsorbed Fn surface [42]. In this study, Ab and Fn adsorb in a complex fashion on the HAp surface, as judged from the $\Delta D$–$\Delta f$ plots, and subsequently, Ab interacts with Fn because of the interfacial changes in elasticity with the two-component adsorption. Vroman et al. described the exchange adsorption of the proteins as follows: the adsorbed fibrinogen was progressively replaced by the other serum proteins, indicating the importance of the molecular weight of the protein [8]. In this study, no exchange adsorption was detected in the...
ΔD–Δf plot and by the Voigt-based viscoelastic analysis; however, the viscoelastic properties of the two-component adlayers were significantly different from those of the one-component adlayers. It is speculated that the two different proteins simultaneously adsorbed on the HAp surface in a complex fashion. At an Ab/Fn ratio of 100, which is similar to that in the serum protein solution generally used in cell culture (e.g. 10 vol% fetal bovine serum), the property of the adlayer in the cell culture was successfully clarified. These interfacial changes would significantly affect the cell adhesion.

4. Conclusions

The competitive adsorption of Fn and Ab proteins from their solutions on the HAp nanocrystals was analyzed in situ using the QCM-D technique. During the adsorption from a one-component solution, the Fn adlayer showed a larger mass and higher viscoelasticity than the Ab adlayer. During the adsorption from a two-component solution, the adlayer properties changed from viscoelastic to elastic with increasing concentration of Ab in the solution, indicating an increasing interaction between Ab and Fn. Regardless of the Ab concentration, the adsorbed mass was similar to the case of Fn in the one-component solution, and the tan δ and d values decreased with increasing Ab concentration. The specific binding mass of anti-Aβ to the two-component adlayers increased with increasing Ab concentration, whereas that of anti-Fn decreased, indicating that Ab was preferentially adsorbed on the upper surface in the two-component adlayers. Therefore, the interfacial viscoelastic properties under the condition of competitive protein adsorption were successfully elucidated, and the revealed phenomena will affect the cell adhesion behavior.

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