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FOCUS REVIEW

In situ QCM-D study of nano-bio interfaces with enhanced biocompatibility

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When biomaterials are implanted into an animal body, the body fluid proteins initially adsorb and then cells recognize these surfaces. Adherent cell functions respond differently to diverse biomaterial surfaces with different properties. Thus, an understanding of cellular responses to biomaterials is crucial for effective control of biomaterial – cell interactions. I have researched how to clarify interfacial phenomena via protein adsorption and subsequent cell adhesion to hydroxyapatite nanocrystals using a quartz crystal microbalance with a dissipation technique. In this review, I focused on the current understanding of enhanced biocompatibility by exploring the roles of protein mediation at the interface. The most promising nano-bio interfaces are explained, and different protein adsorption and cell adhesion processes are highlighted depending on their interfacial states. This approach will clarify several ambiguities of interfacial phenomena between biomaterials and cells and will help in the design of novel biomaterials that can be implanted.
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INTRODUCTION

Reflecting the broad scope and rapid development of biomaterial sciences, dozens of papers have recently been published in this field. Although biomaterials include many types of materials such as metals, ceramics, polymers and inorganic–organic composites, native materials are widely used in the field, such as titanium for dental implants, stainless steel for orthopedic implants, poly(tetrafluoroethylene) for blood vessel replacement, poly(dimethylsiloxane) for internal drainage and poly(methylmethacrylate) for intraocular lenses. Native material surfaces are unsophisticated as compared with biomolecular architecture on living tissue surfaces. To obtain a positive and selective interaction with biomaterial surface architecture, these surfaces should seek to match certain recognition sites on corresponding biological surfaces to form biocompatible hybrid interfaces.

Cell adhesion is involved in various natural phenomena such as embryogenesis, maintenance of tissue structure, wound healing, immune responses, metastasis and tissue integration of biomaterial. Accordingly, biocompatibility of biomaterials is very closely related to cell behavior upon contact. For example, Figure 1 shows three possible processes that can occur after the implantation of biomaterials into the body.[1] First, ions and molecules reach the biomaterial surfaces and interact and bind depending on the surface properties. The hydration layers on the surface are an important factor that influences proteins. Second, water-soluble proteins have hydration shells, and the interactions of surface water layers with the protein water shells influence the fundamental kinetics and thermodynamics of subsequent protein adlayer formation. These interactions determine the structures of the protein adlayers, such as whether they are denatured, orientation and coverage. Last, when cells arrive at the surface, they recognize the structures of the protein adlayers for adherence, spread and form an interface on the surface. Thus, initial cell adhesion behavior is strongly affected by both surface properties and the structures of the protein adlayers. Accordingly, the interface layers are the dominant factors affecting biocompatibility.[2–4]

A possible interfacial phenomenon between a protein adlayer and a cell on a biomaterial surface is shown in Figure 2. After implantation into the animal body, multiple proteins in the body fluid immediately and competitively adsorb on the surface. Accordingly, the behavior of 'protein at interfaces' is an important research area that is classified into conformational changes and adsorbed protein structures, surface exchange of proteins, protein-rejecting surfaces and biological inactivation/activation. In particular, albumin (Ab) and immunoglobulin (IgG) are the larger mass fractional protein components in body fluid, whereas extracellular matrix (ECM) proteins, which determine cell activities, are relatively minor components. In the protein adlayer, the structure of the ECM containing the arginine-glycine-asparagine (RGD) sequence has a role at the interface between the surface and the cell.[5–7] The conformation and denaturation of the protein adlayers and the orientation of the RGD sequence strongly governs biocompatibility,[8,9] which is attributed to cell activities and functions, such as adhesion, proliferation, migration, differentiation, expression and survival. The ECM is classified into collagen, non-collagen glycoprotein, elastin and proteoglycan groups.[10–12] The non-collagen glycoprotein group includes fibrinogen (Fgn), fibronectin (Fn),

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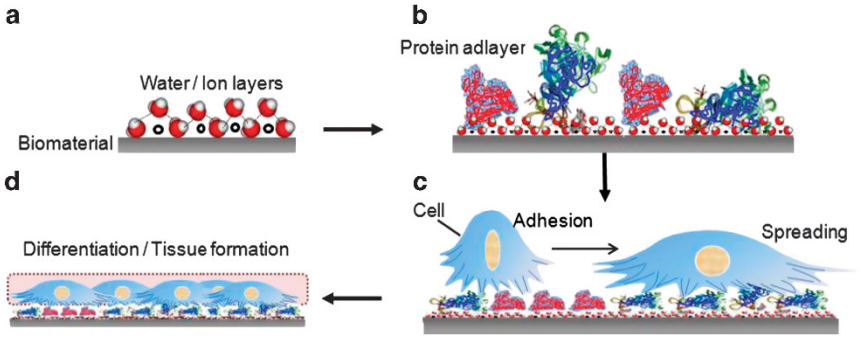

Figure 1 Diagram of successive events on biomaterial surfaces after implantation; (**a**) formation of water and ion layer; (**b**) protein adsorption; (**c**) cell adhesion/spreading; and (**d**) differentiation/tissue formation. Reprinted with permission from Tagaya *et al.*[47]

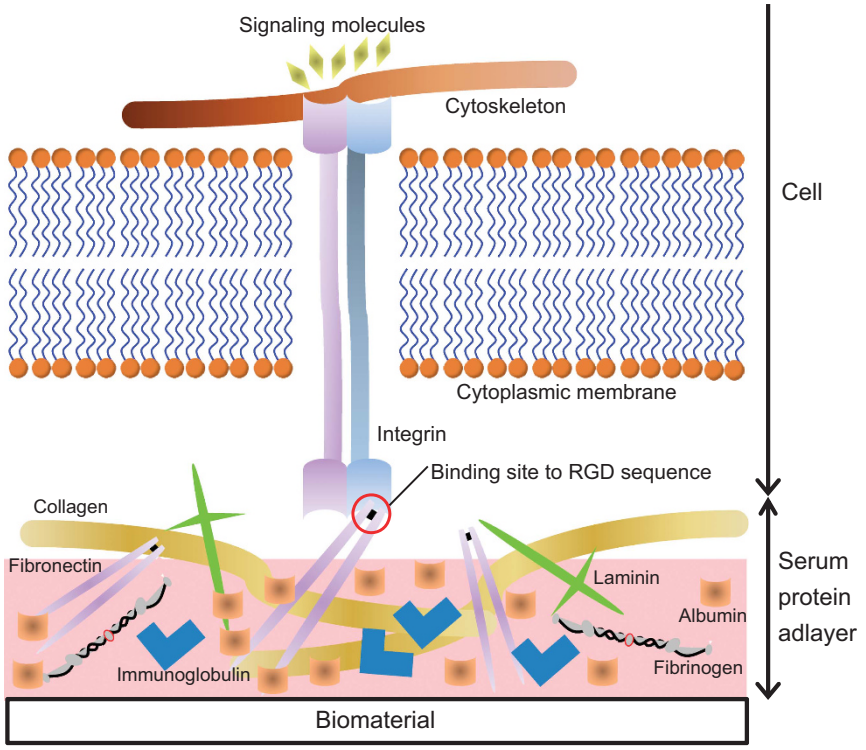

Figure 2 Diagram of the interface between the serum protein adlayer and a cell on the biomaterial surface: an overview of the structure at the interface in which the cell membrane containing integrin binds to the extracellular matrix-protein adlayers. Reprinted with permission from Tagaya *et al.*[47]

laminin, vitronectin, thrombospondins and tenascins, all of which significantly affect the initial cell adhesion behavior as well as cell functions. On the other hand, integrins in the cell membrane directly mediate the attachment between a cell and the RGD sequence of the protein adlayer.[13,14] The cytoskeleton, which is firmly combined with the integrin inside the cell membrane, is altered and subsequently forms adhesion points at the interface. Along with forming these points, signal transduction molecules through the cytoskeleton are successively communicated to determine cell functions. Simultaneously, the cell produces ECM at the interface to consolidate the interfacial junction. The interfacial region at a nanometer scale, including ECM-integrin-cytoskeleton, significantly affects cell activities. Thus, an *in situ* monitoring technique at the interface between the material surface and cell is important for controlling cell functions.

The chemical composition and physical structure of a biomaterial surface have profound scientific importance; their characterization leads to insight into cell–biomaterial interactions. For example, surface morphology and protein structure are known to be attractive features for controlling cell functions. Surface morphology at the nano- and micrometer scales and subsequent protein adsorption are known to affect later cellular activities (proliferation, survival and gene expression).[15–17] The later activities are significantly affected by initial cellular activities (adhesion, spreading). Thus, to evaluate whether the surfaces of engineered biomaterials can induce desirable initial cell interactions, *in vitro* cell culture is performed because there are no applicable universal basic rules to predict cell behaviors on the basis of certain material surface properties. There has been no clarification of the relationships between physicochemical properties and initial cell behavior;[18–20] the interfacial phenomena related to the ECM proteins

have not been clarified for the initial stage. One reason for this lack of clarification is that techniques for *in situ* monitoring of the interfacial phenomena in a solution state have not been established. Therefore, it is indispensable to clarify the phenomena between material surfaces and cells during the initial stage with new techniques to understand biocompatibility at the interface.

In the present focused review, the quartz crystal microbalance with dissipation (QCM-D) technique, which can monitor the interfacial phenomena between biomaterials and cells, is introduced, and the importance of the interfacial phenomena is highlighted. In particular, phenomena on hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAp) surfaces are discussed, which is very important for the design of novel bioactive and biocompatible composites. HAp[21] has been widely investigated for use as a bone filling material with collagen[22–25] and as a drug delivery carrier.[26–30] Protein adsorption and cell adhesion on the surface are critical issues and have been investigated.[31,32] However, the detailed features attributed to the interfaces have not been clarified. Thus, *in situ* monitoring and understanding of the interfaces are of great importance for clarifying the nature of biocompatibility. This paper also clarifies several ambiguities of the interfacial phenomena between biomaterials and cells.

PROTEIN ADSORPTION ON BIOMATERIALS

Mono-component

Protein adsorption behavior on a solid substrate depends on surface properties such as wettability, free energy, charge and roughness.[33,34] The mono-component protein adsorption process and any conformational changes have been investigated with the QCM-D technique.[35,36] The ions in the solvent are known to adsorb on surfaces to form hydration structures, which influence fundamental protein adsorption kinetics. Thus, studies evaluating adsorption dependent on the solvent have been described by the batch method to clarify Ab adsorption on HAp in phosphate-buffered saline,[37] Ab adsorption on silica–titania hybrids in phosphate-buffered saline and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid,[38] and Ab, IgG, Fgn and lysozyme adsorptions on germanium in phosphate-buffered saline or Tris-HCl.[39] The conformations of the Ab adlayer on gold[40] and the Fn adlayer on HAp[41] involving the binding of a monoclonal antibody have also been discussed.

In situ monitoring of conformational changes by the QCM-D technique on biomaterials is desirable. In general, QCM-D measurements are performed by monitoring frequency shifts (Δf (Hz)) and dissipation shifts (ΔD ($\times 10^{-6}$)). The measured Δf is divided by a harmonic overtone (n) at the fundamental frequency of 5 MHz. Only a few studies examining the saturated $\Delta D/\Delta f$ value ($\Delta D_{sat}/\Delta f_{sat}$) from the ΔD–Δf plot based on the measured Δf and ΔD curves have evaluated adsorption behavior and conformation.[42–46] Detailed QCM and QCM-D applications for the adlayer have already been described and reviewed in other journals.[47] Experimental methods for the QCM-D are described in Supplementary Experimental Method S1.

The detectable height (l) of D in the QCM-D system, which can be applied for this discussion of nano-bio interfaces, can be represented by Equation (1) as follows:[46]

$$l = \sqrt{\frac{\eta}{\pi \rho f}} \tag{1}$$

where the η and ρ values are the viscosity and density on the sensor surface, respectively. Thus, the viscous liquid and adlayer on the sensor surface exhibit the higher l value. The value of Δf and ΔD can be represented by Equations (2 and 3), respectively.[46]

$$\Delta f \cong \frac{1}{\rho_q d_q} d_{ad} \rho_{ad} f \left(1 + \frac{2 {d_{ad}}^2 \chi}{3 l^2 (1+\chi^2)}\right) \tag{2}$$

$$\Delta D \cong \frac{2 \rho_{ad} f {d_{ad}}^3}{3\pi f_0 \rho_q d_q} \left(\frac{1}{l^2(1+\chi^2)}\right) \tag{3}$$

Here, $1/\tan\delta$ is replaced by χ, and d_{ad} and ρ_{ad} are the thickness and density of the adlayer. The f_0 is the fundamental frequency, and the d_q and ρ_q are the thickness and density of the quartz.

Figure 3 shows the ΔD–Δf plots of different protein adsorption behaviors and possible adsorption structures.[48] From the Δf and ΔD curves, the adsorption equilibrium time can be obtained to plot the ΔD–Δf in the region between the injection and plateau states. The slope of $\Delta D/\Delta f$ at the plateau state can be defined as $\Delta D_{sat}/\Delta f_{sat}$. From Equations (2 and 3), the higher and lower $\Delta D_{sat}/\Delta f_{sat}$ values approximately denote the viscous and elastic properties of the adlayer at the equilibrium states, respectively. Specifically, plot 1 exhibited a higher $\Delta D_{sat}/\Delta f_{sat}$ value than plot 2, as shown in in Figure 3a, indicating the relatively loose structure and viscous properties of plot 1 depicted in Figure 3b. This tendency can be demonstrated on the basis of Equations (2 and 3), and the $\Delta D/\Delta f$ value is affected only by d_{ad} and $\tan\delta$. Thus, the adlayer of plot 1 also indicated higher d_{ad} and/or $\tan\delta$ values as compared with plot 2. On the basis of previous reports on hydration[42] and friction,[44] the possible structures of the adlayers from plots 1 and 2 can be represented as shown in Figures 3b and c, respectively.

The $\Delta D_{sat}/\Delta f_{sat}$ value is an excellent variable to evaluate the viscoelasticity and structure of the adlayer, and the value has been verified by experiments. Ozeki *et al.*[42] revealed a significant relationship between the $\Delta D_{sat}/\Delta f_{sat}$ value and the amount of hydration. Rodahl *et al.*[44] revealed that the $\Delta D_{sat}/\Delta f_{sat}$ value is significantly related

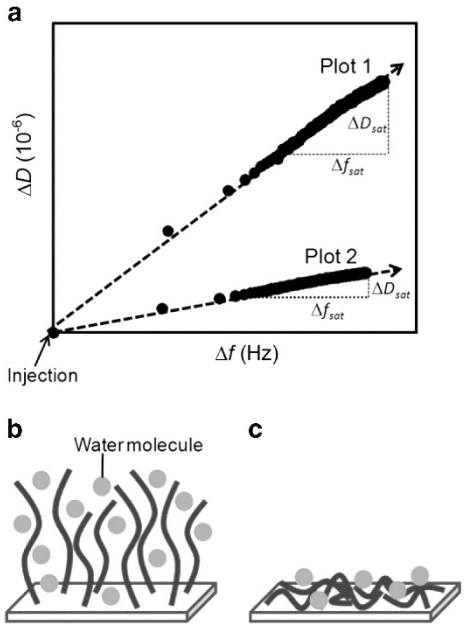

Figure 3 Diagrams of (**a**) $\Delta D - \Delta f$ plots (plots 1, 2) with different protein adlayers, and (**b**, **c**) structures of the adlayers. The $\Delta D/\Delta f$ value at the saturated stage ($\Delta D_{sat}/\Delta f_{sat}$) in plot 2 is lower than that in plot 1, indicating the possible (**b**) viscous and (**c**) elastic structures for plots 1 and 2, respectively. Reprinted with permission from Tagaya *et al.*[47]

to the inverse of the friction coefficient between the adlayer and the sensor surface by defining the protein adlayer as a Newtonian liquid. Monkawa *et al.*[43] reported that the $\Delta D_{sat}/\Delta f_{sat}$ value of the Fgn adlayer successfully corresponds to the conformation with an additional FT-IR analysis. On the basis of this evaluation, Yoshioka *et al.*[45] investigated the conformations of various acidic and basic protein adlayers on HAp using the $\Delta D_{sat}/\Delta f_{sat}$ values. Therefore, the $\Delta D_{sat}/\Delta f_{sat}$ value is of great importance for evaluating the structure of the protein adlayer.

Adsorption behavior dependent on characteristic protein structure is detected by the QCM-D technique. Fgn and Ab are often employed for QCM experiments as model proteins. Fgn is a structural glycoprotein in blood plasma with an isoelectric point of 5.5 and is of a moderate molecular weight (340 kDa) and size (45 nm length).[49–53] A central hydrophobic *E*-domain of the Fgn molecule is connected to two hydrophobic *D*-domains by a coiled-coil chain. These hydrophobic domains are negatively charged under neutral pH conditions. The αC-domains with Arg and Lys residues are positively charged and are substantially more hydrophilic than the *E*- and *D*-domains. On the other hand, the Ab molecule is globular with a pI at 4.7 and a molecular weight of 66.5 kDa and has an asymmetric heart-like structure in which three main domains are divided into six subunit domains.[54] The protein surface has many carboxyl groups and 19 imidazole groups. These groups affect the effective binding to calcium ions and represent a negatively charged surface due to the dissociation of side chains of acidic amino acids, such as glutamic acid, under experimental pH conditions. Figure 4 shows the ΔD–Δf plots and possible conformational changes with the Fgn and Ab adsorptions on the HAp in phosphate-buffered saline.[55] The ΔD–Δf plots in Figures 4a and c show the two-step change for Fgn and the linear change for Ab, indicating the two-step conformational change and monomolecular adsorption on the surface. The $\Delta D_{sat}/\Delta f_{sat}$ value of Ab on the HAp sensors was 1.0×10^{-8}, which is much lower than that of Fgn (34.5×10^{-8}).[43,55] Thus, the Ab was only slightly absorbed on the HAp surface compared to FgnHAp. The adsorption behavior of Fgn on HAp with its dumbbell-like structure has already been described.[43,55] One of the αC domains of Fgn, which is positively charged, is bonded to the negative sites of the HAp surface similar to phosphate and/or hydroxyl ions in an 'end-on' model in Figure 4b. On the contrary, Ab has an asymmetric heart-like structure in which three main domains are divided into the already mentioned six subunit domains. The low amount of Ab adsorption as compared with Fgn indicates that the adsorption model of Ab could be 'side-on' at the monolayer in Figure 4d, and the dissociated carboxyl and imidazole groups interact with the positively charged calcium ions on the HAp surface. Thus, the differential adsorption behaviors of Fgn and Ab with similar pI values can be attributed to their secondary structure and realization of different adsorption models such as 'side-on' and 'end-on'.

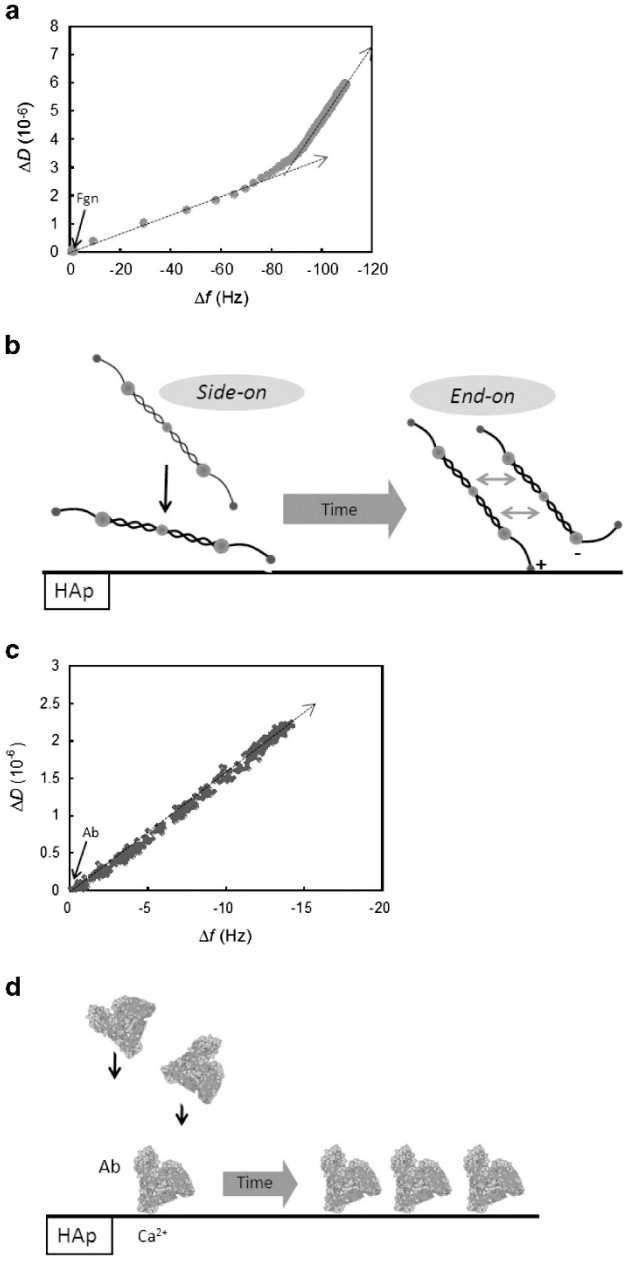

Figure 4 (**a**, **c**) ΔD–Δf plots and (**b**, **d**) possible diagrams of the conformational changes with (**a**, **b**) Fgn and (**c**, **d**) Ab adsorption on the HAp surface. Fgn adsorbs on the HAp, changing from 'side-on' to 'end-on' vs time, whereas Ab adsorbs on the monolayer. Reprinted with permission from Ikoma *et al.*[55]

Multi-component toward serum protein study

In the body fluid, multiple serum proteins immediately and competitively adsorb on the surface.[2–4] The protein adlayers govern biocompatibility. Thus, investigation of interfacial protein–material interactions is important for designing superior biocompatible materials. Although the adsorption of proteins has widely been studied,[56–59] their complex behavior during multiple-protein adsorption at the interface has not yet been elucidated. The substitution of adsorbed fibrinogen on the surface with other abundant proteins in a serum solution is known as the 'Vroman effect'.[60] Several studies characterized the multiple-protein adsorption of Ab and immunoglobulin G (IgG) labeled with radioactive ^{125}I and ^{131}I on poly(ethylene) terephthalate surfaces,[61,62] and Ab, fibrinogen and IgG on a poly (styrene) surface from a plasma solution.[63]

The possible multi-component protein adsorption mechanism on the surface can be divided into the three time-dependent processes. First, the hydrated protein in the liquid interacts with the hydration layer on the surface, and adsorption on the surface occurs with disruption of the hydration structure.[1] Second, the protein repeatedly adsorbs and desorbs on the surface to thermodynamically stabilize the structure.[64] During the adsorption of multi-component proteins, the adsorbed proteins simultaneously exchange with other proteins.[60,65,66] Last, the adsorbed proteins change their conformation to become

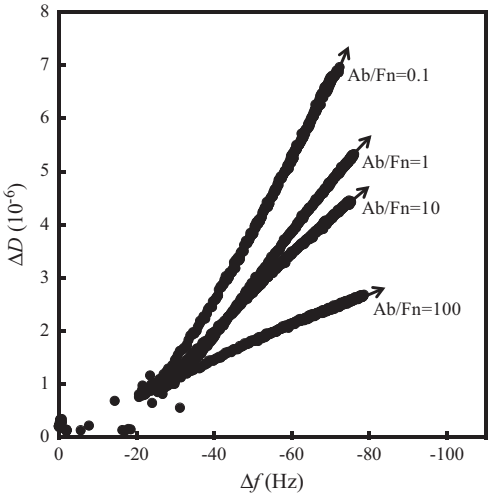

Figure 5 ΔD–Δf plots of the adsorption from the two-component solution for different albumin/fibronectin ratios. With an increase in the albumin concentration, the $\Delta D_{sat}/\Delta f_{sat}$ value decreased from $-14.5 \pm 1.9 \times 10^{-8}$ to $-6.3 \pm 1.6 \times 10^{-8}$, indicating that the viscoelastic behavior of the An-fibronectin compound adlayer changed to elastic with increasing albumin concentration. Reprinted with permission from Tagaya *et al.*[70]

stable higher-order structures on the surface, and the stabilization indicates an equilibrium state.[67] During the adsorption of multi-component proteins, other proteins may subsequently co-adsorb on the stabilized protein surfaces. These interfacial changes are very important for understanding phenomena in body fluids, but only a few studies examining adsorption behavior and conformational changes have been published.[56–59] Therefore, the QCM-D technique is one of the suitable techniques for *in situ* analysis of interfacial phenomena by multi-component protein adsorption.

The two major proteins in the serum are Ab and globulin. Ab is the largest mass fractional protein component in the blood and is known to eliminate cell attachment and block non-specific binding.[68,69] On the other hand, Fn, collagen (Col) and other subtle proteins (osteopontin, laminin, vitronectin and so on) are obligate adhesive proteins for integrin-receptor-based cell adhesion and spreading on surfaces. Thus, the ratio of nonadhesive Ab to adhesive Fn selectively adsorbed on the HAp surface from a multi-component solution (for example, serum) is an important parameter for improving cell adhesion on surfaces. Grainger *et al.*[20] reported that fibronectin (Fn) conformation adsorbs on a poly(tetrafluoroethylene) surface from Fn and Ab mixtures, as determined using an anti-Fn antibody, indicating that Ab masked the adsorbed Fn, and found that the binding of Anti-Fn to the two-component adlayer is suppressed by the presence of Ab. For the HAp surface, Ab and Fn adsorption in a complex fashion has been reported by two-component adsorption.[70] As judged from the ΔD-Δf plots in Figure 5, Ab interacts with Fn due to interfacial changes in elasticity using Voigt-based viscoelastic analysis and an antibody-binding technique.

PROTEIN-MEDIATED CELL ADSORPTION

Formation of nano-bio interfaces

Cell adhesion behaviors depend on surface properties such as topography, wettability and charge and on the protein adlayers.[2–4] The conformational changes, denaturation and RGD sequences of the adsorbed proteins on the surface govern biocompatibility, which is attributed to the adhesion, proliferation, migration and differentiation of cells.[1,9] The ECM with the RGD sequence affects cell adhesion.[5] The integrin of a cell binds to the RGD sequence in the ECM,[13] and subsequent actin cytoskeleton is produced and the associated proteins form focal adhesion points on the surface.[14] When cells adhere and spread on material surfaces, interfacial reactions and morphological changes occur as shown in Figure 1. Thus, *in situ* monitoring of these interfacial phenomena with initial cell behaviors on surfaces is of great importance for controlling cell functions.

Protein adsorption and subsequent cell adhesion on surfaces were reviewed by Anselme.[4] The adsorption of different amounts of fetal bovine serum (FBS) on nanobioceramics causes different cell adhesion behaviors.[71] It is known that bone is a composite material in which collagen fibrils[72,73] form a scaffold for the highly organized arrangement of uniaxially oriented apatite crystals.[74] The process is believed to be directed by the highly acidic ECM proteins; however, the role of the collagen matrix during bone mineralization remains unknown.[75–78] The proliferation and mineralization of osteoblasts on a HAp sintered body with and without pre-adsorbed type I Col were investigated. As a result, various phenotype and gene expression patterns were found to be different from cells on PS dishes.[16,17] Cell functions are determined not only by interfacial proteins but also by substrate surface properties. An understanding of cell adhesion and spreading processes on interfacial protein layers adsorbed on a substrate surface is of significant importance.

Various physical parameters were measured to evaluate interfacial phenomena with initial cell adhesion, such as resistance,[79–85] impedance,[86,87] transient decay time, maximum oscillation amplitude[88] and rheometry.[89] Li *et al.*[90] recently described the viscoelastic properties of a fibroblast cell monolayer on gold using a thickness shear mode quartz crystal resonator with a transfer matrix model. They calculated the μ_{ad} values of 21–39 kPa, η_{ad} values of 0.92–1.56 mPa s and tanδ values of 1.2–2.3 at a 5-MHz resonance frequency. Fernández *et al.*[89] investigated the viscoelastic properties of a fibroblast cell monolayer with a rheometer and obtained G', G'' and tanδ values at 10 Hz of 400, 150 and 0.3 Pa, respectively, indicating that the cell monolayer is an elastic body. Palmer *et al.*[91] suggested that the elastic shear modulus dominates the viscoelastic properties of cells because of their rigid cytoskeletons at lower frequencies, but that the viscous modulus dominates viscoelasticity attributable to the cytoplasmic fluid at the higher frequencies. Actin networks at the high frequency of 1 MHz exhibit a liquid-like property and those at the low frequency of 10 Hz exhibit an elastic property. Their viscoelastic parameters are attributed to the measured frequency or detectable region on the surface.

The high frequency of QCM causes the viscosity behavior of the adherent cells due to the dependence of the actin network property on the frequency. The different viscoelastic properties attributed to the different actin network structures close to the surface can be characterized using QCM-D. Equation (1) indicates that a higher η value for the adlayer can detect a higher l region. The l of a resonating wave in culture medium at 37 °C with a ρ of 0.993 g cm^{-3} and an η of 0.692 mPa s[86] was calculated to be ~100 nm at 15 MHz. The FBS adlayer with measured viscoelastic values demonstrated an l of 100–200 nm at 15 MHz. The l of the adherent cell layers with measured viscoelastic values was also 100–200 nm at 15 MHz. The values of l are a great deal lower than the actual heights of the cells, which are on the micrometer scale. This suggested that QCM-D measures the lower portions of the cells close to the protein adlayer on the sensor surfaces.

The interface between the material surface and cell in QCM-D effectively causes differences in the viscoelastic properties of the

adherent cells. The QCM-D technique focuses on the energy dissipation shift,[66,92–94] allowing the monitoring of cell adhesion and spreading behaviors on surface-oxidized poly(styrene) (PSox),[95–99] tantalum (Ta),[98–100] chromium (Cr),[100] titanium (Ti) and steel.[101] The QCM-D technique has also been used to understand the effects of the pre-adsorption of proteins on cell adhesion;[98–101] for example, on Ta and Cr with and without the pre-adsorption of FBS[100] and on Ti, TiO_2 and steel with the pre-adsorption of Fn or Fgn.[101]

Initial cell adhesion processes and changes in interfacial viscoelasticity using the QCM-D technique have been reported.[102–105] As HAp has good biocompatibility for fibroblasts,[106,107] the adhesion and morphological changes of the fibroblasts on HAp with pre-adsorbed FBS compared to those on PSox have also been reported.[102] Adhesion behaviors that are dependent on surface properties are attributed to cell–surface interactions. QCM-D was also used *in situ* to analyze the adhesion behavior of osteoblast-like cells on PSox and HAp as shown in Figure 6.[105] From the ΔD–Δf plots, the cell adhesion and spreading behaviors depended on the differences between the PSox and HAp surfaces with pre-adsorbed FBS, which are explained in detail in Supplementary Discussion S1. The adhesion speed on PSox was faster than on HAp, which may cause the difference in the ΔD–Δf plots, indicating that pre-adsorbed FBS on the surfaces effectively governs the cell adhesion process. Therefore, the recognition of cells results in different adhesion processes and interfacial viscoelastic properties depending on the surface, which has been successfully elucidated by the QCM-D technique.

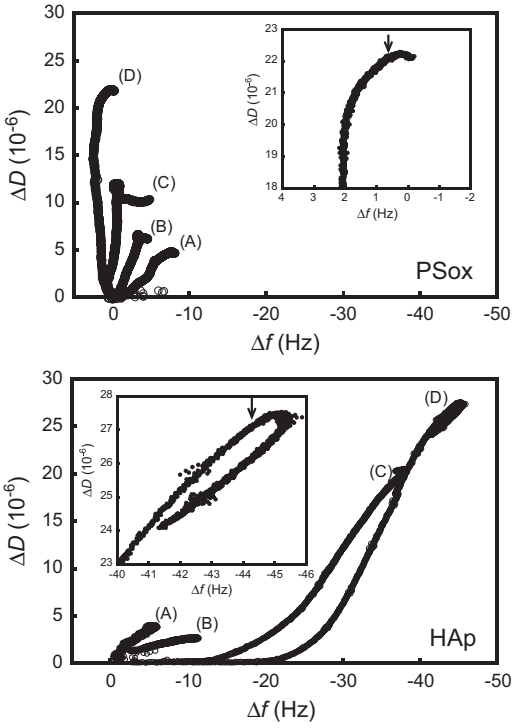

Figure 6 ΔD–Δf plots of osteoblast-like cells adhered on PSox and HAp for 2 h; the seeding densities were (A) 1.0×10^3, (B) 2.5×10^3, (C) 5.0×10^3 and (D) 1.0×10^4 cells per cm^2. The inset figures show the expanded plots of the cells at 1.0×10^4 cells per cm^2. The arrows indicate the point at 1 h. The Δf values were transformed into the values for the fundamental frequency of 5 MHz by dividing by 3, which was the harmonic overtone number. Reprinted with permission from Tagaya *et al.*[105]

Effects of interfacial proteins

The investigation of interfacial phenomena between cells and surfaces modified by various serum proteins is important for controlling cell functions. The pre-adsorption of three proteins (Ab, Fn, collagen (Col)) and subsequent adsorption of FBS (to form the FBS-Ab, FBS-Fn, FBS-Col adlayers), and the adhesion of cells have been reported.[103,104,108] The ΔD–Δf plots of the osteoblast-like cells on FBS, FBS-Ab, FBS-Fn and FBS-Col for 2 h are shown in Figure 7.[108] The FBS-Col surface showed an increase in Δf with a decrease in ΔD, whereas the other surfaces showed a decrease in Δf with an increase in ΔD during the initial 1 h on the FBS, 1.5 h on the FBS-Ab and 0.5 h on the FBS-Fn and subsequent increase in the Δf with a decrease in ΔD, indicating that cell spreading with interfacial reactions such as dehydration and binding caused decreases in the mass and ΔD. The Δf and ΔD values at 2 h were -41.9 ± 2.1 Hz and $+24.2\pm3.3\times10^{-6}$ on the FBS, -9.9 ± 1.5 Hz and $+6.1\pm2.1\times10^{-6}$ on the FBS-BSA, -29.2 ± 1.5 Hz and $+9.2\pm1.6\times10^{-6}$ on the FBS-Fn and $+20.9\pm2.5$ Hz and $-11.5\pm1.1\times10^{-6}$ on the FBS-Col, respectively.

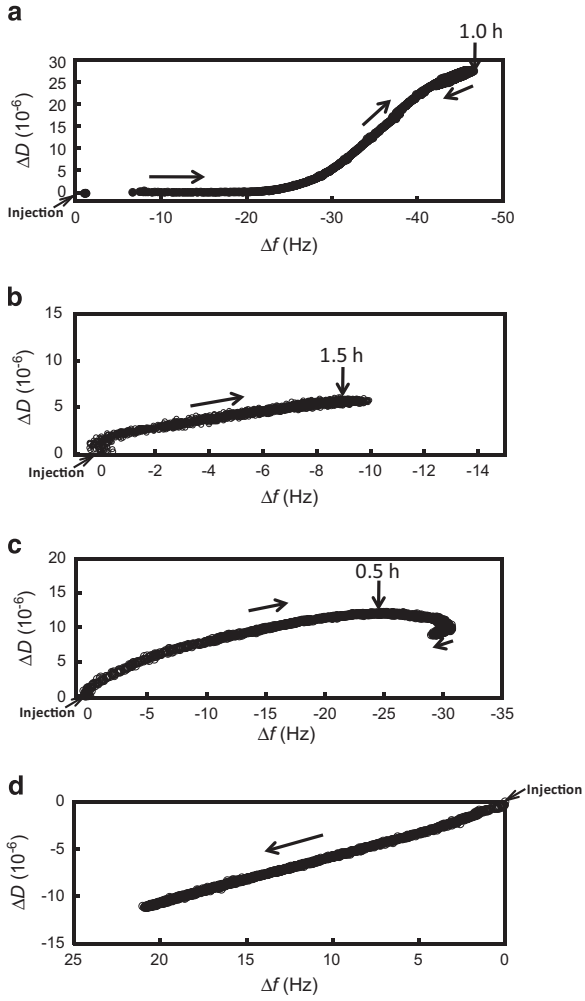

Figure 7 ΔD–Δf plots of cells on HAp modified with (**a**) FBS, (**b**) FBS-albumin, (**c**) FBS-fibronectin and (**d**) FBS-collagen for 2 h. The arrows indicate changes in direction and the culture time at the maximum ΔD values. The ΔD value of the ΔD–Δf plot increased to the maximum during the initial 1 h on the FBS, at 1.5 h on the FBS-albumin, and at 0.5 h on the FBS-fibronectin. Reprinted with permission fromTagaya *et al.*[108]

The ΔD–Δf plots for the HAp surface without the pre-adsorption of the proteins significantly indicated that the mass slightly increased and ΔD also increased during the 2 h, taking into account the rapid kinetics of FBS protein adsorption as compared with cell adhesion. These results support the fact that a different adhesion process occurs on modified surfaces, and the pre-adsorption of Col effectively induces cell-surface reactions. The ΔD–Δf plots for the adhesion and spreading behaviors correspond to the tendencies of fibroblast and hepatocyte adhesion behaviors.[103,104] Accordingly, different cell adhesion processes depending on surface pre-adsorbed proteins are successfully monitored *in situ* by the QCM-D technique. Confocal laser scanning microscope images of adherent cells also demonstrated different morphologies and pseudopods on the surfaces as shown in Figure 8. The cells that adhered on the surfaces modified by Fn and Col had significantly uniaxially expanded shapes and fibrous pseudopods, whereas those modified with Ab had a round shape. Therefore, different cell–protein interactions cause the arrangement of the extracellular matrix and changes to the cytoskeleton at interfaces, and these phenomena can be successfully detected.

CONCLUSION AND FUTURE PERSPECTIVES

In this review, the complexity of the phenomena occurring with cell – material interactions at nano-bio interfaces and, in particular, the effects of proteins on cell adhesion to HAp nanocrystals, which govern subsequent cell behavior at the interface, were highlighted. *In situ* monitoring of the interfacial protein adlayers formed between biomaterials (for example, HAp) and cells have been found to be crucial for controlling cell functions and understanding biocompatible phenomena.[47] The analysis of interfacial interactions with protein adsorption and initial cell adhesion was demonstrated with the QCM-D technique.

The above points, summarized on the basis of *in vitro* experiments, have revealed some of the critical events for biomaterials to stimulate an interface in body fluid. The events that were modeled *in vitro* would be likely to take place *in vivo*. Protein adsorption on surface-interfaces leads to differing degrees of conformational changes at the interface. It is worth estimating the conformational changes and qualitative aspects of protein adsorption with suitable parameters. Studies of qualitative characterization techniques significantly indicate that the 'interfacial heterogeneity effect' does exist, which has also been shown for hydrogel surfaces.[109–112] Models that exist for interfacial protein adlayers that define appropriate heterogeneity parameters for subsequent cell adhesion/spreading are very important.[113]

Future efforts will incorporate the influence of interfacial heterogeneity in protein adsorption studies. Characterization of its influence on the mediation of subsequent reactions at the interface is urgently required, not only to provide novel physical insights into the adsorption process but also to provide a more realistic picture of the events occurring in body fluid. The investigation of interfacial heterogeneity via the analysis of protein adsorption and cell adhesion should then provide an initial and useful framework for analyzing biointerface studies. Obviously, interfacial studies (for example, *in situ* monitoring of conformational changes) can easily be applicable for polymerization processes in a liquid state.[114–116] This framework will build up more predictive techniques to analyze not only the quantitative but also the qualitative aspects of interfacial phenomena.

Further research can bring significant improvements to existing experimental methods to prepare and characterize useful nanobiomaterials and to measure their dynamics and kinetics with mesoscopic scale materials.[30,117–120] The development of surface science for *in situ* measurements at interfaces will take place through interactions with the adjacent fields of surface physics and chemistry, biology and

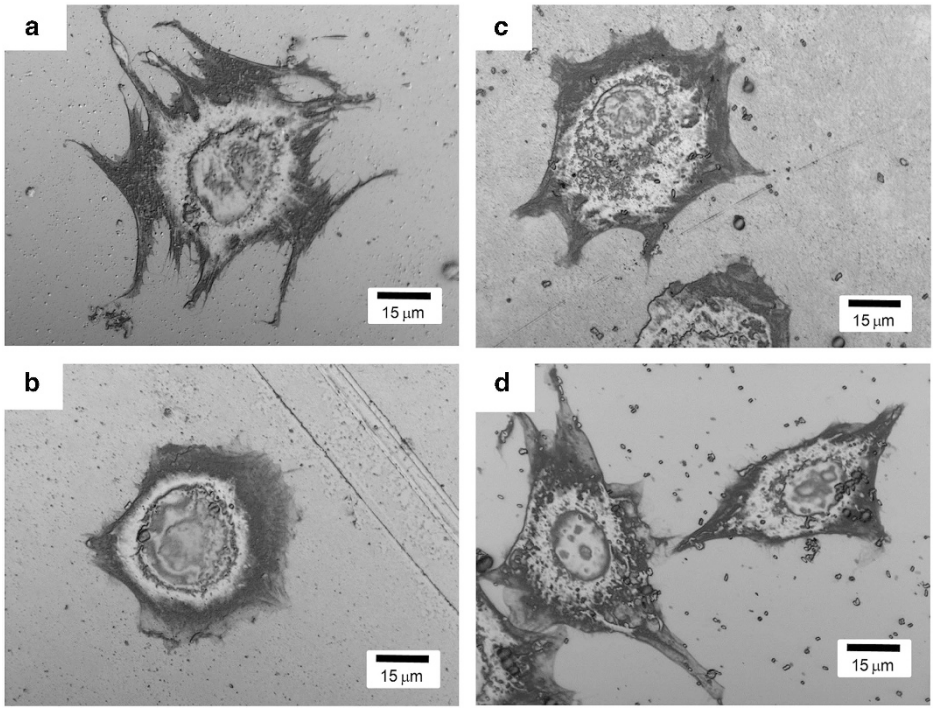

Figure 8 Confocal laser scanning microscope images of cells adhered on (**a**) FBS, (**b**) FBS-albumin, (**c**) FBS-fibronectin and (**d**) FBS-collagen adsorbed on HAp, which were incubated for 120 min. Reprinted with permission from Tagaya *et al.*[108]

polymer sciences. These studies will lead to a deep understanding of nano-bio interfaces.

To overcome existing scientific challenges, mutual interactions should be explored by developing novel detection techniques for interfacial interactions. Proper understanding of cell behavior during contact with implanted biomaterial is essential for attaining adequate health and safety. In particular, the development of tissue engineering techniques requires greater consideration of cell adhesion properties, whether for the improvement of the surfaces by adsorption or grafting of specific adhesion factors,[121] or for the development of hybrid materials for autologous bone cells and materials. Therefore, interface studies have great potential to inform the development of superior biomaterials that are applicable for medical implants, biosensors and biochips for diagnostics, bioelectronics and biomimetics.

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