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Hydration and Energy Dissipation Measurements of Biomolecules on a Piezoelectric Quartz Oscillator by Admittance Analyses

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By using a 27-MHz piezoelectric quartz oscillator connected with a vector network analyzer, we obtained resonance frequency decreases ($-\Delta F_{water}$) and energy dissipation increases (ΔD_{water}) during binding of biotinylated bovine serum albumin, biotinylated ssDNA, biotinylated dsDNA, and biotinylated pullulan to a NeutrAvidin-immobilized 27-MHz quartz crystal microbalance (QCM) plate in aqueous solution, as well as in the wet air phase (98% humidity, $-\Delta F_{wet}$ and ΔD_{wet}) and in the dry air phase ($-\Delta F_{air}$ and ΔD_{air}). $-\Delta F_{water}$ indicates the total mass of the molecule, bound water, and vibrated water in aqueous solutions. $-\Delta F_{wet}$ indicates the total mass of the molecule and bound water. $-\Delta F_{air}$ simply shows the real mass of the molecule on the QCM. In terms of results, $(-\Delta F_{wet})/(-\Delta F_{air})$ values indicated the bound water ratios per unit biomolecular mass were on the order of pullulan (2.1−2.2) > DNAs = proteins (1.4−1.6) > polystyrene (1.0). The $(-\Delta F_{water})/(-\Delta F_{air})$ values indicated the hydrodynamic water (bound and vibrated water) ratios per unit biomolecular mass were on the order of dsDNA (6.5) > ssDNA = pullulan (3.5−4.4) > proteins (2.4−2.5) > polystyrene (1.0). Energy dissipation parameters per unit mass in water ($\Delta D_{water}/(-\Delta F_{air})$) were on the order of pullulan > dsDNA > ssDNA > proteins > polystyrene. Energy dissipation in the wet and dry air phases (ΔD_{wet} and ΔD_{air}) were negligibly small, which indicates even these biomolecules act as elastic membranes in the air phase (without aqueous solution). We obtained a good linear relationship between $[(-\Delta F_{water})/(-\Delta F_{air}) - 1]$, which is indicative of hydration and $\Delta D_{water}/(-\Delta F_{air})$ of proteins. The aforementioned values suggest that the energy dissipation of proteins was mainly caused by hydration and that proteins themselves are elastic molecules without energy dissipation in aqueous solutions. On the contrary, plots in cases of denatured proteins, DNAs, and pullulans were relatively deviant toward the large hydration and energy dissipation from the theoretical line as perfect elastic materials, meaning that the large energy dissipation occurs because of viscoelastic properties of denatured proteins, linear DNAs, and pullulans in the water phase, in addition to energy dissipation due to the hydration of molecules. These two parameters could characterize various biomolecules with structural properties in aqueous solutions.

Viscoelastic and hydration properties of biomacromolecules such as proteins, DNAs, and polysaccharides have generated much interest, because these properties result in the structure and function of biomolecules such as stabilities, folding, structural changes, molecular recognition, and enzymatic activities. The viscoelasticity of these molecules in aqueous solutions has been studied by ultrasonic absorbance measurements,[1] atomic force microscopy,[2] and surface force apparatus.[3] The hydration of biomolecules in aqueous solutions has been widely studied by various methods such as calorimetry,[4] osmotic pressure analyses,[5] dielectric spectroscopy,[6] and NMR techniques.[7] However, more sensitive and time-resolved detection methods of viscoelasticity and hydration of biomolecules are still required.

The piezoelectric quartz crystal microbalance (QCM) is a very sensitive mass measuring device and has been used as a mass sensor in aqueous solutions.[8−10] In many cases, when biomolecules immobilize on the QCM plate, they behave as elastic membranes, and the resonance frequency (ΔF_{water}) decreases linearly with increasing mass (Δm) on the QCM plate; the phenomenon has

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been described by the Sauerbrey equation.[11] However, when these biomolecules are highly hydrated, ΔF_{water} decreases with a different slope or deviates from the linear slope and quantification becomes difficult. If substances on the QCM are highly hydrated, a larger $-\Delta F_{water}$ value than expected is obtained due to the hydration of water.[12−15]The energy dissipation (D factor), reciprocal of the quality factor (Q factor), has become a popular measure that indicates energy loss from the viscous layer against the total vibration energy of the QCM plate. The D factor has been reported to indicate the energy loss between viscous molecules and media in cases of DNA hybridization[15] and adsorption and the following structural changes of large vesicles,[16] or proteins[17] on the QCM plate. Although QCM measurements represent one of some candidates to detect the hydration and viscoelasticity of biomacromolecules, there are few studies that separately or comprehensively explain the hydration and energy dissipation (viscoelasticity) of biomacromolecules, such as proteins, DNAs, and polysaccharides in aqueous solutions.

In this paper, we measured resonance frequency decreases ($-\Delta F_{water}$) and energy dissipation increases (ΔD_{water}) for the binding of biotinylated bovine serum albumin (BSA), biotinylated ssDNA or dsDNA, and biotinylated pullulan to a NeutrAvidin-immobilized 27-MHz QCM connected with a vector network analyzer in aqueous solutions. After the binding equilibrium, $-\Delta F_{wet}$ and ΔD_{wet} or $-\Delta F_{air}$ and ΔD_{air} values were also obtained in the humid air phase (98% humidity) or in the dry air phase, respectively. We first achieved sensitive and real-time measurements of ΔF_{water} within ±4 Hz and ΔD_{water} within 0.5×10^{-6} per each second independent of media. $-\Delta F_{water}$ indicates the total mass of the molecule, bound water, and vibrated water. $-\Delta F_{wet}$ indicates the mass of the molecule and the bound water. $-\Delta F_{air}$ simply shows the real mass of the molecule independent of media. Thus, we expect to separate the resonance frequency decrease ($-\Delta F_{water}$) obtained in the water phase from the mass of the molecule, the binding of water, and the vibrating of water. We discussed relationships between the amount of water hydration [$(-\Delta F_{water})/(-\Delta F_{air})$ −1] and energy dissipation [$\Delta D_{water}/(-\Delta F_{air})$] to separate the energy dissipation caused by the hydration and the energy dissipation caused by the viscoelasticity of biomolecules such as proteins, DNA, and polysaccharide in aqueous solutions. This relation can be also applied to conformation changes of proteins and DNA.

EXPERIMENTAL SECTION

Materials. Polystyrene (10 kDa) was purchased from Toso Co. Ltd. (Tokyo, Japan). Biotinylated oligonucleotides were purchased from Qiagen Co. Ltd. (Tokyo, Japan). NeutrAvidin (60 kDa) and EZ-LinkSulfo-NHS-LC−LC-Biotin was purchased from Pierce Co. (Tokyo, Japan). Pullulan (22−440 kDa) was purchased from Showadenko Co. Ltd (Tokyo, Japan). BSA (66 kDa) was purchased from Amersham Pharmacia Biotech Co. Ltd. (Tokyo, Japan). All reagents were used without further purification.

Admittance Analyses of a Piezoelectric Quartz Oscillator. The admittance analysis of a 27-MHz piezoelectric quartz crystal was accomplished with a vector network analyzer measuring the current at a known applied voltage over a specified range of frequencies (Figure 1A). When the conductance (G) is plotted against the swept frequency, the maximum point appears as an inflection point, which is too noisy to allow for the determination of a precise value. Therefore, we defined the maximum point (the resonance frequency, F) as eq 1, where F_1 and F_2 values could

$$F = (F_1 + F_2)/2 \tag{1}$$

be obtained precisely as the half-height of the curve (Figure 1B).

When the mass is loaded on the QCM plate, the F value shifts to a lower frequency, and the frequency change is obtained as outlined in eq 2.

$$\Delta F = F_b - F_a \tag{2}$$

When the viscous layer is deposited on the QCM plate, the frequency shifts to a lower frequency and the bandwidth (F_2-F_1) of the G curve becomes larger from $(F_2 - F_1)_a$ to $(F_2 - F_1)_b$ as shown in Figure 1B. The energy dissipation (D) and its change (ΔD) are defined as eqs 3 and 4, respectively.

$$D = \frac{F_2 - F_1}{(F_1 + F_2)/2} \tag{3}$$

$$\Delta D = \left(\frac{F_2 - F_1}{(F_1 + F_2)/2}\right)_b - \left(\frac{F_2 - F_1}{(F_1 + F_2)/2}\right)_a \tag{4}$$

A 27-MHz QCM Apparatus. Affinix Q[4] was used as a QCM instrument (Initium Co. Ltd., Tokyo, Japan; http://www.initium 2000.com). The QCM instrument had four 500-μL cells that were equipped with a 27-MHz QCM plate (a 8.7-mm diameter of a AT-cut shear-mode quartz plate and an area of 0.049 cm^2 of an Au electrode) at the bottom of the cell along with a stirring bar and a temperature controlling system.[9] We connected a vector network analyzer (model R3754B, Advantest, Co. Ltd., Tokyo, Japan) with Affinix Q[4] through a π network (Sansei Denshi Co. Ltd., Tokyo, Japan) as shown in Figure 1A, in which the oscillation circuit and the network analyzer could be switched. In the network analysis, three-term calibrations, "open", "short", and "load" (50 Ω), were

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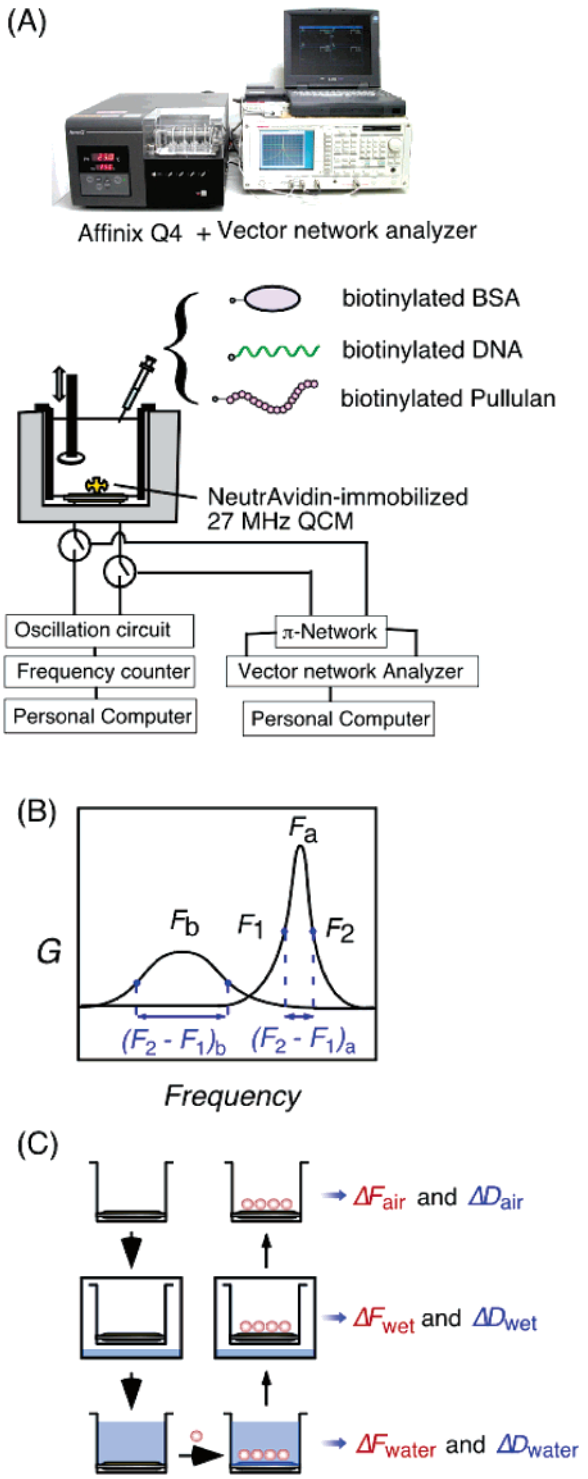

Figure 1. (A) Schematic illustrations of Affinix Q4 connected with a vector network analyzer and (B) admittance analyses of resonance frequencies (F) and energy dissipation (D) by frequency sweep measurements. When the elastic mass was added on a piezoelectric crystal oscillator, the frequency peak shifted from F_a to F_b. When the viscous mass was added, both the frequency shift from F_a to F_b and the broadening of the peak from $(F_2 - F_1)_a$ to $(F_2 - F_1)_b$ occurred. (C) Procedure of measurements for each ΔF and ΔD value.

used. The admittance curve was obtained using a linear frequency sweep with 201 data points in frequency spans of 50 or 10 kHz near the 27-MHz resonance frequency region for measurements in water, in the 98% humid air phase, or in the dry air phase, respectively. Each admittance curve was treated with 16 time averages. One F and D point could be obtained at each 1 s. The frequency noise was less than ±4 Hz, and the dissipation noise was less than $\pm 0.5 \times 10^{-6}$ in the buffer solution. This is the first example that observes both ΔF_{water} and ΔD_{water} values in these sensitivities per every second in the aqueous solution by admittance analyses. F_{air} and D_{air} or F_{wet} and D_{wet} were obtained in dried air on silica gels or in the 98% relative humidity atmosphere over a saturated K_2SO_4 aqueous solution, respectively.[18]

Immobilization of Polystyrene or NeutrAvidin on the QCM Plate. Polystyrene (average M_w 10 200) was spin-coated on a bare Au electrode of 27-MHz QCM plate by dropping 10 μL of a chloroform solution of polystyrene (0.35−1.4 mg mL^{-1}). ΔF_{water}, ΔF_{wet}, and ΔF_{air} values were obtained by subtraction of F values measured before and after coating in the water phase, in the 98% humid air phase, and in the dry air phase, respectively. ΔD_{water}, ΔD_{wet}, and ΔD_{air} values were also obtained by the same procedure.

NeutrAvidin (60 kDa) was covalently immobilized on the QCM plate as follows (see Figure 2):[19] To the cleaned bare Au electrode, 3,3′-dithiodipropionic acid was immobilized, and then carboxylic acids were activated as *N*-hydroxysuccinimidyl esters on the surface. NeutrAvidin was reacted with activated esters by mounting aqueous solutions on the QCM plate. Each F and D value before and after immobilization in the water phase, in the 98% humid air phase, and in the dry air phase were also measured similarly.

Binding Behaviors of Biotinylated BSA, ssDNA, and Pullulan to the NeutrAvidin-Immobilized QCM. Biotinylated BSA was prepared as follows (see Figure 2): Primary protein amino groups were reacted with EZ-linkSulfo-NHS-LC−LC-Biotin. One biotin group was confirmed to be introduced into one protein by MALDI TOF-MS analyses.[20] Biotinylated pullulans (22 kDa, 112 kDa, and 404 kDa) were prepared by reacting reducing ends with biotinamidocaproyl hydrazides as previously described.[21] Biotinylated ssDNA was purchased from Qiagen Co., Ltd., and biotinylated dsDNA was prepared by hybridization with its complementary DNA.

The binding behaviors of biotinylated BSA, pullulan, ssDNA, and dsDNA to the NeutrAvidin-immobilized QCM were followed in TE buffer (pH 7.8, 10 mM Tris-HCl, 200 mM NaCl) solutions or Milli-Q water at 20 °C, and ΔF_{water} and ΔD_{water} values were measured over time. After reactions had reached equilibrium, reaction media were aspirated, and the QCM plate was kept in the 98% humid air phase, then in the dry air phase to obtain values for F_{wet} and D_{wet}, and then F_{air} and D_{air}, respectively. Then, ΔF_{wet} (ΔD_{wet}) or ΔF_{air} (ΔD_{air}) values were calculated by subtraction of F (D) values of NeutrAvidin-immobilized QCM in the 98% humid air phase or in the air phase.

Preparation of Denatured BSA. S−S linkages of biotinylated BSA were reduced by incubating in 0.35 M mercaptoethanol containing buffer solution (0.5 M Tris-HCl, 7 M GnHCl, 10 mM EDTA, pH 8.7), bubbling with N_2 gas at 37 °C for 4 h. Then reduced SH groups were carboxymethylated by the addition of

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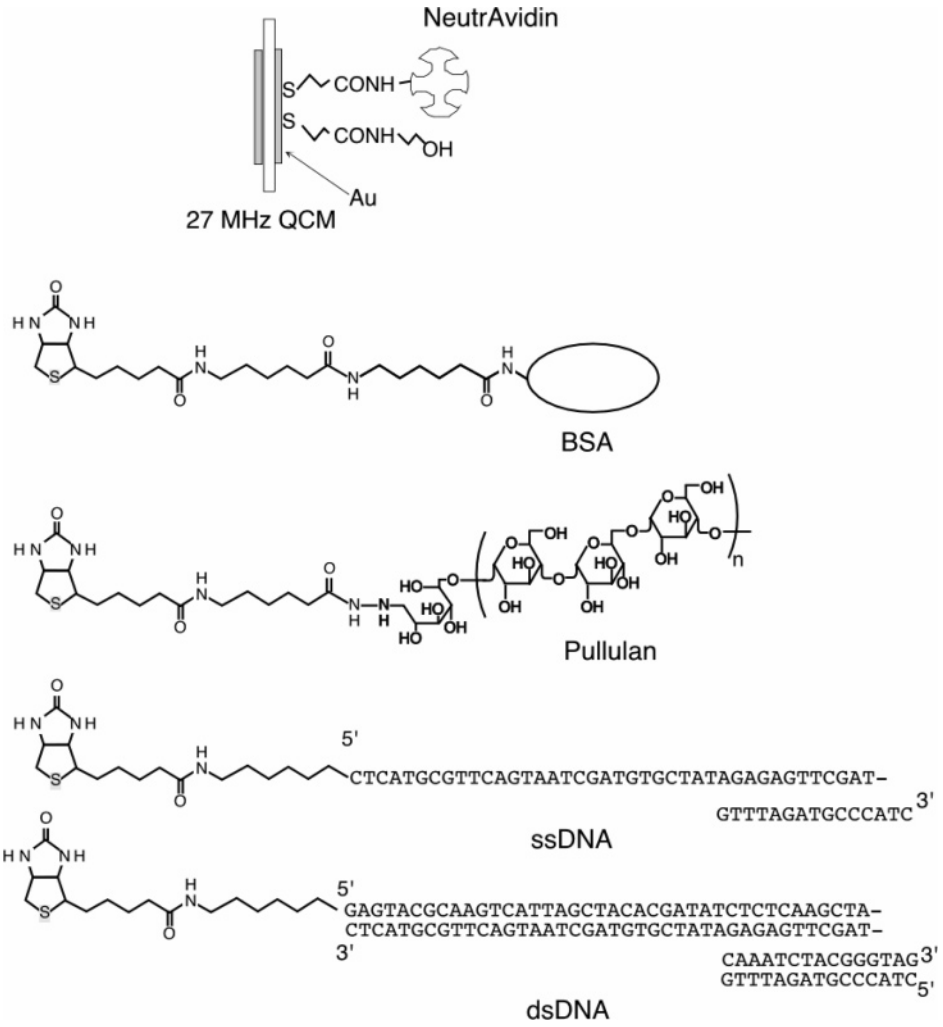

Figure 2. Chemical structures of the NeutrAvidin-immobilized QCM and biotinylated BSA, biotinylated polysaccharides, biotinylated ssDNA, and biotinylated dsDNA.

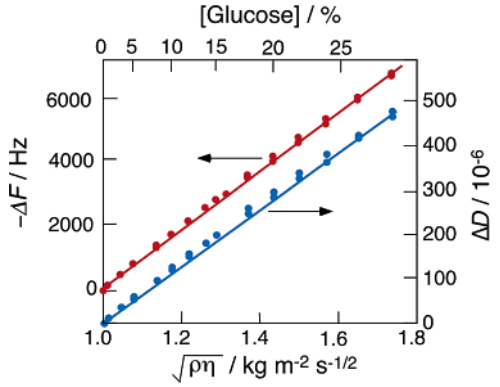

Figure 3. ΔF_{water} and ΔD_{water} changes depending on the increase of the viscosity (η) and density (ρ) of the aqueous solution by the stepwise addition of glucose (0−28 wt % at 20 °C).

iodoacetic acid (final concentration 18.7 mM) at 25 °C for 2 h. The denatured biotinylated BSA was recovered by precipitation in acetone three times.[22]

RESULTS AND DISCUSSION

Confirmation of Validity of ΔF and ΔD Values. Figure 3 shows ΔF_{water} and ΔD_{water} changes in solution, when the concentration of glucose was changed in the range of 0−28 wt % at 20 °C. ΔF_{water} decreased and ΔD_{water} increased linearly with increasing $(\rho\eta)^{1/2}$ values, where ρ and η are density and viscosity of the solution, respectively. The $(\rho\eta)^{1/2}$ value corresponds as a mass of Newtonian liquids loaded on a QCM surface,[23] and the linearity in Figure 3 indicates that ΔF_{water} and ΔD_{water} would be a linear function in this experimental condition. The slope of $-\Delta F_{water}$ against $(\rho\eta)^{1/2}$ was obtained to be 9080 Hz (kg m^{-2} s$^{-1/2}$)$^{-1}$, which was consistent with Kanazawa's eq 5 obtained by the oscillation

$$\Delta F_{water} = -\frac{F_0^{3/2}\sqrt{\rho\eta}}{\sqrt{\pi\rho_q\mu_q}} \quad (5)$$

circuit method, where $F_0 = 27$ MHz.[24] The slope of ΔD_{water} against $(\rho\eta)^{1/2}$ was 6.7×10^{-4} (kg m^{-2} s$^{-1/2}$)$^{-1}$, which was consistent with the theoretical eq 6 obtained by the decay method of the oscillation circuit by Rodahl et al.,[25] where $F_0 = 27$ MHz.

Immobilization of Polystyrene and NeutrAvidin on the QCM. Polystyrene film as a typical elastic and rigid membrane

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$$D_{water} = \frac{2F_0^{1/2}\sqrt{\rho\eta}}{\sqrt{\pi\rho_q\mu_q}} \quad (6)$$

was spin-coated from the chloroform solution by different concentrations on the QCM plate. Frequency decreases due to increase in the coated amount of polystyrene were measured in dry air ($-\Delta F_{air}$), in 98% humid air ($-\Delta F_{wet}$), and in the water phase ($-\Delta F_{water}$). The energy dissipation was also determined in dry air (ΔD_{air}), in 98% humid air (ΔD_{wet}), and in the water phase (ΔD_{water}). $-\Delta F_{water}$ and ΔD_{water} were plotted against $-\Delta F_{air}$ values that indicated the real mass on the QCM plate, as shown in Figure 4A. Slopes of $(-\Delta F_{water})/(-\Delta F_{air})$ and $(-\Delta F_{wet})/(-\Delta F_{air})$ were 1.0 in the wide range of frequency changes (10^2–10^4 Hz), which indicates that the frequency decrease in the water and in the humid air phase, meaning that in the air phase. This means that polystyrene is not hydrated and the mass on the QCM plate could be calculated simply by the Sauerbrey equation. Slopes of $\Delta D_{water}/(-\Delta F_{air})$, $\Delta D_{wet}/(-\Delta F_{air})$, and $\Delta D_{air}/(-\Delta F_{air})$ were calculated as 0.16×10^{-8} Hz^{-1}, which represents a negligibly small amount of energy dissipation. The aforementioned result means that polystyrene is an elastic and rigid membrane and its oscillation hardly dissipates even in the water phase.

NeutrAvidin (60 kDa) was also covalently immobilized on the Au electrode of the QCM by using an amine-coupling method.[19] The Au electrode was covered with the activated ester of succinimidyl dithiopropionate, and NeutrAvidin was immobilized stepwise through amine-coupling reactions of NeutrAvidin on the QCM plate. $-\Delta F_{water}$ and ΔD_{water} values were obtained in Milli-Q water. $-\Delta F_{wet}$ and $-\Delta F_{air}$ were obtained in 98% humid air and in the air phase, after the water phase was aspirated from the QCM surface. Both $-\Delta F_{water}$ and $-\Delta F_{wet}$ values linearly increased with increasing $-\Delta F_{air}$ values (Figure 4B) and the $-\Delta F_{air}$ saturated near 500 ± 10 Hz, which corresponds to a mass of 310 ± 10 ng cm^{-2} according to the Sauerbrey equation. NeutrAvidin was estimated to be a spherical protein with a diameter of ~6 nm, and its mass was calculated to be 320 ng cm^{-2} when NeutrAvidin covered the Au electrode (area, 0.049 cm^2) as a monolayer. The calculated immobilized amount is consistent with the maximum binding amount of NeutrAvidin as a monolayer. The slope $(-\Delta F_{water})/(-\Delta F_{air})$ was calculated to be 2.4, which indicates that NeutrAvidin interacts with water and the apparent mass increased 2.4 times compared with that in the air phase. The slope of 1.6 for $(-\Delta F_{wet})/(-\Delta F_{air})$ indicates that the apparent mass increased 1.6 times due to the binding of water around NeutrAvidin in the wet air phase compared with that in the dry air phase.

ΔD_{water} plotted against $-\Delta F_{air}$, ΔD_{water} increased with increasing $-\Delta F_{air}$ and the slope was 1.4×10^{-8} Hz^{-1}, which was larger than 0.16×10^{-8} Hz^{-1} for the polystyrene coating (Figure 4), indicating that NeutrAvidin seems to be a slightly dissipated membrane as compared with polystyrene. However, $\Delta D_{wet}/(-\Delta F_{air})$ and $\Delta D_{air}/(-\Delta F_{air})$ were negligibly small ($<0.1 \times 10^{-8}$ Hz^{-1}, data not shown), which means that NeutrAvidin acts as a rigid membrane in the air phase regardless of whether it was dry or wet. The values obtained are summarized in Table 1.

Binding Behaviors of Proteins, DNAs, and Pullulans. Typical time courses of binding behaviors of biotinylated BSA, biotinylated ssDNA, and biotinylated pullulan to the NeutrAvidin-immobilized QCM are shown in Figure 5. We could monitor

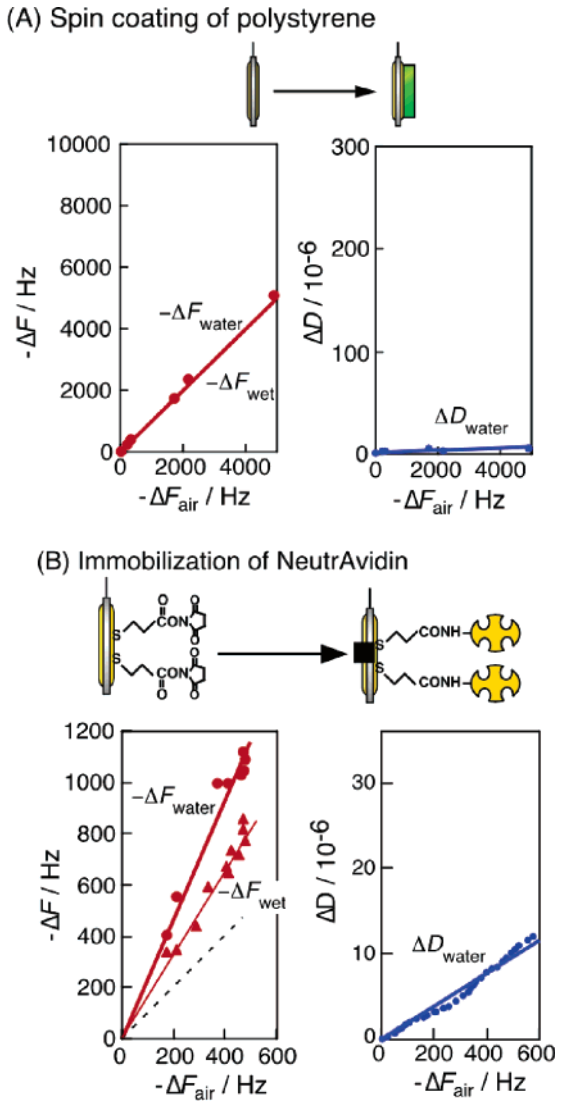

Figure 4. Linear plots of $-\Delta F$ and ΔD against $-\Delta F_{air}$ values (the real mass) of (A) the spin coating of polystyrene (average M_w 10 200) and (B) the covalent immobilization of NeutrAvidin (60 000) on a 27-MHz QCM plate. $-\Delta F_{water}$ and ΔD_{water} were obtained in Milli-Q water at 20 °C, $-\Delta F_{wet}$ and ΔD_{wet} were obtained in the 98% humid air phase, and $-\Delta F_{air}$ and ΔD_{air} were obtained in the dry air phase on silica gel at 20 °C. ΔD_{wet} and ΔD_{air} were not shown, because of the negligibly small values ($<0.3 \times 10^{-6}$). The dotted line in (B) indicates the slope of 1 obtained for the polystyrene coating.

$-\Delta F_{water}$ and ΔD_{water} values with a low noise level ($\Delta F_{water} < \pm 4$ Hz and $\Delta D_{water} < \pm 0.5 \times 10^{-6}$) per every second. When globular BSA proteins bound to the QCM surface, $-\Delta F_{water}$ increased largely due to apparent mass increase on the QCM and ΔD_{water} values slightly increased, indicating that the energy dissipated upon binding was relatively small. On the contrary, when linear single-stranded DNA (ssDNA) and pullulan bound to the QCM surface, both $-\Delta F_{water}$ and ΔD_{water} values increased greatly. Thus, the large energy dissipation occurred together with an increase in mass when these viscoelastic linear molecules bound to the QCM surface.

Hydration Ratio and Energy Dissipation of Biomolecules. During following the binding process in aqueous solutions

Table 1. Hydration and Energy Dissipation Of Proteins, Polysaccharides, And DNAs

	Binding water ratio[a] $(-\Delta F_{wet})/(-\Delta F_{air})$	Hydrodynamic water ratio[b] $(-\Delta F_{water})/(-\Delta F_{air})$	Energy dissipation $\Delta D_{water}/(-\Delta F_{air}) / 10^{-8}$ Hz^{-1}
Polystyrene	1.0	1.0	0.16
Neutravidin (60 kDa)	1.6	2.4	1.4
Calmodulin (17 kDa)	-	1.8	0.76
BSA (66 kDa)	1.4	2.5	1.6
Denatured BSA (66 kDa)	-	4.7	8.3
Pullulan (ca. 140 mer, 22 kDa)	2.1	3.6	11
Pullulan (ca. 710 mer, 112 kDa)	2.2	4.4	20
Pullulan (ca. 2600 mer, 404 kDa)	2.2	3.5	23
ssDNA (55 mer, 17 kDa)	1.6	4.2	11
dsDNA (55 bp, 34 kDa)	-	6.5	19

[a] Contains (bound water + biomolecule) per unit mass. The amount of bound water is expressed as $[(-\Delta F_{wet}) - (-F_{air})]/(-\Delta F_{air})$. [b] Contains (bound water + vibrated water + biomolecule) per a unit mass. The amount of (bound water + vibrated water) is expressed as $[(-\Delta F_{water} - (-F_{air})]/(-\Delta F_{air})$.

$(-\Delta F_{water})$, we obtained stepwise $-\Delta F_{wet}$, and ΔD_{wet} values in the 98% relative humidity atmosphere after aspirating media and washing with Milli-Q water, in addition to the $-\Delta F_{air}$, and ΔD_{air} in the dry air phase on silica gels. $-\Delta F_{water}$ has been reported to increase 9 times as expected from the Sauerbrey equation when biomolecules such as dsDNA were immobilized on a QCM plate in an aqueous solution.[15] The result was simply explained as "hydration" of biomolecules. However, that is larger than ~1.4 of the amount ratio of bound water for dsDNA obtained by a gravimetric study.[26] Thus, we should consider separately the bound water (hydration, generally) and vibrated water (with a QCM oscillation) for molecules. Therefore, $-\Delta F_{air}$, $-\Delta F_{wet}$, and $-\Delta F_{water}$ values in this study would indicate the real mass of molecules, the mass of molecules with the bound water, and the total mass of molecules with bound and vibrated water in the aqueous phase, respectively.

The $-\Delta F_{water}$ and $-\Delta F_{wet}$ values for bound BSA, ssDNA, and pullulan are plotted against $-\Delta F_{air}$ in the left columns of Figure 6, compared with those of polystyrene coatings as shown by dotted lines. ΔD_{wet} and ΔD_{air} were negligibly small, and the data were not shown. The slope of $(-\Delta F_{wet})/(-\Delta F_{air})$ in the linear correlation indicates the bound water ratio per unit biomolecular mass and $(-\Delta F_{water})/(-\Delta F_{air})$ reflects the hydrodynamic water (bound water + vibrated water) ratio per unit biomolecular mass. Values obtained from each biomolecule are summarized in Table 1.

ΔD_{water} values increased linearly with increasing $-\Delta F_{air}$ and then deviated downward from the linear line. The aforementioned results indicate that biomolecules vibrate independently and that energy dissipation per unit mass $[\Delta D_{water}/(-\Delta F_{air})]$ are constant when the immobilized density is low on the QCM plate. When the immobilized density increases, biomolecules interact with each other on the QCM plate and the energy dissipation deviates downward due to an increase in the elasticity of the packed molecules. ΔD_{water} values were less than 15×10^{-6} in our all measurements, which were relatively small (1−2%) compared with the D values of our instrument ($(7.4 \pm 0.078) \times 10^{-4}$), and hardly affected the measurements. The linear parts of $\Delta D_{water}/(-\Delta F_{air})$ showing the energy dissipation of the independent molecule are also summarized in Table 1.

Pullulan with different molecular weights (22 000, 112 000, and 404 000) and dsDNA were also measured in terms of $(-\Delta F_{water})/(-\Delta F_{air})$, $(-\Delta F_{wet})/(-\Delta F_{air})$, and $\Delta D_{water}/(-\Delta F_{air})$ in the same manner, and the results are also summarized in Table 1. The $(-\Delta F_{wet})/(-\Delta F_{air})$ of dsDNA was not obtained as a constant value possibly because of denaturation in the wet air phase.

The $(-\Delta F_{wet})/(-\Delta F_{air})$ value indicates the bound water ratio per unit mass was on the order of pullulan (2.1−2.2) > BSA (1.4) = NeutrAvidin (1.6) = ssDNA (1.6) > polystyrene (1.0). The amount of bound water is defined as $[(-\Delta F_{wet}) - (-\Delta F_{air})]/(-\Delta F_{air})$, and that of BSA (0.4) was consistent with the hydration amount (0.29−0.34) for BSA in the aqueous solution obtained by various methods.[4,27] The bound water ratio per unit mass of pullulan was independent of the molecular length (140, 710, and 2600 mer). These results suggest that ΔF_{wet} would reflect an

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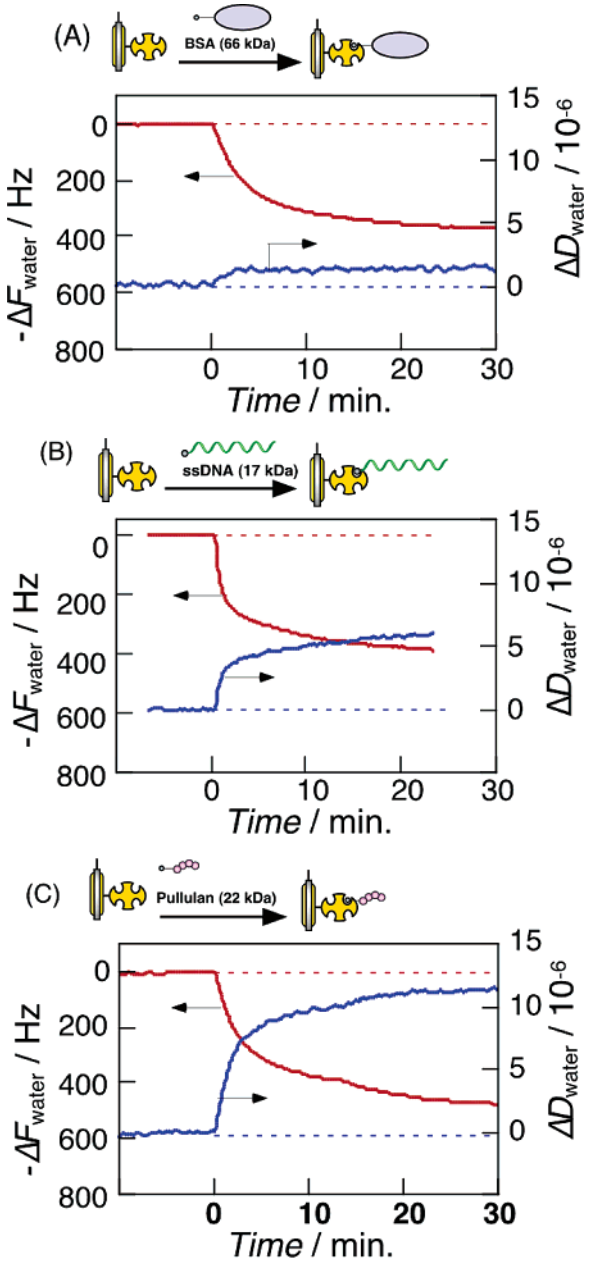

Figure 5. Time courses of $-\Delta F_{water}$ and ΔD_{water} values, responding to bindings of (A) biotinylated BSA (66 kDa), (B) biotinylated ssDNA (55 mer, 17 kDa), and (C) biotinylated pullulan (140 mer, 22 kDa) to the NeutrAvidin-immobilized 27-MHz QCM in a buffer solution (20 °C, 10 mM Tris, 200 mM NaCl).

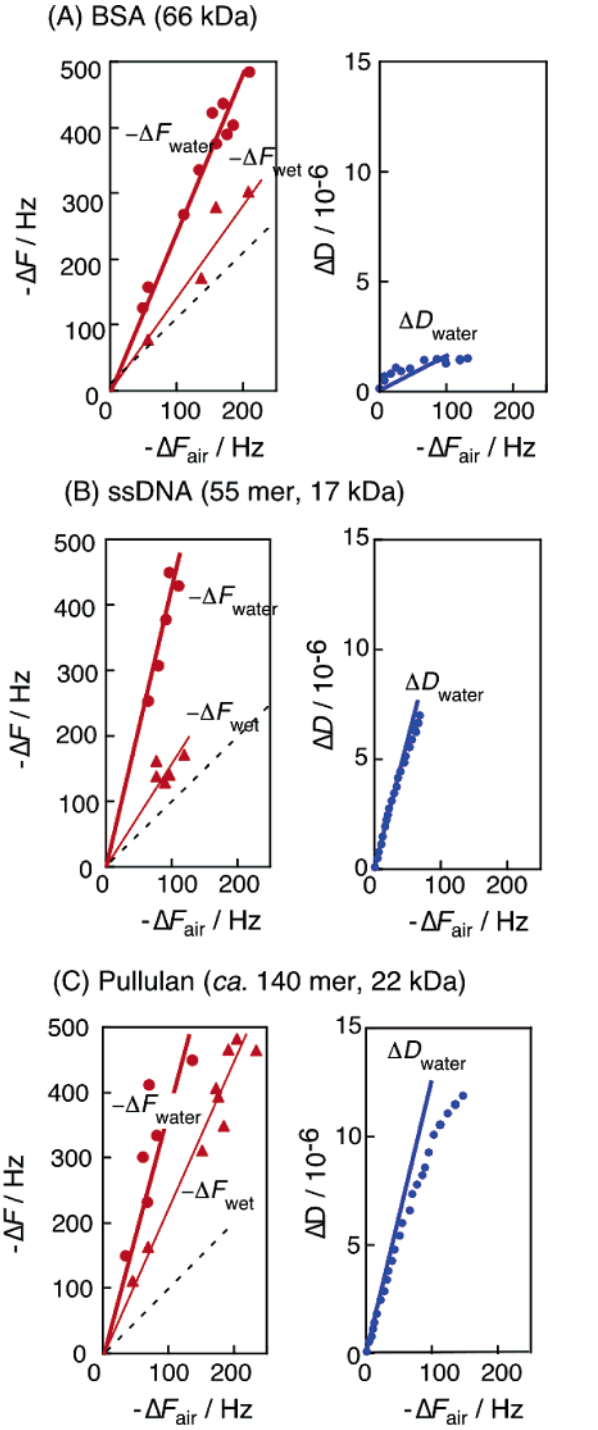

Figure 6. Linear plots of $-\Delta F$ and ΔD against $-\Delta F_{air}$ values (the real mass) of bound (A) biotinylated BSA, (B) biotinylated ssDNA, and (C) biotinylated pullulan. $-\Delta F_{water}$ and ΔD_{water} were obtained in a buffer solution (10 mM Tris, 200 mM NaCl) at 20 °C, $-\Delta F_{wet}$ and ΔD_{wet} were obtained in the 98% humid air phase, and then $-\Delta F_{air}$ and ΔD_{air} were obtained in the dry air phase on silica gel at 20 °C. ΔD_{wet} and ΔD_{air} are not shown, because of the negligibly small values ($<0.3 \times 10^{-6}$). Dotted lines indicate the slope of 1 obtained for the polystyrene coating.

amount of directly bound water with a surface of biomolecules. Hydrophobic polystyrene did not have any bound water or hydration in the humid air phase as well as in the water phase. The tendencies of $(-\Delta F_{wet})/(-\Delta F_{air})$ values for various molecules are reasonable depending on their molecular hydroproperties.

On the contrary, $(-\Delta F_{water})/(-\Delta F_{air})$ values indicated the hydrodynamic water (bound water + vibrated water) ratios per unit biomolecular mass are slightly complex and depend on molecular structures and lengths. The hydrodynamic water ratio of double-stranded DNA $[(-\Delta F_{water})/(-\Delta F_{air}) = 6.5]$ was larger than that of the single-stranded DNA (4.2). The dsDNA has been reported to stretch upon hybridization when compared with entangled ssDNA on the substrate.[28] Therefore, stretched dsDNA vibrates with more water than that of ssDNA. The $\Delta D_{water}/(-$

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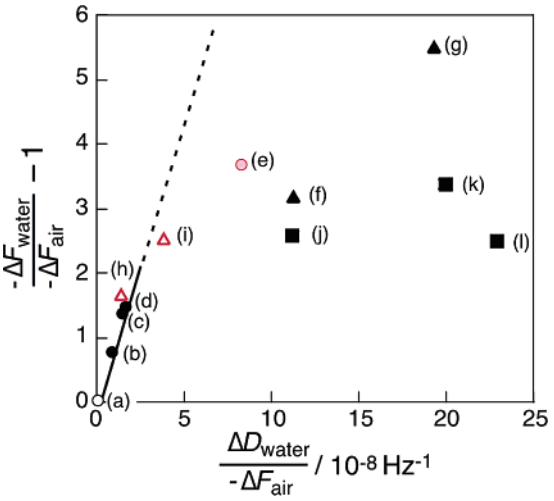

Figure 7. Relationship between the hydrodynamical water amount per unit mass [$(-\Delta F_{water})/(-\Delta F_{air}) - 1$] and the energy dissipation per unit mass [$\Delta D_{water}/(-\Delta F_{air})$] of (a) Polystyrene, (b) calmodulin, (c) Neutravidin, (d) BSA, (e) denatured BSA, (f) ssDNA, (g) dsDNA, (h) compact ssDNA, (i) compact dsDNA, (j) pullulan (22 kDa), (k) pullulan (112 kDa), and (l) pullulan (404 kDa).

ΔF_{air}) showing energy dissipation of the stretched dsDNA was also higher than that of the entangled ssDNA through DNA−water interactions ($\Delta D_{water}/(-\Delta F_{air}) = 19 \times 10^{-8}$ and 11×10^{-8} Hz^{-1} for dsDNA and ssDNA, respectively).

The $(-\Delta F_{water})/(-\Delta F_{air})$ values of pullulans were in the range of 3.5−4.4, which did not simply depend on molecular weight, and the values are in the middle of those for DNAs and proteins. However, $\Delta D_{water}/(-\Delta F_{air})$ values simply increased with increasing their molecular lengths. Thus, the longer pullulan shows the largest loss of energy in the water phase.

In the case of proteins, $(-\Delta F_{water})/(-\Delta F_{air})$ values were in the range of 2.4−2.5, which were smaller than those for DNAs and polysaccharides. The $(-\Delta F_{water})/(-\Delta F_{air})$ value of calmodulin (17 kDa) was 1.8 and also obtained in the same manner in the water phase. In contrast, $(-\Delta F_{water})/(-\Delta F_{air})$ values of the denatured BSA that might have a linear form due to reductions of SH groups were larger than the native BSA ($(-\Delta F_{water})/(-\Delta F_{air}) = 4.7$ and 2.5 for denatured BSA and native BSA, respectively). The energy dissipation of proteins was relatively small [$\Delta D_{water}/(-\Delta F_{air}) = (0.76-1.6) \times 10^{-8}$ Hz^{-1}], which indicates that small or globular proteins are rigid compared with linear DNAs and polysaccharides. When BSA was denatured, the $\Delta D_{water}/(-\Delta F_{air})$ value became larger (8.3×10^{-8} Hz^{-1}) than that of the native BSA (1.6×10^{-8} Hz^{-1}). These results indicate that not only ΔD_{water} but also ΔF_{water} would be a parameter depending on molecular structures and hydrophobicity/hydrophilicity of molecules and that could be used as a novel parameter to characterize biomolecules in aqueous solutions.

Relationships between Hydration and Energy Dissipation. On the basis of the results obtained in this study, the relationships between the hydrodynamic water amount and the energy dissipation for various materials are introduced. Relationships between $[(-\Delta F_{water})/(-\Delta F_{air}) - 1]$ (the hydrodynamic amount of water per unit mass) and $\Delta D_{water}/(-\Delta F_{air})$ (the energy dissipation per unit mass) are shown in Figure 7. The solid line in Figure 7 indicates the theoretical plots, in which hydrophilic and elastic materials are immobilized on the QCM surface and the energy dissipation is caused only by the hydrodynamic water of the hydrophilic elastic materials (see Appendix). The nonhydrated and elastic polystyrene was plotted near the zero point. Results for proteins were plotted on the theoretical line and a good linear relationship was observed in proteins (b−d), which indicates proteins have almost elastic properties independently of a molecular size. Energy dissipation in the air phase (ΔD_{air}) was negligibly small as mentioned above. The aforementioned results revealed that energy dissipation in proteins is mainly caused by interactions with water molecules. When BSA was denatured by breaking S−S linkages, the plot deviated from the theoretical line due to increases of the hydration and large energy dissipation (plots d and e). This is explained by increases of the solvent-accessible surface area and viscoelastic properties of a linear-like form of the denatured BSA.

In the case of DNAs, plots of (f) and (g) deviated from the theoretical line toward both the large hydration and large energy dissipation, indicating that linear DNA molecules are largely hydrated and energy dissipation occurs due to the viscoelastic properties of the structures in addition to large hydrations. The deviation of dsDNA (g) was larger than that of ssDNA (f), corresponding to the relatively extended duplex of dsDNA compared with the entangled structure of ssDNA. DNAs are well known to form compact structures by binding oligoamines such as spermidine and multivalent cations such as $Co(NH_3)_6^{3+}$.[29,30] When 0.4 mM $Co(NH_3)_6^{3+}$ was added to the dsDNA- or ssDNA-immobilized QCM in the aqueous solution, the plots of (f) ssDNA and (g) dsDNA moved onto the theoretical line of proteins as (h) ssDNA and (i) dsDNA. This result indicates that entangled DNA dehydrates and adopts elastic globular structures, like proteins.

In the case of pullulans, the plots of (j), (k), and (l) also deviated toward the large energy dissipation from the theoretical line, depending on the molecular weight. The aforementioned shows that the amount of pullulan hydration is independent of the molecular length, but the energy dissipation increases upon an increase in the molecular length of the pullulan due to its viscoelastic properties.

SUMMARY

We measured resonance frequency decreases ($-\Delta F_{water}$) and energy dissipation increases (ΔD_{water}) during the binding of proteins, DNAs, and polysaccharides in the water phase, together with $-\Delta F_{wet}$ and ΔD_{wet} in the humid air phase and $-\Delta F_{air}$ and ΔD_{air} in the dry air phase. ΔF_{water}, ΔF_{wet}, and ΔF_{air} indicate the total mass of molecules, the bound water, and vibrated water, the total mass of molecules and the bound water, and the mass of molecules, respectively. ΔD_{water} indicates the energy dissipation due to the hydration or viscoelasticity of molecules. ΔD_{wet} and ΔD_{air} were negligibly small, which indicates biomolecules behave as elastic materials in air phase. From the linear plots of the hydration amount and the energy dissipation for proteins, proteins were found elastic and energy dissipations of proteins were mainly caused by hydration. In cases with linear DNAs and pullulans, plots deviated from the theoretical line largely due to their hydrated and viscoelastic structures. On the other hand, DNA in

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the presence of multivalent cations such as $Co(NH_3)_6^{3+}$ dehydrates and adapts to elastic structures like proteins.

Recently, when hydrophilic biomolecules such as peptides, proteins, and DNA were immobilized on the 27-MHz QCM plate, we needed to calibrate frequency changes in aqueous solutions (ΔF_{water}) compared with those in air phase (ΔF_{air}).[33] When the NeutrAvidin-immobilized or short 55-bp dsDNA-immobilized QCM were employed in air phase, ΔF_{air} indicated simply the real mass and the constant of Sauerbrey equation was obtained as $\Delta m = 0.62 \pm 0.02$ ng cm^{-2} Hz^{-1}. When the NeutrAvidin- or DNA-immobilized 27-MHz QCM was soaked in aqueous solutions, ΔF_{water} were larger than ΔF_{air}, and there was a good linear correlation between ΔF_{water} and ΔF_{air}: $\Delta F_{water} = 2.4 \times \Delta F_{air}$ for the NeutrAvidin immobilization and $\Delta F_{water} = 6.5 \times \Delta F_{air}$ for the 55-bp DNA immobilization. Thus, the factor of Sauerbrey's equation in aqueous solutions can be determined to be $-0.62/2.4 = -0.26$ ng cm^{-2} Hz^{-1} for avidin and be $-0.62/6.5 = -0.095$ ng cm^{-2} Hz^{-1} for the short dsDNA. When the long dsDNA (500−1000 bp) was immobilized on the QCM, however, the linear relation between ΔF_{water} and ΔF_{air} decreased, because of the energy dissipation of the long DNA strand in aqueous solutions. Therefore, when the long linear biomolecules such as DNA or polysaccharide was immobilized on the QCM plated, we should consider more careful calibration between ΔF_{water} and ΔF_{air}.

We propose that admittance analyses of a piezoelectric crystal to obtain both ΔF and ΔD values in the water and air phase will become a useful technique to characterize biomolecules using hydration and viscoelasticity in aqueous solutions and/or to study conformational changes of various biomolecules.

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APPENDIX

Here, we explain how to separate the energy dissipation due to the hydrated water of molecules from the energy dissipation due to both the hydration and the viscoelastic property of molecules on a quartzcrystal oscillator in aqueous solutions. When a quartzcrystal oscillator contacts with thin elastic membrane, frequency changes can be surmised using an energy-transfer model. The frequency change can be expressed by a simple equation using the kinetic energy (E) of an oscillator and the transferred energy to adsorbed molecules (eq 7).[23,31,32]

$$\frac{\Delta F_{air}}{F} = -\frac{\Delta E}{2E} = -\frac{\Delta E_f^t}{2E_q^s} \tag{7}$$

where E_q^s is the energy stored in the quartz oscillator vibration before the adsorption of foreign molecules and E_f^t is the energy transferred from the quartz to adsorbed molecules. The frequency change (ΔF_{air}) of adsorbed molecule in air phase without the energy dissipation is according to eqs 7. The classical Sauerbrey's equation can be given from eqs 7.

When elastic molecules without the energy dissipation adsorbed on the quartz resonator surface are in aqueous phase, some of the energy stored in the quartz resonator is transferred to the molecule. This transferred energy is shared between stored and dissipated energy, because of its elastic properties of the adsorbed molecule, the energy dissipation occurred through vibration water of the molecule only. Thus the following energy balance is established.

$$E_q^s = E_q^{s\prime} + E_f^t + E_l^d \tag{8}$$

where $E_q^{s\prime}$ is the remaining energy stored in the quartz resonator after the deposition of a foreign molecule, E_f^t is the vibrational energy transferred from the quartz to the molecule, and E_l^d is the vibrational energy dissipated through hydrodynamic water. In this case, the frequency change can be expressed eqs 9.

$$\frac{\Delta F_{water}}{F} = -\frac{\Delta E}{2E} = -\frac{\Delta E_f^t + \Delta E_l^d}{2E_q^s} = -\frac{\Delta E_f^t}{2E_q^s} - \frac{\Delta E_l^d}{2E_q^s} \tag{9}$$

where the dissipation factor is defined as the dissipated energy against stored energy.

$$\Delta D = \frac{\Delta E_{dissipated}}{2\pi E_{stored}} \tag{10}$$

The relation between frequency change and dissipation factor change is given by eqs 9 and 10.

$$\frac{\Delta F_{water}}{F} = -\frac{\Delta E_f^t}{2E_q^s} - \frac{2\pi\Delta D_{water} \times E_q^s}{2E_q^s} = -\frac{\Delta E_f^t}{2E_q^s} - \pi\Delta D_{water} \tag{11}$$

Therefore,

$$\Delta F_{water} = -F\frac{\Delta E_f^t}{2E_q^s} - F\pi\Delta D_{water} \tag{12}$$

where eq 13 is a combination of the loaded mass (ΔF_{air}) according to Sauerbrey's equation and the contribution of the hydrodynamic water. The frequency change due to the loaded elastic molecule with hydrodynamic water is given as eq 14.

$$\Delta F_{water} = \Delta F_{air} - F\pi\Delta D_{water} \tag{13}$$

$$\frac{\Delta F_{water}}{\Delta F_{air}} - 1 = -\frac{F\pi\Delta D_{water}}{\Delta F_{air}} \tag{14}$$

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Thus, there is a linear correlation between $[(-\Delta F_{water})/(-\Delta F_{air}) - 1]$ and $\Delta D_{water}/(-\Delta F_{air})$, when the elastic molecules are immobilized on a QCM plate (see Figure 7).

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