Simultaneous anomalous reflection and quartz-crystal microbalance measurements of protein bindings on a gold surface†

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Protein bindings onto a gold surface were detected simultaneously by QCM (ΔF_{water}) and anomalous reflection (ΔR) of gold on the same surface in aqueous solutions; the obtained $\Delta F_{water}/\Delta R$ values correlated with surface areas and viscosity of proteins.

Viscoelastic and hydration properties of proteins have generated much interest, because these properties result in the structure and function of proteins such as stabilities, folding, structural changes, molecular recognition, and enzymatic activities. A piezoelectric quartz-crystal microbalance (QCM) is a very sensitive mass measuring device and has been used as a mass-sensor in aqueous solutions.[1–3] In many cases, when solid and hydrophobic materials are immobilized on the QCM plate, they behave as elastic membranes, and the resonance frequency (ΔF) decreases linearly with increasing mass (Δm) on the QCM plate; the phenomenon has been described by the Sauerbrey equation.[4] However, in a liquid system, frequency shifts (ΔF_{water}) correlate not only with the mass but also with the viscosity of the liquid as described by the Kanazawa–Gordon equation.[5] Therefore, when hydrated and viscoelastic proteins are immobilized, ΔF_{water} decreases with a different slope or deviates from the linear slope and quantification becomes difficult.[6] If substances on the QCM are highly hydrated, a larger $-\Delta F_{water}$ value than expected is obtained due to the hydration.[7–9]

In order to solve this complexity of the QCM, its combination with an optical sensing method such as surface plasmon resonance (SPR) measurement has been studied, because SPR is supposed to simply reflect surface mass changes without effects of water.[10–15] Simultaneous detections with QCM and SPR have been studied by two different methods: a dual probed device with one gold surface, in which SPR signals can be obtained from a corrugating grating prepared on the gold surface of a QCM plate,[10,11] and a parallel measurement device using a single cell with different gold surfaces for SPR and QCM, respectively.[12–15] In the simultaneous detection method, however, there are still some difficulties in detecting SPR signals on the vibrating QCM gold plate, because the grating of the QCM gold surface affects the vibration of the QCM in water.

In this paper, we propose a new simultaneous sensing device using an optical fiber sensor based on anomalous reflection (AR) of a gold surface of the QCM electrode (Fig. 1). Gold performs as a dielectric rather than a metal for blue or violet light and causes a significant reduction in reflectivity by adsorption of a monolayer on its surface.[16–18] The AR technique does not require complicated optical geometry, a very smooth and thin gold surface, and expensive instrumentation (laser system) as seen in SPR measurements.[17,18] We designed a 27-MHz QCM flow cell equipped with an optical fiber to detect AR of the gold surface, as shown in Fig. 1. A 27-MHz piezoelectric crystal (a square quartz plate: 2 × 4 mm, a gold electrode: 3.3 mm^2) was set at the bottom of a flow cell made of a transparent acrylic resin (cell volume: 4 μL). A multimode optical fiber (diameter: 1.2 mm) with a blue LED (λ = 470 nm) as a light source was set vertically at the top of the QCM electrode at a distance of 1.1 mm. The reflected light was coupled into the fiber and detected with a spectrometer.

We followed the immobilization process (amine coupling reactions) of catalase (232 kDa) as a protein onto carboxyl groups of 3,3′-dithiodipropionic acid that was activated with *N*-hydroxysuccinimide on the QCM gold surface (see illustration in Fig. 2).[6] Fig. 2A shows typical time courses of the catalase immobilization onto the QCM surface followed by both frequency decreases (ΔF_{water}) of the QCM and reflectivity decreases (ΔR) of

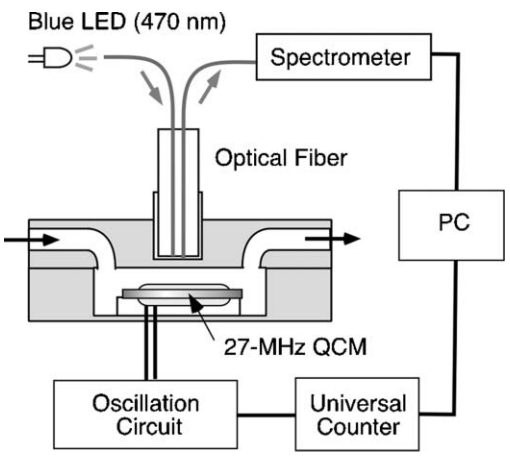

Fig. 1 A schematic illustration of the simultaneous detection of protein bindings to a gold surface of a 27-MHz quartz-crystal microbalance (QCM) and an optical fiber sensor based on an anomalous reflection (AR) of gold. Volume of flow cell: 4 μL; flow rate 100 μL min^{-1}; area of QCM plate: 2 × 4 mm; area of gold electrode: 3.3 mm^2; diameter of multimode optical fiber: 1.2 mm.

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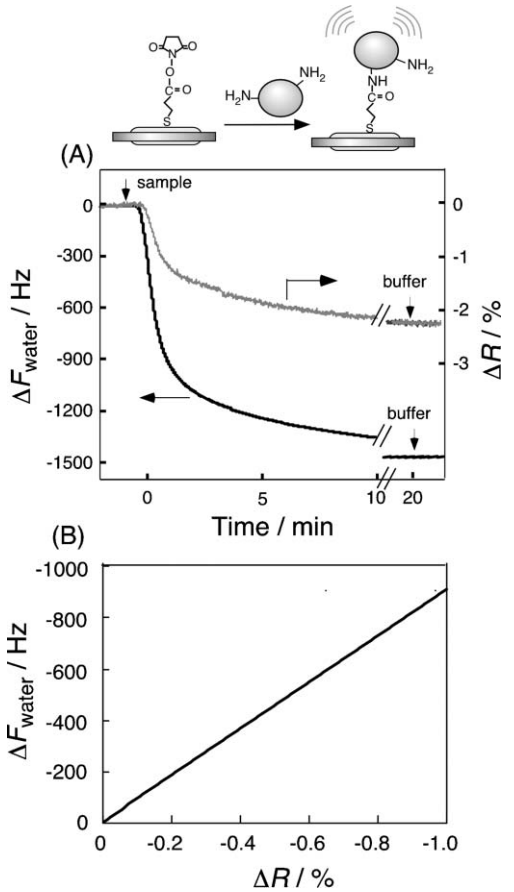

Fig. 2 (A) Typical time courses of the catalase immobilization by amine coupling reactions with the activated carboxyl groups on the QCM plate (pH 7.4, 20 mM HEPES, 200 mM NaCl, flow rate 100 μL min^{-1}, 25 °C). Sample was injected at −23 s and reached the cell at 0 s. The sample solution was exchanged to the buffer solution at the second arrow (at 20 min). (B) Linear correlation between ΔF_{water} and ΔR along the initial time course (0–70 s).

the AR. In Fig. 2B, ΔF_{water} is plotted against ΔR along initial time courses.

The ΔR values give the layer thickness of adsorbed molecules.[17] When the octadecanethiol monolayer (C_{18}-SH) was immobilized in a water phase (reflective index n_1 = 1.33), ΔR was obtained as −1.1% corresponding to a layer thickness of 2.3 nm, if the reflective index and the density of the layer are assumed to be n_2 = 1.5 as typical organic molecules and d = 1.0, respectively.[17] The obtained layer thickness (2.3 nm) was consistent with the theoretical value (2.2 nm, 167 ng cm^{-2}). In this system, we also calibrated ΔR directly by the mass of various proteins and polystyrene beads adsorbed on the Au surface in the air phase (ΔF_{air}). ΔF_{air} was measured by drying the cell in the air phase, after obtaining ΔF_{water} and ΔR in the flow cell. Regardless of the substances, ΔR values showed good linearity related to ΔF_{air}. Therefore, the influence of reflective index differences of proteins and their solution used here is very little, if any, in this system.

From the result of the calibration, the change of ΔR = −2.2% in Fig. 2A corresponded to ΔF_{air} = −686 Hz, which corresponds to a mass increase of 425 ng cm^{-2} according to Sauerbrey equation.[4,6] In other words, the frequency change of catalase in water (ΔF_{water} = −1550 Hz in Fig. 2A) was 2.3 times larger than ΔF_{air} = −686 Hz, due to the protein hydration and the viscoelasticity of the hydrated protein. Thus, the following calibration was obtained: ΔR (%) = 312 × ΔF_{air} (Hz) and ΔR = −1.0% corresponds to 193 ng cm^{-2} of the mass change. This is in relatively good agreement with the monolayer experiment of 1% ΔR = 167 ng cm^{-2}.[17]

After flowing out of the sample solution and changing to the buffer solution at 20 min, no decreases of ΔR and ΔF_{water} values were observed (see Fig. 2A). This reveals that almost all protein molecules bound to the substrate are covalently immobilized on the surface. Therefore, the fluctuation of ΔR and ΔF_{water} values would respond to the identical surface reaction.

Immobilizations of 9 proteins were carried out to follow both ΔF_{water} and ΔR values with time, according to Fig. 2A, and $\Delta F_{water}/\Delta R$ values for each protein are summarized in Table 1, together with their molecular weights. ΔR values simply reflect the membrane thickness (mass) and the larger $\Delta F_{water}/\Delta R$ indicates the larger effect of hydration and viscosity of proteins. The order of $\Delta F_{water}/\Delta R$ was not simply dependent on the molecular weight of the proteins, as shown in Table 1.

Fig. 3A shows the correlation between $\Delta F_{water}/\Delta R$ and solvent accessible surface area of proteins calculated from Surface Racer3.0® and PDB files,[19] and demonstrates a good linearity. Thus, proteins having a larger surface area such as catalase vibrate with the larger amount of water around the protein showing a larger $\Delta F_{water}/\Delta R$ value.

Gekko and coworkers obtained adiabatic compressibility (β_s) of various globular proteins by means of sound velocity measurements.[20,21] β_s values reflect the flexibilities and therefore the volume fluctuations of proteins in aqueous solutions. Fig. 3B shows plots of $\Delta F_{water}/\Delta R$ values against the compressibility β_s values, and a good linear correlation was obtained. Thus, proteins having larger flexibility showed larger $\Delta F_{water}/\Delta R$ values, indicating the viscoelasticity of proteins.

In conclusion, we have simultaneously obtained both frequency changes of the QCM (ΔF_{water}) and the AR changes (ΔR) of a gold surface of the same QCM plate with time courses of globular protein bindings. ΔF_{water} reflects the effects of the hydration, the viscoelasticity, and the mass of proteins; in contrast, ΔR simply reflects the membrane thickness (mass). The $\Delta F_{water}/\Delta R$ showing effects of hydration and viscoelasticity of proteins had a good linearity with the solvent accessible surface area of proteins and the compressibility of proteins. The simultaneous detection by both QCM and AR methods on the same gold plate will become a

Table 1 $\Delta F_{water}/\Delta R$ values of various proteins[a]

Proteins	M_w/kDa	$\Delta F_{water}/\Delta R$
NeutraAvidin	60	1200 ± 50
Ovalbumin	45	1100 ± 130
BSA	68	1000 ± 80
Catalase	232	860 ± 90
Ribonuclease A	13.7	590 ± 70
α-Chymotrypsin	25.7	510 ± 30
Avidin	67	490 ± 130
Trypsin	23	480 ± 60
Horse radish peroxidase	44	430 ± 10

[a] pH 7.4, 20 mM HEPES, 200 mM NaCl, flow rate 100 μL min^{-1}, 25 °C.

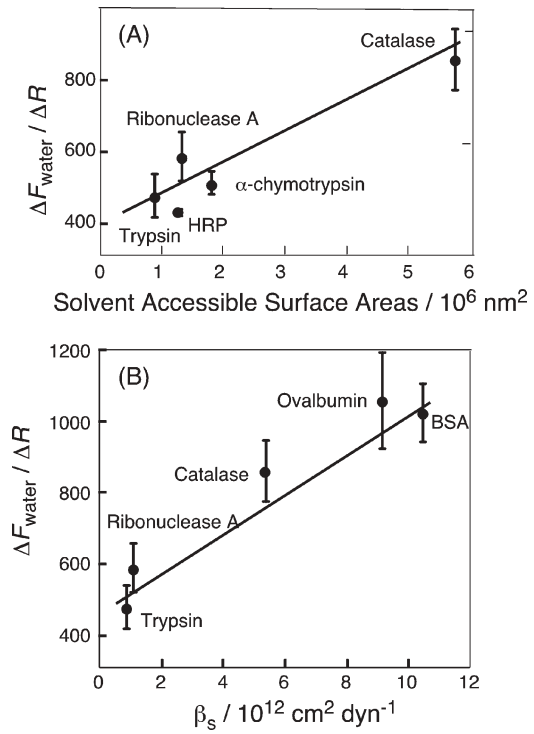

Fig. 3 Linear correlations between $\Delta F_{water}/\Delta R$ and (A) solvent accessible surface area (from ref. 19) and (B) adiabatic compatibility β_s (from refs. 20 and 21) of various proteins.

useful tool to assess the effects of hydration and viscoelasticity of biomolecules such as DNA/RNA and polysaccharides, in addition to proteins.

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