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Aggregation Behavior of Amyloid β_{1-42} Peptide Studied Using 55 MHz Wireless-Electrodeless Quartz Crystal Microbalance

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A homebuilt wireless-electrodeless high frequency quartz crystal microbalance is adopted for long-time monitoring of the aggregation behavior of amyloid β_{1-42} peptide in a flow-cell system. The monomer amyloid peptides are immobilized on both surfaces of the crystal, and an amyloid- β solution is injected. The monotonic frequency decrease indicates aggregation on the crystal, which yields aggregation rate. Aggregation is observed even at a peptide concentration as low as 550 nM.

1. Introduction

The amyloid β ($A\beta$) peptide has been recognized as the primary pathogenic agent of Alzheimer disease (AD).¹ $A\beta$ is extracted from the parental amyloid precursor protein by enzymes or enzyme complexes,² and its concentration is normally controlled by specific enzymes and antibodies in the cortical area of the brain.³ The full-length $A\beta$ peptides produced are $A\beta_{1-40}$ and $A\beta_{1-42}$, and the latter is more hydrophobic to cause fibril formation⁴ and is more neurotoxic.⁵ Therefore, understanding of the aggregation behavior of the $A\beta_{1-42}$ peptide is necessary to clarify the aggregation mechanism and for the development of drugs for AD.

In this study, we focus on using a quartz crystal microbalance (QCM) biosensor for the real-time monitoring of the aggregation behavior of the $A\beta_{1-42}$ peptide. The QCM has shown a pronounced ability for studying recognition behavior among biochemical molecules through changes in the resonance frequency of the quartz plate. A surface-modified quartz plate adsorbs the target molecule, resulting in an increase in the effective mass of the oscillator and then in a decrease in its mechanical resonance frequency. Resonance frequency is monitored during a binding reaction between the protein immobilized on the surface and the target protein in real time without any labeling, yielding thermodynamic binding constants.^{6,7} Because the QCM is a mass-sensitive biosensor, its sensitivity deteriorates when detecting a low-molecular-mass protein such as the $A\beta$ peptide (~ 4.5 kDa). Thus, it is necessary to improve QCM sensitivity. The most promising approach for this is to increase the fundamental resonance frequency f or decrease the quartz-plate thickness d , because QCM sensitivity, which is equivalent to the change in the resonance frequency Δf , is proportional to the square of the fundamental resonance frequency ($\Delta f \propto f^2$) or inversely proportional to the square of the thickness ($\Delta f \propto d^{-2}$). Recently, we have proposed wireless-electrodeless oscillators⁸⁻¹⁰ and achieved advanced QCM systems with much higher fundamental frequencies of up to 85 MHz.¹¹⁻¹⁵ Au or Pt electrodes have been adopted for the QCM, but they have much higher mass densities than quartz, causing signifi-

cant inertia resistance because of the maximum acceleration at the surface. We theoretically and experimentally demonstrated that QCM sensitivity is deteriorated by the presence of the electrodes.^{12,15}

Here, we apply the 55 MHz wireless-electrodeless QCM to study biochemical reactions related to the $A\beta_{1-42}$ peptide. First, $A\beta_{1-42}$ monomers are immobilized on both the crystal surfaces, which is confirmed by treatment with the anti- $A\beta_{1-42}$ antibody. Second, a solution containing $A\beta_{1-42}$ monomers is injected to monitor the aggregation of the analyte onto the immobilized monomers. The result shows strong hydrophobic bonding even for a low-concentration solution.

2. Experimental Procedure

30- μ m-thick AT-cut blank quartz plates with a 3 mm diameter are used throughout this study. Their fundamental resonance frequency is approximately 55 MHz, which is significantly higher than the conventional QCM frequency (~ 10 MHz).^{6,7} We deposit 9-nm-thick Au films on both surfaces of the quartz plates to immobilize the peptide using the gold-alkanethiol binding reaction, not for electrodes (atomic-force microscopy confirmed that the deposited surfaces were very smooth with a roughness of $R_a=0.4$ nm). The crystals are cleaned in piranha solution (98% H_2SO_4 :33% H_2O_2 =4:1) for 10 min, and after rinsing with ultrapure water several times, they are immersed in a 10 μ M 5-carboxy-1-pentanethiol/ethanol solution for 24 h. The sensor surfaces are then activated using a 100 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (EDC) solution and a 100 mM N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) solution for 1 h at 37 $^{\circ}C$. The crystals are immersed in a 5.5 μ M $A\beta_{1-42}$ /phosphate buffer solution (PBS; pH 7.4) for 24 h at 4 $^{\circ}C$, and after the rinsing procedure with PBS, they are immersed in a 50 mM glycine/PBS solution to block the remaining activated sites to avoid nonspecific binding.

The sensor crystal is then set in the handmade sensor cell^{12,15} and the cell is incorporated in the homebuilt flow-injection system, as shown in Fig. 1. The microp-

ump pumps the carrier solution of PBS at a flow rate of 50 $\mu\text{L}/\text{min}$. The switching valve selects a solution to be injected, which flows into the sensor cell through the degasifier. A 3 m Teflon tube column maintains the solution temperature at 37 $^{\circ}\text{C}$. When the $\text{A}\beta_{1-42}$ solution (550 nM) is selected as an analyte, it is returned to the injection vial via the valve after the QCM cell (Fig. 1) and is circulated for long-time monitoring of the aggregation behavior.

A burst signal ($\sim 100 V_{pp}$) is applied to the antenna for generation of the electromagnetic field, exciting the shear-horizontal vibration of the quartz plate without contact. The piezoelectric effect allows the noncontacting detection of vibrational amplitude ($\sim 0.1 V_{pp}$) and phase by the detection antenna,⁹⁾ and the detected reverberating signal is fed to a superheterodyne spectrometer, where the phase and amplitude of the resonance vibration are extracted.¹⁶⁾ The phase value is continuously measured to monitor the frequency change through the linear relationship between frequency and phase near resonance frequency.¹⁴⁾ The distance between the antenna and the quartz plate is about 1 mm. The effect of the surrounding solution on driving efficiency is insignificant.

First, we inject the anti- $\text{A}\beta_{1-42}$ antibody at a concentration between 67 pM and 67 nM to confirm the reactivity of the $\text{A}\beta_{1-42}$ peptides immobilized on the quartz surfaces. Then, using another identical crystal, we inject the $\text{A}\beta_{1-42}$ peptide/PBS analyte for long-time monitoring of the frequency change.

The $\text{A}\beta_{1-42}$ peptide (No. A9810) and a monoclonal anti-human $\text{A}\beta_{1-42}$ antibody (No. A1349) are from Sigma-Aldrich Japan. The 5-carboxy-1-pentanethiol (No. C387) and EDC (No. W001) are from Dojindo Laboratories. Sulfo-NHS (No. 56485) is from Sigma-Aldrich Japan. Glycine (No. 2709) is from Wako Pure Chemical Industries.

3. Results and Discussion

First, we evaluate the activity of the immobilized $\text{A}\beta_{1-42}$ peptide using its antibody. The amino terminal of the $\text{A}\beta_{1-42}$ peptide covalently combines with a self-assembled monolayer through EDC and sulfo-NHS, and this tight binding may affect the hydrophobic nature of the peptide. Figure 2 shows the resonance-frequency change caused by the injection of the anti- $\text{A}\beta_{1-42}$ antibody. In a typical antigen-antibody reaction, the frequency decreases exponentially with an exponential coefficient of $k_a C_A + k_d$, where k_a and k_d denote the reaction-rate constants for association and dissociation, respectively, and C_A is the concentration of the analyte (anti- $\text{A}\beta_{1-42}$ antibody).^{12,15)} Figure 2 shows that the change in the frequency obeys the exponential function and the exponential coefficient depends on the concentration of the analyte injected, confirming the antigen-antibody reaction. This antibody recognizes amino acids 17-24 in the middle part of $\text{A}\beta_{1-42}$. Considering that major hydrophobic amino acids are present in the middle part, the successful recognition by the antibody suggests that the inherent hydrophobic property of the peptide remains unchanged. Furthermore, the hydrophobic amino acids near the carboxyl end will remain active. Thus, the im-

mobilization of the $\text{A}\beta_{1-42}$ monomers and its hydrophobic nature are confirmed.

Next, we investigate the aggregation behavior of the peptide by injecting the $\text{A}\beta_{1-42}$ monomer solution of the concentration at a 550 nM. As shown in Fig. 3(a), the resonance frequency continues to decrease from the arrival of the analyte at the sensor cell; it decreases exponentially for 400 min and then linearly without being saturated. This behavior is different from that observed in an antigen-antibody reaction, where the frequency change reaches an equilibrium value with a balance between association and dissociation. Thus, the unsaturated frequency response reflects the aggregation of the $\text{A}\beta_{1-42}$ peptide onto the $\text{A}\beta_{1-42}$ monomers immobilized on the surfaces. Previous studies have indicated the critical concentration K_c of the $\text{A}\beta$ peptide over which the aggregation becomes significant. The reported K_c values are between 10 and 100 μM (100,¹⁷⁾ 50,¹⁸⁾ and 10 μM ¹⁹⁾, which are much higher than the concentration used in this study (550 nM). This is an important result because our study demonstrates that the aggregation occurs significantly even for a low-concentration $\text{A}\beta$ peptide.

In our QCM, the frequency fluctuation rate is typically on the order of 10^{-7} in a steady flow, and the frequency fluctuation shown in Fig. 3(a) is larger. The larger fluctuation can also be confirmed by comparison with the smooth frequency change shown in Fig. 2. Therefore, we consider that the fluctuation observed in Fig. 3(a) originates from the specific aggregation behavior of the $\text{A}\beta_{1-42}$ peptide. Kellermayer et al.¹⁹⁾ reported a stepwise growth of the $\text{A}\beta$ peptide and suggested that the discontinuous assembly mechanisms may be a general feature of amyloid growth. The frequency fluctuation may therefore reflect the discontinuous growth of the $\text{A}\beta_{1-42}$ peptide, which will be a topic for a future study.

We calculate the total amount of the peptide adsorbed due to the aggregation reaction using the Sauerbrey equation,²⁰⁾ which relates the frequency change Δf to the adsorbed mass Δm via $\Delta f = \Delta m/M_q f$, where M_q and f are the mass of the crystal and the resonance frequency, respectively. Figure 3(b) shows the result. (A small part of the edge of the quartz plate is held by a fixture ($\sim 2\%$ of the total area), but almost the entire area on both surfaces can be used as the sensing region, which was used to determine the adsorbed mass from the area.) The frequency change may also be caused by the change in viscosity during the adsorption. However, the viscosity effect becomes insignificant compared with the mass-loading effect at high frequencies because the viscosity load is proportional to $f^{1.5}$ whereas the mass load is proportional to f^2 .²¹⁾ We observed no marked vibrational-amplitude change during the aggregation reaction. Thus, we calculated the adsorption mass considering that the frequency change is caused only by the mass-loading effect.

The linear mass increase region gives an aggregation rate of 1.38×10^{-15} mol/min. This indicates that about 62 peptides aggregate in an area of 1 nm^2 every year.

4. Conclusions

A wireless electrodeless QCM with a 30- μ -m thick AT-cut quartz plate was developed to study the aggregation rate of A β_{1-42} . Monomers of A β_{1-42} were immobilized on both crystal surfaces, which was confirmed by the antibody of A β_{1-42} . The aggregation behavior was then monitored by injecting 550 nM A β_{1-42} . A continuous and unsaturated frequency decrease was observed, yielding an aggregation rate of 62 peptides/nm²/year. Such a low-concentration aggregation is first observed in real time. The frequency change during the aggregation suggested a discontinuous growth of the peptide. Future works will include the systematic study of the aggregation behavior of the peptide at various PHs and temperatures as well as a study on the frequency fluctuation during aggregation.

5. Acknowledgement

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Figure Captions

Fig. 1 Homebuilt flow-injection system for the real-time monitoring of biochemical reactions and aggregation behavior of peptides.

Fig. 2 Resonance frequency change caused by injection of anti-human $A\beta_{1-42}$ antibody at concentrations of 0.067, 33, and 67 nM. The horizontal axis is the time from the arrival of the antibody solutions.

Fig. 3 (Color online) (a) Resonance frequency decrease caused by the injection of a 550 nM $A\beta_{1-42}$ solution. (b) Change in the adsorbed mass of the $A\beta_{1-42}$ peptide on the crystal surfaces obtained by the Sauerbrey equation. The broken red line indicates the aggregation rate during the stable aggregation stage of the $A\beta_{1-42}$ peptide.

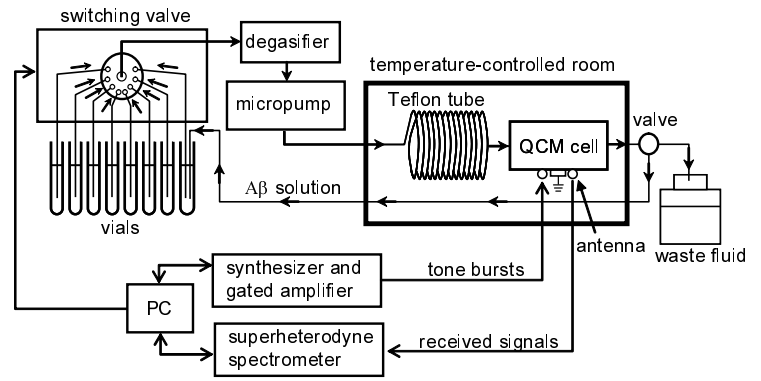


Fig. 1.

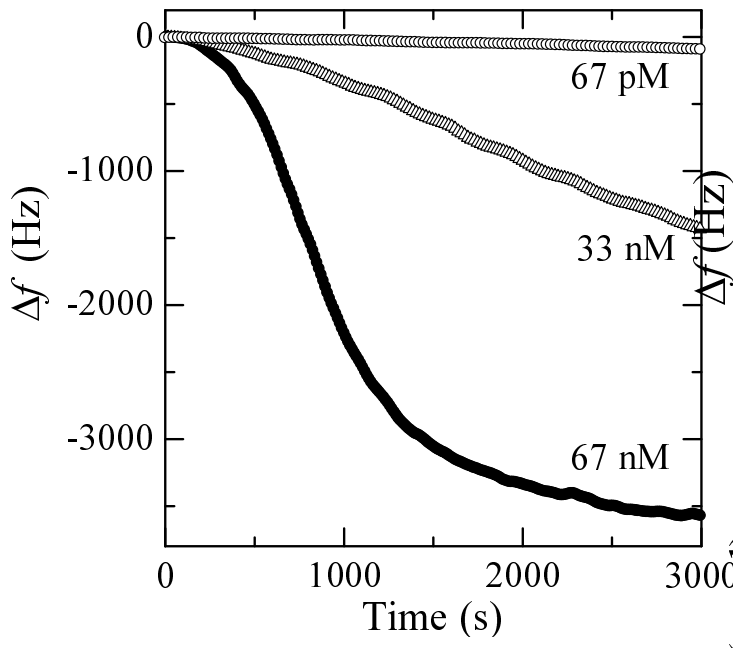


Fig. 2.

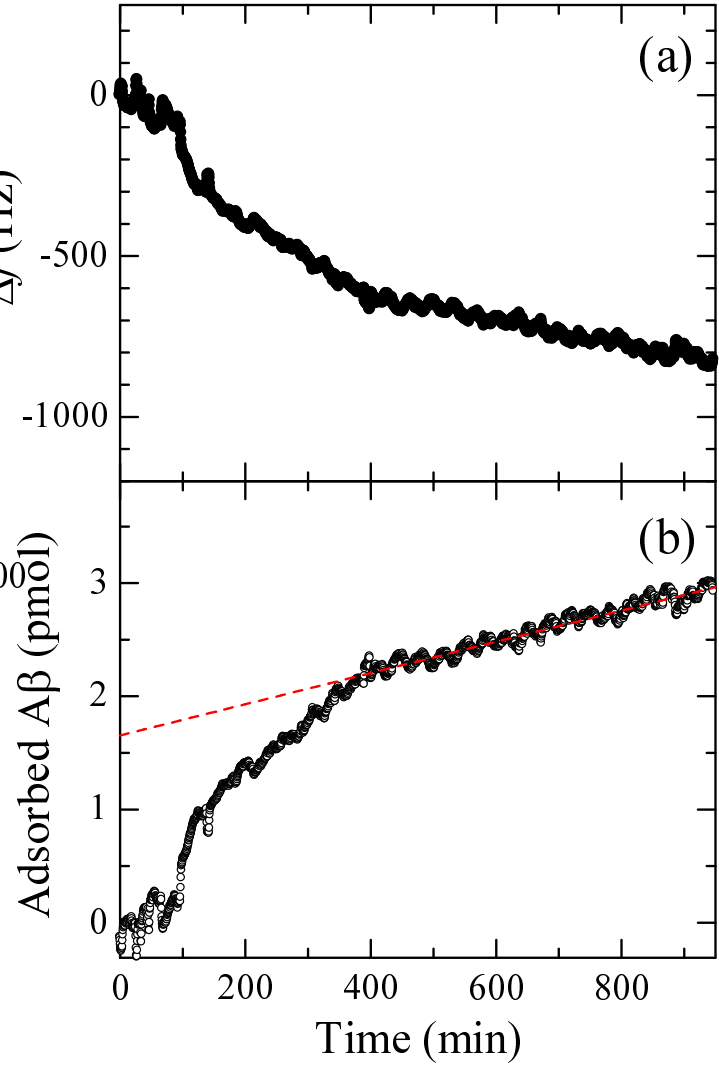


Fig. 3.