

Viscoelasticity Response during Fibrillation of Amyloid β Peptides on a Quartz-Crystal-Microbalance Biosensor

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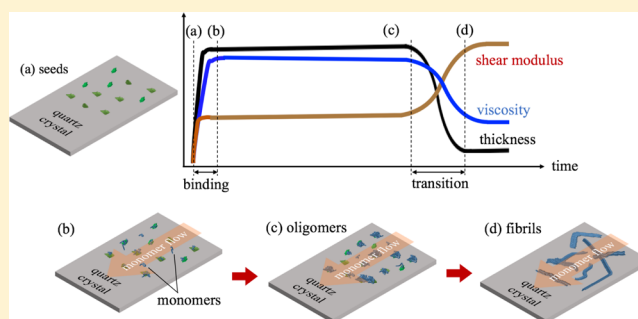
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Supporting Information

ABSTRACT: Unlike previous in vitro measurements where Amyloid β ($A\beta$) aggregation was studied in bulk solutions, we detect the structure change of the $A\beta$ aggregate on the surface of a wireless quartz-crystal-microbalance biosensor, which resembles more closely the aggregation process on the cell membrane. Using a 58 MHz quartz crystal, we monitored changes in the viscoelastic properties of the aggregate formed on the quartz surface from monomers to oligomers and then to fibrils, involving up to the 7th overtone mode (406 MHz). With atomic-force microscopy observations, we found a significant stiffness increase as well as thinning of the protein layer during the structure change from oligomer to fibrils at 20 h, which indicates that the stiffness of the fibril is much higher. Viscoelasticity can provide a significant index of fibrillation and can be useful for evaluating inhibitory medicines in drug development.



INTRODUCTION

Amyloid β ($A\beta$) peptides consist of 36–43 amino acid sequences, which result from the amyloid precursor protein through the cleaving procedure by β and γ secretases.^{1,2} The $A\beta$ peptides relate strongly to pathogenesis of Alzheimer's disease (AD); their aggregates are main components of amyloid plaques in brains of AD patients.^{3,4} The $A\beta$ peptides show self-assembling behavior and form the amyloid fibrils through a specific aggregation pathway, involving oligomers. The amyloid fibril is composed of the β -sheet structures and is highly stable,⁵ and it is considered as the dead end of the aggregation pathway. The $A\beta$ oligomers appear before the fibril state and therefore are called intermediates of the pathway (Figure 1). The oligomers^{6,7} and fibrils^{8,9} show neurotoxicity, and they are

recognized as pathogenicity factors for AD. Therefore, clarifying the transition behavior from oligomers into fibrils is not only helpful in understanding the aggregation mechanism (kinetic study of $A\beta$ fibril formation^{10–12}) but also in drug development.¹³

Although the aggregation phenomenon of the $A\beta$ peptides has been studied for decades, the aggregation reactions were usually performed in bulk liquids,^{8,10,13–15} where critical problems always arose: the $A\beta$ aggregation reaction in bulk solution proceeds with many interactions among aggregates (monomer, oligomers, and fibrils). These could cause products different from those formed on the surface by the interaction only between monomers and nuclei. Because the in vivo aggregation reaction proceeds with the deposition of $A\beta$ monomers on the $A\beta$ nuclei immobilized on the membrane surface, it is preferable to study the aggregation reaction on the surface not in the bulk solution.

It is expected that the structural change from oligomers to fibrils will cause a significant change in viscoelasticity of the aggregate layer.^{16,17} This is because the amyloid fibrils show stiffness much higher than standard protein aggregates.¹⁸ Because a high-frequency quartz-crystal microbalance (QCM) is sensitive not only to the mass of the aggregates on the surface but also to their viscoelasticity,^{19,20} we expect that such a

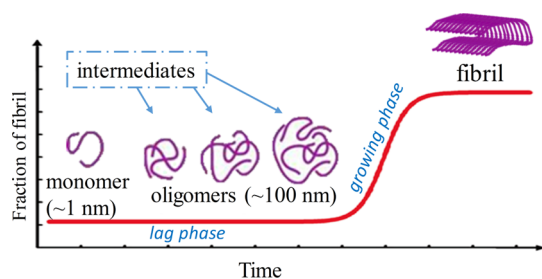


Figure 1. Typical aggregation pathway of the $A\beta$ peptide from the monomer to the fibril dead end.

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structural transition can be detected with a high-frequency QCM measurement.

Viscoelastic models in a QCM assay^{21–23} have been built for various surroundings such as gas, water,^{24,25} and amphiphilic overlayer.^{26–28} Most studies have used dissipation of the resonance vibration for this purpose,^{29,30} which is, however, affected not only by protein viscoelasticity but also by many ambiguous conditions such as the solution flow rate, the holding condition of the sensor chip, and so on. On the other hand, the frequency change, which can also be used for evaluating the viscoelasticity, is highly stable and much more robust to ambiguous measurement conditions. It is thus desirable to use only the frequency responses, but a QCM with sufficiently higher frequency is needed for this to improve the sensitivity to viscoelasticity.¹⁹

In this study, the aggregation reaction of the $A\beta_{1-40}$ peptide was promoted on a wireless-electrodeless (WE) QCM chip packaged in a microchannel, and its progress was monitored in real time through frequency responses, up to the 7th overtones. (The fundamental resonance frequency of our QCM is about 58 MHz, so that the 7th mode exceeds 400 MHz). This very-high-frequency QCM improves the mass sensitivity for smaller particles like $A\beta$ (molecular weight: 4500 Da)³¹ and allows determining simultaneously the protein-layer thickness, viscosity, and shear stiffness.

It has been shown that $A\beta_{1-42}$ seeds immobilized on a surface capture $A\beta_{1-40}$ monomers, resulting in oligomeric layers.^{32,33} Here, the aggregation reaction of $A\beta_{1-40}$ monomers on the $A\beta_{1-42}$ seeds was then studied. This binding system is practically important for two reasons. First, $A\beta_{1-42}$ peptides show much higher hydrophobicity than $A\beta_{1-40}$,³⁴ and they easily form the aggregation nuclei on the membrane surface. Second, the number of $A\beta_{1-40}$ are highest in the human brain,^{35–37} and they will accumulate on the $A\beta_{1-42}$ seeds.

Besides the overtone measurements, we performed the aggregation reaction on a bare quartz crystal without packaging to study the correlation between the viscoelasticity change and the surface morphology with an atomic-force microscope (AFM). We succeed in detecting, for the first time, the stiffening phenomenon in the protein layer after a long lag time on the surface. In addition, we reveal that this stiffening is caused by the transition from oligomers into fibrils.

■ VISCOELASTIC MODEL

We assume that a continuous and homogenous protein layer grows on the quartz oscillator between the solution layer and quartz surface. The resonance frequency of such a multilayer system principally depends on four parameters: thickness, viscosity, stiffness of the protein layer, and the overtone index n of the resonance.^{19,20,25} We then simultaneously measured the frequency change for $n = 1, 3, 5$, and 7 to extract the remaining three parameters inversely by the least-squares method.

In the model,^{19,20} we assigned different materials to each layer: first, the WE-QCM is regarded as purely elastic (α -quartz). Next, the protein ($A\beta$) layer is regarded as a viscoelastic Kelvin–Voigt material,²⁸ where an elastic spring and a dashpot are connected in parallel. Last, the solution layer is considered as a Newtonian fluid. Considering the free boundaries at the upmost and the downmost boundaries and continuities of stress and displacement at each interface, we obtain the frequency equation and then the frequency change

$$\Delta f_n \approx \frac{1}{2\pi\rho_q h_q} \operatorname{Im} \left(\kappa_p \xi_p \frac{1 - A e^{2\xi_p h_p}}{1 + A e^{2\xi_p h_p}} \right) \quad (1)$$

where

$$A = \frac{\kappa_p \xi_p + \eta_s \xi_s \tanh(\xi_s h_s)}{\kappa_p \xi_p - \eta_s \xi_s \tanh(\xi_s h_s)}, \quad \kappa_p = \frac{\mu_p^*}{j\omega_n},$$

$$\xi_p = \sqrt{-\frac{\rho_p \omega_n^2}{\mu_p^*}}, \quad \xi_s = \sqrt{\frac{j\rho_s \omega_n}{\eta_s}},$$

$$\mu_p^* = \mu_p + j\omega_n \eta_p$$

Here, h , ρ , μ , and η denote thickness, mass density, shear modulus, and viscosity, and ω_n is the angular frequency of the n th overtone. The subscripts s , p , and q represent solution, protein, and quartz layers, respectively. We assumed ρ_p to be 1100 kg/m³ to reduce the ambiguity of calculation because of its noncritical behavior compared with other parameters.¹⁹ The three parameters (h_p , μ_p , and η_p) are then inversely determined from four overtone frequencies measured simultaneously.

■ EXPERIMENTAL SECTION

All measurements and sample preparation were done at room temperature.

Preparation of $A\beta_{1-42}$ Seed Solution. $A\beta_{1-42}$ monomer (4349-v, Peptide Institute, Ibaraki, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO) with magnetic stirring for 10 min at 150 rpm. It was then diluted in acetate buffer solution (ABS, pH = 4.6) to a concentration of 50 μ M, where the volume ratio of DMSO to ABS was 1 to 5. This seed solution was then stirred for 24 h at 1200 rpm to form the fibrils.^{15,32} The solution was then exposed to 200 W ultrasonic irradiation under 26 kHz to fragmentate the fibrils to obtain the seeds. The solution was diluted by ABS to obtain the seed solution with a concentration of 16 μ M total monomer. The formation of fibril seed was confirmed by thioflavin-T (ThT) fluorescence experiment;¹⁵ the ThT level of the seed solution was higher than that of the monomer solution by a factor of ~ 100 .

Preparation of $A\beta_{1-40}$ Flowing Solution. $A\beta_{1-40}$ monomer (4307-v, Peptide Institute, Ibaraki, Osaka, Japan) was dissolved in DMSO and was diluted in ultrapure water until a final concentration of 10 μ M was reached. The ratio of DMSO to ultrapure water was 1:20. Note that the flowing solution should be prepared right before its injection into the QCM measurement.

Preparation of MEMS QCM Chips. A bare AT-cut quartz chip of 1.7 mm $\times$ 2.5 mm with a thickness of 28 μ m was placed into the Si microchannel, which was sandwiched by two glass plates as shown in Figure 2a. The solution volume is about 0.8 μ L, and the quartz crystal was lightly supported with micropillars and walls, without tight clamping (Figure 2b). The package was sealed by an anodic-bonding procedure.^{31,38}

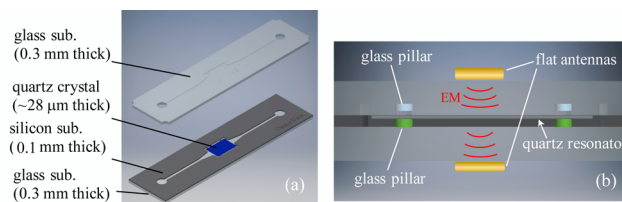


Figure 2. (a) Appearance of the wireless MEMS QCM before the packaging. (b) Mechanism of wireless excitation and detection of the packaged MEMS QCM via the flat antennas through the electro-magnetic wave.

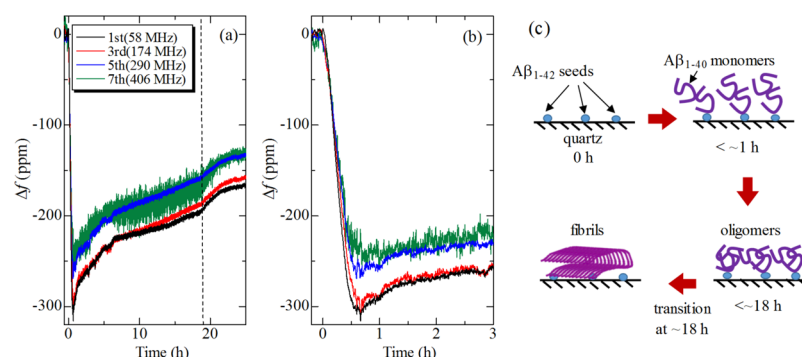


Figure 3. Overtone experiment for fundamental, 3rd, 5th, and 7th modes under $A\beta_{1-40}$ monomer-solution flow over the seeds immobilized on the packaged MEMS QCM. (a) Entire experimental results, with the dashed line, indicating the frequency ramp. (b) Close-up figure of (a). (c) Possible structural change on the surface.

The micro-electromechanical system (MEMS) QCM chip was first washed with piranha solution (7:3 of H_2SO_4 to H_2O_2) for 10 min, rinsed with ultrapure water (until pH 7), and cleaned with UV–ozone for 15 min. It was then immersed with the $A\beta_{1-42}$ seed solution and incubated for ~ 20 h at $4^\circ C$ to immobilize the seeds on the quartz crystal by nonspecific adsorption. The chip was washed with ultrapure water and then set into the sensor cell with two flat copper antennas (Figure 2b).

Preparation of Nonpackaged WE-QCM. The nonpackaged WE-QCM was used to study the surface morphology of the $A\beta$ aggregate using AFM. The bare quartz chip of $1.7\text{ mm} \times 2.5\text{ mm}$ area and $28\text{ }\mu\text{m}$ thickness was washed with piranha solution for 10 min, rinsed with ultrapure water, and cleaned with UV–ozone for 15 min. Next, the chip was immersed in the $A\beta_{1-42}$ seed solution for 20 h at $4^\circ C$. The chip was then sandwiched by silicon rubber sheets and set into the handmade sensor cell with a volume of 0.3 mL , and two antennas were embedded on both sides. The details are given in refs 39 and 40.

Experiment Setup for the MEMS QCM for Viscoelasticity. Two flat antennas were connected to a network analyzer (ZNB, Rohde & Schwarz), and the transmission signal (S12) was monitored. A micropiezoelectric pump was used to induce solution flow.

Initially, ultrapure water flowed into the sensor cell over the quartz surfaces as the carrier solution. When the four frequencies became stable, the $A\beta_{1-40}$ flowing solution was injected with the flow rate $20\text{ }\mu\text{L}/\text{min}$. (The solution would fill the microchannel in 2.4 s). We monitored the resonance frequencies of the fundamental (58 MHz), 3rd (174 MHz), 5th (290 MHz), and 7th (406 MHz) modes simultaneously.

AFM Observation. The aggregation reaction that occurred on the surface of the nonpackaged WE-QCM system was stopped at representative stages (before injection, initial binding, just before the structural transition, and after the structural transition), and AFM images on the quartz-chip were taken. A tapping-mode using a silicon cantilever with a bending stiffness of 40 N/m was used for the measurements. The resonance frequency of the cantilever was near 300 kHz . The scan frequency rate was 1 Hz . The AFM system was produced by Shimadzu Co. Ltd. (SPM A9600).

RESULTS

Overtone Experiment. A typical result for the overtone measurement of $A\beta_{1-40}$ binding to seeds on the MEMS QCM is shown in Figure 3a,b. (Other independent experimental results are shown in Supporting Information, Figure S1). As shown in Figure 3b, the $A\beta_{1-40}$ flow solution arrives at the sensor chip at time 0, and all overtone frequencies drastically drop by $250\text{--}300\text{ ppm}$ within 1 h. Then, the frequencies gradually increase until 18 h with slopes of 2.1, 2.8, 2.5, and 1.7 ppm/h between 10 and 15 h for fundamental, 3rd, 5th, and 7th modes, respectively. However, the resonance frequencies significantly increased between 18 and 20 h (we call this a

frequency ramp), and their slopes also increased to 8.0, 6.6, 6.1, and 10 ppm/h , respectively. Afterward, the frequencies became stable again with slopes of 3.0, 3.5, 3.1, and 3.6 ppm/h , respectively.

Viscoelasticity Calculation. Here, we evaluate the evolution of thickness (h), viscosity (η), and shear modulus (μ) of the $A\beta$ layer from the four (fundamental, 3rd, 5th, and 7th) resonance frequencies using the least-squares method.

The results are shown in Figure 4 for the experiments in Figure 3. After a few minutes from the arrival of the solution at

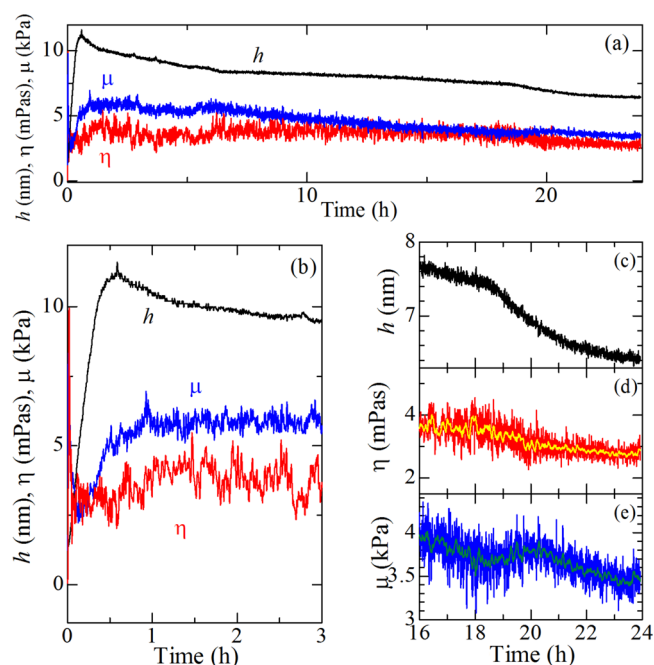


Figure 4. (a) Evolutions of layer thickness, viscosity, and shear modulus of $A\beta$ layer derived from the results in Figure 3. (b) Their changes in the early stage. Panels (c–e) show changes of thickness, viscosity, and shear modulus at the transition phase.

the quartz surfaces, the thickness increases up to about 12 nm (Figure 4b) and then decreases gradually. Its decremental rate, however, raises between 18 and 21 h (Figure 4c). A similar trend is observed in the viscosity change (Figure 4b,d). The shear modulus increases at first (Figure 4b), then gradually decreases, and increases again between 18 and 21 h (Figure 4e). The viscosity and the shear modulus change in a different direction in the period between 18 and 21 h .

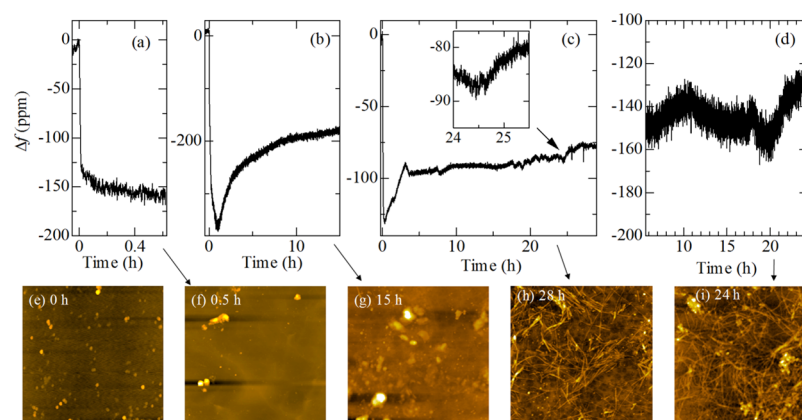


Figure 5. WE-QCM experimental results with only the fundamental mode stopped at (a) 0.5, (b) 15, and (c) 28 h. Panel (d) shows another experiment focusing at the frequency ramp. Note that these are completely independent measurements. Panel (e) shows an AFM image before the injection, and panels (f–i) are AFM images taken after the experiments (a–d), respectively.

Correlation between the Frequency Change and the Surface Structure. Figure 5a–d shows a series of independent QCM experimental results, and Figure 5e–i shows their corresponding AFM images. (AFM images for other experiments are shown in Supporting Information, Figures S2 and S3). Before the injection (0 h), we see many dots with ~ 100 nm diameters (Figure 5e), indicating immobilized $A\beta_{1-42}$ seeds on the WE-QCM surface. After a few minutes of the injection (0.5 h), the frequency dropped (Figure 5a), and the corresponding AFM image, Figure 5f, shows larger particles than those seen at 0 h. The measurement in Figure 5b shows the gradual increase of the frequency after the initial binding. The AFM image at 15 h (before the ramp) shows many amorphous-like structures, where the particles grew bigger than before (Figure 5g). In Figure 5c, the ramp appears at 24.5 h where the slope increased from 0.5 to 15 ppm/h (see the enlarged figure). Figure 5d confirms the observation of the ramp in another independent experiment at ~ 20 h. The AFM images after the ramp clearly show many fine fibrils in the entire region of the quartz surfaces (Figure 5h,i). Therefore, the frequency ramp near 20 h indicates the transition from oligomers to amyloid fibrils.

DISCUSSION

In considering the time-dependent evolution of the viscoelastic parameters and surface structure, we separate the results into three phases. First is the binding phase. It is the period starting from the injection of monomer solution until the maximum frequency decrement is reached. Second is the lag phase, which is the long and relatively stable interval from 0.5 h (after the binding phase) to ~ 18 h (before the frequency ramp). Last is the transition phase from 18 to 20 h in case of Figure 3a.

Before the arrival of the $A\beta_{1-40}$ flowing solution, we see dots with diameters of about ~ 100 nm on the quartz surface in the AFM image (Figure 5e), which we recognize as $A\beta_{1-42}$ seeds immobilized on the quartz chip. In the binding phase, the significant drops in the frequencies cause a large increase in the thickness, viscosity, and shear modulus in the $A\beta$ layer (Figures 3b and 4b), indicating that the flowing $A\beta_{1-40}$ monomers are captured by the $A\beta_{1-42}$ seeds, making the layer thicker and stiffer.

In the early stage of the lag phase (from 1 to 6 h in Figure 4a), the frequencies increase and the thickness decreases, and one may attribute these changes to the detachment of weakly

captured monomers from the surface. However, this would not be a principal cause because the viscosity and shear modulus remain unchanged in this period (Figure 4a,b); our QCM sensing region is about 20 nm from surface,¹⁹ and the averaged shear modulus and viscosity in this region are deduced from the inverse calculation. Because the parameters (stiffness, viscosity, and thickness) depend on the body layer of the system, they should be taken into account together. From the binding-phase response ($< \sim 0.5$ h), it is expected that the three parameters increase simultaneously when monomers attach to the seeds immobilized on the sensor surface. Therefore, detachment of monomers should make all three parameters drop. However, the stable viscoelastic properties (shear modulus and viscosity) contradict this prediction. Therefore, the unchanged average shear modulus and viscosity with decrease in the thickness of the adlayer indicates increases in the stiffness and viscosity of the protein aggregates. It is also shown in ref 33 that the $A\beta$ particle size increases gradually on the surface, which contradicts detachment of weakly captured monomers from the surface. Thus, we consider that the condensation of $A\beta$ aggregates occurs in this period.

After this period in the lag phase, both thickness and shear modulus decrease gradually, while the viscosity remains unchanged until just before the ramp (Figures 3a and 4b). We consider that $A\beta$ capturing and aggregate condensation³³ continue during this period. Unchanged viscosity again argues against the contribution of detachment of monomers. The shear-modulus decrease suggests that the protein stiffness is nearly unchanged during this period, so that the averaged modulus gradually decreased. This view is confirmed by AFM images (Figure 5g), which show that the aggregates grow on the quartz surface; the deposited $A\beta$ peptides continue to form dense aggregates, but their structure is not yet fibril.

At the transition phase (18–21 h in Figures 3a and 4c–e), a larger increase, compared to the lag phase, occurred in frequency slopes, which results in the stiffness increase and the thickness decrease. This indicates the transition from oligomer to fibril because amyloid fibrils show stiffness much higher than standard protein aggregates.¹⁸ It is verified by AFM images shown in Figure 5h,i, where dense aggregate structures are replaced by fibril-like structures. Because we failed to find any fibrils just before the ramp, we think that fibrillation occurs simultaneously over nearly the whole area of the quartz crystal. (As shown in Supporting Information, Figure S4, the dense

fibril structure was observed in different areas of the quartz chip after the ramp). The significant decrease of the $A\beta$ layer thickness in the phase can be caused by the ordering of the aggregates to form the dense fibril. The average shear modulus simultaneously increases, reflecting the much higher stiffness of the formed fibrils. Furthermore, the stiffness increase is also enhanced by the formation of the cross-networking fibril structure as shown in the AFM images (Figures S5h,i and S3b,c).

The viscoelastic model used in this work is greatly simplified; the model regards the $A\beta$ layer as a continuous and homogenous layer despite the fact that the protein layer includes not only the aggregates but also the surrounding solution. However, because the viscoelasticity of the aggregate significantly changes, the overall property of the protein layer will be sufficiently affected. The mechanical properties related to the fibril formation can then be evaluated even macroscopically, providing a significant insight. A more appropriate mode, which considers the island growth of $A\beta$ aggregates around specifically adsorbed nuclei, will be developed in our future studies.

CONCLUSIONS

Analysis of $A\beta$ aggregation was carried out on a quartz surface, and the conformational change correlation to frequency response was verified by AFM. It is a label-free methodology of monitoring the mechanical variation of adsorbed proteins on the surface in real time. The viscoelasticity model of $A\beta$ aggregation was successfully established, where we were able to characterize $A\beta$ aggregation in detail, showing it to be a decisive method for analyzing the aggregation phenomenon. The model may be applied in further analysis of other fibrillation characteristics, such as duration, rate, and interval of the fibrillation upon introduction of impurities such as inhibitors and different solvents.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.8b00639.

Three more overtone experiments, two more non-packaged-QCM experiments, and a few extra AFM images (PDF)

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Author Contributions

Y.-T.L. performed overtone and correlation experiments and viscoelasticity calculation and wrote this paper. H.O. invented the WE-QCM system, produced this study, and wrote the paper. K.N. prepared the solutions and fabricated the MEMS QCM. F.K. fabricated the MEMS QCM.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ABS, acetate buffer solution; $A\beta$, amyloid β ; AD, Alzheimer's disease; AFM, atomic-force microscopy; DMSO, dimethyl fluoride; ThT, thioflavin-T; MEMS, micro-electromechanical system; WE-QCM, wireless-electrodeless quartz-crystal microbalance

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