A Rapid, Sensitive, and Specific Detection of Aggregated α-Synuclein by a Liposome-Immobilized Cantilever Sensor

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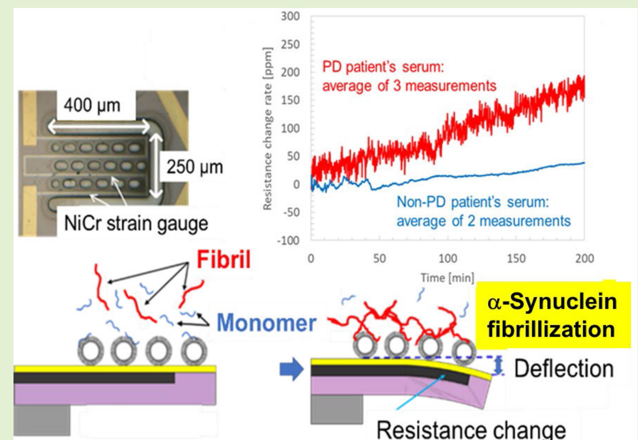
***Abstract*—Parkinson's disease (PD) is characterized by dopaminergic cell loss and the formation of Lewy bodies, of which the main component is aggregated and fibrillized α-synuclein (aSyn). Recent studies suggested that ultratrace amounts of aSyn aggregates are also present in biofluid specimens, and they can serve as a biomarker for PD. Because aSyn has been shown to possess a prion-like property, we attempted to enhance the sensitivity and specificity of a cantilever microsensor to detect aSyn aggregates by exploiting the properties of self-templating assembly and lipid interaction on the surface of liposome-immobilized cantilever sensor. We found that the liposome-immobilized cantilever sensor was able to successfully detect aSyn fibrils at a very low concentration (100 pg/mL), and the addition of aSyn monomers, which were converted into fibrils in the presence of aSyn aggregates and further acted as a template for fibrillization, lowered the detection limit to 10 pg/mL. The sensitivity of this cantilever sensor was comparable to or slightly superior to that of enzyme-linked immunosorbent assay (ELISA). Moreover, the lag time for the detection of aSyn fibrils has been significantly reduced to 100–120 min, compared to the tens of hours needed in conventional ELISA, real-time quaking-induced conversion (RT-QuIC), and protein misfolding cyclic amplification (PMCA) assays. Finally, preliminary measurements of aSyn aggregates showed the possibilities of discriminating serum from PD and non-PD patients. The liposome-immobilized cantilever sensor could serve as a promising tool for the early or preclinical diagnosis of PD.**



***Index Terms*—α-synuclein (aSyn), cantilever sensor, liposome, Parkinson's disease (PD), prion–lipid interaction, self-templating assembly.**

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I. Introduction

PARKINSON'S disease (PD) is the second most popular neurodegenerative disorder next to Alzheimer's disease. Unfortunately, neither disease-modifying therapies nor accurate early diagnostic biomarkers exist for PD [1], [2], [3], [4]. The pathological hallmarks of PD include dopaminergic cell loss and the formation of α-synuclein (aSyn) aggregates. The latter is also observed in other neurological disorders, such as dementia with Lewy bodies (DLBs) and multiple system atrophy (MSA), and these are termed α-synucleinopathies. Recently, aSyn was demonstrated to possess the prion-like property of self-templating assembly [5], [6]. The self-assembly of aSyn initiates at the nucleation phase, wherein small seeds of aggregates (oligomers) are formed, followed by the exponential growth phase, wherein aSyn oligomers grow into larger aggregates (fibrils), and finally reaches the plateau phase [4], [7], [8]. If small aggregate seeds are already present in the reaction solution, the nucleation phase, which is

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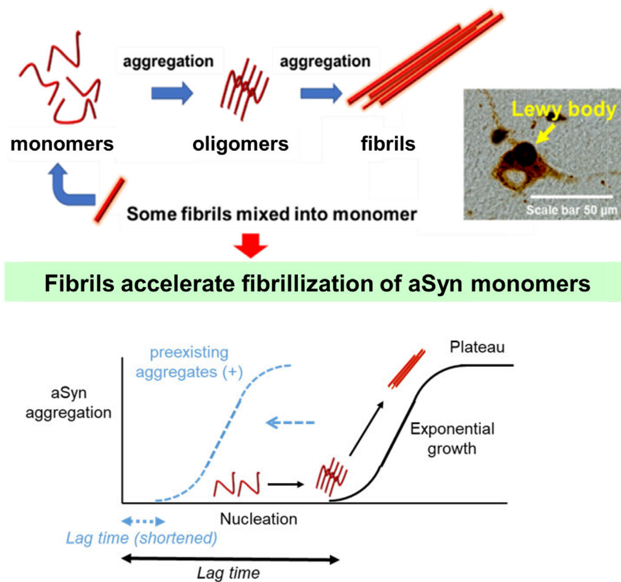

Fig. 1. Asyn species (monomers, oligomer, and fibrils) and the three phases in the aSyn fibrillization process (nucleation, exponential growth, and plateau phase).

a rate-limiting step, can be skipped, thereby greatly reducing the lag time to the growth phase (Fig. 1).

To date, there is no validated biomarker for PD. A noninvasive biochemical marker with high sensitivity and specificity is essential for monitoring the disease progression and early identification of individuals in the early stage before irreversible neuronal loss in the substantia nigra and associated motor symptoms. The detection of soluble aSyn aggregates in biological fluids could contribute to the diagnosis of PD [2], [3]. Indeed, several important observations linking aSyn aggregate formation and the PD pathogenesis have been presented in detail elsewhere [1], [2], [4].

Several methods, such as enzyme-linked immunosorbent assay (ELISA), real-time quaking-induced conversion (RT-QuIC) assay [9], and protein misfolding cyclic amplification (PMCA) assay [10], [11], [12], can be used to detect aSyn aggregates in biofluid samples. The sensitivity and specificity of the detection by ELISA depends on those of the antibody to the target molecule. However, there are no antibodies with high specificity for aggregated aSyn. Furthermore, some undesirable drawbacks remain unresolved, such as technical difficulties and time-consuming measurements. In the RT-QuIC and PMCA assays based on the same principle, the aggregated aSyn seeds are shaken in aSyn monomer solution to amplify them by a self-template mechanism, followed by the detection with the amyloid-binding fluorescent dye, such as thioflavin T (ThT) [13], [14]. It is highly sensitive but requires a long time, tens of hours. In addition, spontaneous formation and consequent amplification of the aSyn aggregates without aSyn seeds in the samples is often observed and is a major problem. Other methods to sense the aggregation/fibrillization of aSyn aggregates have also been devised, developed, and explained elsewhere [15], [16].

In this study, based on the self-templating property of prion-like proteins, we focused on the detection of aSyn aggregates using a cantilever sensor that is label-free, simple, inexpensive, and realizes a relatively short detection time [17], [18]. Detection of aSyn by mechanical sensors such as cantilevers has been little studied except for QCM. The interactions of molecules generate surface stress on the cantilever, which is converted into the mechanical deflection and detected. Typical interactions between biochemical counterparts, such as protein–protein interaction and DNA–DNA hybridization, are recognized and detected on the cantilever [19], [20], [21]. For example, we achieved sensitive detection of amyloid β aggregates using liposome-immobilized cantilever sensors [15]. Notably, cantilever sensors can be integrated with microfluidic and nanofluidic systems in an array configuration to simultaneously detect different types of targets. These multianalyses will be a target for development for biosensor technology in the near future.

Lipid–protein interactions in the membrane have been so far vigorously investigated [22]. The interaction between the lipid and prion proteins including aSyn has also been precisely reported [23], [24], [25], [26], and the potential mechanisms were briefly described as follows [24]. When the prion protein binds to lipids such as unilamellar liposomes, alanine/arginine/glutamine (ARQ) at codons 136, 154, and 171 of the prion protein is switched from its normal structure to a β-sheet-rich structure (prion conversion). Thus, a similar mechanism is expected to further promote aSyn aggregation and fibrillation on liposomes.

By combining the above two key features with the static-mode cantilever sensor, we sought to detect extremely low concentration of aSyn aggregates specifically amplified by the addition of aSyn monomers. Finally, we preliminarily evaluated the serum from PD and non-PD patients. From our related conference paper [27], one of the results was discussed more and newly compared with those using our RT-QuIC as different measurement techniques, showing intrinsic merits of the cantilever sensor, and importantly, it was newly applied for the evaluation of clinical biospecimen of PD patient as above.

II. Materials and Methods

A. Preparation of aSyn Monomers/Fibrils and Patients-Derived Biofluid Samples

Mouse aSyn was expressed in Escherichia coli BL21 (DE3) (BioDynamics Laboratory) and purified as previously described [28]. To generate aSyn fibrils, purified aSyn monomers (7 mg/mL) were incubated in the fibrillation buffer containing 30-mM Tris–HCl and 150-mM KCl at pH 7.5 in a quaking incubator at 1000 r/min, 37 °C for 120 h. Next, aSyn fibrils were pelleted by ultracentrifugation at 186 000 g for 20 min, stored at −80 °C, and resuspended in phosphate-buffered saline (PBS) when necessary.

Serum from PD and non-PD patients and cerebrospinal fluid (CSF) from healthy controls were prepared at Kyoto University Hospital with the approval of the Ethics Committee of Graduate School and Faculty of Medicine, Kyoto University, Kyoto, Japan. Venous blood was processed within 1 h of collection and allowed to clot for 15 min prior to centrifugation at 1500 g to obtain serum. CSF was centrifuged at 1500 g for 15 min, and the supernatant fraction was collected as CSF sample and stored at −80 °C until use.

B. Phospholipid Liposome

We selected 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) as the main phospholipid in the liposome to be used as a sensing biosupermolecule because DPPC is a major component of phospholipids in human (brain) cells. Its hydrophilic group PC is neutral, not charged, which is necessary because charged one has electrostatic interaction with charged proteins, other than aSyn, in a biofluid solution for measurement. Also added phosphatidylethanolamine (PE) and embedded by 1% volume in the DPPC to bond covalently with the self-assembled monolayer (SAM) formed on the cantilever [29], [30], [31]. The liposomes were prepared at a concentration of 30 mM using DPPC supplemented with PE via a freeze–thaw process. Those constituent molecules of liposome were purchased from NOF Corporation (Tokyo, Japan). The chemicals for SAM were purchased from Sigma Aldrich (St. Louis, MO, USA). Their preparation procedures and substances are reported previously [30], [31]. This time, the liposome was prepared as small as about 40 nm in diameter, aiming to increase its mechanical stability, while the previous size was about 100 nm.

C. Cantilever Microsensor

A microcantilever with a NiCr thin-film strain gauge was fabricated using a Si microelectromechanical system; the surface micromachining process has been described in detail [33], [34], [35]. The cantilever bends by its surface stress change that arises from molecular interactions among targeted and sensing biomolecules, including the initial change in mass on the cantilever surface, as previously explained [15]. As a sensing supermolecule, a liposome is immobilized on the cantilever surface released from the substrate. When the target protein aSyn approaches and interacts with the liposome immobilized on the cantilever, liposomal deformation induces surface stress [36] in addition to the mass change that occurs due to the target molecules. Mass is one of the important origins of surface stress; however, other origins become more important, typically for the case that the number of the target molecule is much smaller than that of the sensing liposome. To date, liposomes have been understood to deform and cause stress during interactions. Therefore, the variation in the static deflection of the cantilever, in such case, originates from that of liposomes as sensing biomolecules, other than from that in mass. Such surface stress enables deflection of the cantilever and changes in gauge resistance. The rate of change in resistance is proportional to that of surface stress as per the relationship derived from both Stoney's equation and the gauge factor one. Therefore, the interaction between aSyn and liposome can be evaluated according to the change in resistance. From the viewpoints of limit-of-detection (LOD) for an optical static-mode cantilever sensor, the measurable target mass obtained from surface stress is expected to be as low as 200 pg/mL [37]. For electrical static-mode operation, on the other hand, the noises from both the piezoresistive film and the electronic circuit used usually become serious origins to limit the LOD.

To reduce the thermal drift (noise) in the static-mode operation of the sensor, stabilizing temperature is indispensable. This is because: 1) the drift fluctuates the gauge resistance, even if the change ratio of the resistance against temperature is smaller than 10–100 ppm/°C for NiCr used and 2) more importantly, the phase stability of phospholipid depends on the temperature, which soft molecules naturally fluctuate, especially for DPPC used here, between gel and ripple phases in room temperature in the measurement. Here, we stabilized the temperature at 23.0 °C by a developed Peltier substrate programmed to actively control it within the range of $\pm$0.1 °C, where its thermal capacitance was significantly larger than that of the sensor, as reported elsewhere [38]. As a consequence, the stabilized temperature of the sensor successfully reduced the fluctuation of the output signal.

To avoid evaporation of the solution during chronological detection, a droplet-sealing structure using polydimethylsiloxane (PDMS) was fabricated to achieve complete sealing, as reported elsewhere [15]. The sealed reservoir could avoid the solution from evaporating for more than a day. To observe the change in resistance over time, after introducing the aSyn added in PBS into the PDMS reservoir, the gauge resistance was measured using a high-precision digital multimeter controlled by the LabVIEW 2012 software [38], [39].

D. Liquid-Environment AFM

It is so important to check whether the aggregation and/or fibrillization of aSyn really occurs on the cantilever surface. Therefore, we tried to observe the phenomena by a liquid-environment atomic force microscopy (AFM). Bruker NanoWizard III NW3-XS-O was used as the AFM apparatus with contact mode, which probe had the spring constant of 0.09 N/m and the intrinsic oscillation frequency of 110 kHz. The surface measured by the AFM was exactly the same as that of the measured cantilever sensor, where target aSyn fibril was added on the liposome immobilized on the cantilever in the solvent of PBS and high-concentrated aSyn monomer (500 μg/mL). Note that, different from the gauge resistance measurement of the cantilever sensor, the concentration of added aSyn fibril was as high as 100 and 1000 μg/mL.

III. Results and Discussion

A. Liquid-Environment AFM Observation of aSyn Aggregates on Liposome

Before measurements by the cantilever sensor, AFM surface views (top and bird's-eye) were obtained for aSyn fibrils (100 μg/mL) on liposomes, which were immobilized on a cantilever (Fig. 2). Aggregates observed on the surface of liposomes were considered as aSyn fibrils, and these aggregates increased monotonously with time after supplying aSyn fibril solution to the liposomes, but these aggregates were observed only at high concentrations of aSyn fibrils (100, 1000 μg/mL) within 60 min. Although it was difficult to determine whether they were bound or simply deposited on liposomes, we could at least confirm that the aggregates were definitely immobilized on the liposomes immobilized on the cantilever surface.

B. Time Course Measured by Liposome-Immobilized Cantilever Sensor

We attempted to detect trace amounts of aSyn fibrils utilizing the prion-like property of aSyn. First, a high concentration

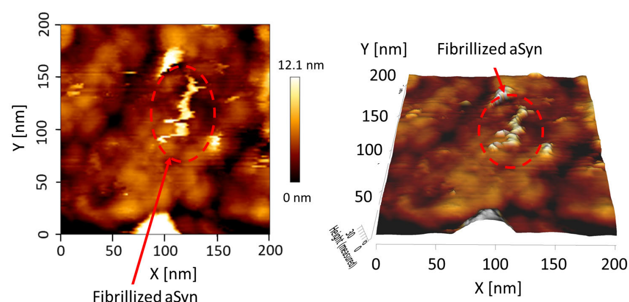

Fig. 2. AFM surface views obtained in a liquid cell for fibrillized aSyn (100 μg/mL) on liposomes immobilized on a cantilever 60 min after its addition. Top views in left and bird's-eye views in right.

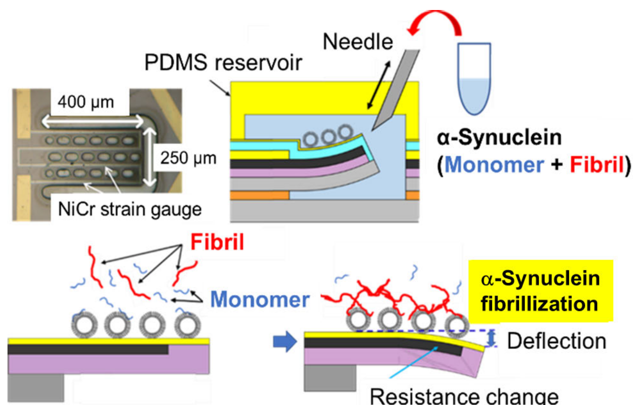

Fig. 3. Protocol for the sensitive detection of aSyn fibrils utilizing their property of self-templating assembly on the cantilever sensor.

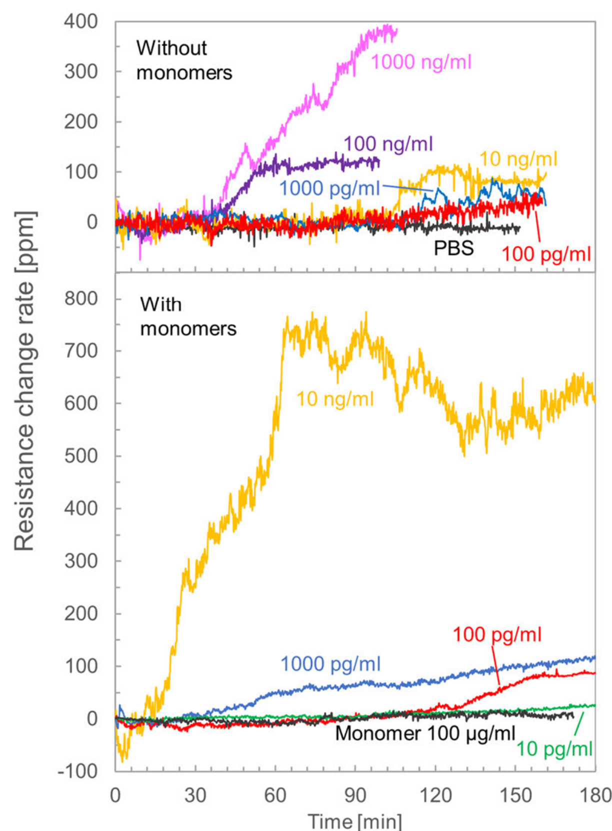

Fig. 4. Chronological change rate of piezogauge resistance of the cantilever sensor for recombinant aSyn fibrils. (Top) Without monomers (0, 100, and 1000 pg/mL; 10, 100, and 1000 ng/mL, respectively) in PBS. (Bottom) With monomers (0, 10, 100, and 1000 pg/mL and 10 ng/mL, respectively) in 100-μg/mL monomeric aSyn solution.

of aSyn monomer solution (100 μg/mL) in PBS was introduced into the reservoir. Then, aSyn fibrils (down to 10 pg/mL) were applied and mixed several times with a syringe. Finally, the signal from aSyn fibrils amplified by the addition of aSyn monomers was measured. We employed the aSyn monomers at 100 μg/mL, which is the minimum concentration commonly used in RT-QuIC assays. The protocol for the cantilever sensor is illustrated and summarized in Fig. 3. Before the main measurements, we first attempted to determine the LOD of aSyn fibrils in PBS by the liposome-immobilized cantilever sensor without aSyn monomers. Fig. 4 (top) shows the time course of the change in the piezoresistive gauge resistance of the sensor with different concentrations of recombinant aSyn fibrils (0, 100, and 1000 pg/mL; 10, 100, and 1000 ng/mL) in PBS. In PBS solvent without aSyn fibrils, chronological signal changes were negligible. The rate of change in resistance was demonstrated to increase, and the lag time to the onset of this increase was also shortened in a concentration-dependent manner [Fig. 4 (top)]. In contrast, we could not detect any signal change in the CSF from a healthy individual, who presumably had only aSyn monomers (not shown). In this setting using the static-mode cantilever sensor, the LOD was determined to be approximately 100 pg/mL, whereas the LOD for the electrical static mode was reported to be 1–10 ng/mL [40], suggesting that the interaction of aSyn fibrils with liposome improved detection sensitivity. When 10–1000 ng/mL of aSyn fibrils were added in Fig. 4 (top), the output level increased monotonically and reached a plateau phase as in Fig. 1, although below 10 ng/mL, the increase seemed to be in progress.

After preliminary measurements, the time course of gauge resistance change at different concentrations of aSyn fibrils with 100-μg/mL aSyn monomers was measured in Figs. 4 (bottom) and 5. Fig. 5 displays an enlarged view of Fig. 4 (bottom), with a focus on the data of 10-pg/mL aSyn fibrils. The change was negligible for aSyn monomers (100 μg/mL) without aSyn fibrils, suggesting that there was almost no surface stress between aSyn monomers and DPPC phospholipids. The addition of aSyn monomer, as shown in Fig. 5, allowed us to detect 10 pg/mL of aSyn fibrils. With the addition of aSyn monomers, the output level seemed to reach plateau phase for 10 ng/mL of aSyn fibrils, as in the case without aSyn monomers. However, below that level, a plateau phase was not reached. The self-templating nature of aSyn has been utilized to enhance the sensitivity and specificity to detect aSyn aggregates in human biofluid samples [9], [41], [42]. For example, aSyn aggregates could be specifically amplified by the addition of aSyn monomers into biological samples from α-synucleinopathies (PD, DLB, and MSA) but not from the other neurodegenerative disorders involving other prionoid proteins, such as amyloid β and tau [43]. Furthermore, the cantilever sensor was much more sensitive than the fluorophore, allowing for more rapid detection of aSyn fibrils. Given that the liquid target mass measurable by the cantilever sensor was reported to be 10 pg/mL in dynamic mode [36], the LOD of 10 pg/mL by the static mode would be excellent and the

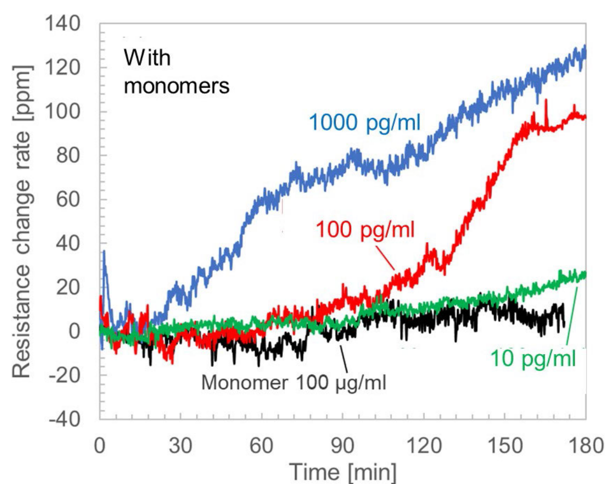

Fig. 5. Enlarged view of Fig. 4 (Bottom: With monomers) along the vertical axis, with a focus on the data for 10-pg/mL aSyn fibrils.

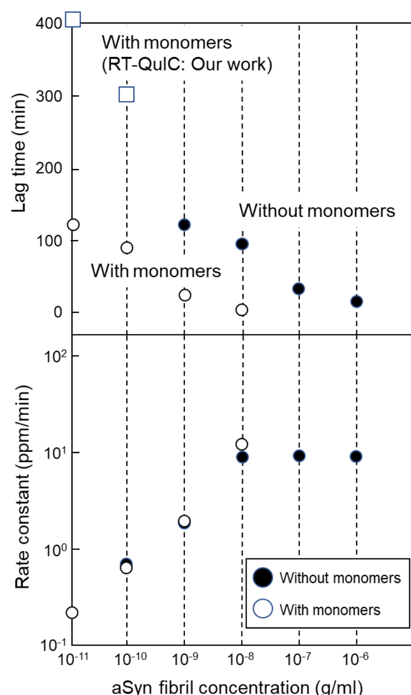

Fig. 6. Lag time (top) and rate constant (bottom) of the output of the cantilever sensors for aSyn fibrils with and without aSyn monomers, calculated and replotted from Fig. 4. The lag time with monomers is also compared between by the cantilever sensor and RT-QuIC.

sensitivity is expected to be increased by the addition of aSyn monomers.

C. Comparison of Lag Time and Rate Constant Between With and Without aSyn Monomers

Amyloid fibril has generally been shown to grow with time in an exponential manner and reach the plateau phase, as seen in Fig. 1. In accordance with this, the lag time and rate constant were extracted from Fig. 4 and replotted in Fig. 6, respectively. As shown in Fig. 6 (top), the lag time increased monotonically with the decrease in added aSyn fibril concentrations with and without aSyn monomers. Moreover, the lag time needed to detect the same aSyn fibril concentration was reduced by about two orders of magnitude with the

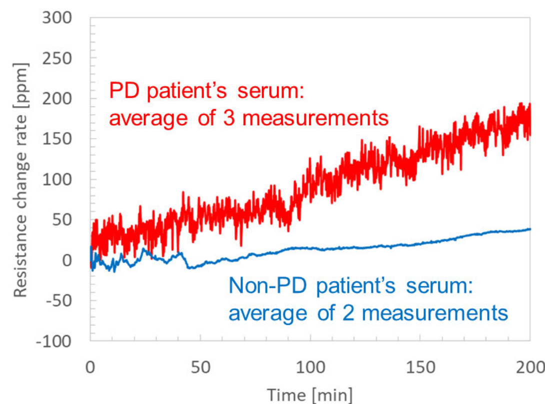

Fig. 7. Chronological change rate of averaged piezogauge resistance of the cantilever sensor after three consecutive measurements of serum from one PD patient and two consecutive measurements of serum from one non-PD patient.

addition of monomers. This indicates the effect of the self-templating phenomenon, in which fibril seeds are amplified by aSyn monomers. Next, the rate constant was extracted by the first-order approximation from the initial increase in the output level of the cantilever sensor in Fig. 4 with and without aSyn monomers. Fig. 6 (bottom) showed that the rate constant decreased almost in an exponential manner with the decrease in the concentration at relatively low concentrations, whereas it was almost the same at high concentrations. Note that, at the same concentration, the rate constant was almost the same with and without aSyn monomers. This also indicates that the intrinsic rate of aSyn fibrillization might be approximately the same, regardless of the addition of aSyn monomers. Compared the results of 10 ng/mL and 1000 pg/mL in Fig. 4 (bottom) with those in Fig. 6, the change in resistance change rate in Fig. 4 seems to be larger. It has been reported that when there is only a very low concentration of template (aSyn aggregates) near the detection limit, not only the lag time but also the time to reach plateau is prolonged and the amount of final aSyn aggregates produced tends to decrease [44]. Within the measurement time in this study, a plateau was actually not reached with a 1000 pg/mL of template, and the amount of aSyn aggregates produced was also considered to be decreased. Therefore, it may be observed that the rate of change in resistance is very smaller for the 1000 pg/mL of template than for the 10 ng/mL.

Furthermore, the lag times in our RT-QuIC assay for 10 and 100 pg/mL of aSyn fibrils were plotted in Fig. 6, for comparison with those in the cantilever sensor. The lag time of 6–7 h for 10 pg/mL of aSyn fibrils was comparable to one of the best results in RT-QuIC assay in the literature [44], but about three times longer than those in the cantilever sensor, despite the addition of high salt or SDS in RT-QuIC assay for increased sensitivity. This would also suggest the strong interaction between fibrils and liposome membranes.

D. Preliminary Evaluation for Serum From PD and Non-PD Patients by the Cantilever Sensor

We preliminarily evaluated the aSyn aggregates in serum from PD and non-PD patients by the cantilever sensor. In Fig. 7, the average of three measurements in a PD patient

and two measurements in a non-PD patient were chronologically plotted, and their difference seems to be relatively clear. Although the increased number of samples is necessary to be analyzed, it would be suggested that the aggregation and fibrillization of prion-like proteins such as aSyn can dominate in causing the surface stress change on the cantilever sensor over the matrix effect of the serum.

For the specificity of the aSyn fibril amplification in the aSyn monomer solution, this is guaranteed by the fact that the homologous seeding is much more efficient than the heterologous seeding (amplification of prionopid proteins seeded by different prionoid aggregates, also called cross-seeding). In fact, aSyn monomers RT-QuIC assays have been shown not assemble into fibrils in the presence of amyloid β, tau, and other prion protein seeds [9], [12], [45], [46], [47], [48].

For the sensitivity of the aSyn fibril detection, one of the most sensitive RT-QuIC assays detects 1 pg/100 μL (=10 pg/mL) of aSyn fibrils in less than 10 h [44]. The cantilever in this article detects 10 pg/mL of aSyn fibrils in about 2 h [Figs. 4 (bottom) and 5], which is not inferior in terms of sensitivity. Furthermore, in RT-QuIC and PMCA assays, spontaneous self-assembly of aSyn monomers without aSyn seeds is often observed after prolonged incubation [49], [50], [51]. The cantilever sensor can detect aSyn fibrils with higher sensitivity than ThT dyes in RT-QuIC assays, potentially reducing the probability of false positives due to the spontaneous self-assembly of aSyn monomers.

In the future, specificity can be further improved by introducing antibodies, sugar chains, proteins, and cholesterol into the liposome membrane, and/or by using other phospholipid components. We are also considering an array approach that uses different types of monomers, such as amyloid β and tau, for simultaneous and multiple analyses to help diagnose multiple proteinopathies.

IV. Conclusion

We have successfully combined the self-templating and lipid interaction properties of aSyn species with a liposome-immobilized cantilever microsensor. This enabled the rapid and specific detection of aSyn fibrils, with an LOD of 10 pg/mL and a lag time of approximately 120 min, which is shorter than PMCA/RT-QuIC assays. Furthermore, the sensitivity was comparable or slightly superior to that of conventional ELISA for aSyn aggregates. Finally, preliminary measurements have shown the possibilities of discriminating serums in PD and non-PD patients. Our findings indicate that the liposome-immobilized cantilever sensor is a promising technology for the diagnosis of early or prodromal PD and can also contribute to the development of disease-modifying therapies for PD.

Acknowledgment

A series of new results are added by different measurement methods; then, the contents are totally reconsidered and summarized.

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