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Label-Free, Chronological and Selective Detection of Aggregation and Fibrillization of Amyloid β Protein in Serum by Microcantilever Sensor Immobilizing Cholesterol-Incorporated Liposome

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Abstract:

To facilitate the early diagnosis of Alzheimer's disease and Mild Cognitive Impairment patients, we developed a cantilever-based microsensor that immobilized liposomes of various phospholipids to detect a trace amount of amyloid beta (Aβ) protein, and investigated its aggregation and fibrillization on model cell membranes in human serum. Three species of liposomes composed of different phospholipids of 1,2-dipalmtoyl-*sn*-glycero-3-phosphocholine (DPPC), DPPC/phosphatidyl ethanolamine (PE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylglycerol (DPPG) having varied hydrophilic groups were applied, which showed different chronological interactions with Aβ(1-40) protein and varied sensitivities of the cantilever sensor, depending on their specific electrostatic charged conditions, hydrophilicity, and membrane fluidity. 1,2-ditetradecanoyl-*sn*-glycero-3-phosphpcholine (DMPC) having short hydrophobic carbon chains confirmed to show a large interaction with Aβ(1-40) and a high sensitivity. Furthermore, the incorporation of cholesterol into DMPC was effective to selectively detect Aβ(1-40) in human serum, which effect was also checked by

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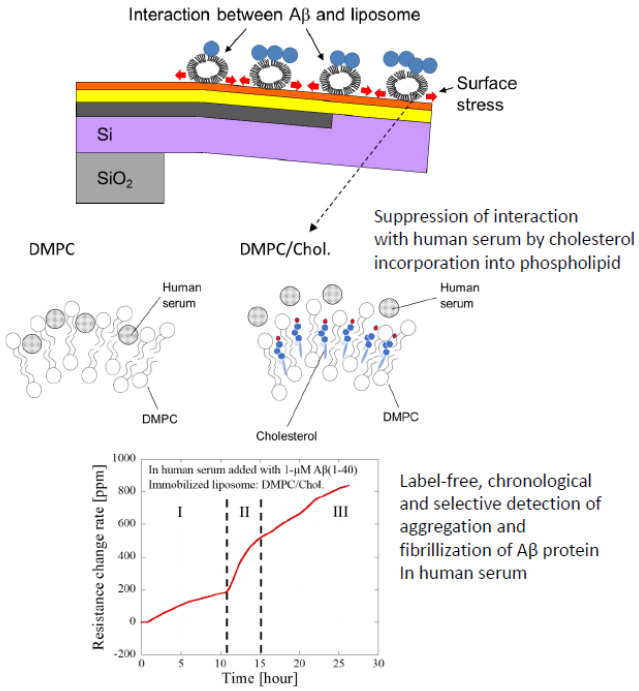
quartz crystal microbalance (QCM). Finally, Aβ detection of 100-pM order was expected selectively in the serum by using the developed biosensor.

Graphical Abstract

We have developed the liposome-immobilized microcantilever sensor for detection of Aβ fibril, thereafter newly confirmed the effectiveness of cholesterol addition into the phospholipid to improve the selectivity of the detection of Aβ aggregation/fibrillization in human serum.

It is also suggested that optimizing the deformation of liposome might induce more the perturbation of membrane structure of DPPC enough to improve its interaction with Aβ.

Finally, Aβ detection of 100-pM order was expected selectively in the serum by using the developed biosensor.



Keywords: cantilever; biosensor; liposome; amyloid-β; serum;

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1. Introduction

Much attention has been paid extensively on Alzheimer's disease (AD), which is one of dementia and serious amyloidosis [1, 2]. Many researches have claimed that amyloid beta (Aβ) protein and/or peptide is a causative factor for AD [3-5]. In biomedical field, an enzyme-linked immunosorbent assay (ELISA) has been still one of the most practical methods that can detect pM-ordered Aβ protein [6,7]. However, a number of serious issues exist in ELISA, including a lack of knowledge of the label-fibril binding stoichiometries, which varies for different types of aggregates or as a function of extrinsic conditions, complicated procedures, highly trained skills, long measurement time, expensive measurement facilities etc. Furthermore, ELISA can only detect the existence of target molecules because ELISA is based on typically antigen-antibody reaction such as sandwich method. As for other prospective methods for detecting Aβ aggregation and/or fibrillization, fluorescence spectroscopy combined with molecularly specific dyes, including thioflavin T (ThT) and Congo red, and the other ones such as circular dichroism (CD), nuclear magnetic resonance (NMR), X-ray diffraction (XRD), were examined and found to be excellent detecting principles but difficult to detect and characterize very small amount of Aβ aggregation [8,9]. Label-free technique such as surface plasmon resonance (SPR) and surface-enhanced Raman scattering (SERS) are very attractive and can detect around pM-order or less. But it is necessary to set up a precise and complicated optical detection system to operate the above main label-free

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methods. This is difficult to apply for a simple diagnosis required on the spot with a small and portable device or equipment in near future. On the other hand, some of the label-free methods are combined with nanofluidic device structure [10], so the situation of miniaturization and scalability would progress gradually. Recently, PET [11] and MALDI-TOF MS [12] are recognized ultrasensitive (less than pM-order) to detect selectively a trace amount of protein fragments. Meanwhile, those techniques need also complicated procedures, highly trained skills, very large and expensive equipment.

Compared to the above technique, mechanical sensor of cantilever has been investigated that are simple, comprehensible in principle [13] and shown highly sensitive miniaturized transducers for label-free detection of biomolecules [14-17]. The molecular interactions on the cantilever surface generate stress then directly transduced into its mechanical deflection, which is precisely detected. We should note that cantilevers are not limited by mass sensitivity but operate via an entirely different principle, detecting tiny changes in in-plane forces, called surface stress. The factors and the phenomena responsible for the surface-stress response during molecular recognition still remain ambiguous and confusing for biological adsorption, due to the complexity of the interactions involved. While at the same time, typical paired interactions between biomolecular partners have been realized and detected on the cantilever such as DNA–DNA hybridization [14], protein–DNA interactions [15,18], and protein–protein interactions [19]. Furthermore, it should be noted that other important

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merits for the cantilever sensor exist in terms of parallelization; arrayed configuration for simultaneous and different species of target molecules, and integration with micro- and/or nanofluidic systems [9].

As an effective biosensor to detect Aβ aggregation and fibrillization, we proposed a microcantilever sensor using phospholipid liposomes (model cell membranes) as Aβ-sensing biomolecules [20-22]. This sensor can discriminate the fibrillization process of Aβ (e.g., molecular configuration of monomer, oligomer to protofibril, and fibril). The cantilever sensor is one of the promising Aβ biosensors to chronologically detect the trace amount of Aβ for establishing early AD diagnostics and to elucidate AD mechanisms. For this purpose, to detect fibrillizing Aβ having a concentration of less than 1 nM is required, because the fibrillizing Aβ, not monomeric one, is most toxic and its concentration of AD patients is reported to be pg/mL ordered [23,24]. At the present level [20-22], the concentration should be improved by more than 10 times for Mild Cognitive Impairment (MCI) patients than that for usual AD ones.

When Aβ protein combines on “raft” on the membrane, it is explained that usual α-helix and/or random coil structured Aβ changes to β-sheet one that tends to be amyloid fibril. The raft is a microdomain which composition is different and phase-separated from the surrounding membrane, and some of the raft is found to be membrane-protein-localized domain rich in sphingolipid, cholesterol or fatty acid. So far, we investigated the effect of different hydrophobic groups in phospholipids on the interaction between Aβ(1-40) and liposomes as well as on the sensitivity of the cantilever sensor using

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1,2-dipalmtoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1-palmtoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), respectively [22]. These liposomes are recognized as *non-raft* membrane. Therefore, the mimicking of raft-like membrane would be promising for improving the sensitivity and selectivity of Aβ.

In this work, firstly, we focused on the hydrophilic groups other than phosphocholine (PC) to make clear their effects on sensing characteristics and performance and to mimic the biomembrane-like environment. We employed 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylglycerol (DPPG) and DPPC containing 30 wt% phosphatidyl ethanolamine (PE). The hydrophilic PG group is negatively charged [25], while PE and PC are electrically neutral. Secondly, the cantilever sensor immobilized by liposomes incorporated with cholesterol was examined especially for DMPC and DPPC, where DMPC improved more its sensitivity against Aβ [22]. We note that not only the detection of low Aβ concentration but Aβ measurement in human blood (serum) is essential. Therefore, thirdly, we attempted to detect Aβ in human serum in order to apply this sensor practically for AD diagnosis, thereafter investigated whether Aβ can be selectively detected in human serum using the effect of cholesterol on membranes.

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2. Materials and Methods

2.1. Biochemical Molecules

In this work, Aβ(1-40) was selected as a target biomolecule. Aβ(1-40) in non-aggregated state with the molar mass of 4250 g/mol was purchased from Peptide Institute (Osaka, Japan). DPPC was selected as a major phospholipid molecule for the liposome because its bilayer membrane has the phase-transition temperature at about 42 ^{o}C from gel to liquid phase [22], and DPPC liposomes can be immobilized in stable gel state at room temperature. To have covalent bonding between the phospholipid and self-assembled monolayer (SAM) formed on the Au surface of cantilever, phosphatidyl ethanolamine (PE) was also incorporated into the phospholipid membrane. DPPC, DPPG, PE and DMPC were all purchased from Avanti Polar Lipids (Birmingham, Wales, UK). Cholesterol for inclusion in the phospholipid was purchased from Sigma Aldrich (St. Louis, MO, USA). For preparation of SAM, 16-mercaptoundecanoic acid, N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'- ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Normal human serum was obtained from Kohjin Bio (Sakado, Japan). The components of the serum include total protein (73 mg/mL), cholesterol (1.6 mg/mL), and triglycerides (820 μg/mL). Polydimethylsiloxane (PDMS) was purchased from Dow Corning Toray (Tokyo, Japan).

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2.2. Preparation of phospholipid liposome

We prepared five different liposomes by freeze-thaw process, i.e., DPPC, DPPC (containing 30 wt% PE), DMPC, DMPC incorporated with cholesterol and DPPG, respectively, with concentration of 30 mM throughout the experiments. The preparation procedures for the former four are described elsewhere [26,27]. All the liposomes were modified with 1 wt% PE, respectively, because the modification enhanced the immobilization of liposomes on the SAM/Au surface of the cantilever [22,27]. In the experiment, cholesterol was incorporated in the preparation process of liposome when dissolving phospholipids in chloroform.

2.3. Preparation of Aβ(1–40) solution

At first a 300-μM Aβ(1-40) solution was prepared by dissolving the non-aggregated Aβ(1-40) powder thoroughly into ammonia water (0.1 wt%). Then, the prefabricated Aβ(1-40) solution was diluted ranging from μM to nM-order with ultrapure water or normal human serum. It is noted that the Aβ serum solutions were used to mimic the plasma condition of patients with AD.

2.4. Cantilever sensor immobilized with liposome

Basically, deflection (bending) of the cantilever beam changes due to the interaction-induced surface stress. We can inquire the several parameters that decide the bending for a single beam from the basic equations of material mechanics, one of which is the Stoney's formula that relates the surface

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stress and curvature of the beam. As piezoresistive change is translated to strain (deflection) that is induced from the curvature by the mechanics, the surface stress can be obtained through the Stoney's formula.

As one of the origin of surface stress, mass is most important but the other origin would exist simultaneously, especially when the concentration of target biomolecule is much lower than that of the interacted sensing biomolecule. It has been understood so far that liposome deforms and would give the stress during the interaction. It would be therefore reasonable that in such cases the change in static deflection of cantilever is mainly originated from that due to the liposomes as sensing biomolecules.

We fabricated a liposome-immobilized cantilever sensor with NiCr strain gauge on silicon on insulator (SOI) wafer via Si microelectromechanical systems (MEMS) processes, while a polydimethylsiloxane (PDMS) droplet-sealing structure was also applied to the cantilever sensor [20,27].

As reported elsewhere [20], the films of NiCr and Au were deposited successively on a silicon-on-insulator (SOI) wafer by rf sputtering and then patterned by photolithography, where the NiCr film (40 nm thickness) is piezoresistive strain gauge. The cantilever structure was formed by etching a buried Si oxide sacrificial layer. The length and width of the cantilever are 370 and 220 μm, respectively.

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The micro-cantilever surface was coated with Au ultrathin film (~8 nm) by vacuum evaporation, thereafter SAM was formed on the surface via thiol-metal bond by immersing in ethanol solution of 16-mercaptoundecanoic acid. Thereafter, the carboxyl radicals on the SAM surface were activated by N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide in the mixed solution of 1,4-dioxane (90 vol%) and ultrapure water (10 vol%) in order to attach the amino radicals of other biomolecules via amide linkage. Finally, the liposomes containing 1 wt% PE phospholipid molecules which have amino radicals were immobilized on the SAM surface by immersing in the liposome suspension for at least 12 h. The micro-cantilever with immobilized liposomes was kept in ultrapure water before being used. In order to suppress the evaporation of the droplet, a droplet-sealing PDMS structure was fabricated with a 2 mm-diameter hole of solution reservoir, which was adhered on the cantilever chip [27].

Fig. 1 shows a microscopic image of the cantilever as well as the principle for detecting Aβ before and after the interaction between liposomes and Aβ. The bending of the cantilever is driven by the surface stress changes arising as a result of specific interactions between the biomolecules on the cantilever surface. Prior to main measurements with adding target Aβ protein, control experiments without the target proteins were done in air and ultrapure water, respectively. The primary signal level of resistance change with liposome immobilized on SAM/Au cantilever was confirmed to be chronologically almost stable and less than 5-10 ppm for 1 h in air and ultrapure water, respectively

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[20]. It should be noted that, without the liposome/SAM on the cantilever, the signal was also stable and less than 10 ppm after addition of Aβ protein. This indicates that both physical deposition and nonspecific binding of Aβ protein would be negligible without the liposome/SAM.

In our experiments the supplied droplet filling the PDMS reservoir is much larger than that of cantilever and the sealed reservoir can avoid evaporation of solution for more than 24 h, therefore the setup of the target solution in the reservoir would not affect the sensor readings within the measurement time range.

As mentioned before, Aβ is known to aggregate on phospholipid membranes which are similar to neuro cell membranes. It generally takes more than 24 h for Aβ(1-40) to become mature fibril through intermediated-aggregation [25,28]. The long-time measurement of the interaction between Aβ(1-40) and phospholipid membranes of liposomes becomes very informative for obtaining the related mechanism and early diagnosis of AD. Fortunately, it is possible to measure the gauge resistance of the cantilever sensor for more than 24 h because the PDMS droplet-sealing structure above the cantilever can successfully keep the Aβ solution from evaporation.

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3. Results and Discussion

3.1. Interaction between Aβ and liposomes with different hydrophilic groups

After introducing the each solution with Aβ(1-40) into the PDMS droplet-sealing structure of the cantilever sensor, the resistance of its piezoresistive gauge was measured using the LabVIEW 2012 software. As we have studied previously the difference in the effect of hydrophobic group of liposome on sensing behavior of Aβ(1-40) [22], we started to investigate how the liposomes composed of different hydrophilic groups interact with Aβ. Fig. 2(a) shows the chronological resistance change rates of the cantilever sensor using liposomes which consisted of phospholipids of DPPG, DPPC, and DPPC containing 30 wt% PE (DPPC+PE), respectively. The resistance change rate for DPPG was clearly the highest among the three different liposomes. Although the gauge resistances of the cantilevers immobilized by DPPC and DPPC+PE were stable from the beginning to around 8–9 h (the first stage of the interaction between Aβ and phospholipid membranes), the resistance for DPPG increased until 8–9 h. In essence, the sensors immobilizing liposomes can detect the dynamic behavior of Aβ(1-40) within chronologically different stages of Aβ aggregation and fibril growth [21,22]. It is considered that the resistances for liposomes of DPPC and DPPC+PE are stable when Aβ is monomer (the 1st stage). The interaction between non-aggregated Aβ and liposomes is mostly an electrostatic one [25]. The resistance was stable until 8–9 h, because the electrostatic interaction between monomeric Aβ and the liposome of DPPC or DPPC+PE did not arise, where both PC and PE groups are neutral. On the other

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hand, the resistance for DPPG liposome was increased gradually and largely, compared with the cases of DPPC and DPPC+PE. DPPG is negatively charged [25]. Furthermore, it was reported that Aβ is charged positively in acid solution [29,30] and the solutions including hydrophilic PG groups show weak acidity [29]. Therefore, monomeric Aβ electrically interacted with hydrophilic PG groups. It is reasonable that the electrostatic interaction between monomeric Aβ and DPPG liposomes increased the resistance of the cantilever until 8–9 h. At the present stage, we consider that the electrostatic interaction is dominant and much larger for DPPG than the other hydrophobic interaction for DPPC or DPPC+PE with neutral hydrophilic group because positively charged molecules other than the Aβ are all interacted in the measured solution.

At the second stage of the interaction between Aβ and phospholipid membranes (from 8–9 to 16 h), the resistances for all the cases of DPPG, DPPC and DPPC+PE increased as shown in Fig. 2(a). The previous study demonstrated that the rapid increase in the resistance of the cantilever immobilized by PC liposomes is driven by hydrophobic force [22], which facilitates the interaction between Aβ and hydrophobic groups of the liposomes. Moreover, we believe that this rapid increase could be majorly related to Aβ aggregation, which makes the hydrophobicity of Aβ stronger than that for Aβ monomer [31]. The more Aβ becomes aggregated, the more hydrophobic interaction between Aβ and liposomes prevails than the electrostatic interaction [25].

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The increase rate in the resistance for DPPG is extremely large, compared with those for DPPC and DPPC+PE. Given that both DPPC and DPPG have the same hydrophobic group and their transition temperatures are nearly close to 41 °C, little difference would occur in the interaction with Aβ(1-40) from the viewpoint of membrane fluidity. This suggests that, even at the 2nd stage, not only hydrophobic but also electrostatic interaction extremely increased the resistance.

At the third stage of the interaction between Aβ and membranes after 16 h, the resistance change rates for DPPG, DPPC, or DPPC+PE were all saturated, because Aβ became almost mature fibril (which was less poisonous than Aβ aggregations [32-34]). The final amount of Aβ fibril became extremely larger for DPPG than that for DPPC or DPPC+PE, because the resistance change rates for DPPG and DPPC were around 1300 and 80 ppm, respectively. The amount of Aβ fibril for DPPG was about 15 times larger than that for DPPC. This might indicate for the second stage of DPPG case that coexisting electrostatic force would enhance the hydrophobic interaction between the Aβ fibril and membrane. Next we continued to consider the interactions between Aβ and liposomes having both PC and PE hydrophilic groups (DPPC+PE). At the second stage (from 8–9 to 16 h), similar to DPPC, the resistance for DPPC+PE increased clearly. From the slope of the curve, the resistance change for DPPC+PE was slightly smaller than that for DPPC. In the hydrophobic interaction between those liposomes and Aβ aggregates, it is suggested that the lipid membrane with PE group interacted less with Aβ than that with PC group. As one of the reasons, it is reported that PE molecules interfere with

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the interaction between Aβ and lipid membranes [35]. At the third stage (after 16 h), the change rate for DPPC was almost saturated, while that for DPPC+PE started to decrease. This implies that the surface stress on the cantilever was decreased and/or released due to some mechanisms rather than Aβ fibril growth. It is reported that membranes containing PE lipid are easier to collapse than PC lipid membranes via the interaction with Aβ [36]. Therefore, the decrease in the rate at the third stage might be related to the collapse of PE lipid.

3.2. Detection of low-concentrated Aβ(1-40) by DMPC with short hydrophobic carbon chain

As mentioned above, the negatively charged DPPG will detect the other positively charged molecules in foreign substances in the measured solution, other than charged Aβ protein as a target molecule. Therefore, DPPG cannot detect specifically the targeted Aβ protein. Except for DPPG, DMPC with neutral hydrophilic group had showed highest sensitivity and DMPC incorporated with cholesterol showed the selectivity in crude human serum. As the chronological result for DMPC is shown in Fig. 2(b) for 10-μM Aβ, we can compare Figs. 2(a) and (b) and find that the sensitivity for DMPC is in the range of 30-40% of that for DPPG and by about one order of magnitude larger than those of the other phospholipid with neutral hydrophilic group. So we chose to use DMPC rather than DPPG. Previously, DMPC showed a larger sensitivity against Aβ(1-40) due to its short hydrophobic carbon chain and resulting large membrane fluidity [22]. We further examined the interaction between low-concentrated Aβ (10 nM) and DMPC in PBS, thereafter found a qualitatively different behavior

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compared to DPPC with the same hydrophilic group. Fig. 2(b) shows chronological resistance change rates of the cantilever sensor immobilized by DMPC liposomes in both 10 μM and 10 nM Aβ(1-40) solutions. The maximum of the resistance change rate for DMPC (about 500 ppm) was greatly larger than that for DPPC (about 80 ppm as shown in Fig. 2(a)). We reported that the phospholipids composed of short saturated carbon chains increase the sensitivity of the cantilever sensor against Aβ [22]. The improvement is relevant to the membrane fluidity (i.e., structural instability) of liposomes that promotes the interaction between Aβ and membranes [37,38]. At the first stage (until 8–9 h), the resistance for DMPC increased from the beginning, although those for DPPC or DPPC+PE were stable as shown in Figs. 2(a) and (b). Such increase in the first stage implies that DMPC interacted with Aβ(1-40) monomers, even though it also has the neutral hydrophilic PC group. DMPC is electrically neutral, so the main interaction mechanism with Aβ(1-40) would not be electrostatic interaction but hydrophobic one, whereas several researchers have also reported that monomeric Aβ interacts with DMPC [39,40]. It would be reasonable that the increase from the first stage is related to membrane fluidity as mentioned above.

For 10-μM, until about 16 h, the resistance of the cantilever immobilized by DMPC liposomes increased monotonously. After about 16 h, the resistance change rate for DMPC was saturated, which means that Aβ fibrillization process became almost stable. Different from the case of DPPC, it is

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summarized that DMPC recognized not only Aβ aggregation and fibrillization but also Aβ monomer at its initial stage.

For a low-concentrated Aβ of 10-nM, on the other hand, it should be noted that the increase for the second stage (Aβ fibrillization) seems to occur at around 18 h and saturate at around 32 h. These onsets were delayed because the association between the low-concentrated Aβ molecules and membrane needed more time, compared to the case for high concentration of 10-μM. Finally, the rate reached approximately 200 ppm. We could detect 10-nM Aβ, which is still larger by 1–2 orders of magnitude than the concentration of average AD patients. More specifically, however, the result indicates that the sensor with DMPC could detect Aβ with 100 pM order, because we can measure the change rate as small as 1–10 ppm by using low-pass filtering as in Figs. 3(a) and 5. The rate for 10-nM Aβ was 100 ppm or more in Fig. 2(b).

3.3. Interaction between different type of liposomes and human serum

Up to now, two kinds of phospholipids has been found to be effective to improve interaction and resultant sensitivity against Aβ(1–40) fibril [22], which are 1) DMPC with short hydrophobic groups and 2) DPPC incorporated by cholesterol. As our final purpose to apply the sensor in human blood (serum) for diagnosis of AD and MCI patients, it is absolutely necessary to examine the interaction between the investigated phospholipids of DPPC(+PE), DPPG, DMPC, or those incorporating cholesterol and human serum which includes many important proteins. Fig. 3(a) plots

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time course of resistance change rates of the cantilever immobilized by liposomes of DPPG, DMPC, and DMPC incorporating cholesterol (33% molar ratio) in undiluted crude human serum (, where the noise level of output signal became reduced with a low-pass filter compared to Figs. 2). It is clear that the resistance change rates for DPPG and DMPC increased monotonously with time, suggesting that DPPG and DMPC interacted with some proteins and/or other molecules in the serum.

On the other hand, it was observed previously that the resistance for DPPC changed negligibly with time in human serum [21], although phospholipid such as DPPC might have various interactions with proteins or other molecules in the serum. This could suggest that the interactions between DPPC and the proteins or others in human serum chronologically changed little on the cantilever surface, possibly because DPPC is neutral (not charged). From a viewpoint of membrane fluidity by carbon chains (hydrophobic group) of lipid, transition temperatures of DP-lipids of DPPG and DPPC are the same as 41 °C. Therefore, DPPG and DPPC would interact similarly with proteins or others in human serum, whereas DPPG interacted markedly with proteins or others in serum as shown in Fig. 3(a). This indicates that the increase in the resistance change rate for DPPG would be caused by the electrostatic interaction between charged hydrophilic PG group in DPPG and charged proteins or others in human serum.

As the reason why large change occurred for DMPC, we expect that the lipid phase of DMPC liposomes at room temperature is related to the large interaction. Given that DMPC exhibits high

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fluidity and soft membrane due to its liquid phase at room temperature, proteins in human serum are easier to penetrate into DMPC membrane than DPPC and interact with it, as illustrated in Fig. 3(b).

On the other hand, the resistance change rate for DMPC liposomes incorporated with cholesterol became almost stable as also shown in Fig. 3(a). It was revealed that incorporating cholesterol into liposomes can suppress the interaction with proteins, where cholesterol has a role of suppressing membrane fluidity. Such effect is illustrated in Figs. 3(b) and (c) and explained as one of "dual effect" [41,42]: The incorporated cholesterol suppresses the fluidity in liquid phase but enhances it in ripple or gel phase. Given that DMPC is in liquid phase at room temperature, it becomes difficult for proteins in human serum to interact with DMPC liposomes incorporated with cholesterol. Accordingly, the resistance would become nearly stable with time. Therefore, it is reasonable that incorporating cholesterol into liposome of DMPC is effective for suppressing the interaction with human serum.

Moreover, Fig. 4 shows top and bird's-eye views by AFM(Pacific Nanotechnology Nano-R) of DMPC incorporated with cholesterol and DMPC immobilized on SAM/Au after introducing into human serum for 5 h. The diameter of the as-prepared liposomes was approximately 100 nm. The diameter increased, and the shape seemed to deform only for pure DMPC liposomes, whereas they were negligibly changed for those containing cholesterol. This indicates that the interaction could occur between pure DMPC and some proteins and/or other molecules in human serum.

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The result for cholesterol-incorporated DMPC liposome in Fig. 3(a) shows that the chronological change was less than 20 ppm then much small. Therefore, it is reasonable to compare the chronological result in serum with that in PBS with the same Aβ concentration.

Next, it becomes essential whether the DMPC incorporating cholesterol can detect effectively the dynamic behavior of Aβ aggregation and fibrillization in human serum. Fig. 5 shows the time course of the resistance change rate for DMPC liposomes incorporating cholesterol in human serum added with 1-μM Aβ(1-40). The chronological curve can be divided into three stages, as similar to Fig. 2(a). This curve suggests that the cantilever sensor was able to detect the aggregation and fibrillization of Aβ even in human serum, in spite of the condition of suppressed fluidity as explained above.

3.4. Evaluation on the effect of cholesterol incorporation by QCM

Fig. 6 plots time course of frequency shift of QCM (initium AFFINIX QN μ: sensitivity of 30 pg/Hz) as a function of molar % ratio of cholesterol incorporated in DPPC. The measurement started just after adding a target monomeric Aβ(1-40) solution (4 μM) diluted in PBS into a reservoir on the electrode. Different from the chronological curves for the cantilever sensor, every curve showed monotonous decrease from the initial and gradual saturation. The frequency shift became almost saturated at around 5000 s or more. It is considered that after addition of monomeric Aβ(1-40), it starts to interact and bind with liposome immobilized on the Au surface of the QCM oscillator, therefore its mass increases then the frequency shift decreases. It should be noted that the molecules physically

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deposited on the surface of the oscillator would be continuously removed by rotating a magnetic stirrer in the solution. Due to the memory size of this system, the measurement time could not reach the time range of the fibril growth (the 2nd stage: 8-9 h). But it is clear that mass change due to the interaction with monomeric Aβ(1-40) depended on the cholesterol ratio. It should be noted that the shift with 10 molar % ratio is smaller than that with no incorporation, which mechanism is unclear at the present stage. Moreover, the shift became monotonically increased (not decreased) with time for 45 molar % ratio (no figure).

The membrane stiffness (or membrane fluidity) is one of the important factors to determine the interaction between protein and liposome membrane. DPPC indicates the gel phase under the physiological condition like the present experimental conditions. The incorporation of cholesterol into gel-phase DPPC liposome perturbs the membrane structure, which results in the increased fluidity (dual effect). The cholesterol molecules are likely to be dispersed or form the domain-like region in the membrane with increased fluidity. It is well-known that the cholesterol domain strongly detect the target Aβ relative to the dispersed cholesterol, that is, the sensitivity of DPPC/30% cholesterol > DPPC10% cholesterol. In contrast to DPPC/10% cholesterol, pristine DPPC liposomes with gel phase immobilized on the QCM electrode appeared to deform a bit. This deformation might induce the perturbation of membrane structure of DPPC enough to improve its interaction with Aβ. Thus, this deformation effect of DPPC liposome due to its immobilization without cholesterol onto the QCM

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electrode is a possible reason with respect to the question that the QCM frequency shift of DPPC with 10% cholesterol plotted in Fig. 6 was less than pristine DPPC. As discussed above, cholesterol has the 'dual effect', then its interaction strength would tend to show some optimum behavior dependent on membrane fluidity. From the result, the ratio of 33 % is adequate to increase the interaction capability of cholesterol-incorporated DPPC. Based on the results, we used the ratio for the cholesterol incorporation in the liposomes in Figs. 3(a), 4 and 5.

From the above QCM results, we should consider that it is desirable to make a liposome-immobilized QCM sensor like the surface of the cantilever sensor. At the present stage, however, it is much difficult to include the QCM measuring system within a microsensor similar to the mentioned static-mode cantilever sensor. In addition, we estimate that it would be difficult for QCM to detect the deformation of liposome dependent on its species because QCM can measure mass only.

4. Conclusions

We have developed the liposome-immobilized microcantilever sensor for detection of Aβ fibril, thereafter newly confirmed the effectiveness of cholesterol addition into the phospholipid to improve the selectivity of the detection of Aβ aggregation/fibrillization in human serum. It was revealed that charged hydrophilic group in DPPG liposome that has electrostatic interaction showed much large sensitivity against charged Aβ target protein, although selective detection is difficult among other coexisting charged foreign substances in the solution. On the other hand, neutral hydrophilic group in

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DMPC liposome that has high membrane fluidity then large interaction strength, showed good sensitivity against the Aβ protein as low as 10 nM in ultrapure water, also detection of 100 pM-order was expected by using a low-pass filtering, but it showed the interaction signal in human serum of healthy persons, indicating that some molecules in the serum such as proteins had interacted. However, DMPC incorporated cholesterol showed significantly suppressed interaction with the serum, leading to detect Aβ selectively in the serum. We believe this is due to the 'dual effect' of cholesterol incorporation. From QCM results, the interaction was most increased and effective for 33 % ratio of cholesterol/DPPC, suggesting that optimizing the deformation of liposome might induce more the perturbation of membrane structure of DPPC enough to improve its interaction with Aβ.

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FiguresFig. 1. (a) A microscopic image of a cantilever. The principle for detecting Amyloid β(Aβ) (b) before and (c) after the interaction between liposomes and Aβ. Some liposome incorporates cholesterol in this work.

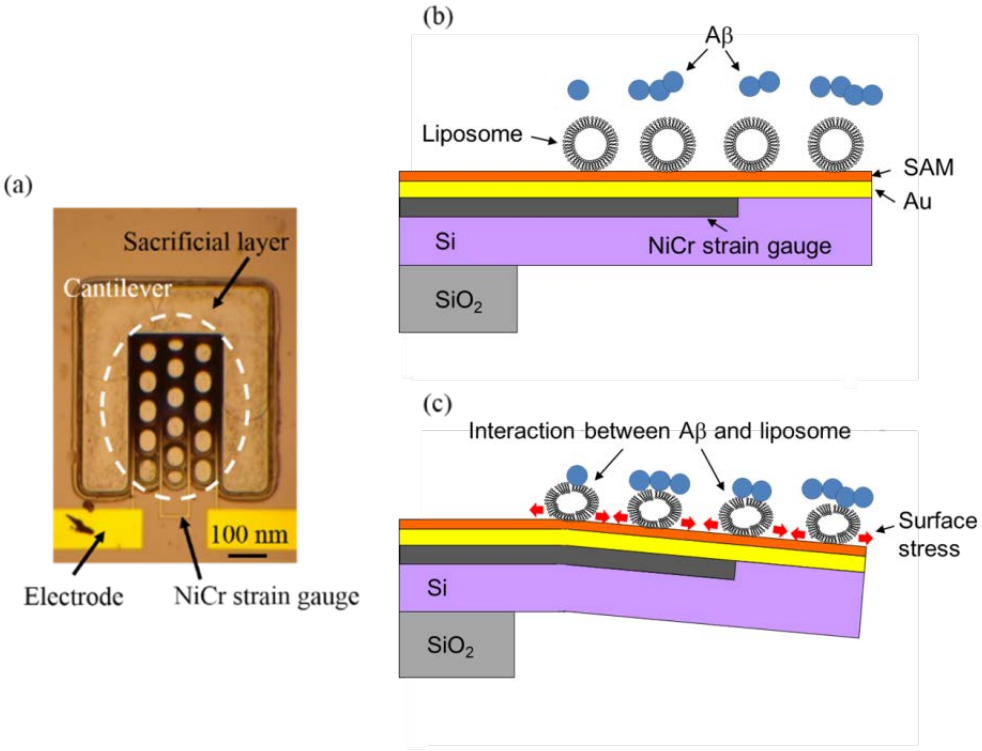

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Fig. 2. (a) Chronological resistance change rates of cantilever sensors using liposomes having different hydrophilic groups in 10-μM Aβ(1-40) solution. The inset is enlarged curves for DPPC and DPPC+PE.

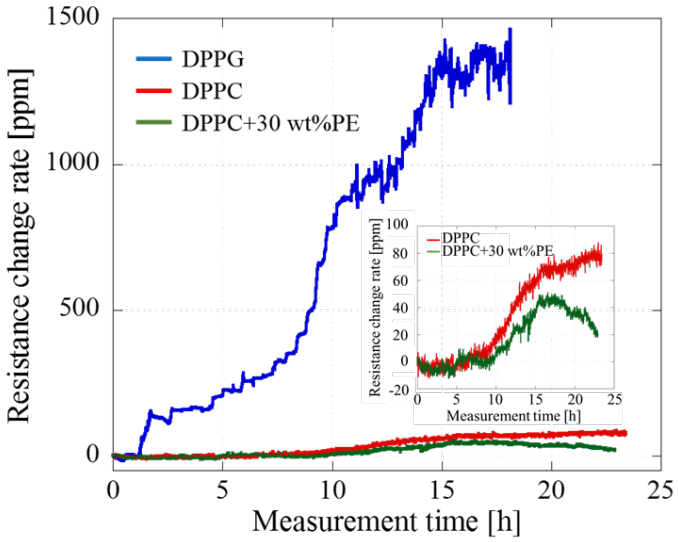

Fig. 2. (b) Chronological resistance change rates of the cantilever sensor immobilized by DMPC liposomes in Aβ(1-40) solutions of 10-μM (blue line) and 10-nM (red line).

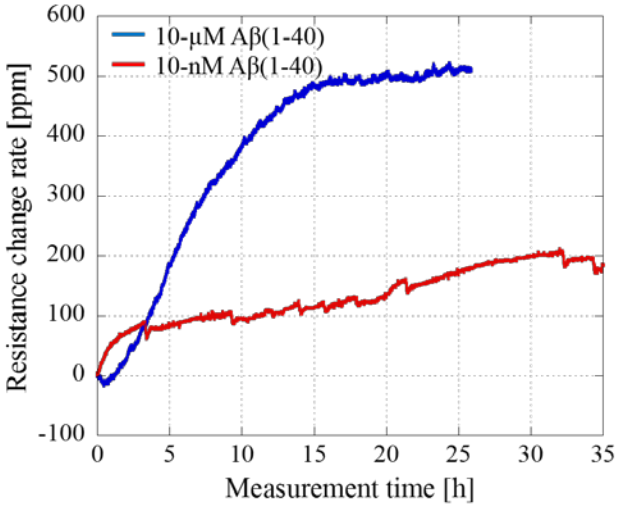

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Fig. 3. (a) The time course of resistance change rates of the cantilever immobilized by liposomes of DPPG, DMPC, and DMPC incorporating cholesterol in human serum measured with low-pass filtering.

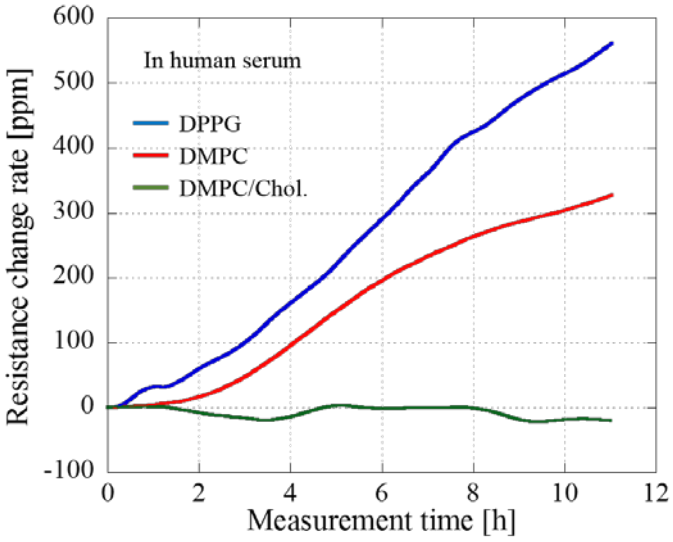

Fig. 3. Interaction between (b) DMPC or (c) DMPC incorporating cholesterol and proteins in human serum (molecular weight 66.4 kDa).

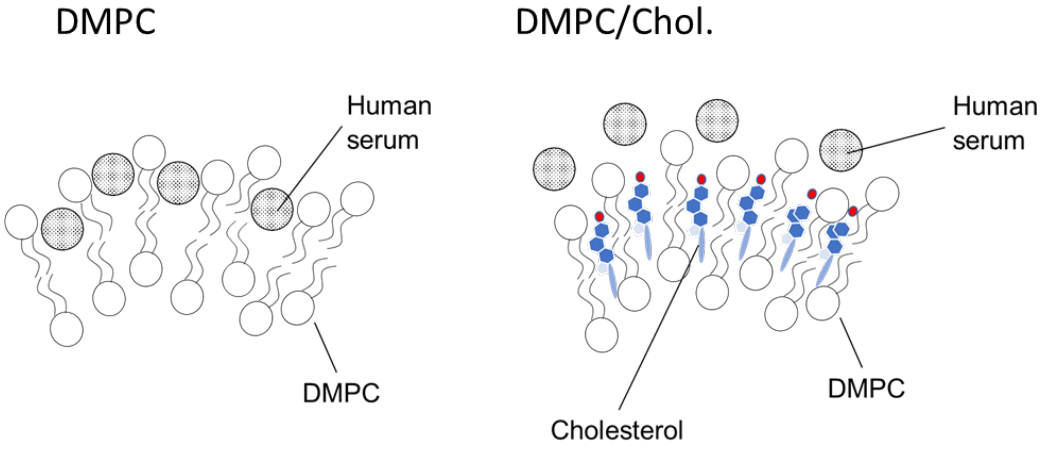

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Fig. 4. AFM images of the immobilized liposomes: (a) top view and (b) bird's-eye view of DMPC incorporating cholesterol, (c) top view and (d) bird's-eye view of DMPC after introducing into human serum for 5 h.

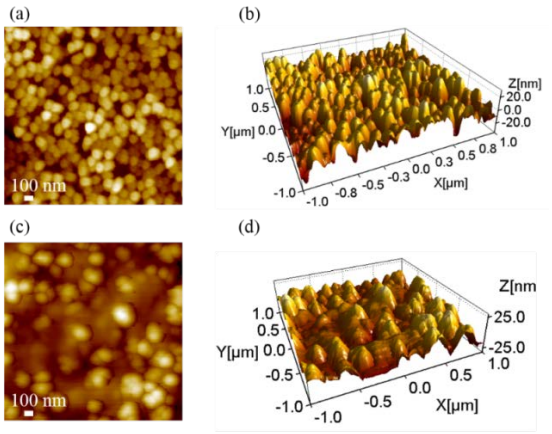

Fig. 5. Time course of resistance change rate with DMPC liposome incorporating cholesterol in human serum added with 1-μM Aβ(1-40) measured with low-pass filtering.

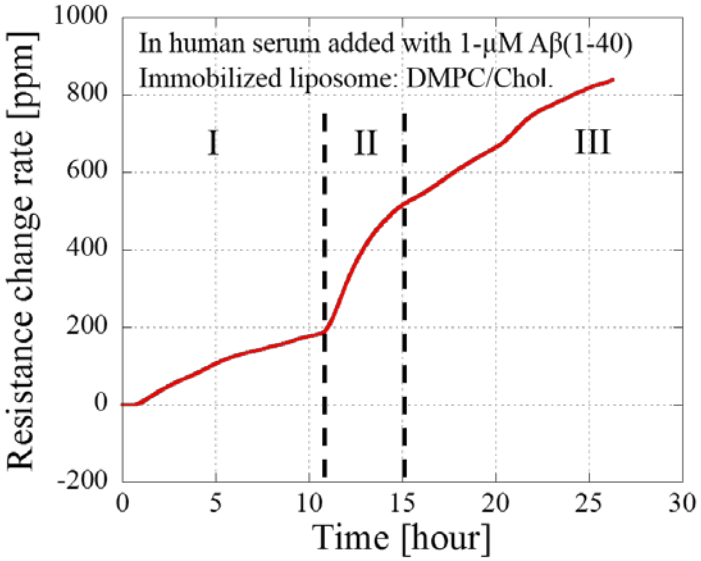

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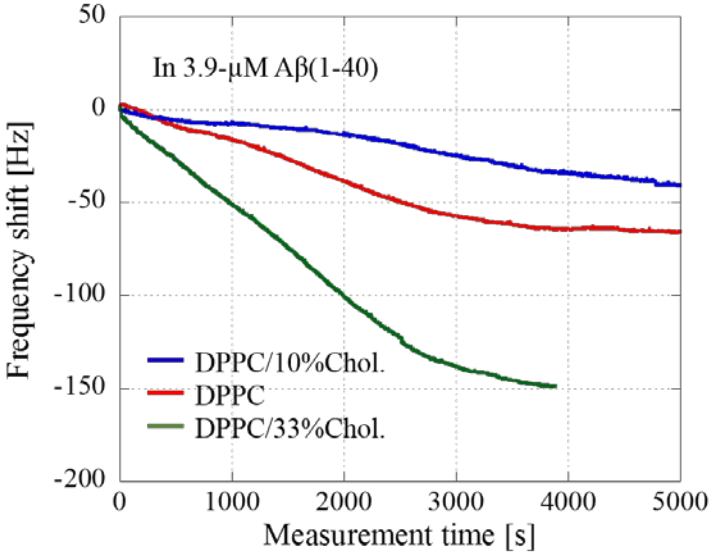
Fig. 6. Time course of QCM frequency shift after addition of target monomeric Aβ(1-40) as a function of incorporated cholesterol molar % ratio to DPPC.



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