Small Liposomes Accelerate the Fibrislation of Amyloid β (1–40)*

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The deposition of amyloid β (Aβ) peptides is a pathological hallmark of Alzheimer disease. Aβ peptides were previously considered to interact specifically with ganglioside-containing membranes. Several studies have suggested that Aβ peptides also bind to phosphatidylcholine membranes, which lead to deformation of membranes and fibrillation of Aβ. Moreover, the role of membrane curvature, one type of deformation produced by binding of proteins to a membrane, in the binding and fibrillation of Aβ remains unclear. To clearly understand the relationship between the binding, consequent membrane deformation, and fibrillation of Aβ, we examined the amyloid fibrillation of Aβ-(1–40) in the presence of liposomes of various sizes. Membrane curvature increased with a decrease in the size of the liposomes. We used liposomes made of 1,2-dioleoyl-sn-glycero-3-phosphocholine to eliminate electrostatic effects. The results obtained showed that liposomes of smaller sizes (<50 nm) significantly accelerated the nucleation step, thereby shortening the lag time of fibrillation. On the other hand, liposomes of larger sizes decreased the amount of fibrils but did not notably affect the lag time. The morphologies of fibrils, which were monitored by total internal reflection fluorescence microscopy, atomic force microscopy, and transmission electron microscopy, revealed that the length of Aβ-(1–40) fibrils became shorter and the amount of amorphous aggregates became larger as liposomes increased in size. These results suggest that the curvature of membranes coupled with an increase in water-accessible hydrophobic regions is important for binding and concentrating Aβ monomers, leading to amyloid nucleation. Furthermore, amyloid fibrillation on membranes may compete with non-productive binding to produce amorphous aggregates.

Results: Small vesicles accelerated Aβ fibrillation, whereas large vesicles promoted amorphous aggregation.

Conclusion: Moderate membrane-curvature-dependent interaction is an important factor for Aβ aggregation.

Significance: Two types of membrane binding, one leading to amyloid nucleation and the other to non-productive binding, may be common to various amyloidogenic proteins.

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The deposition of amyloid β (Aβ) peptides is a pathological hallmark of Alzheimer disease. Aβ peptides were previously considered to interact specifically with ganglioside-containing membranes. Several studies have suggested that Aβ peptides also bind to phosphatidylcholine membranes, which lead to deformation of membranes and fibrillation of Aβ. Moreover, the role of membrane curvature, one type of deformation produced by binding of proteins to a membrane, in the binding and fibrillation of Aβ remains unclear. To clearly understand the relationship between the binding, consequent membrane deformation, and fibrillation of Aβ, we examined the amyloid fibrillation of Aβ-(1–40) in the presence of liposomes of various sizes. Membrane curvature increased with a decrease in the size of the liposomes. We used liposomes made of 1,2-dioleoyl-sn-glycero-3-phosphocholine to eliminate electrostatic effects. The results obtained showed that liposomes of smaller sizes (<50 nm) significantly accelerated the nucleation step, thereby shortening the lag time of fibrillation. On the other hand, liposomes of larger sizes decreased the amount of fibrils but did not notably affect the lag time. The morphologies of fibrils, which were monitored by total internal reflection fluorescence microscopy, atomic force microscopy, and transmission electron microscopy, revealed that the length of Aβ-(1–40) fibrils became shorter and the amount of amorphous aggregates became larger as liposomes increased in size. These results suggest that the curvature of membranes coupled with an increase in water-accessible hydrophobic regions is important for binding and concentrating Aβ monomers, leading to amyloid nucleation. Furthermore, amyloid fibrillation on membranes may compete with non-productive binding to produce amorphous aggregates.

Alzheimer disease is one of the most deleterious neurological diseases. The main cause of this disease has been attributed to the deposition of amyloid β (Aβ) peptides in the brain (1–3); therefore, a large number of studies have examined the mechanism underlying the fibrillation of Aβ peptides in vivo and in vitro. Aβ peptides are produced by the sequential cleavage of the amyloid precursor protein with β and γ secretases. Although Aβ peptides are initially disordered in solution, they fold to form an oligomeric nucleus, possibly with a large amount of cross-β structures. Elongation then occurs, resulting in the formation of mature amyloid fibrils (3–5). The structures and toxicities of oligomers, which are directly responsible for cytotoxicity, were the recent targets of studies on Alzheimer disease as well as other amyloidosis (6). In a previous study using NMR, it was reported that one of the initial structures of oligomers was a non-β-sheet structure containing 3–10 helix in solution (7).

Aβ peptide concentrations in vivo were shown to be a few nM (~5 nM) (8–11), whereas fibrillation in vitro requires an order of magnitude higher concentration (3, 12). To clarify the reason for this difference, many studies proposed that Aβ peptides might interact with lipid membranes, which decreases diffusion dimensions and increases the local concentration of Aβ peptides (13–15). Various studies showed that specific membrane components (e.g. cholesterol, sphingomyelin, ganglioside, etc.) interact specifically with Aβ peptides, and this interaction has an important role in Aβ fibrillation (13, 16–20); however, other studies challenged this proposal (21, 22). Recently, a two-step mechanism of membrane disruption by Aβ peptides has been proposed in which (i) Aβ oligomers bind to the membrane to form ion-permeable pores, and (ii) the process of Aβ fibrillation causes membrane disruption mainly due to the interaction of Aβ fibrils with gangliosides (20, 23).

Amyloid fibrillation of various proteins, other than Aβ peptides, in lipid membranes has been extensively studied. Lipid
membranes are known to be involved in the fibrillation of α-synuclein and human islet amyloid polypeptide, which are associated with Parkinson disease and type 2 diabetes, respectively (24–27). Various kinds of lipids that affect amyloid fibrillation have also been studied, and the findings obtained revealed that electrostatic interactions between proteins and membranes facilitated the binding of proteins to the membranes as well as their fibrillation (25, 28, 29). The size or radius of liposomes may also be an important factor for determining these interactions; α-synuclein binds to small unilamellar vesicles, and islet amyloid polypeptide interacts with high curvature membranes (30–32).

However, the role of membrane properties on the fibrillation of Aβ peptides has not yet been elucidated in detail. Previous studies using giant unilamellar vesicles of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) suggested that Aβ peptide binding caused membrane deformation (33, 34). A simulation study revealed that the accumulation of Aβ peptides deformed membranes to produce highly curved membranes (35).

Thus, the deformation of membranes may be one of the key factors that accelerate fibrillation. Moreover, the presence of Aβ peptides in the synapses in vivo caused a dysfunction in synaptic vesicles with a diameter of ~40 nm (36). Because the binding of Aβ peptides to a membrane and the subsequent deformation are a linked phenomenon, the presence of deformed membranes may accelerate the binding and fibrillation of Aβ. Membrane curvature, one possible type of membrane deformation, can be controlled by changing the radius of liposomes; therefore, studies are needed to verify the relationship between membrane deformation, binding, and fibrillation.

We herein examined the effects of liposomes of various diameters on the amyloid fibrillation of Aβ-(1–40) to determine whether the fibrillation depends on the membrane curvature and consequent increase in water-accessible hydrophobic regions. We chose DOPC and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) as the liposome components because Aβ binding was reported to bend phosphocholine membranes (35), and phosphocholine, a zwitterionic lipid, allowed us to focus on the effects of membrane curvature while minimizing the effects of electrostatic interactions (16, 37). We previously observed that although Aβ-(1–40) is relatively easy to keep in monomeric states, Aβ-(1–42) forms nuclei for spontaneous fibrillation much faster than Aβ-(1–40) (38). Consequently, explosive fibrillation of Aβ-(1–42) resulted in a large number of short fibrils, minimizing the interaction with the surface. Thus, to address the effects of membranes, we chose Aβ-(1–40), although it will be important to compare Aβ-(1–40) and Aβ-(1–42) to obtain further insight into the interactions of Aβ with membranes.

The results of the present study revealed that membrane curvature is an important factor for determining the amyloid fibrillation of Aβ-(1–40). Liposomes of larger sizes decreased the length of amyloid fibrils, and this was accompanied by an increase in the amount of amorphous aggregates. These results suggest that amyloid fibrillation on membranes competes with amorphous aggregation, thereby providing an insight into the mechanism underlying synaptic vesicle impairments.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lyophilized Aβ-(1–40) purchased from Peptide Institute Inc. (Osaka, Japan) was dissolved in a 0.05% (w/w) ammonia solution at a concentration of 100 μM and stored at −80 °C. Phospholipids DOPC, POPC, 1,2-dioleoyl-sn-glycero-3-phospho-1-serine (DOPS), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-DOP) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Thioflavin T (ThT) and 8-anilino-1-naphthalenesulfonate (ANS) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Sigma, respectively. All other reagents were obtained from Nacalai Tesque (Kyoto, Japan).

**Preparation of Liposomes**—Liposomes were prepared as previously described (39). Briefly, lipids (~10 mM) were stored in methanol stock solutions. Lipid films were prepared by drying appropriate amounts of the stock solution in a stream of dry nitrogen followed by desiccation for at least 1 h to ensure removal of the organic solvent. Appropriate buffer solutions were added and vortexed to rehydrate the lipid films. After 10 freeze-thaw cycles, lipid suspensions were extruded 20 times through 50-, 100-, or 200-nm polycarbonate membranes (Whatman, Clifton, NJ) using a minietruoder (Avanti Polar Lipids, Inc., Alabaster, AL) just before use. Liposomes with a diameter of 30 nm were prepared by an ethanol injection method with slight modifications (40). Appropriate lipids (~100 mM) in methanol were diluted to 10% (v/v). After vortexing, the lipid suspension in the tubes were set on a water bath-type ultrasonic transmitter with a temperature controller (ELESTEIN SP070-PG-M, Elekon Sci. Inc., Chiba, Japan) that applied repetitive ultrasonication pulses to samples from three directions (41). The temperature was set to 37 °C, and ultrasonic pulses were applied with repeats of 1 min of ultrasonication and 9 min of quiescence for 3 h. After ultrasonication, this suspension was centrifuged to precipitate multilamellar liposomes or/and aggregations of lipids followed by the collection of the supernatant. As this supernatant may contain methanol, it was dialyzed against an appropriate buffer twice. After dialysis, the concentration of lipids was quantified. Liposome sizes were measured by dynamic light scattering using DynaPro Titan (Wyatt Technology Co., Goleta, CA). Liposomes containing the fluorescence lipid (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl (Rho-DOPES)) (0.1% mol) or DOPS were prepared using the same procedure as described above.

**Analytical Ultracentrifugation**—The sedimentation velocity data were obtained by a Beckman-Coulter Optima XL-A analytical ultracentrifuge (Beckman Coulter, Miami, FL). The samples were first centrifuged at 3000 rpm (700 g) for 5 min to stabilize the absorbance and temperature. Next, centrifugation was performed at 60,000 rpm (250,000 × g) and 4 °C. Absorbance data at 220 nm were collected at an interval of 20 min at the radial increment of 0.003 cm in the continuous scanning mode. The absorbance data were analyzed by using sedfit to obtain the sedimentation coefficients. The parameters of solvent conditions (partial specific volume, density and viscosity) were calculated by using sednterp.
Membrane-dependent Fibrillation of Amyloid β

**Total Internal Reflection Fluorescence Microscopy (TIRFM), Atomic Force Microscopy (AFM), and Transmission Electron Microscopy (TEM)—**The prism-type TIRFM system was used to observe individual amyloid fibrils. The details of this system were described previously (42, 43). Briefly, an aliquot (14 μl) of sample solution was deposited on a quartz glass slide, and a fibril image was obtained with TIRFM. We used an argon laser (532 nm) and a helium-cadmium laser (442 nm) to excite ThT and rhodamine, respectively. AFM images were obtained using a Digital Instruments Nanoscope IIIa scanning microscope (Veeco Instruments Inc., Plainview, NY) as reported previously (44). TEM images were obtained using a HITACHI H-7650 transmission microscope (Hitachi, Tokyo, Japan) at 20 °C with a voltage of 80 kV and magnification of 30,000. The sample solution (5 μl) was spotted onto a collodion-coated copper grid (Nissin EM Co., Tokyo, Japan). After 1 min, the remaining solution was removed with filter paper, and 5 μl 2% (w/w) uranyl acetate was spotted onto collodion-coated copper grids. After 1 min, the remaining solution was removed in the same manner.

**Amyloid Fibrillation and ThT Fluorescence Assay—**The formation of Aβ-(1–40) fibrils was examined at 10 μM in 10 mM sodium phosphate buffer (pH 7.5) containing 5 μM ThT, 100 mM NaCl, and DOPC or POPC liposomes at various concentrations. These solutions (200 μl) were added to the 96-well microplates (Greiner-Bio-One, Tokyo, Japan), and a seal was affixed (PowerSeal CRISTAL VIEW, Greiner-Bio-One). The plates were set on a microplate reader (SH-9000, Corona Electric Co., Ibaraki, Japan), and amyloid fibrillation was monitored by ThT fluorescence. It is noted that, although the morphology of Aβ fibrils is likely to depend on agitation of solution (45), we used the fibrillation conditions without agitation. The fluorescence intensity of ThT at 490 nm was measured with an excitation wavelength of 450 nm at 37 °C. The lag time of fibrillation was defined as the time at which ThT fluorescence reached 10% of the maximum.

**ANS Assay to Evaluate Hydrophobicity—**ANS fluorescence was measured using the Hitachi fluorescence spectrophotometer F4500 with the excitation wavelengths at 350 nm. The fluorescence of ANS was monitored by adding 2 μM ANS to the solution. The concentrations of liposomes and amyloid fibrils were 2 μM.

**Isothermal Titration Calorimetry (ITC)—**Isothermal titration calorimetry (ITC) of Aβ-(1–40) monomers to the two different sizes of DOPC liposomes (30 nm and 100 nm) in 10 mM phosphate buffer (pH 7.5) containing 100 mM NaCl was performed by using a VP-ITC instrument (GE Healthcare) at 37 °C. The concentrations of Aβ-(1–40) in the syringe and liposomes in the cell were 100 μM and 5 mM, respectively. The reference power for the base line was set to 10 μcal/s, and the initial delay was 1800 s. Titration of Aβ-(1–40) was comprised of 23 injections with the spacing time of 900 s or 300 s and the filter period of 2 s. The injection volume was 12 μl for each, and the stirring speed was 242 rpm.

The heat of dilution (and mixing) of Aβ-(1–40) titrated to the same buffer was also measured. The apparent values of the change in enthalpy ($a_{bind}^{app}$) and the association constant ($a_{Ka}^{app}$) were obtained by the fit of the binding isotherms to the one-site binding model incorporated in Origin 7.1. By using the relationships $\Delta G_{bind}^{app} = -RT \ln(a_{bind}^{app}) = a_{Ka}^{app} - T \Delta S_{bind}^{app}$, the apparent thermodynamic values of the change in Gibbs free energy ($\Delta G_{bind}^{app}$) and entropy ($\Delta S_{bind}^{app}$) were further calculated. The apparent dissociation constant ($a_{Ka}^{app}$) was obtained based on the relation of $a_{Ka}^{app} = 1/a_{bind}^{app}$. It should be noted that $a_{Ka}^{app}$ values are more susceptible to the fit than $a_{bind}^{app}$ values.

**RESULTS**

**Preparation of Liposomes and Aβ Solution—**To investigate the effects of the lipid size on the fibrillation of Aβ-(1–40), we prepared liposomes of four sizes (~200, 100, 50, and 30 nm) and analyzed the kinetics of fibrillation monitored by ThT fluorescence. We first confirmed the size of DOPC liposomes with TEM (Fig. 1, A–D) and dynamic light scattering (Fig. 1, E–H). The results obtained indicated that we could prepare liposome samples of distinct sizes with relatively small distributions. We call the respective preparations 30-, 50-, 100-, and 200-nm liposomes.

To check whether Aβ-(1–40) samples before the fibrillation experiments were monomeric, we conducted a sedimentation velocity measurement (Fig. 1, I and J). The sedimentation coefficient distribution for Aβ-(1–40) solution revealed a sharp peak at ~0.3 S. Using this value, we calculated the molecular weight to be ~4700, consistent with that of Aβ-(1–40) monomer, 4300.

The absence of oligomeric aggregates was further confirmed by native-PAGE followed by Western blotting with antibodies to Aβ-(1–40) monomers (data not shown). In addition, we observed the kinetics of Aβ-(1–40) fibrillation with samples with and without ultracentrifugation to remove possible aggregates, where we also checked the effects of 30- and 100-nm liposomes. We did not observe significant differences between Aβ-(1–40) samples with and without ultracentrifugation (data not shown). Therefore, we used Aβ-(1–40) preparations without ultracentrifugation assuming that we can address fibrillation of Aβ-(1–40) monomers.

**Dependence of Fibrillation on the Liposome Concentration—**We monitored the fibrillation of 10 μM Aβ-(1–40) in 10 mM phosphate buffer (pH 7.5) containing 100 mM NaCl and 5 μM ThT at 37 °C using amyloid-specific ThT fluorescence (46). It has been reported that Aβ-(1–40) fibrils with 2-fold symmetry with twisted morphology predominate when fibrils are grown in vitro with agitation of the Aβ-(1–40) solution, whereas fibrils with 3-fold symmetry with straight ribbon morphology predominate when fibrils are grown without agitation (45). Throughout the paper, we prepared the fibrils without agitation because agitation might affect the morphologies of liposomes.

Spontaneous fibrillation without agitation occurred with a lag time of 12 h (Fig. 2A). The straight and long morphology of mature fibrils was confirmed by TIRFM, AFM, and TEM (see below). These results were consistent with the findings of our previous studies (38, 47).

To examine the effects of liposomes on the fibrillation of 10 μM Aβ-(1–40), we added 30- or 100-nm liposomes at 10, 50, 100, 250, 500, 750, and 1000 μM (Fig. 2, A and D). We observed a significant acceleration in fibrillation in the presence of 30-nm liposomes at 10 μM as revealed by a decrease in the lag

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FIGURE 1. Preparation of liposomes of various sizes and Aβ monomers. A–D, TEM images of liposomes of various diameters: 30 (A), 50 (B), 100 (C), and 200 nm (D). The scale bar represents 100 nm. E–H, distribution of liposome diameters determined by TEM and dynamic light scattering (DLS). The liposome diameters prepared were 30 (E), 50 (F), 100 (G), and 200 (H) nm. I, sedimentation boundary profiles of Aβ monomers. A sedimentation pattern was recorded at 60,000 rpm and 4 °C by monitoring the absorbance at 220 nm, and fitted traces at intervals of 20 min are shown. J, sedimentation coefficient distributions derived from sedimentation boundary profiles of Aβ monomers.

FIGURE 2. Effects of 30 and 100 nm DOPC liposomes on amyloid fibrillation of Aβ-(1–40) at 37 °C without shaking. A and D, kinetics monitored by ThT fluorescence in the absence and presence of 30 nm (A) or 100 nm (D) DOPC liposomes at various concentrations at 37 °C. The concentrations of the liposomes are described in each figure. Amyloid fibrillation at the respective liposome concentrations was monitored at three wells, and the representative kinetics is shown. The insets show the average lag times. B, C, E, and F, dependences of the relative lag time (B and E) and maximal ThT amplitude (C and F) on the 30-nm (B and C) or 100-nm (E and F) DOPC liposome concentrations. Dotted lines are the values obtained in the absence of liposomes. The error bars and gray zones indicate the S.D. among three experiments.
time from \( \sim 12 \) h to \( \sim 7.6 \) h (Fig. 2A, inset): the relative lag time in 30-nm liposomes at 10 \( \mu \)M was 0.6 (Fig. 2B). Further increases in the liposome concentration slightly decreased the lag time. We compared the amount of fibrils by the ThT intensities at the end of the measurements (i.e. final ThT intensities at about 40 h). When the ThT intensity decreased after the maximum, we used the maximal ThT intensity. The final ThT intensity was similar to that in the absence of liposomes at all the liposome concentrations examined (Fig. 2C). In contrast, the presence of 100-nm liposomes at various concentrations slightly decreased the lag time (Fig. 2D, inset, and E). The final ThT intensity in the presence of 100-nm liposomes was independent of the liposome concentration within an acceptable level of error (Fig. 2F).

These results suggested that 10 \( \mu \)M liposomes were sufficient to examine the effects of liposomes on the fibrillation of 10 \( \mu \)M A\( \beta \)-(1–40). The binding equilibrium between A\( \beta \)-(1–40) and liposomes that affected amyloid fibrillation may have been established even at low concentrations of liposomes. Thus, we used 10 \( \mu \)M DOPC liposomes to examine the effects of liposomes of various sizes on the fibrillation of A\( \beta \)-(1–40).

**Dependence of Fibrillation on the Liposome Size**—The kinetics of A\( \beta \)-(1–40) fibrillation was examined in the presence of 10 \( \mu \)M DOPC liposomes of various diameters (i.e. 30, 50, 100, and 200 nm) (Fig. 3A). The size of DOPC liposomes notably affected both the lag time and final ThT intensity (Fig. 3, C and D). The lag time was the shortest for the 30-nm liposomes and increased with the size of liposomes, returning to the value in the absence of liposomes at 100- or 200-nm liposomes (Fig. 3). On the other hand, the maximal ThT intensities observed in the presence of 30- or 50-nm liposomes were similar to that in the absence of liposomes and were larger than those in the presence of 100- or 200-nm liposomes (Fig. 3D). The slope of the elongation process after the lag time was slightly less steep in the presence of 100- or 200-nm DOPC liposomes (Fig. 3A). These results suggested that small liposomes accelerated fibrillation without changing the final amount of fibrils, whereas the larger liposomes decreased the amount of fibrils without an apparent decrease in the fibrillation rate.

We performed the same experiments with POPC liposomes at 10 \( \mu \)M (Fig. 3B). Both the lag time and final ThT intensity revealed similar dependences on the size of liposomes to those observed for DOPC liposomes (Fig. 3, B, inset, C, and D), although the final ThT intensities of POPC liposomes were smaller than those of DOPC liposomes. The smaller final ThT intensities of POPC liposomes might be caused by the difference of the membrane surface properties due to the difference of acyl chains or of the temperature of gel-liquid crystalline phase transition; transition temperatures are \(-17\) and \(-2\) \( ^\circ\)C.
for DOPC and POPC membranes, respectively. Although there were some differences between DOPC and POPC, we could observe a similar effect of liposome size on the kinetics of Aβ-(1–40) fibrillation. These results suggested that the size-dependent effects of liposomes may be common to various liposomes. We then attempted to address why 30- and 50-nm liposomes significantly promoted the nucleation reaction, whereas larger liposomes reduced the maximal ThT value without notable effects on the lag time.

Hydrophobic Surface of Membranes Exposed to a Solvent—To clarify the mechanism underlying the promotion of nucleation by small liposomes, we assumed a simplified model in which the inner leaflet was ignored, and the lipid of the outer leaflet was represented by a rectangle. According to this model, the inside circumference was \( \pi(R-2l) \), and the number of rectangles was represented by \( n = \pi(R-2l)/d^{0.5} \), where \( R \), \( l \), and \( d \) are the diameter of a liposome, the height of a lipid, and the number of rectangles, respectively. Consequently, \( d \) was calculated by \( d = \pi R/n - A^{0.5} \). The estimated value of \( d \) was plotted against \( R \) (Fig. 4C), which showed that \( d \) was inversely proportional to \( R \). The dependence of \( d \) on the diameter of liposomes was consistent with that of the ANS fluorescence.
Fibril Morphology—We examined the morphologies of fibrils prepared in the presence of DOPC liposomes with various diameters using TIRFM, AFM, and TEM (Fig. 5). We used liposomes in which rhodamine-labeled lipids were included for TIRFM so that we could also monitor the location of the liposomes. Liposomes were deformed by interaction with surfaces; therefore, the exact morphology of liposomes could not be detected by our measurements. The three methodologies covered a wide range of dimensions from nm to μm.

In the absence of liposomes, Aβ-(1–40) fibrils were straight with a length up to several μm. They were well dispersed without clustering. Fibrils were still long and dispersed in the presence of 30- or 50-nm liposomes, although some clusters and fibril clumps were observed. In the TIRFM images, the locations of liposomes revealed by rhodamine fluorescence always overlapped with amyloid fibrils, which indicated that the liposomes were the sites of amyloid nucleation. The sizes of liposomes were markedly smaller than those of the fibrils.

AFM images indicated that fibrils became shorter and amorphous aggregates accumulated, with an increase in the size of liposomes, as was evident from AFM images obtained in the presence of 100- and 200-nm liposomes. TEM images in the presence of 100- and 200-nm liposomes revealed that fibrils associated with the ruptured liposomes. TIRFM images in the presence of 100- and 200-nm liposomes showed the significant clustering of fibrils. TEM images in the presence of large liposomes showed thick and short fibrils. Taken together, the morphologies of Aβ-(1–40) fibrils observed by TIRFM, AFM, and TEM depended on the size of the coexisting liposomes, which indicated that fibrils became shorter and clustered with an increase in the size of the liposomes, and the amount of amorphous aggregates increased.

Binding of Aβ-(1–40) to Liposomes Characterized by ITC—To examine the interactions between Aβ-(1–40) and DOPC liposomes, we performed ITC measurements (Fig. 6). Liposomes with the sizes of 30 nm and 100 nm were chosen to address the effects of high and low curvatures, respectively, on Aβ-(1–40) fibrillation. Consecutive titrations of Aβ-(1–40) to 30-nm liposomes in the ITC cell showed the positive ITC peaks with gradual decreases in the intensity (Fig. 6A). The heats of dilution and mixing of Aβ-(1–40) to buffer were much smaller than those of Aβ-(1–40) titrations to liposomes (Fig. 6A, inset). Thus, endothermic reactions indicated intermolecular interactions between Aβ-(1–40) and 30-nm liposomes.

On the other hand, ITC thermogram of Aβ-(1–40) titrations to 100-nm liposomes showed exothermic peaks that decreased gradually in amplitude (Fig. 6B). The extents of heat were larger than those observed when Aβ-(1–40) was titrated to 30-nm liposomes (Fig. 6A). These data suggested that although Aβ-(1–40) was bound to both 30- and 100-nm liposomes, the detailed mechanisms were distinct.

To obtain further insights into interactions between Aβ-(1–40) and liposomes, we evaluated apparent thermodynamic parameters based on analyses of binding isotherms. The values of \( K_d \) of Aβ-(1–40) for 30-nm and 100-nm liposomes were 67.1 μM and 36.4 μM, respectively. These values were smaller than those of α-synuclein binding to the liposomes, which mimicked presynaptic membranes \( K_d = 261 \) μM, or the...
These indicated that binding of Aβ-(1–40) to 100-nm liposomes was slightly stronger than that to 30-nm liposome. Interestingly, the sign and amplitude of $\Delta G_{\text{bind}}^{\text{app}}$ and $\Delta S_{\text{bind}}^{\text{app}}$ were different depending on the size of liposomes. The Aβ-30-nm liposome binding was driven by the positive entropy change ($T \Delta S_{\text{bind}}^{\text{app}} = 14.3$ kcal/mol) to overcome the unfavorable endothermic reaction ($\Delta G_{\text{bind}}^{\text{app}} = 8.4$ kcal/mol). On the contrary, the Aβ-100-nm liposome binding was favored by the negative enthalpy change ($\Delta G_{\text{bind}}^{\text{app}} = -62.0$ kcal/mol) to overcome the entropy loss ($T \Delta S_{\text{bind}}^{\text{app}} = -55.7$ kcal/mol). The large value of $\Delta G_{\text{bind}}^{\text{app}}$ in the Aβ-100-nm liposome binding may imply the complex mechanism in comparison with the Aβ-30-nm liposome binding. Similar complicated ITC results were observed for the α-synuclein binding to liposomes consist of 1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (53).

Moreover, biphasic patterns of each ITC peak in titration of Aβ to 100-nm liposomes suggested apparent two processes; the fast process with a sharp and intense peak was followed by the slow process with a broad and low intensity peak (Fig. 6B). These indicated that binding of Aβ to 100-nm liposomes was not a simple intermolecular interaction, although further studies are required to clarify the details of the liposome size-dependent binding of Aβ.

As can be seen in the ITC thermograms (Fig. 6), the binding reactions between Aβ-(1–40) and liposomes did not saturate, which brought uncertainty of the fitting. Thus, the error values of $K_a$ were large. Calculation of other thermodynamic parameters by using $K_a$ with the large error further increased uncertainty. To obtain a saturated curve, which can decrease errors, it was required to increase the concentrations of Aβ-(1–40) in the syringe. However, the concentration of Aβ-(1–40) (100 μM) used was already high, and it was not practical to further increase the Aβ-(1–40) concentration due to the formation of nonspecific aggregations. Although decreasing temperature to delay nonspecific aggregation might be an alternative possibility at higher Aβ-(1–40) concentrations, there were concerns about changing the binding mode and/or aggregation pathway compared with those at 37 °C. Thus, we conclude that although the errors were large, the ITC results clearly showed distinct bindings of Aβ-(1–40) to the two kinds of liposomes and suggested that the binding mode and aggregation pathway of Aβ-(1–40) depend on the degree of membrane curvature.

Effects of Anionic Phospholipids on the Fibrillation—To address the role of electrostatic interactions between Aβ-(1–40) and membranes in fibrillation, we examined the effects of liposomes composed of DOPC and DOPS at a ratio of DOPC: DOPS = 1:1 (i.e. 50% DOPS liposomes). DOPS is one of anionic phospholipids reported to accelerate fibrillation of Aβ-(1–40), and it has been suggested that phosphatidylserine groups of DOPS liposomes interact with positive-charged Lys residue at the C terminus of Aβ-(1–40) (37). However, the dependence on the size of liposomes has not been examined. We monitored the fibrillation Aβ-(1–40) in the presence of 50% DOPS lipo-
We then prepared the 30-nm liposomes composed of 100% DOPC, 50% DOPS, or 100% DOPS and monitored the fibrillation of Aβ-(1–40) in the presence of these liposomes (Fig. 7, C and D). The liposomes containing 50% DOPS promoted the nucleation reaction as well as 100% DOPC liposomes. However, the degree of promotion of DOPS liposomes was small.

Thus, although the DOPS liposomes were reported to accelerate the Aβ-(1–40) fibrillation (37), we saw no clear acceleration under our conditions. On the contrary, we observed slightly adverse effects to suppress the accelerating effects produced by small DOPC liposomes. It is possible that, under our experimental conditions, the electrostatic interaction between the negative-charged head group of PS and positive-charged amino acid residues of Aβ-(1–40) tightly trapped the Aβ-(1–40) molecules on liposomes so that further nucleation became difficult.

**DISCUSSION**

We herein showed that DOPC liposomes markedly accelerated the fibrillation of Aβ-(1–40) and that these effects depended on the size of liposomes even at the same total concentration of phospholipids; smaller liposomes more strongly accelerated fibrillation. Acceleration was suppressed by an increase in the liposome size. The amount of fibrils decreased, and that of amorphous aggregates increased.

To understand these distinct effects of liposomes on fibrillation, we assumed two types of interactions between Aβ-(1–40) and liposomes; one was productive binding leading to nucleation and fibrillation, and the other was non-productive binding that formed amorphous aggregates (Fig. 8, A and B). An analysis of the water-accessible hydrophobicity of liposomes probed by ANS binding and estimation of curvature suggested that small liposomes with higher surface curvature had more water-accessible hydrophobic regions and were effective for producing amyloid nucleation. Small liposomes with larger water-accessible hydrophobic regions interact with Aβ monomers weakly and concentrate them adequately to initiate nucleation. Although the binding of Aβ-(1–40) to larger liposomes could also occur, it may be non-productive for amyloid nucleation because the local concentration was not sufficiently high to break supersaturation. Alternatively, although the local concentration was high, bound Aβ-(1–40) molecules were tightly

**FIGURE 7. Effects of DOPS on the DOPC-dependent fibrillation of Aβ-(1–40).** A and B, the kinetics of Aβ-(1–40) fibrillation in the presence of 50% DOPS liposomes of 30 (pink), 50 (yellow), 100 (green), or 200 (blue) nm in diameter or without lipids (black) (A) and the dependence of relative lag time on the liposome size (B). The inset in A represents the average lag time at various liposome sizes. C and D, the kinetics of Aβ-(1–40) in the presence of 100% DOPC (pink), 50% DOPS (purple), and 100% DOPS (blue) liposomes of 30 nm in diameter (C) and the dependence of relative lag time on the DOPC content (D). The inset in C represents the average lag times at different contents of DOPS.
trapped on the surface of membranes and, thus, were unable to diffuse and transform to seed competent conformations. The ITC measurements showed that the affinity of αβ to the 100-nm liposome was slightly stronger than that to the 30-nm liposome, consistent with the tighter binding and trapping of the αβ-(1–40) on the larger liposomes. These two types of interactions may propagate beyond liposome surfaces, leading to amyloid fibrillation or amorphous aggregation in bulk solvent.

The competitive mechanism between productive and nonproductive binding (Fig. 8A) was analogous to the competition between amyloid fibrillation and amorphous aggregation in the absence of liposomes, as represented by a phase diagram of amyloid fibrillation dependent on salt and protein concentrations. Amyloid fibrils have been shown to form in the supersaturated solutions of responsible proteins via a nucleation and growth mechanism (41, 54–56). Supersaturated regions can be classified into two types depending on the stability of supersaturation (Fig. 8C) (41). Supersaturation was remarkably stable in the metastable region, at which the solute concentration was slightly above the solubility, and seeding was essential to break supersaturation and make fibrils. Spontaneous fibrillation could occur after a certain lag time in the labile region, in which the driving force of precipitation increased. Various kinds of agitations such as shaking (57), stirring (58), or ultrasonic irradiation (44, 59, 60) have been shown to effectively force spontaneous fibrillation under these supersaturated regions. One possible mechanism responsible for agitation-induced fibillation is the concentration of amyloidoegenic proteins at the hydrophobic air-water interface produced by agitation (41, 61). Ultrasonication is known to produce cavitation bubbles with hydrophobic surfaces, and stirring or shaking will also increase the hydrophobic air-water interface. The hydrophobic air-water interface adsorbs proteins, concentrates them, and produces various aggregates including seed competent aggregates. Once amyloid fibrillation starts on the seeds, it even propagates to bulk regions without a hydrophobic surface.

Water-accessible hydrophobic surfaces produced by membrane curvature may play a similar role in membrane-assisted amyloid fibrillation to the air-water hydrophobic surface of cavitation bubbles induced by ultrasonic irradiation. αβ monomers may be concentrated effectively by absorption to the hydrophobic surface due to membrane curvature. Thus, the membrane-assisted acceleration of fibrillation might be similar to the agitation-induced acceleration of fibrillation in the labile region (Fig. 8).

In the phase diagram excessively strong precipitation forces produced glassy amorphous aggregates in which molecules were trapped in non-amyloidogenic conformations. One possible mechanism underlying aggregation is that promiscuous interactions between molecules produce various conformational states. In a similar way, various types of interactions on the membrane may trap the monomers, preventing rearrangements to seed-competent conformations. The evenly dispersed charged head groups of phospholipids may contribute to preventing the bound αβ monomers from associating to the seed-competent conformation. Thus, we considered that membrane-assisted amorphous aggregation corresponded to amorphous aggregation under strong forces of precipitation (Fig. 8).

We previously reported the amyloid fibrillation of αβ-(1–40) in real time at a single fibrillar level at pH 7.0 with TIRFM combined with the use of ThT (62). We used the surfaces of various chemically modified substrates. Extensive fibrillation was generally observed on surfaces with negative charges. In contrast, fibril growth was largely suppressed on positively charged or hydrophobic surfaces. A previous study reported
that Aβ fibrillation was facilitated by negatively charged acidic phospholipids rather than neutral phospholipids (63). Because the pI value of Aβ is ~5, the net charge was slightly negative under neutral pH conditions. These results indicated that strong electrostatic attraction tightly trapped the Aβ(1–40) monomers on the surface so that subsequent lateral movement and nucleation were inhibited. The inhibitory role of a strongly hydrophobic surface can be also explained in a similar manner assuming that the hydrophobic surface interacts with the hydrophobic residues of Aβ(1–40). These results support the view that there are two types of membrane binding, one leading to amyloid fibrillation and the other to amorphous aggregation.

Finally, although lipid rafts have been considered to play an important role in Aβ fibrillation (13, 16–19), the underlying mechanism remains unclear (21, 22). It is possible that the lipid raft carries the site of productive binding, as proposed here more than the other lipid membranes. Our proposal that there are two types of membrane binding for amyloidogenic proteins, one leading to amyloid nucleation and the other to non-productive binding and amorphous trapping, may be common for various amyloidogenic proteins. The specific removal of amyloidogenic proteins may be possible by preparing liposomes with only non-productive binding.

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