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Effects of Charged Cholesterol Derivatives on A β 40 Amyloid Formation

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Abstract

Understanding of the mechanistic progress of Amyloid- β peptide (A β) aggregation is critical for elucidating the underlying pathogenesis of Alzheimer's disease (AD). Herein, we report for the first time the effects of two cholesterol derivatives, negatively charged cholesterol sulfate (cholesterol-SO₄) and positively charged 3 β -[N-(dimethylaminoethane)carbamoyl]-cholesterol (DC-cholesterol), on the fibrillization of A β 40. Our results demonstrate that both of the nonvesicle forms of cholesterol-SO₄ and DC-cholesterol moderately accelerate the aggregation rate of A β 40. This effect is similar to that observed for unmodified cholesterol, indicating the importance of hydrophobic interactions in binding of A β 40 to these steroid molecules. Furthermore, we show that the vesicles formed at higher concentrations of anionic cholesterol-SO₄ facilitate A β 40 aggregation rate markedly. In contrast, the cationic DC-cholesterol vesicles show the ability to inhibit A β 40 fibril formation under appropriate experimental conditions. The results suggest that the electrostatic interactions between A β 40 and the charged vesicles can be of great importance in regulating A β 40-vesicle interaction. Our results also indicate that the structural properties of the aggregates of the cholesterol derivatives, including the surface charge and the size of the vesicles, are critical in regulating the effects of these vesicles on A β 40 aggregation kinetics.

Abstract

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Notes

The authors declare no competing financial interests.

Supporting Information.

Aggregation kinetics of A β peptides with cholesterol and the derivatives, salt effect results, and light scattering data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Anomalous protein aggregation and fibril formation is one of the dominant characteristics in the pathogenesis of a number of neurodegenerative diseases, such as Alzheimer's, Parkinson's and Creutzfeldt–Jakob diseases.^{1–3} In Alzheimer's disease (AD), extensive genetic, biochemical and pathological evidence links accumulation and amyloid fibril formation of amyloid- β (A β) peptides (e.g., the major components A β 40 and A β 42), produced by the β - and γ -secretase cleavage of the parental amyloid precursor protein (APP), to the AD phenotype.^{4, 5} A β amyloid fibril contains a typical cross- β -sheet architecture extending in a direction parallel to the fibril axis, identified by high resolution techniques such as solid-state NMR at the molecular level.^{6–8} Moreover, recent evidences suggest that the oligomeric, diffusible assemblies of A β peptides formed in the early stages of aggregation, appear to be highly toxic species in AD.^{9–11}

Although it has been reconciled that both A β oligomers and fibrillar plaques may play roles in the progressive degeneration of neurons,¹² the fundamental mechanism by which the assembly process causes the toxicity leading to cell death is still unclear. A growing body of recent research highlights the importance of cellular membranes in mediating A β self-assembly and the consequent cellular toxicity.^{13–15} Cholesterol is an essential component of the eukaryotic plasma membrane, necessary for membrane fluidity, permeability and receptor function. Elevated levels of cholesterol have been recognized as one important risk factor for AD, and the role of cholesterol in APP processing and A β generation has been supported by recent studies.^{16–18} Sparks et al. reported a dose-dependent A β amyloid accumulation in the brain of rabbits fed with a high-cholesterol diet.¹⁹ Cerebral A β generation was reported to be cholesterol dependent,²⁰ and guinea pigs treated with high doses of simvastatin, a widely used cholesterol-lowering drug, showed a strong and reversible reduction of cerebral A β levels in the cerebrospinal fluid and brain homogenate.²¹ Although the mechanism by which cholesterol modulate A β generation is unclear, lipid rafts, the cholesterol-rich membrane microdomains, appear to promote β - and γ -secretase processing function.^{22, 23} Furthermore, increased free cholesterol in the cytoplasm has also been found to affect the aggregation of A β peptides into fibrils.^{24, 25} These suggest that one of the possible roles for cholesterol in AD may be to directly interact with A β and consequently modulate the amyloidogenic process of A β . However, most of the reports available so far have mainly focused on cholesterol as the component in cellular membranes or lipid bilayer or monolayer model membranes,^{26–29} leaving the direct investigation of the effects of the pure form of cholesterol on A β amyloid formation largely neglected. Although increasing efforts have been provided to put insight into the interactions between cholesterol and A β peptides,^{30–32} a detailed mechanistic view of cholesterol-mediated A β fibrillogenesis is unclear.

Cholesterol, as a neutral and hydrophobic steroid molecule, can be decorated to form a series of derivatives, such as the oxidation metabolite 27-hydroxycholesterol and 24S-hydroxycholesterol. The effects of these derivatives in the pathology of AD have been suggested in recent studies.^{33, 34} Cholesterol sulfate (cholesterol-SO₄, Fig. 1) is one of the most important known steroid sulfates, and has emerged as a significant lipid constituent in a variety of human tissues,³⁵ with a concentration of ~110–170 μ g/mL in human plasma.^{36, 37}

While its definite functions in human physiology remain poorly understood, considering the potential use of the level of A β including the aggregated A β species in plasma as a biomarker for early diagnosis of AD,³⁸ the effect of this cholesterol derivative on A β amyloidogenesis is of physiological interest. The sulfate moiety of cholesterol-SO₄ has a *pKa* of ~3.3, indicating that it is normally ionized under physiological conditions. The presence of this negatively charged moiety makes cholesterol-SO₄ an amphiphilic compound in comparison to a rather rigid hydrophobic cholesterol analog. On the other hand, a cationic cholesterol derivative, 3 β -[N-(dimethylaminoethane)carbonyl]-cholesterol, or DC-cholesterol (Fig. 1), has been commonly utilized for preparation of lipid membranes with cationic charged groups. DC-cholesterol contains a cholesterol moiety attached by an ester bond to a positively charged dimethylethylenediamine group. Although rarely studied to date, recent simulation results suggested the crucial role of hydrophobic force in regulating cholesterol–A β interaction.³² Owing to the additional charges in cholesterol-SO₄ and DC-cholesterol molecules, these cholesterol derivatives may exhibit unique characteristics in mediating A β amyloid formation. Elucidating the effects of these derivatives on A β fibrillization will provide new mechanistic and structural insights into the underlying function of steroid-based molecules in modulating A β aggregation pathway, complementing the current efforts towards a deep understanding of the roles of cholesterol and the mutants in AD.

In the present work, we study for the first time the mechanistic effect of pure cholesterol-SO₄ and DC-cholesterol on aggregation of A β 40 peptide. We employ a combination of fluorescent spectroscopy and atomic force microscopy (AFM) imaging to assess the roles of specific intermolecular forces, e.g., hydrophobic and electrostatic interactions in lipid molecules and A β 40 peptide association and their impact on A β fibrillogenesis. Our results demonstrate that low concentration of non-vesicle cholesterol-SO₄ and DC-cholesterol both moderately accelerate A β 40 fibril formation, similar to that of cholesterol. This implicates the dominating role of hydrophobic interactions between A β 40 and cholesterol derivatives in facilitating the aggregation rate. Furthermore, we show that the vesicle structures formed by high concentrations of anionic cholesterol-SO₄ facilitate A β 40 aggregation rate markedly. In contrast, the vesicles of cationic DC-cholesterol have the ability to inhibit A β 40 fibril formation under appropriate experimental conditions, distinct from those of the cholesterol-SO₄. Our results suggest the critical role of the structural properties of the vesicles of the cholesterol derivatives, including the surface charge properties and the size, in mediating their effects on A β 40 amyloid formation.

EXPERIMENTAL METHODS

Materials

All chemical reagents were obtained from commercial suppliers and used without further purification unless otherwise mentioned. Lipid molecules cholesterol, DC-cholesterol and cholesterol-SO₄ were purchased from Sigma-Aldrich. Solutions of DC-cholesterol and cholesterol-SO₄ were prepared by thoroughly mixing appropriate amounts of compound powder in dimethyl sulfoxide (DMSO), while cholesterol was dissolved in a mixture of

hexane/chloroform/ethanol 11:5:4 v/v/v according to a previously reported protocol by Fantini et al.³⁹

Synthesis and preparation of A β 40 peptide

A β 40 peptide was synthesized on a PS3 solid phase peptide synthesizer (Protein Technologies) using standard Fmoc strategy. The resulting crude peptide was purified by reversed phase high-performance liquid chromatography (RP-HPLC) using a C18 reverse phase column and characterized by matrix-assisted laser desorption ionization mass spectrometry (MALDI). The A β 40 peptide utilized in the kinetic aggregation assay was monomerized as described previously.⁴⁰ Briefly, lyophilized A β 40 powder was dissolved in aqueous NaOH solution (2 mM) and the pH was adjusted to 11 by using 100 mM NaOH solution. The solution was sonicated for 1 h in ice-water bath, then filtered through 0.22- μ m filter (Millipore) and kept on ice before use. The concentration of A β 40 was determined by using the tyrosine UV absorbance at 280 nm ($\epsilon = 1,280 \text{ M}^{-1}\text{cm}^{-1}$).

Kinetic aggregation assay of A β 40 using thioflavin T (ThT)

The aggregation kinetics followed by ThT fluorescence were conducted as described previously.⁴¹ In brief, the monomerized A β 40 peptide solution was diluted to a final concentration of 12 μ M in pH 7.4 phosphate buffer (50 mM Na phosphate, 150 mM NaCl) containing 20 μ M ThT. 100 μ L solution was transferred into wells of a 96-well microplate (Costar black, clear bottom). The plate was sealed with a microplate cover and loaded into a Gemini SpectraMax EM fluorescence plate reader (Molecular Devices, Sunnyvale, CA), where it was incubated at 37 °C. The fluorescence of ThT was measured every 10 min after shaking for 5 s with excitation wavelength of 440 nm and emission wavelength of 480 nm.

For the assays with cholesterol derivatives, a stock solution of DC-cholesterol or cholesterol-SO₄ was prepared by dissolving 5.0 mg of the samples in 1.0 mL DMSO and then different stock solutions were prepared by further dilution. The stock solution of cholesterol was prepared by dissolving 5.0 mg of the sample in a mixed solvent composed of hexane:chloroform:ethanol 11:5:4 v/v/v, and further dilutions were applied using the same mixed solvent to obtain stock solutions with different concentration of cholesterol. A particular amount of stock solutions was added to A β 40 solution (final concentration of 12 μ M) in pH 7.4 phosphate buffer (50 mM Na phosphate, 150 mM NaCl) containing 20 μ M ThT. The solution was then pipetted into the plate reader (100 μ L/well) for the kinetic assay. In consideration of the high sensitivity of A β 40 aggregation rate on the experimental conditions and therefore the day-to-day assay variance of lag time in A β 40 aggregation kinetics, an A β 40-alone sample was always measured in each assay as the control.

The kinetic parameter lag time (t_{lag}) was extracted by fitting the kinetic data using the following equation⁴²:

$$F = \frac{F_{max}}{(1 + ve^{-k(t-t_m)})^{1/v}}$$

where F is the fluorescence intensity at time t , F_{max} is the maximum steady-state fluorescence, k is the apparent rate constant of amyloid accumulation, t_m is the point of the maximum elongation rate, and v represents the asymmetry of the aggregation kinetics curve. The lag time (t_{lag}) is the time at which the tangent at t_m crosses the initial baseline and is given by $t_m - (1 + v)/k$.

Atomic force microscopy (AFM)

Aliquots (20 μ L) of the A β 40 solution were adsorbed onto the surface of freshly cleaved mica (5 $\times$ 5 mm) for 5 min at room temperature. The liquid was wicked off by absorption into filter paper. Salts and unbound materials were removed by three washes with 30 μ L of water. The samples were dried overnight, and AFM images were acquired in tapping mode utilizing an Asylum Research MFP 3D AFM system with MikroMasch NSC15/AI BS cantilevers.

Critical micelle concentration (CMC) determination

The CMC values of the cholesterol derivatives used in this study were measured following a previously reported protocol.⁴³ Briefly, a series of solutions for each lipid derivative were prepared that differ by an order of concentration. The cholesterol derivatives were dissolved in pH 7.4 phosphate buffer (50 mM Na phosphate, 150 mM NaCl) containing 5% DMSO. The final concentration range of the cholesterol derivatives was from 0.50 ng/mL to 0.25 mg/mL ($\sim 9 \times 10^{-10}$ M to 4×10^{-4} M). Using a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon) at slit width of 10 nm and excitation and emission wavelength of 460 nm, light intensity for each solution was measured 3 times and then was averaged. Data were plotted as logarithm of sample concentration versus average light scattering intensity, and CMC values were calculated as the intercept with x -axis of a linear regression line for the concentrations where a rapid increase in light scattering intensity was observed.

Vesicle size measurement using dynamic light scattering

100 μ L aliquots of different concentrations of each cholesterol derivative in pH 7.4 phosphate buffer (50 mM Na phosphate, 150 mM NaCl) containing 5% DMSO were incubated at 37 $^{\circ}$ C for 24 h. Then samples were diluted to 200 μ L with Millipore H₂O and stirred for 30 s. Size measurements were performed using a model Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK) with disposable cuvettes. The average diameters of vesicles were calculated as the mean of three measurements, with 12-15 runs per measurement.

Zeta potential measurement

A 100 μ L aliquot of the samples in pH 7.4 phosphate buffer (50 mM Na phosphate, 150 mM NaCl) containing 5% DMSO was incubated at 37 $^{\circ}$ C for 24 h. Then the sample was 10-fold diluted to 1 mL with Millipore H₂O and stirred for few seconds. Zeta potential measurements were performed using a model Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK) with a disposable polycarbonate folded capillary cell (model DTS1070) with a path length of 4 mm. The values of zeta potential are reported as the mean of three measurements.

RESULTS AND DISCUSSION

Effects of low concentrations of cholesterol derivatives on A β 40 aggregation

Cholesterol and its derivatives, like other lipid molecules, can self-associate and form aggregated structures in aqueous solution above the concentration of the CMC value. In order to study the effect of pure non-aggregating cholesterol derivatives on aggregation of A β 40, we first measured the CMC values of cholesterol-SO₄ and DC-cholesterol following a protocol reported by Avdulov et al.⁴³ As shown in Table 1, the CMC value of cholesterol-SO₄ in pH 7.4 phosphate buffer (50 mM Na-phosphate, 150 mM NaCl) is approximately 12.6 nM (equivalent to 6.2 ng/mL), and the CMC value of DC-cholesterol is approximately 14 nM (equivalent to 7.5 ng/mL). The CMC values for these two derivatives are slightly lower than that of the unmodified cholesterol, which is ~31 nM (equivalent to ~12.1 ng/mL), as reported previously.^{43, 44}

The kinetics of fibrillization of monomeric A β 40 peptide (12 μ M) in phosphate buffer (50 mM Na-phosphate, 150 mM NaCl, pH 7.4) was monitored via a ThT fluorescence experiment. ThT molecule can selectively bind to the fibrillar structure, leading to a significant increase of its fluorescence quantum yield.⁴⁵ As shown in Fig. 2a, the aggregation kinetics of A β 40 peptide had a sigmoidal appearance containing a lag phase associated with nucleation, a fast growth phase related to the propagation of fibrils, and a final stationary phase. This is consistent with previous studies which indicated that A β amyloidogenesis proceeds by a nucleated polymerization mechanism.⁴⁶⁻⁴⁹

The effect of DC-cholesterol on amyloidogenesis of A β 40 was examined using the same ThT fluorescence assay. DC-cholesterol was used at concentrations below its CMC value in this case, allowing to assess the mechanistic effect of non-aggregating DC-cholesterol structures on A β aggregation. Here we reasonably assume that DC-cholesterol mainly exists as monomeric molecules under the current experimental conditions, although one could not exclude the possibility of the formation of oligomeric structures of DC-cholesterol. As shown in Fig. 2a, the presence of a low concentration of DC-cholesterol (0.5 to 2.5 ng/mL) slightly but noticeably facilitates the aggregation rate of A β 40, with a shorter lag phase as compared to that measured without DC-cholesterol. This suggests that DC-cholesterol likely facilitates the early nucleation reactions to form crucial oligomeric intermediates toward the formation of fibrillar structures. The aggregation half time (t_{50}), which is defined as the time at which the ThT fluorescence intensity reaches half of the maximum, is decreased approximately 21% from ~ 10.5 h to ~ 8.3 h in the presence of DC-cholesterol at 2.5 ng/mL (Fig. 2b). This result is comparable to that observed for cholesterol (Fig. S2), wherein the addition of 2.5 ng/mL cholesterol also moderately accelerates the aggregation rate of A β 40, as revealed by an approximately 27% decrease of t_{50} from ~10 h to ~ 7.3 h. These results implicate that the additional moiety attached on the hydrophobic cyclic sterol ring structure, including the presence of the positively charged dimethylethylenediamine residue, does not dramatically change the regulating function of cholesterol on A β fibril formation. This is also consistent with recent simulation studies,^{30, 32} which reported that the hydrophobic interactions between cholesterol and the A β hydrophobic region play a critical role in association between these molecules.

The effect of low concentration of cholesterol-SO₄ on aggregation of A β 40 was also studied. Similarly, the concentrations below the CMC value of cholesterol-SO₄ were used in order to provide insight into the non-aggregating cholesterol-SO₄ mediated A β 40 amyloidogenesis. As shown in Fig. 3, additional cholesterol-SO₄ (0.5 to 2.5 ng/mL) also moderately facilitated the aggregation rate of A β 40, with the aggregation t_{50} value decreased from ~ 11.2 h to ~ 9.8 h. Again, the small difference between the t_{50} values (Fig. 3b) suggests that under the current condition, the kinetic effect of cholesterol-SO₄ on A β 40 aggregation is not significant. This is similar to that of the unmodified cholesterol and DC-cholesterol, suggesting that the negatively charged sulfate moiety of cholesterol-SO₄ does not dramatically influence its function in mediating A β aggregation. Taken together, if non-aggregating cholesterol and the derivatives modify the aggregation of A β via binding with the peptide, as suggested by recent simulation studies,^{30, 32} they presumably interact with A β peptides mainly through hydrophobic interactions. The positively or negatively charged groups in the cholesterol derivatives studied here likely are not involved in critical interactions in this process.

Effects of aggregated vesicles of cholesterol derivatives on A β 40 aggregation

The local concentration of cholesterol-SO₄ in human tissues can be much higher than its CMC value. Therefore, besides addressing its regulating role as a non-aggregating form in A β aggregation, it would be valuable to examine the effect of the aggregated vesicles formed at concentrations above the CMC value on the aggregation of A β . We thus prepared the aggregated vesicles of the pure cholesterol derivatives formed above their CMC values, and examined their kinetic effects on A β amyloidogenesis. As shown in Fig. 4a, addition of large amount of cholesterol-SO₄ (concentration of 0.25 μ g/mL and higher), under which the molecule self-associates to form vesicle structures, facilitates the aggregation rate of A β 40 (12 μ M) in a concentration-dependent manner. The presence of cholesterol-SO₄ of 25 μ g/mL leads to a significantly shorter t_{lag} changed from 12.2 to 3.1 h (~ 75% decrease) (Fig. 4a). With the presence of cholesterol-SO₄ of 250 μ g/mL, the A β 40 kinetic aggregation curve shows an immediate growth phase, with no lag phase observed. Similar results were also observed using a lower concentration of A β 40 (5 μ M), as shown in Fig. S1. These results suggest that the cholesterol-SO₄ vesicles could influence the early oligomerization reaction, facilitating the formation of oligomeric nucleus and/or the propagation of the oligomers into fibrillar structure. The kinetic effect of cholesterol vesicles were also tested for comparison (Fig. S2). Although only a middle concentration (25 μ g/mL) of cholesterol was used here, because of the low solubility of cholesterol under the experimental condition, the results nonetheless demonstrate that the appearance of the cholesterol vesicles increases the aggregation rate of A β 40 (Fig. S2). This is consistent with previously reported results for cholesterol incorporated into the lipid membrane structure.^{27, 50} Moreover, the results also suggest that the accelerating activity of cholesterol vesicles is weaker in comparison to that of the cholesterol-SO₄ (Fig. 4a vs. Fig. S2).

Interestingly, the DC-cholesterol vesicles show an unusual effect on A β aggregation kinetics. In the presence of relatively low concentration of DC-cholesterol while above its CMC value, A β aggregates faster with a shortened lag phase (Fig. 4b). However, when high amount of DC-cholesterol, e.g., 25 μ g/mL, was added, the aggregation of A β was

dramatically interfered. The increase of ThT fluorescence is delayed significantly with the t_{lag} value increased from ~12.4 to 23.6 h. The final stationary fluorescence intensity also decreases dramatically. When 250 $\mu\text{g/mL}$ DC-cholesterol was used in the kinetic assay, there is no observable ThT fluorescence at the ending time point of the kinetics experiment, suggesting a strong inhibiting activity on A β fibrillization. In comparison to that of the cholesterol-SO₄, these results indicate distinct roles of these two vesicles in the aggregation of A β 40 under high concentration conditions.

The effect of the lipid vesicles on A β 40 aggregation was further assessed using AFM imaging. AFM images of A β 40 (30 μM) in the absence or presence of either cholesterol-SO₄ or DC-cholesterol were acquired after incubating the samples for 17 h on a speci-mix aliquot mixer at 37 °C. In the absence of cholesterol derivatives, characteristic fibrillar amyloids were produced (Fig. 5a). Fibers were also observed in the presence of 50 ng/mL cholesterol-SO₄ (Fig. 5b). Formation of fibers still occurs with a high concentration of cholesterol-SO₄ of 500 $\mu\text{g/mL}$ (Fig. 5c), consistent with the non-inhibiting effect of this derivative on A β aggregation, as suggested by the aggregation kinetic results. For DC-cholesterol, A β fibers were observed in the image of A β 40 (30 μM) co-incubated with low concentration of DC-cholesterol (50 ng/mL) (Fig. 5d). However, as shown in Fig. 5e, in the presence of 500 $\mu\text{g/mL}$ DC-cholesterol, no fiber structures were found in the image. This is consistent with our observations from the kinetic results, suggesting an inhibitory activity of the DC-cholesterol species under high concentration condition. These results demonstrate that the DC-cholesterol vesicles formed at high concentration of the molecules may play different roles in A β aggregation, in comparison to cholesterol-SO₄ vesicles. In view that one of the major structural differences between these two molecules is their opposite charge, the results may suggest the potential effects of electrostatic interactions in mediating interactions between the vesicles and A β . However, we do not exclude other factors, including hydrophobic interactions and the properties of particle structures, on impacting the functions of cholesterol derivatives in modulating A β aggregation.

Interactions between A β 40 and the cholesterol derivatives

The importance of cholesterol, as a crucial component of cellular membrane, in modulating the aggregation of A β peptide has long been recognized.¹⁸ However, most of the previous *in vitro* studies on the cholesterol-mediated A β amyloidogenesis were performed using mixed cholesterol-phospholipid model structures. Studies towards clarification of the particular interactions between A β peptide and the cholesterol molecule and the mechanistic effect of cholesterol on regulating A β amyloidogenesis are scarce. Nonetheless, direct binding of cholesterol to APP and A β amyloid fibrils has been indicated in some recent studies.^{31, 51} Computational approaches have started to be applied recently to shed light on the interaction details between free cholesterol and A β peptide. Di Scala et al. studied the binding between cholesterol and A β peptide via both docking modelling and surface pressure measurements of Langmuir monolayers, and their results suggested that a hydrophobic fragment of amino acids 22-35 of A β can serve as a potential cholesterol-binding domain, primarily forming hydrophobic interactions with the apolar part of cholesterol.³² Using all-atom molecular dynamic simulations, Zhou et al. reported an important role of steroid-benzyl interaction in cholesterol-mediated aggregation.³⁰ More specifically, the amino acid Phe19 was found to

be critical for this interaction. Our experimental results are in accord with these simulation results. At concentrations below the CMC values, where cholesterol-SO₄ or DC-cholesterol remains as nonvesicle forms, presumably mainly monomeric structures, they moderately accelerate the aggregation rate of A β , similar to that of the unmodified cholesterol. This suggests that none of the negatively or positively charged groups in these mutants could dramatically influence the interactions between A β and cholesterol derivatives, suggesting that no critical electrostatic interactions were formed between these charged moieties and A β molecule. In other words, we could reasonably speculate that the hydrophobic interactions between the non-aggregating cholesterol derivatives and the A β peptide, especially its hydrophobic sequence region, are dominant in the regulating mechanisms on A β amyloidogenesis. Recently it has been reported that cholesterol can stimulate the formation of oligomeric A β channel structures in membrane,^{52, 53} and a cholesterol-like compound bexarotene can efficaciously occupy the cholesterol-binding domain in A β peptide, thus interfering with the formation of amyloid pore channels.⁵⁴ Given the similarity of the structures of cholesterol and cholesterol-SO₄ or DC-cholesterol derivatives, it would not be surprising that these derivative molecules could bind with A β peptide at the same cholesterol-binding site and mediate the oligomerization and fibrillization of A β *via* a similar mechanism.

Our results demonstrate that the negatively charged cholesterol-SO₄ vesicles dramatically facilitate the aggregation of A β 40. Vesicles formed by lipid molecules, such as amphipathic phospholipids, normally contain a liquid crystalline bilayer structure with the hydrophilic head group facing the surrounding aqueous solvent, sequestering the hydrophobic tail regions in the micelle center. Lipid vesicles thus provide an interface that can act as a catalytic or inhibiting surface, and can influence the A β aggregation kinetics in similar manner as nanoparticles.⁵⁵⁻⁵⁸ It has been suggested that the head group charge of the phospholipids contributes to the association between the membrane and the A β peptide via electrostatic interactions. However, the exact effects of head group charges on A β peptide aggregation are somewhat controversial.⁵⁹ Kremer et al. reported that the affinity of A β 40 to the zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) is weaker than for the negatively charged 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), supporting the view that the charged head groups mediate binding.⁶⁰ On the contrary, results from Hellstrand et al. suggested that there is no significantly different effect on A β aggregation kinetics when increasing amount of negative charge was incorporated into phospholipid vesicles.⁵⁵ These contradictory results suggest the sensitivity of the kinetic roles of lipid vesicles on their specific structural and surface properties.

The typical steroid structure of cholesterol-SO₄ possibly makes it less favourable to form ordered gel- or liquid crystalline-like vesicle structures like other phospholipids. Nonetheless, the size of the vesicles was analysed using dynamic light scattering method assuming a sphere model. The results indicate that the size of the vesicles is dependent on the concentration of cholesterol-SO₄ (Fig. 6a and Fig. S3), with larger vesicles formed at higher concentrations. The difference of the width of the size distribution suggests the polydisperse feature of the particles of varied sizes. Considering the amphipathic nature of cholesterol-SO₄, it would be conceivable to speculate that considerable amount of negatively charged sulphate ion moieties may still tend to be exposed to face the solvent on the surface

of the vesicles. The measured negative zeta potential, i.e., -53 mV for cholesterol- SO_4 at a concentration of $250\text{ }\mu\text{g/mL}$, also suggests the presence of negative charges on the surface of the formed vesicles. Therefore, besides the plausible hydrophobic interactions, it is quite possible that the sulfate group may also play a role in guiding the binding between A β and the cholesterol- SO_4 vesicles via electrostatic interactions. This is indicated by a stronger facilitating effect of cholesterol- SO_4 on the aggregation kinetics of A β , in comparison to that of the unmodified cholesterol. One possibility is that the positively charged residues along the A β sequence at neutral pH used in this study, including Arg, Lys, and His residues, may interact with the negatively charged sulfate groups exposed on the surface of the vesicles. This will lead to the adsorption of the monomeric A β on the surface of vesicles, thus increasing local concentration of A β and favouring the formation of critical nucleus for further propagation (Fig 7).

The surface of DC-cholesterol vesicles is speculated to contain positive charges, opposite to that of cholesterol- SO_4 . The zeta potential is approximately $+32$ mV for DC-cholesterol at a concentration of $250\text{ }\mu\text{g/mL}$, consistent with the proposed surface charge properties of DC-cholesterol vesicles. At low concentrations while above its CMC value, the formed DC-cholesterol vesicles also facilitate the amyloid formation of A β . Again, here one would attribute the significantly accelerating effect of DC-cholesterol vesicles partly to the vesicle-aided nucleus formation. The positive charges on the surface of the particles may exhibit as a favourable factor for facilitating the adsorption of A β on the vesicles, as A β also contains considerable amount of negatively charged residues (i.e., 3 Asp and 3 Glu). With a theoretical isoelectric point $\text{pI} \sim 5.31$, the overall net charge of A β_{40} at pH 7 is approximately -3 , and therefore the overall electrostatic attractions between A β_{40} molecule and DC-cholesterol vesicles is expected to be quite favourable. Surprisingly, the vesicles formed at higher concentrations of DC-cholesterol, i.e., $25\text{ }\mu\text{g/mL}$ and above, starts to inhibit the aggregation of the peptide. We found that relatively large vesicle structures with diameters of ~ 300 nm were formed at low concentrations of DC-cholesterol (Fig. 6b). These vesicles may provide favourable surface area for local binding with A β_{40} , thus facilitating the formation of oligomeric nucleus structures for elongation to form fibrils. With increased concentration of DC-cholesterol, the diameter of the formed vesicles becomes surprisingly smaller. At the highest concentration of $250\text{ }\mu\text{g/mL}$ used in the study, the size of the particle is approximately 100 nm (Fig. 6b). It has been previously reported that the size and the curvature properties of the liposome vesicles are important factors in determining the binding between the vesicles and amyloidogenic proteins such as A β and α -synuclein.⁶¹⁻⁶³ Considering the small radius and high curvature properties of positively charged DC-cholesterol vesicles formed at high concentrations, and the overall negative net charge of A β_{40} , these small vesicles may strongly bind with A β monomers, as depicted in Fig. 7. Therefore it would be likely that strong attractive electrostatic interactions, together with hydrophobic interactions, may prevent the diffusion, conformational adjustment, and the self-association of the monomers along the vesicle surface. This will inhibit the formation of critical nucleus on vesicle surface and the further propagation to form fibrillar structures. In addition, because of the large amount of the small vesicles formed under the high concentration condition, such binding will dramatically reduce the concentration of

monomeric A β 40 in solution, thus also possibly leading to retardance or complete inhibition of amyloid fibril formation of A β 40 peptide.

The effect of DC-cholesterol on A β 40 amyloidogenesis was also investigated with different NaCl salt concentration. The increase of NaCl salt concentration (50-300 mM) did not significantly change the aggregation kinetics of A β 40 alone (Fig. S4). However, increase of concentration of NaCl led to dramatic decrease of the kinetic difference between A β 40 alone and A β 40 with DC-cholesterol (Fig. S4). This suggests the sensitivity of the interaction of A β 40 and DC-cholesterol on the ionic strength, indicating the critical role of electrostatic force in A β 40 and DC-cholesterol interactions. On the other hand, increase of NaCl concentration slightly increases the facilitating effect of cholesterol-SO₄ on the kinetics of A β 40 aggregation, possibly because of the screening effect of the salt on repulsive electrostatic interactions between the negatively charged residues in A β 40 and the sulfate group of cholesterol-SO₄.

The specific structural properties of the vesicles could dramatically influence their interactions with A β 40 peptide. It was reported that small unilamellar gel phase vesicles can show faceted structure,⁶⁴ where the edges between the facets are highly curved which may be possible that peptides accumulate in such areas.⁵⁵ Our results show that the vesicles of cholesterol derivatives can affect the aggregation of A β peptide, and these effects are highly dependent on the structural properties of the vesicle system. However, it is not trivial to clarify the detailed structural characteristics of these vesicles at molecular level. Some previous studies indicated that cholesterol can form irregular structures under certain conditions.^{44, 65} Future studies for clarifying the detailed structural characteristics of the vesicles formed by the cholesterol derivatives will be critical for understanding the mechanistic reasons of the distinct regulating roles of the vesicles on A β aggregation discovered in this research.

A β 40 was used as an amyloidogenic model protein in this study, since A β 40 shows a more moderate aggregation propensity than another aggressive component of the amyloid plaques in AD, A β 42, which contains two more residues (Ile-Ala) at the C-terminus. This allows the feasibility to carefully investigate the kinetic effects of the cholesterol derivatives on aggregation of A β 40 peptide. Due to the fast aggregation kinetics of A β 42 itself, the kinetic effect of the cholesterol derivatives on A β 42 amyloidogenesis is not as significant as that on A β 40 (Fig. 4 vs. Fig. S5a). Comparison of the fluorescence intensity of the final stationary phase (Fig. S5b) of the kinetic traces shows that cholesterol-SO₄ can facilitate A β 42 fibrillization, while high concentration of DC-cholesterol interferes with the formation of amyloid fibrils. The results suggest that these cholesterol derivatives may interact with A β 42 in a mechanism similar to that with A β 40.

CONCLUSION

In summary, we report for the first time the kinetic effects of both non-aggregating forms and aggregated vesicle structures of cholesterol-SO₄ and DC-cholesterol on A β 40 amyloid formation. Our results demonstrate that the non-aggregating forms of cholesterol-SO₄ and DC-cholesterol accelerate the aggregation rate of A β 40 moderately, similar to that observed

for the unmodified cholesterol, indicating the importance of hydrophobic interactions in binding of these steroid molecules and A β 40. Furthermore, we show that the vesicles formed at high concentrations of pure anionic cholesterol-SO₄ facilitate A β 40 aggregation rate markedly, whereas the cationic DC-cholesterol vesicles have the ability to inhibit A β 40 fibril formation under appropriate experimental conditions, distinct to that of the cholesterol-SO₄. Our results implicate that the characteristics of the protein-vesicle interface, including the surface charge properties and the size of the cholesterol derivative vesicles, are critical in regulating the effects of the vesicles of these cholesterol derivatives on A β 40 amyloid formation. Our findings not only provide new insights into the mechanistic effect of natural cholesterol derivatives on A β amyloidogenesis, but also may illuminate design of specific molecular particles as inhibitors of A β fibril formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Chemical structures of cholesterol and the two derivatives.

Figure 2.

Effect of low concentration of DC-cholesterol (below the CMC value) on the aggregation of Aβ40. (a) The aggregation kinetics of Aβ40 (12 μM) in the absence or the presence of different amount of DC-cholesterol measured by ThT fluorescence at 37 °C. (b) Growth phase half time (t_{50}) of the aggregation kinetics of Aβ40 in the absence or the presence of DC-cholesterol. Data are reported as means $\pm$ the standard deviation of triplicate results.

Figure 3.

Effect of low concentration of cholesterol-SO₄ (below the CMC value) on the aggregation of Aβ40. (a) The aggregation kinetics of Aβ40 (12 μM) in the absence or the presence of different amount of DC-cholesterol measured by ThT fluorescence at 37 °C. (b) Growth phase half time (t_{50}) of the aggregation kinetics of Aβ40 in the absence or the presence of cholesterol-SO₄. Data are reported as means $\pm$ the standard deviation of triplicate results.

Figure 4.

Effect of high concentration of cholesterol-SO₄ (a) or DC-cholesterol (b) on the aggregation of Aβ₄₀. The aggregation kinetics of Aβ₄₀ (12 μM) in the absence or the presence of different amount of cholesterol derivatives were measured by ThT fluorescence at 37 °C. The concentrations of the cholesterol derivatives used were above their respective CMC values. The inset shows t_{lag} values of the kinetic aggregation traces with data reported as means ± standard deviation of triplicate results.

Figure 5.

Tapping mode atomic force microscopy images of A β 40 fibrillization reactions. (a) A β 40 (30 μ M) in pH 7.4 phosphate buffer (50 mM Na phosphate, 150 mM NaCl) incubated on a speci-mix aliquot mixer at 37 °C. Images were also taken for A β 40 (30 μ M) in the presence of 50 ng/mL cholesterol-SO₄ (b), 500 μ g/mL cholesterol-SO₄ (c), 50 ng/mL DC-cholesterol (d), and 500 μ g/mL DC-cholesterol (e). Samples were incubated for 17 h before imaging.

Figure 6. Size of cholesterol-SO₄ (a) and DC-cholesterol (b) vesicles formed at different concentrations of the compounds. The diameters values were determined by dynamic light scattering.

Figure 7.
Schematic representation of the possible interactions between Aβ40 and the negatively charged cholesterol-SO₄ vesicles and the positively charged DC-cholesterol vesicles with different size.

Table 1

CMC value of the cholesterol derivatives.

Lipid molecule	CMC value (ng/mL)
Cholesterol-SO ₄	6.2 ± 1.6
DC-cholesterol	7.5 ± 2.6
Cholesterol *	12.1

*
Data from ref. 43.