Cholesterol catalyses Aβ42 aggregation through a heterogeneous nucleation pathway in the presence of lipid membranes

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Supplementary Information

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Methods

All chemicals and reagents were purchased from Sigma Aldrich (UK) Ltd. unless otherwise stated.

Preparation of Aβ peptides

The recombinant Aβ(M1-42) peptide (MDAEFRHDSGY EVHHQKLVFF AEDVGSNKGA IIGLMVGGVV IA), here called Aβ42, was expressed in the *E. coli* BL21 Gold (DE3) strain (Stratagene, CA, U.S.A.) and purified as described previously with slight modifications²⁷. Briefly, the purification procedure involved sonication of *E. coli* cells, dissolution of inclusion bodies in 8 M urea, and ion exchange in batch mode on diethylaminoethyl cellulose resin followed by lyophilisation. The lyophilised fractions were further purified using Superdex 75 HR 26/60 column (GE Healthcare, Buckinghamshire, U.K.) and eluates were analysed using SDS-PAGE for the presence of the desired protein product. The fractions containing the recombinant protein were combined, frozen using liquid nitrogen, and lyophilised again.

Preparation of lipid vesicles

All lipids used in this paper, and cholesterol, were purchased from Avanti Polar Lipids, Inc. For all the experiments, the preparation of lipid vesicles was achieved using sonication (Bandelin, Sonopuls HD 2070, 5 x 5 min, 50% cycles, 10% maximum power) on ice and subsequent centrifugation at 15000 rpm for 30 min at 25°C. We found that lipid vesicles prepared by extrusion or sonication resulted in the same increase in aggregation kinetics of Aβ42, thus showing that the method of the lipid preparation does not have a significant effect on the kinetics of Aβ42 (Supplementary Fig. 8).

The DMPC:cholesterol vesicles used in this study contained different levels of cholesterol, defined as the fraction of cholesterol out of the total lipid concentration used together with DMPC to prepare the vesicles. For example, DMPC:cholesterol vesicles containing 5% cholesterol indicate that the vesicles were prepared from lipid mixtures containing 95% DMPC and 5% cholesterol on molar level.
**Sample preparation for kinetic experiments**

Solutions of monomeric peptides were prepared by dissolving the lyophilised Aβ42 peptide in 6 M guanidinium hydrochloride (GuHCl). Monomeric forms were purified from potential oligomeric species and salt using a Superdex 75 10/300 GL column (GE Healthcare) at a flowrate of 0.5 mL/min, and were eluted in 20 mM sodium phosphate buffer, pH 8 supplemented with 200 μM EDTA and 0.02% NaN₃. The centre of the peak was collected and the peptide concentration was determined from the absorbance of the integrated peak area using $\varepsilon_{280} = 1490$ L mol⁻¹ cm⁻¹. The monomer obtained in this way was diluted with buffer to the desired concentration and supplemented with 20 μM Thioflavin T (ThT) from a 1 mM stock. All samples were prepared in low binding eppendorf tubes on ice using careful pipetting to avoid introduction of air bubbles. Each sample was then pipetted into multiple wells of a 96-well half-area, low-binding, clear bottom and PEG coated plate (Corning 3881), to give 80 μL per well, in the absence and the presence of lipid vesicles at different [L]/[P] ratios.

**Kinetic assays**

Assays were initiated by placing the 96-well plate at 37 °C under quiescent conditions in a plate reader (Fluostar Omega, Fluostar Optima or Fluostar Galaxy, BMGLabtech, Offenburg, Germany). The ThT fluorescence was measured through the bottom of the plate with a 440 nm excitation filter and a 480 nm emission filter. The ThT fluorescence was followed for three repeats of each sample.

**ThT spectral scan**

Fluorescence spectral scans were measured with 20 μM ThT in the wavelength range of 470 - 550 nm using a 440 nm excitation filter. Aβ42 fibrils were prepared by incubating monomeric solutions of Aβ42 at 2 μM in phosphate buffer, pH 8 in the absence or the presence of DMPC vesicles or DMPC:cholesterol vesicles containing 15% cholesterol at a [L]/[P] ratio of 10. Spectra were averaged from independent measurements.
**Fluorescence polarisation measurements**

Stock solutions of 2 mM DPH and laurdan were prepared by solubilising them in 100% ethanol (EtOH) and 100% DMSO respectively overnight with stirring at room temperature (RT). The dyes were then each diluted into stock solutions of the prepared vesicles, and the mixture was incubated for 30 min at 45 °C; the final DMSO/EtOH content of this mixture was kept below 0.5%. The different solutions were placed in 96-well half-area, low-binding, clear bottom and PEG coated plates (Corning 3881). The fluorescence polarisation of the dyes was then monitored using a plate reader (Polarstar Omega, BMG Labtech, Aylesbury, UK) under quiescent conditions at temperatures ranging from 25 °C to 50 °C. A 355-nm excitation filter and two matched emission filters, 430 nm for DPH and 480nm for laurdan, were used.

**Circular dichroism spectroscopy**

CD experiments were carried out using a Jasco J-810 spectropolarimeter equipped with a Peltier holder in 20 mM sodium phosphate pH 8.0. To reduce the general artefacts caused by the contribution of the lipids due to optical rotation and absorption of the membrane\(^{59}\), CD spectra were measured at a protein concentration of 0.1 mg/ml, in the presence of DMPC or DMPC:cholesterol vesicles containing 15% cholesterol at a [L]/[P] ratio of 10. Measurements were performed with a scanning speed of 50 nm/min and a data pitch of 1 nm. Spectra were averaged from five scans and smoothed using the “means-movement” smoothing procedure implemented in the Spectra Manager package. The contribution of the buffer solution was subtracted from experimental spectra, and the mean ellipticity values per residue (MRE) were calculated as:

\[
MRE = \frac{3330m\Delta A}{lCN}
\]

where \(l\) is the path length (0.1 cm), \(m\) the molecular mass (4,645 Da) and \(C\) the protein concentration expressed in mg/mL. Time-induced structural transitions were monitored in the 190 - 250 nm range and the MRE at 218 nm was plotted as a function of time.
**Dynamic light scattering**

Dynamic light scattering (DLS) experiments were performed with a Zetasizer Nano-S (Malvern) at 25°C using 100 μM lipid samples in 20 mM phosphate buffer at pH 8. The hydrodynamic radii were deduced from the translational diffusion coefficients using the Stokes-Einstein equation. Diffusion coefficients were inferred from the analysis of the decay of the scattered intensity autocorrelation function. All calculations were performed using the software provided by the manufacturer.

**Differential scanning calorimetry**

The thermograms were acquired using a Microcal VP-DSC calorimeter (Malvern Instruments) with a scanning rate of 1 °C min⁻¹ from 10 °C to 65 °C. 1 mM lipid samples were degassed (20 min, RT) before mixing and acquisition of the DSC thermograms. All differential scanning calorimetry (DSC) thermograms were corrected by subtracting the thermogram of the phosphate buffer, and were subjected to concentration normalisation.

**Atomic force microscopy**

Atomic force microscopy (AFM) was performed on positively functionalised mica substrates. The mica surface was cleaved and was incubated for 1 min with 10 μl of 0.5% (v/v) (3-aminopropyl)triethoxysilane (APTES, from SIGMA) in Milli-Q water. Then, the substrate was rinsed three times with 1 ml of Milli-Q water and dried by a gentle stream of nitrogen gas. An aliquot of 10 μl of a 2uM sample of Aβ42 monomers or fibrils were prepared in phosphate buffer, pH 8 either in the absence, or the presence of DMPC or DMPC:cholesterol vesicles containing 15% cholesterol at a [L]/[P] ratio of 10 was deposited on the positively functionalised surface. The droplet was incubated for 10 min, rinsed with 1 ml of Milli-Q water and dried by a gentle stream of nitrogen gas. The preparation was carried out at room temperature. AFM maps were realised by means of a JPK nanowizard2 system operating in tapping mode and equipped with a silicon tip (µmasch, 2 Nm⁻¹) with a nominal radius of 10 nm. Images were flattened using the SPIP software (Image Metrology).
Cryo-electron microscopy

Samples of 10 µM Aβ42 monomers and 85%DMPC/15%Cholesterol vesicles (100 µM total lipid) were incubated at 37°C in non-binding microfuge tubes (Axygen) for 24 hrs. The fibrillation process was also followed in a parallel for ThT-containing samples using fluorescence to ensure the fibrillation plateau had been reached. Samples of 10 µM Aβ42 monomer without vesicles were incubated at 37°C in PEGylated plates (Corning 3881) in a plate reader and collected after reaching the plateau in ThT fluorescence. Specimens for electron microscopy were prepared in a controlled environment vitrification system (CEVS) to ensure stable temperature during sample preparation. The specimens were prepared as thin liquid films, <300 nm thick, on lacey carbon filmed copper grids and plunged into liquid ethane at -180°C. This leads to vitrified specimens, avoiding component segmentation and rearrangement, and the formation of water crystals, thereby preserving original microstructures. The vitrified specimens were stored under liquid nitrogen until measured. A Fischione Model 2550 cryo transfer tomography holder was used. For samples formed in the presence of vesicles we used a JEM 2200FS microscope, equipped with an in-column energy filter (Omega filter), which allows zero-loss imaging. The acceleration voltage was 200kV and zero-loss images were recorded digitally with a TVIPS F416 camera using SerialEM under low dose conditions. For samples formed in the absence of vesicles we used a microscope (Philips CM120 BioTWIN Cryo) equipped with a post-column energy filter (Gatan GIF100). The acceleration voltage was 120 kV. The images were recorded digitally with a CCD camera under low electron dose conditions.

Fourier transform infrared spectroscopy

Attenuated total reflection infrared spectroscopy (ATR-FTIR) was performed using a Bruker Vertex 70 spectrometer equipped with a diamond ATR element. Spectra were acquired with a 375 µM sample of Aβ42 in phosphate buffer, pH 8, either alone or in the presence of DMPC or DMPC:cholesterol vesicles containing 15% cholesterol at a [L]/[P] ratio of 10. The resolution was 4 cm⁻¹ and all spectra were processed using Origin Pro software. The spectra were first averaged (3 spectra with 128 co-averages) and then the second derivative was calculated applying a Savitzky-Golay filter (2nd order, 12 points).
Section 1: Aβ42 aggregation in other lipid systems

We have found that, unlike DMPC vesicles, DMPC:cholesterol vesicles accelerate Aβ42 aggregation. To assess the changes in the physico-chemical properties of the DMPC vesicles resulting from the presence of cholesterol, and that could explain the ability of these vesicles to accelerate Aβ42 aggregation, we carried out a series of biophysical measurements on the lipid vesicles and Aβ42 fibrils (Supplementary Figs. 6-7).

Recent data have shown that the chemical properties of lipids are key regulators of the interplay between the functional and aberrant behaviour of α-synuclein in the presence of vesicles. We therefore monitored the thermotropic properties of the lipid vesicles used in this study using differential scanning calorimetry (DSC) (Supplementary Fig. 6). In the absence of cholesterol, the DSC scan of DMPC is characterised by a sharp transition the melting temperature, $T_m$, of 23±1 °C (Supplementary Fig. 6), which is associated with the transition of the membrane from the gel to the liquid state; this value is in agreement with previously reported values. In the presence of increasing concentrations of cholesterol up to 15%, the peak broadens and gradually decreases in area, and the DSC data are consistent with phase diagram and previous calorimetric studies for the same and similar systems. We further characterised the acyl-chain order of the DMPC:cholesterol membranes using two different fluorescent dyes, laurdan (6-Dodecanoyl-2-Dimethylaminonaphthalene) and DPH (1,5-Diphenyl-hexa-1,3,5-triene), the optical characteristics of which are highly dependent on their environments, such that binding to membranes results in a sharp increase of the fluorescence signal (Supplementary Fig. 7). At 37 °C, the fluorescence polarisation of both dyes solubilised within the DMPC:cholesterol membranes were shown to increase with increasing concentrations of cholesterol, indicating that the presence of the latter decreased the acyl-chain order of the membrane (Supplementary Fig. 7).

As the incorporation of cholesterol into the lipid bilayers has also been reported to affect the size of lipid vesicles, we measured the effects of cholesterol on the
DMPC:cholesterol vesicles using dynamic light scattering (DLS), finding that their size increased by 65% when the concentration of cholesterol reached 15% (Supplementary Fig. 6). We then performed ThT-based kinetic aggregation measurements of Aβ42 in the presence of DMPC:cholesterol vesicles containing 0 or 10% cholesterol vesicles of larger sizes and found no measureable effect of the size of the vesicles on the aggregation kinetics of Aβ42 (Supplementary Fig. 8).

We also performed experiments with model systems composed of DMPC and DMPE (20%) with or without added cholesterol (Supplementary Fig. 5). DMPE is a zwitterionic lipid with smaller headgroup compared to DMPC and it forms lamellar phases with a melting transition around 50.2 °C. In the mixed DMPC-DMPE bilayer at 37 °C, most DMPE is expected to be present in the fluid phase. Interestingly, no changes could be observed in the aggregation rate of Aβ42 when the peptide was incubated in the presence of DMPC:DMPE vesicles when compared to DMPC vesicles (Supplementary Fig. 5).
Figure 1: Effects of DMPC:cholesterol vesicles on the aggregation kinetics of Aβ42. Kinetic profiles of the aggregation reaction of 2µM Aβ42 in the absence and the presence of DMPC vesicles (a) and DMPC:cholesterol vesicles containing 5% (b) 10% (c), and 15% (d) cholesterol at increasing [L]/[P] ratio.
Figure 2: Effects of DMPC:cholesterol vesicles at low [L]/[P] on the aggregation kinetics of Aβ42. (a) Kinetic profiles of the aggregation reaction of 2μM Aβ42 in the absence and the presence of low [L]/[P] ratios of DMPC:cholesterol vesicles. (b) Normalised half-times, $t_{1/2}$, of the aggregation kinetics of Aβ42 as a function of the [L]/[P] ratio, as derived from the data in (a).
Figure 3: Effects of DMPC:cholesterol vesicles with high cholesterol content on the aggregation kinetics of $\text{A}\beta 42$. Kinetic profiles of the aggregation reaction of 2µM $\text{A}\beta 42$ in the absence and the presence of increasing $[L]/[P]$ ratios of DMPC:cholesterol vesicles containing 20% (a) and 40% (b) cholesterol. (c) Normalised half-times, $t_{1/2}$, of the aggregation kinetics of $\text{A}\beta 42$ as a function of $[L]/[P]$ ratio, as derived from the data in (a) and (b).
Figure 4: Effects of POPC and DOPC vesicles on the aggregation kinetics of Aβ42. Kinetic profiles of the aggregation reaction of 2μM Aβ42 in the absence and the presence of POPC vesicles (a), POPC:cholesterol vesicles containing 25% cholesterol (b), DOPC vesicles (c), and DOPC:cholesterol vesicles containing 25% cholesterol (d), at increasing [L]/[P] ratio. (e) Normalised half-times, $t_{1/2}$, of the aggregation kinetics of Aβ42 as a function of [L]/[P] ratio, as derived from the data in (a)-(d).
Figure 5: Effects of DMPC:DMPE:cholesterol vesicles on the aggregation kinetics of Aβ42. Kinetic profiles of the aggregation reaction of 2µM Aβ42 in the absence and the presence of DMPC vesicles (a), DMPC:DMPE vesicles containing 20% DMPE (b), and DMPC:DMPE:cholesterol vesicles containing 20% DMPE and 20% cholesterol (c), at increasing [L]/[P] ratio. (d) Normalised half-times, $t_{1/2}$, of the aggregation kinetics of Aβ42 as a function of [L]/[P] ratio, as derived from the data in (a)-(c).
Figure 6: Biophysical characterisation of the effects of cholesterol on DMPC vesicles. (a) DSC heating thermograms of the lipid vesicles used in this study; the melting temperature ($T_m$) is similar for all vesicles (about $23 \pm 1$ °C). (b) Size distribution of the lipid vesicles (prepared by sonication) as measured by DLS; the diameter of the vesicles increases with increasing amounts of cholesterol.
Figure 7: Fluorescence polarisation (FP) measurements showing the effect of cholesterol on the acyl-chain order of the DMPC:cholesterol vesicles. Fluorescence polarisation of either laurdan (a) or DPH (b) solubilised in either DMPC vesicles (sky blue), or DMPC:cholesterol vesicles containing 5% (purple), 10% (teal), and 15% (pink) cholesterol. The chemical structures of the fluorescent dyes are shown.
**Figure 8: Effects of the size of the lipid vesicles on Aβ42 aggregation kinetics.**

(a) Particle size distribution of all lipid vesicles as measured by DLS. Extrusion with a 30 nm pore produces vesicles of a distribution centered at a diameter of 72 nm, while extrusion with a 50 nm pore produces vesicles of a distribution centered at a diameter of 82 nm. (b,c) The effect of DMPC vesicles (b) or DMPC:cholesterol vesicles containing 10% cholesterol (c) with different sizes on the aggregation kinetics of a 2 μM solution of Aβ42 were monitored using ThT fluorescence. (d) Normalised half-times, $t_{1/2}$, of the aggregation kinetics of a 2 μM sample of Aβ42 in the presence of vesicles of different sizes, as derived from data in (b), (c) and Supplementary Fig. 1.
Figure 9: Time-course measurements of Aβ42 aggregation in the presence of vesicles using CD spectroscopy. CD spectra of 20 µM Aβ42 in sodium phosphate buffer, pH 8 in the absence (a), or in the presence of either DMPC vesicles (b) or DMPC:cholesterol vesicles containing 15% cholesterol (c) at a [L]/[P] ratio of 10 as a function of time (represented in different colours). Note the increased negative values of the mean residue ellipticity at 218 nm (MRE\(_{218\text{nm}}\)). (d) Normalised mean residue ellipticity at 218 nm as a function of time starting from 20 µM Aβ42 monomer at time zero either alone, or supplemented with DMPC vesicles at a [L]/[P] ratio of 10. (e) Normalised mean residue ellipticity at 218 nm as a function of time starting from 20 µM Aβ42 monomer supplemented at time zero with DMPC vesicles or DMPC:cholesterol vesicles containing 15% cholesterol at a [L]/[P] ratio of 10.
Figure 10: Effect of the lipid vesicles on the properties of Aβ42 fibrils. (a) Infrared spectra of Aβ42 fibrils are formed in the absence or the presence of DMPC or DMPC:cholesterol vesicles containing 15% cholesterol at a [L]/[P] ratio of 10. (b) ThT fluorescence spectra of Aβ42 fibrils formed in the absence or the presence of DMPC or DMPC:cholesterol vesicles containing 15% cholesterol at a [L]/[P] ratio of 10.
Figure 11: Effects of DMPC:cholesterol vesicles on the aggregation of Aβ42 in the presence of Brichos. (a) Kinetic profiles of the aggregation reaction of 1.2 μM Aβ42 in the absence of vesicles, and in the absence and presence of one molar equivalent of Brichos (1.2 μM). (b) Change in the apparent rate constant of surface-catalysed secondary nucleation in the presence of one molar equivalent of Brichos. Note the significant decrease in $k_2$ in the aggregation of Aβ42 in the absence of vesicles, due to the presence of Brichos. (c) Change in the apparent rate constant of primary nucleation of Aβ42 in the presence of one molar equivalent of Brichos and DMPC:cholesterol lipid vesicles containing 10% cholesterol at [L]/[P] ratios of 50 and 100. The rate constants were derived from the curves in Fig. 4c.
Figure 12: Quantitative chemical kinetics analysis of the effect of DMPC:cholesterol vesicles on the aggregation of Aβ42. (a) Kinetic profiles of the aggregation reaction of 2 µM Aβ42 in the absence and the presence of DMPC:cholesterol vesicles containing 5% cholesterol at increasing [L]/[P] ratios (represented by different colours). The solid lines show predictions for the resulting reaction profiles when the rates of the primary and secondary pathways are modified to include the lipid-induced nucleation process. (b) Evolution of the apparent reaction rate constants with increasing [L]/[P] ratios of DMPC:cholesterol vesicles containing 5% cholesterol. $k_n'$ is the apparent rate constant of primary nucleation in the presence of vesicles, $k_+'$ is the apparent rate constant of elongation in the presence of vesicles, and $k_2'$ is the apparent rate constant of secondary nucleation in the presence of vesicles; $k_{\text{lipids}}/k$ represents in each case either $k_n'k_+'/k_nk_+$ or $k_2'k_+'/k_2k_+$, where $k_{\text{lipids}}$ is the rate constant in the presence of lipids.
**Figure 13: Effects of DMPC:cholesterol vesicles on the aggregation of Aβ42 at different protein concentrations.** Kinetic profiles of the aggregation reaction of 1.5 μM (a) and, 3 μM Aβ42 (b) in the absence and the presence of DMPC:cholesterol vesicles containing 10% cholesterol at increasing [L] (represented by different colours). (c,d) Normalised half-times, $t_{1/2}$, of the aggregation kinetics of 1.5 μM (c) and, 3 μM (d) Aβ42 in the presence of DMPC:cholesterol vesicles containing 10% cholesterol, as derived from data in (a) and (b).
Figure 14: Effects of bexarotene on the aggregation kinetics of Aβ42 in the absence and presence of DMPC:cholesterol vesicles. (a) Kinetic profiles of a 2 μM solution of Aβ42 in the absence or the presence of 3 molar equivalents of bexarotene in the absence or the presence of DMPC:cholesterol vesicles containing 10% cholesterol at a [L]/[P] ratio of 50. (b) Normalised half-times, t₁/₂, of the aggregation kinetics of Aβ42 as derived from the data in (a).