Amyloid-β oligomers have a profound detergent-like effect on lipid membrane bilayers, imaged by atomic force and electron microscopy

David C. Bode,1 Mark Freeley,1 Jon Nield,1 Matteo Palma,1 and John H. Viles*1

School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK

Running Title: Imaging the impact of Amyloid-β on lipid membranes

*To whom correspondence should be addressed: Dr J.H. Viles, School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK  j.viles@qmul.ac.uk

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ABSTRACT

Amyloid-β peptide’s (Aβ) ability to disrupt membrane integrity and cellular homeostasis is believed to be central to Alzheimer’s disease pathology. Aβ is reported to have various impacts on the lipid bilayer, but a clearer picture of Aβ influence on membranes is required. Here we use atomic force and transmission electron microscopies to image the impact of different isolated Aβ assembly types on lipid bilayers. We show that only oligomeric Aβ can profoundly disrupt the bilayer, visualized as widespread lipid extraction and subsequent deposition, which can be likened to an effect expected from the action of a detergent. We further show that Aβ oligomers cause widespread curvature and discontinuities within lipid vesicle membranes. In contrast, this detergent-like effect was not observed for Aβ monomers and fibers, although Aβ fibers did laterally associate and embed into the upper leaflet of the bilayer. The marked impact of Aβ oligomers on membrane integrity identified here reveals a mechanism by which these oligomers may be cytotoxic.

Introduction

The amyloid cascade is a widely accepted description for the molecular basis of Alzheimer’s disease (AD). This hypothesis is centred on a small hydrophobic peptide, amyloid-β, which is typically 40 or 42 amino acids in length (Aβ40/42). The self-association of Aβ, and subsequent interaction with the lipid bilayer is thought to cause disrupted synaptic function, the resulting loss of cellular homeostasis ultimately leads to hyper-phosphorylation of tau, cell death and dementia.(1) Aβ fibrous plaques are abundant in the AD brain and have high lipid content.(2,3)

The interaction of Aβ with lipid membranes has been expertly reviewed,(4-6) and draws parallels with the mechanism of toxicity established for anti-microbial peptides.(6) Importantly, Aβ42 oligomers are reported to perforate cellular membrane by forming single, discrete ion channel pores.(7) Alternatively, a carpeting effect of Aβ has been proposed, which is thought to result in a general increase in membrane conductance either by membrane thinning or a lateral spreading of lipid
head-groups.(8-10) Studies have also revealed that Aβ42 can extract and incorporate lipid into growing Aβ42 fibres by an effect likened to the action of a detergent, which has the potential to rupture the lipid bilayer.(11) Some of these effects have been observed for other amyloid forming proteins, and include: amylin;(12-14) alpha-synuclein;(15) and a Prion protein fragment,(16) which suggests a shared mechanism of membrane disruption. Nevertheless, a clear picture of the impact of Aβ on membranes is lacking, perhaps in part due to insufficient assembly-state characterization and separation of Aβ oligomers from monomers and fibres.

Atomic Force Microscopy (AFM) is particularly well placed to probe the topography of supported lipid bilayers.(17) However, many AFM studies have focused on how the lipid bilayer can influence Aβ fibre assembly.(5,18-21) AFM studies which investigate the impact of Aβ on the bilayer structure are only starting to be explored. (22-25)

Here we report a systematic investigation of the effects of both Aβ40 and Aβ42 isoforms on lipid bilayers. The Aβ isoforms have been studied in three broad assembly states: monomers; prefibrillar heterogeneous oligomeric assemblies, and fibres. We have employing AFM and Transmission Electron Microscopy (TEM) to show that these different Aβ structures have profoundly different impacts on membrane integrity, which can explain the diverse effects reported.

Results and Discussion

Lipid bilayers were deposited onto muscovite mica surfaces from an aqueous mixture of phosphatidylcholine (PC), cholesterol and GM1-ganglioside (68:30:2 by weight, a ratio chosen to mimic the typical composition of the extracellular face of a cellular membrane). A liposomal suspension in aqueous buffer was incubated with the negatively charged mica, allowing the lipid head groups to adhere and fuse with the mica substrate to form a double-leaflet lipid bilayer. The remaining suspension was then blotted off the mica and air-dried. AFM images indicate this to be an effective method of generating flat, continuous patches of lipid bilayer with a sharp ‘cliff-edge’ boundary between the membrane patch and lipid-free mica, as shown in supplemental Figure S1. A mean bilayer height has been measured at 4.6±0.1 nm, as would be expected for this lipid composition.

To understand the nature of the interactions between monomeric, oligomeric and fibrillar Aβ40/42 with lipid bilayers, it is important to isolate and characterize the different Aβ assembly types, see Experimental procedures, Figure S2 and S3 for details. We first deposited size exclusion chromatography-purified monomeric Aβ40 and Aβ42 preparations (10 µM) on to the lipid bilayer. After 60 mins on the bilayer surface, the Aβ solution was blotted off, and the mica was allowed to air-dry for imaging. It is clear that neither monomeric Aβ42 nor Aβ40 have a detectable impact on the integrity of the bilayer, as seen in Figure 1a and Figure S4a. There is an even distribution of lipid with minimal disruption on both the lipid surface and also at the lipid edge, where a very sharp ‘cliff-edge’ transition between the lipid-bilayer and the mica is observed. Moreover, the bilayers seen in these images are indistinguishable from the bilayer treated with aqueous buffer.

In contrast, prefibrillar heterogeneous oligomeric preparations of Aβ42 and Aβ40 (10 µM monomer equivalent) have a very disruptive impact on the integrity of the bilayer (see Figure 1b and S4b respectively). In particular there is widespread extraction of lipid from bilayer islands, suggesting a very strong detergent effect by Aβ oligomers. Furthermore, small clumps of lipid appear deposited on the surrounding mica in close-proximity to the more substantial lipid islands. This is evident in membrane height cross-sections (see Figure 1d and Figure S4d). In contrast, the impact of Aβ42 and Aβ40 mature amyloid fibres was much less pronounced than seen for the oligomeric preparations, with almost no Aβ-fibre induced lipid extraction and deposition (no detergent-effect). The lipid bilayers remained predominantly intact in the
presence of Aβ fibres, as shown in Figure 1c and S4c.

We aimed to quantify the extent of the detergent effect for the three Aβ assembly types. This was achieved by measuring lipid coverage at the lipid-edge interface between regions of bilayer and lipid-free mica for multiple (n=20) preparations (Figure 1e and S4e). This data is presented as percentage area lipid coverage versus distance from an island of lipid (for details see Figure S5). The mean lipid coverage data confirms that neither monomer or fibre had an impact on the sharp ‘cliff edge’ transition between the lipid bilayer and mica substrate. In contrast, a very poorly defined transition between the lipid bilayer and the mica was consistently observed upon the addition of Aβ oligomers. The highly disrupted islands of lipid bilayer were surrounded by small deposits of lipid that become less abundant and dispersed at further distances from the main island of lipid, suggesting an extraction of lipid away from the bilayer origin. We observed this consistently in numerous bilayers and Aβ preparations; mean values from typically 20 measurements are shown for each Aβ type. Further examples are shown for Aβ42 and Aβ40 in supplemental Figures S6 and S7 respectively. Previous studies have utilised Aβ-reconstituted liposomes to form an Aβ infused mica-supported bilayer, leading to membrane-spanning pore-like annular-oligomer structures, but with this method of bilayer preparation the extraction and resulting deposition of lipid was not observed.(26-28)

In addition to the widespread disruption of lipid at the edges of the bilayer, extraction of lipid by Aβ oligomers also resulted in large holes in the membrane which span the bilayer, as previously reported.(22) Quantification of these holes indicates a typical diameter of 50 nm (see Figure 2). A similar range of hole sizes in the bilayer is observed for Aβ40, (see Figure S8). These holes, caused by lipid extraction, should not be conflated with the formation of ion-channel pores. The diameter of Aβ ion-channels have been shown to be between 1.7 and 2.4 nm based on measurements of their ionic conductance,(7) imaging of annular Aβ oligomers which resemble channel structures,(26-28) and also modelling of Aβ barrel-like structures in the bilayer.(29,30) The holes observed in Figure 2 are much larger and would be indistinguishable from membrane-patch break-down in ionic conductance measurements.(7) These large holes are also very occasionally observed upon the addition of Aβ fibres, however their occurrence is relatively rare and may be explained by the presence of a small number of oligomers in the Aβ fibre preparation.

We were also interested in quantifying the extent by which the amyloid fibres embed into bilayers. AFM is particularly suited to measuring lipid-bilayer heights; measured to be 4.6±0.1 nm, while single Aβ fibres are typically 5.3±0.1 nm in height (see Figures 3, S9 and S10). Measurements of the height of fibres laterally associated to the surface of the membrane indicate the extent to which the fibres embed and displace lipids within the bilayer. Fibres that laterally associate onto the surface of the membrane, consistently protrude to a height of 2.5±0.05 nm above the lipid bilayer surface. This indicates that the fibres do indeed embed into the membrane to a depth where the upper leaflet of the bilayer is displaced, as shown in Figure 3c. It seems that fibres do not displace lipid from the lower leaflet of the supported lipid bilayer, nor do fibres rest on the surface of the membrane to leave the bilayer unperturbed. Lateral association of amyloid fibres on the surface of a lipid bilayer has been reported using a range of techniques, including EM and fluorescence microscopy. (11,13,31-33) However, this is the first clear evidence that laterally associated Aβ fibres are capable of embedding and displacing the upper leaflet of the bilayer, rather than simply residing on the charged surface of the bilayer.

To investigate the extent in which oligomers and curvy-linear protofibrils embed into the lipid bilayer, we used AFM to record both topographical and phase-contrast information. Oligomers and curvy-linear proto-fibrils generated here are typically at least 4 nm in diameter. Phase-imaging can be used to distinguish differences in viscoelastic properties that in turn can be explained by different surface
properties even when there is little change in height topology. In this way, Aβ oligomers can be observed even when they are fully embedded into the membrane (see Figure S11). Phase imaging of bilayers exposed to Aβ oligomer revealed dark regions within the bilayer which are indicative of increased probe adhesion to membrane inserted Aβ; these oligomers were otherwise poorly visualized by height topography. The oligomer and curvy-linear proto-fibrils have a widespread presence in the membrane and are sufficiently buried to be similar height to the surrounding bilayer, displacing both leaflets of the membrane which might then facilitate the observed lipid extraction.

We further investigated the impact of Aβ assemblies on the structure of lipid membranes within large unilamellar vesicles (LUVs) by negative-stain TEM imaging. In agreement with the AFM data, monomers of Aβ have little impact on the membrane, as the vesicles show little difference in appearance from vesicles imaged in the absence of Aβ, Figure 4a and S12. Aβ42 oligomers clearly disrupt the shape of the vesicles, causing numerous discontinuities and curvatures within the bilayer (Figures 4b and S12). Peptide induced membrane curvature has also been reported for anti-microbial peptides whose toxic action is linked to a detergent effect dependent on membrane insertion.(34,35) In contrast, Aβ fibres cause less distortion in the vesicle appearance, with only the ends of the fibres causing membrane curvature (Figure 4c and S13). This behaviour is also apparent for β2-microglobulin and amylin amyloid fibres imaged by TEM.(13,32)

In summary, nanoscale imaging of supported lipid bilayers have revealed very different impacts on the membrane when challenged with Aβ monomers, heterogeneous-oligomers or fibres, Figure 5. In the presence of isolated monomers of Aβ40 or Aβ42, the lipid membrane remains unperturbed, while fibres laterally embed into the bilayer to displace the upper leaflet. Notably, only Aβ oligomers cause extraction of lipid from the membrane, explaining the high levels of lipid deposited within extracellular Aβ plaques in the AD brain. In vivo, this detergent effect is likely to impact cellular homeostasis and integrity, and is in line with the relative cytotoxicity of Aβ oligomers compared to fibrillar assembly states.(36,37) (38) Cell viability assays report cytotoxicity for both Aβ40 and Aβ42, although Aβ40 is less potent than Aβ42.(38) Here we show there is not a significant difference in membrane disruption caused by Aβ40 oligomers compared to Aβ42, while only Aβ42 oligomers form ion-channels.(7) The differences in ion-channel formation and lipid bilayer disruption (Figure 5) suggest there may be two or more mechanisms by which Aβ can disrupt neuronal homeostasis and trigger the cascade of events which culminate in dementia. Multiple modes of toxic action present numerous therapeutic targets; our study indicates therapeutic molecules, that prevent insertion of Aβ oligomer into membranes may circumvent both mechanisms of toxicity.

Experimental procedures

Aβ Peptides and Solubilisation

Lyophilised Aβ40 and Aβ42 peptides were purchased from Cambridge Research Biochemicals, and EZBiolab Inc. Aβ peptides were synthesised using solid-phase F-moc (N-(9-fluorenyl)methoxycarbonyl) chemistry, producing a single elution band in HPLC with correct mass verified by mass spectrometry. Unless otherwise stated all other chemicals were purchased from Sigma-Aldrich.

Peptides were solubilised in ultra-high quality (UHQ) water (0.7 mg/ml at pH 10) and left at 4 °C for 12 h. Samples were then centrifuged for 15 min at 16,000 g at 4 °C, to remove any high molecular weight aggregates. Aβ concentration was determined by absorbance of stock solutions at 280 nm (ε = 1280 M⁻¹cm⁻¹).

Purification of Monomeric Aβ by Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) was utilised to separate Aβ monomer from amyloid assemblies of higher molecular weight. Superdex 75 10/300 GL column (GE Healthcare) was used to purify and elute a
monomeric fraction of solubilised Aβ preparations using AKTA FPLC. Stock Aβ solution (90 μM) in UHQ water was loaded onto the column and the monomer peak was eluted with a solution of NaCl (160 mM), HEPES (30 mM) using a 0.5 ml/min flow rate at 4 °C. Eluted monomer had a typical concentration of 30 μM.

SEC-purified Aβ was deemed to be seed-free, evidenced by a single elution band and an absence of observable assemblies by negative-stain TEM. Seed-free Aβ, termed here as monomeric, also had no ThT fluorescence signal and exhibited a clear lag-phase in the nucleation polymerisation reaction (see Supplemental Figure S2). Monomeric samples were used directly after SEC elution or immediately stored at -80 °C.

**Aβ Oligomer and Fibre Preparations**

**Fibre Preparations:** Amyloid fibre preparations of Aβ40 and Aβ42 (10 μM monomer-equivalent) were generated in a 96-well in aqueous buffer containing NaCl (160 mM) and HEPEs (30 mM) at pH 7.4, as previously described (39), see supporting information for further details. Assembly kinetics were monitored with Thioflavin T (ThT) fluorescence (10 μM). Adjacent sample-wells with no ThT added were used to generate assemblies for use in all experiments. To remove any low molecular weight oligomers, fiber preparations were centrifuged at 16,100 g for 5 min. The supernatant, which contained small diffusible oligomers, was removed, and the fiber pellet was re-suspended. At equilibrium, Aβ assemblies had the typical amyloid fibrous appearance according to AFM and TEM, see Supplemental Figure S3. Fibre preparations have a low level of Aβ monomer and oligomer content.

**Oligomer Preparations and Characterisation:** Similarly, Aβ40 and Aβ42 assemblies with predominantly circular oligomeric structure were obtained from the well plate towards the end of the lag-phase, as monitored by ThT fluorescent dye in separate wells. Oligomeric samples were used immediately or stored at -80 °C to halt further assembly. The heterogeneous lag-phase mixture contains a range of oligomer sizes, while appreciable monomeric Aβ is still present, as indicated by SEC elusion profiles, Supplemental S2d. According to TEM these oligomeric preparations are heterogeneous in nature, but contain a large number of circular oligomeric assemblies that range in size, with typical diameters between 10 and 12 nm, identified by TEM and AFM, see Supplemental Figure S3.

TEM micrographs of negatively stained prefibrillar oligomeric assemblies of Aβ42 were further characterized using single-particle-analyses. A total data set of 3997 single particles were grouped into 25 class averages, see Supplemental Figure S3a. From this data set, 70% were broadly circular in appearance and ranged in diameter between 10 to 12 nm. Using the relationship: Volume (nm^3) = Mass (kDa)/1.27 (nm^3/kDa) and assuming spherical protein assemblies, approximate masses of between 400-700 kDa are indicated. While SEC suggests smaller oligomers are also present (Supplemental Figure S2d), the remaining particles appeared elongated up to 16 nm, with some approaching curvy-linear status. Some much longer curvy-linear protofibrillar assemblies were indeed present, typically 100 nm long. In agreement with our SEC elution profiles (Supplemental Figure S2d) it has been shown much of Aβ remains monomeric in the prefibrillar lag-phase preparations.(40) The nature of the heterogeneous Aβ assemblies present in lag-phase preparations has previously been reviewed.(40)

**Lipid Bilayer Preparation**

Lipid bilayers were prepared using similar methods to those previously described. A lipid mixture of egg phosphatidylcholine (PC) (Avanti Polar Lipids Inc.), monosialotetrahexosyl-ganglioside (GM1) (Avanti Polar Lipids Inc.), and cholesterol (Sigma-Aldrich Ltd.) solubilized to 5 mg/ml in a 2:1 mixture of chloroform and methanol were mixed in a glass vial at a ratio of 68:2:30 (w/v). Organic solvent was then gently removed under a stream of nitrogen, and left overnight in a fume hood to form a lipid film.

For AFM studies, the dried lipid film of PC:GM1:Cholesterol was rehydrated in an aqueous buffer (160mM NaCl, 30mM HEPEs,
pH 7.4) to achieve a 0.6 mg/ml liposome solution. This vesicle suspension was then tip sonicated at low power for 3-times 30 sec periods, until the cloudy lipid solution was clear, ready for deposition onto mica.

For TEM lipid vesicle studies, large unilamellar vesicles (LUVs) were generated by the extrusion method. [31] A 1 mg/ml lipid solution was immediately passed through a lipid extruder (Avanti Lipids Ltd.) which contained a 100 nm polycarbonate filter (Avanti Polar Lipids Inc.), seven times to generate a uniform size, LUVs were typically 100 – 200 nm in diameter.

Preparation of Supported Lipid Bilayers on Mica, and application of Aβ

Mica discs (Agar Scientific ltd.) were cleaved 5 times with sticky tape, and both the mica and the lipid vesicle suspension was heated to 60 °C in a benchtop oven. The heated suspension was then pipetted onto mica discs in a Corning, sterile 6-well plate (Sigma-Aldrich Ltd.). The plate was then sealed in a humid box and incubated at 60 °C for 30 min, before decreasing the temperature to 25 °C at a rate of 5 °C every 15 min until reaching room temperature. Slow cooling of the sample enables large micro-islands of bilayer to form on the mica, Supplemental Figure S1.

The mica was then washed 3 times with UHQ water, ready for exposure to Aβ. Typically 200 μl of Aβ (10 μM) with NaCl (160 mM) and HEPEs (30 mM), at pH 7.4 was applied by pipette to evenly coat the surface of the mica. The Aβ droplet was incubated in a sealed container for 1 h to minimise evaporation and maintain salt/Aβ concentration. The droplet was then removed using filter paper and the mica surface was washed again 3 times with UHQ water. Mica samples were finally left to dry in ambient air conditions under the cover of pierced parafilm. The samples were then imaged by AFM. Aβ was also deposited directly onto the mica in the absence of lipid bilayer using a similar protocol.

Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM); Quantification and Characterization

Details of the measurements made using both AFM and TEM can be found in the supplemental information. In particular: AFM data quantification of the detergent effect; bilayer hole diameters;(41) lateral fibre embedding. and single particle analysis (42) (43) of TEM micrographs.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.
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Figure Legends:

Figure 1. Aβ42 oligomers have a detergent-like effect on the bilayer which is not seen for Aβ42 monomer or Aβ42 fibres.

AFM topographical images are shown for mica-supported lipid bilayers on exposure to:
(a) Aβ42 monomer; (b) Aβ42 heterogeneous oligomer and (c) Aβ42 fibre. Height scale range is 12 nm.
(d) Height cross-sections. (e) Percentage bilayer coverage at the edge of a lipid bilayer, exposed to Aβ42 monomer (green), heterogeneous oligomer (blue) and fibre (red). Each data-point represents an average % bilayer coverage within a 0.1 x 0.5 μm region. Twenty measurements per data point, measured across 3 separate mica-supported lipid bilayer preparations, error bars are standard error.

Figure 2. Aβ42 oligomer causes holes within supported lipid bilayers.
(a) Left: AFM topographical image depicting a region of supported lipid bilayer which has holes formed after incubation with Aβ42 heterogeneous oligomer, height colour scale range is 12 nm. Right: Binary map of holes, generated by height threshold. (b) Height cross-section. Typically, both the upper and lower leaflets of bilayer are extracted. Scale bar = 0.3 μm. (c) Range of hole diameters with a modal value of 50 nm.

Figure 3. Aβ fibres embed into the lipid bilayer, displacing the upper leaflet.
(a) AFM topographical images show Aβ42 fibres on mica (left) and on the surface of a lipid bilayer (right). Scale bar = 1 μm. Height scale range is 12 nm. Each image is accompanied by a height cross-section. (b) Mean Aβ42 fibre height recorded on both mica, and height above the bilayer surface. (c) A scaled schematic showing how Aβ fibres displace the upper leaflet of the membrane as fibres laterally embed into the bilayer.

Figure 4. Aβ oligomer disruption of lipid vesicles.
TEM negative-stain images of LUVs in the presence of: (a) Aβ42 (10 µM) monomer; (b) Aβ42 (10 µM) heterogeneous oligomers; (c) Aβ42 (10 µM) fibres. Scale bars represent 100 nm. Aβ42 oligomers cause widespread curvature and discontinuities of the membrane lipid-bilayer, while vesicles are relatively unaffected by Aβ42 monomers and fibres.

Figure 5: The mechanisms of amyloid-β mediated membrane disruption is dependent on Aβ structure.
Aβ monomers, oligomers and fibres have distinct impacts on membrane integrity. (a) Aβ monomers have little impact on membrane structure. (b) Only oligomeric Aβ42 has the ability to form ion channels pores in cellular membranes. (7) (c) Oligomers of both Aβ42 and Aβ40 have a widespread detergent effect causing lipid extraction, curvature and rupture of the membrane bilayer. (d) The ability of Aβ to form ion channels or have a detergent effect is lost on assembly into amyloid fibres, instead fibres can laterally embed into the membrane and displace the upper leaflet of the bilayer. Fibre structure; PDB 2MXU (44).
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5:
Amyloid-β oligomers have a profound detergent-like effect on lipid membrane bilayers, imaged by atomic force and electron microscopy
David C. Bode, Mark Freeley, Jon Nield, Matteo Palma and John H. Viles

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