On the Physiological/Pathological Link between Aβ Peptide, Cholesterol, Calcium Ions and Membrane Deformation: A Molecular Dynamics Study

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Abstract

The dynamic interplay between Cholesterol, asymmetrically (at physiological condition) or symmetrically (hallmark of aging) distributed in membrane, and β amyloid peptides is investigated by a computational approach. The drawn overall picture, starting from the very appearance of β amyloid peptides and going through their self-assembling into potentially toxic oligomeric species, reinforce some of the experimental and theoretical shots recently reported in literature, while new important molecular hints on the physiological role played by the β amyloid peptide are proposed. The so dreaded formation of amyloid pores selective for the passage of calcium ions could in fact explain their physiological concomitant recruitment in the regulation of synaptic plasticity.

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INTRODUCTION

Secretases cleave the amyloid precursor protein (APP) into three fragments. Sequential cleavage by β-secretase (BACE) and γ-secretase produces first C99 and then the β amyloid peptide (Aβ).

Aβ is the main component of amyloid "plaques" found in the brains of Alzheimer's disease (AD) patients, and despite it has a physiological function at low concentration (picomolar),[1,2] it becomes toxic if overexpressed.

If α-secretase acts on APP first instead of β-secretase, the α/γ cleavage sequence will end with the production of the non-pathogenic middle fragment P3[3], therefore, the control of the cleavage kinetics is of paramount relevance in determining the evolution of the Alzheimer's pathology.

For decades Aβ oligomers and higher concentration of cholesterol (Chol) in the blood have been tightly correlated to AD[4]. However, it is still controversial if Chol dyshomeostasis has a role on the onset of the pathology, by promoting its production and accumulation[5], or Chol dyshomeostasis is just a consequence of Aβ fibrillation[6].

It has been identified a Chol binding site at level of the C99 precursor that could promote the recognition of the cleavage site by γ secretase[7].

The literature suggests several mechanisms by which the interaction between C99 or APP and Chol could promote an increased production of potentially toxic Aβ fragments[7] and consequently amyloidogenesis and AD. For example, a) the C99/APP association with Chol could promote the partitioning of the protein into raft domains where β and γ-secretase are mainly distributed[8,9] conversely to α-secretase; b) Chol binding to C99 may catalyze the substrate recognition and cleavage by γ secretase; c) in the presence of high concentration of Chol the α-secretase activity decreases[5].

Several studies highlighted also a role of brain gangliosides in the pathogenesis of AD.

The possible involvement of monosialotetrahexosylganglioside (GM1) in AD has been first suggested by Yanagisawa et al. because of the finding of Aβ species tightly associated with GM1 in not yet mature plaques (GAβ). They suggested that this association, instead of secreted Aβ peptides, may act as a seed

for Aβ deposition[10].

Matsuzaki group showed that at low Aβ:GM1 ratio Aβ assumes an α-helix conformation, while at a high Aβ:GM1 ratio fibril formation ensues.[11] Aβ is proposed to bind GM1 in a cholesterol dependent manner at level of raft domains. Chol could inhibit or facilitate membrane-Aβ interaction through fine adjustment of glycosphingolipid conformation[12]. Conformational changes or aggregation states of Aβ peptides can be finely tuned trough the selective interaction with specific membrane components, promoting several possible fates (e.g. plaques, pore formation or membrane vesiculation).

Aim of this work is to follow the dynamic interplay between Chol and Aβ peptides starting from the very appearance of Aβ peptides and going through their self-assembling into potentially toxic oligomeric species[13].

Assuming that Aβ is produced thanks to the presence of Chol that promotes the recognition of the binding site by γ secretase in raft domains, we could also reasonably believe that in particular conditions (such as a specific distribution or high concentration of Chol) Aβ never leaves the membrane or, if secreted, the favorable binding to GM1, in turn stabilizing a helical peptide conformation, at low Aβ:GM1 ratio, could promote its consequent insertion into the lipid core of a target cell membrane.

This hypothesis has been put forward by some authors. For example, Liguori et al. have shown by a computational approach that the Aβ42 monomer having helical conformation can be energetically stabilized in a heterogeneous lipid membrane where Chol is asymmetrically distributed, while in absence of Chol peptides are mainly lying down at the lipid/water interface, and at the most symmetric 50%/50% exo/cyto Chol distribution, the N-terminus of the Aβ42 peptide prefers to move out of the membrane.[14]

Recently Rondelli et al. have experimentally shown that unstructured small sized oligomers, most probably monomers, can insert deeper into a supported raft-mimic membrane. [15]

Aβ peptides, as other amyloid peptides, also have strong tendency to interact and aggregate.[16] We have shown that Aβ peptides embedded into a pure POPC membrane give reason to the formation of linear aggregates having helix frustrated conformation.[17]

Moreover, several studies evidenced the tendency of amyloid peptides to form channel-like structures in membrane, permeable to calcium ions.[18]

Di Scala et al.[19] proposed that Chol stabilizes the boundaries of an annular channel composed by 8 Aβ fragments embedded into a lipid bilayer. These octameric channels have an outer diameter of 5.8 nm and pore diameter of 1.8 nm that well reproduce geometric features experimentally measured by atomic force microscopy[20,21].

In their study, however, they use a predetermined number of pre-assembled peptide-cholesterol complexes and they took in consideration only the hydrophobic portion of Aβ (22-35 residues) having higher affinity for the inner core of the bilayer, presuming that the remaining part has less importance since it will remain exposed to water.

If we admit that Aβ, at physiological conditions, should not stand inside a membrane where Chol is symmetrically distributed, or in absence of Chol, then we have to look at the potential physiological fate of Aβ when Chol is asymmetrically distributed. The fundamental question I tried to answer with this work is: *what could happen immediately after the physiological production of the Aβ peptide at membrane level?*

Taking into account all the previous experimental and computational observations, with this study the potential effect of Chol on the initial steps of Aβ1-42 peptides aggregation will be investigated using a combination of coarse grained molecular dynamics (to unveil the dynamics of protein aggregation in membrane) and atomistic simulations (to explore the conformation of assembled proteins). Experiments on membrane interactions of amyloid peptides are challenging and contradicting because of the occurrence of three main parallel processes: membrane binding, peptide aggregation and membrane deformation. A precise control of the experimental conditions is fundamental for the interpretation of the data and the conclusion drawn out of them. In this contest Molecular Dynamics provides the opportunity to study various aspects of membrane-proteins systems at detail not accessible when using experimental techniques.

Unlike the study performed by Di Scala et al., the complete sequence of Aβ42 fragments with helical configuration, which stability in membrane has been already investigated by Liguori et al.[14], will be considered. Also the initial binding of Aβ with Chol will not be imposed, so that Chol and peptides will freely diffuse into the surrounding lipid sea. Moreover, the spontaneous Chol-assisted self-assembling of Aβ peptides will be followed by varying the concentration of peptides, and the transversal heterogeneity of the membrane, by using no Chol, symmetrical and asymmetrical Chol distribution both in the liquid-ordered and in the liquid-disordered state of the membrane. Several investigations in the literature[22,23,24] highlighted the influence of membrane fluidity, in turn related to Chol content[25], on the interaction with Aβ peptides.

The reason of investigating the heterogeneous distribution of Chol is that in biological membranes of young cells Chol is asymmetrically distributed in the membrane, while with aging, the distribution of Chol becomes more symmetrical. So far, changes in Chol asymmetry may contribute to AD.[26]

At this stage, the proposed interaction of GM1 headgroups with Aβ peptides, at the water-membrane interface, should not play any role on the spontaneous self-assembly of peptides already embedded into the membrane.

At last, the reciprocal effect of oligomers on the lipid distribution profiles for that concerns membrane aggregation, poration and membrane's local bending, will be finally explored.

METHODS

A. Coarse grained setup

The following systems were simulated:

a) An Aβ monomer embedded into a bilayer made up of palmitoyloleoylglycero-phosphocholine (POPC) lipids either in presence of:

   i) Chol (50% of the total membrane volume) asymmetrically (30%/70% exo/cyto) distributed. In this case, the total number of POPC lipids has been accordingly

adjusted in order to have the same number of beads in the upper and lower membrane leaflets.

ii) Chol (50% of the total membrane volume) symmetrically (50%/50%) distributed.

iii) In absence of Chol.

All systems were assembled with the insane.py script for lipids as bilayers with a box size of 9 nm in the x and y directions and 12 nm in the z direction.[27]

The choice of such high concentration of Cholesterol, one of the typical features associated with the appearance of AD, is intentional, in order to provoke a response of the system under potentially stressful conditions.

The structure of the Aβ42 peptide in an apolar environment (mimicking the lipid environment) was obtained from the Protein Data Bank (entry 1IYT[28]). The mostly helical structure (with 70% of the sequence in helical configuration) was converted to a coarse-grained representation.

Peptides were then inserted inside the membrane perpendicularly to the membrane surface using the Gromacs tool g_membed. Position restraints are applied to the peptide during the first 10 ns of equilibration in the NVT ensemble, followed by an equilibration simulation run of 30 μs.

In order to investigate mesoscopic phenomena (long range effects) such as peptide-peptide aggregation and membrane deformation, all the previous equilibrated three systems (*i,ii,iii*) have been replicated (lipids/peptide ratio remains constant) along the x,y axes, to have:

b) 8, 9, 16 or 64 Aβ replica embedded into a POPC membrane with again:

i) Chol (50% of the total membrane volume) asymmetrically (30%/70% exo/cyto) distributed.

ii) Chol (50% of the total membrane volume) symmetrically (50%/50%) distributed. As anticipated at point a *ii)* upper and lower membrane leaflets have same final number of beads.

iii) In absence of Chol.

iv) As control, Chol (50% of the total membrane volume) asymmetrically (30%/70% exo/cyto) distributed but with no peptides embedded into the membrane.

All the systems were simulated for 15 μs at two different temperatures, 280 and 330K, in order to reproduce a liquid-ordered and a liquid-disordered phase, respectively.

Since Aβ peptides have a strong tendency to stress the membrane curvature in concomitance to their self-assembling[17], the following simulations have been performed by applying position restrains to lipids along the normal to the membrane for 45 μs at 330K:

c) 64 Aβ replica embedded into a POPC membrane with again

i) Chol (50% of the total membrane volume) asymmetrically (30%/70% exo/cyto) distributed.

ii) Chol (50% of the total membrane volume) symmetrically (50%/50%) distributed.

iii) In absence of Chol.

For each system, the bilayer was inserted in a water box (water layer 5 nm thick, over and down the bilayer along z) in the presence of 0.1 M sodium chloride and sodium as counter-ions. The whole system (membranes, co-ions, and counter-ions) was electroneutral.

Simulations were performed by version 4.6 of the GROMACS simulation package[29] and all Coarse Grained (CG) interactions were described by the Martini force field,[30] which uses a mapping of on average four heavy atoms to one CG interaction site. The force field has been primarily parameterized to match thermodynamic quantities such as the free energy of hydration, solvation, and partitioning between polar and apolar solvents. In this work, we used the literature parameters for water and sodium/chloride ions[30], POPC lipids[30], and cholesterol[31].

For the full topologies, including bonded terms, see http://cgmartini.nl, where topology files in Gromacs format are readily available.

Energy minimization was performed using the method of steepest descents. Equilibration was performed by using the Berendsen weak coupling thermostat and barostat algorithms[32] with coupling constants of 1.0 ps and 3.0 ps, respectively. After equilibration, the temperature was kept constant at 300 K using the v-rescale algorithm[33] with a time constant of 1.0 ps. The Parrinello-Rahman algorithm[34] was applied for semi-isotropic pressure coupling (1 bar). The equation of motion were integrated using a leap-frog algorithm and a timestep of 20 fs. Following standard protocols associated with the Martini force field, the LJ and Coulomb potentials were smoothly shifted to zero between 0 and 1.2 and between 0.9 and 1.2 nm, respectively, using the Gromacs shifting function. The neighbor list was updated every 10 steps with a cutoff of 1.4 nm. Electrostatic interactions were screened implicitly ($\varepsilon = 15$). The center of mass motion was removed every step. Periodic boundary conditions were applied in all directions.

Tab 1. Abbreviations

Liquid ordered (prefix Lo-) / Or Liquid disordered (prefix Ld-)	Chol N° of Aβ peptides	Chol Asymmetrically distributed (AC)	Chol Symmetrically distributed (SC)	No Chol (NC)	Lipid position restraints (suffix *-res)*
	0	AC0			
	1	AC1	SC1	NC1	
	8	AC8	SC8	NC8	
	16	AC16	SC16	NC16	
	64	AC64	SC64	NC64	

B. Backmapping and Atomistic setup

The CG approach provides information about peptide and membrane dynamics, but not about the conformational preferences of membrane-embedded Aβ peptides, since secondary structure elements are constrained. To address this issue, atomistic Molecular Dynamics (MD) simulations of membrane embedded Aβ assemblies were performed. The starting configurations were obtained by extracting a portion of the bilayer containing the corresponding octameric structure, maintaining the same protein/lipid ratio, from the CG simulation and reverted to an atomistic representation[35]. GROMOS 53A6 force field was used in all atomistic MD[36]. Moreover, the SPC (simple point charge) water model[37] was used, the time step was set to 2 fs and the temperature was kept constant at 280K using the Nose-Hoover algorithm[38,39] with a time constant of 0.1 ps. Periodic boundary conditions were applied. The Parrinello-Rahman algorithm[34] was applied for semi-isotropic pressure coupling (1 bar). The particle-mesh Ewald (PME) algorithm[40] was used for electrostatics.

RESULTS AND DISCUSSION

Aβ monomers appear stable inside the membrane either in presence of Chol asymmetrically, symmetrically distributed or in absence of Chol, over 30 μs of CGMD simulation (Fig S1).

The reason of choosing to start with the Aβ monomer completely embedded into the membrane is that, the peptide, physiologically produced at membrane level in helical configuration can be stabilized and forced to go deeper into the lipid hydrophobic core thanks to the interaction with the asymmetrically distributed cholesterol.[14] If Aβ peptides are locally concentrated, due to an overproduction induced by a dyshomeostatis of cholesterol, they will probably start to aggregate since their strong propensity to interact.[17]

1. Phenomena at long spatial range

In any of the systems containing more than 1 replicas, peptides show a strong propensity to self-assemble.

As already predicted and then confirmed by MD simulations, Aβ assemblies into a pure POPC bilayer tend to adopt a linear shape with a peculiar "frustrated helix" arrangement (deformed helix with the polar moiety forced toward water).[17] In presence of Chol asymmetrically distributed, the frustrated helical arrangement persists, but instead to grow linearly it rolls up into a spiral (Fig 1) partially surrounded by tilted Chol molecules (Fig S2).

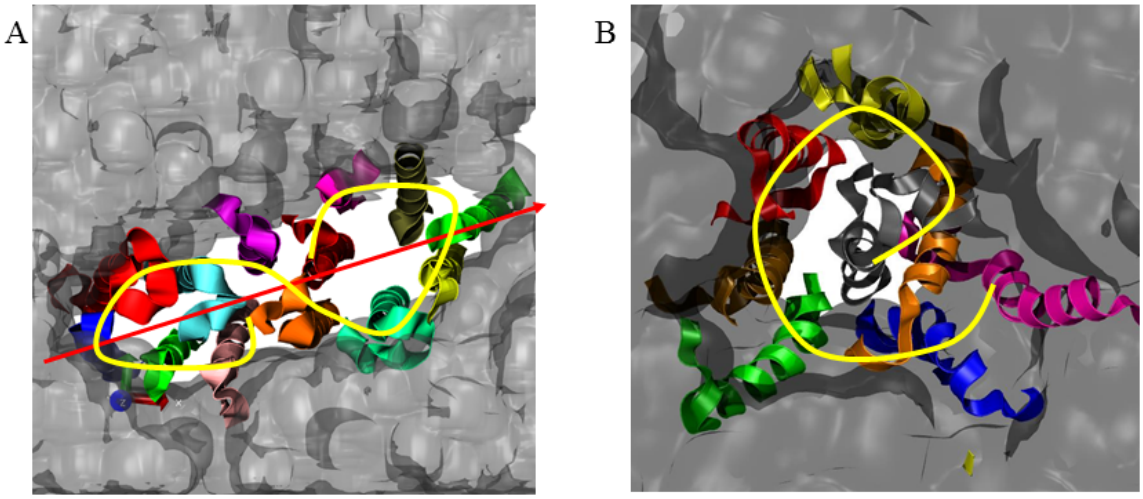

Fig 1. Top View of the assembly composed by Aβ peptides differently colored, embedded into a POPC membrane (gray transparent) in absence of Chol (panel A) or in presence of Chol (Chol not explicitly represented, panel B). White spaces appear in place of peptide lateral chains not explicitly represented.

As consequence, Aβ peptides in presence of variable amount of Chol always aggregate in membrane forming clusters composed by several of small *circular* building blocks, which size is limited to~7-8 peptides each and that keep their own round shape over all the simulation time despite inter-blocks contacts (see Fig 2). These round-shaped species remind the annular channels stabilized by Chol at the boundaries previously modelled by Di Scala et al.[19]

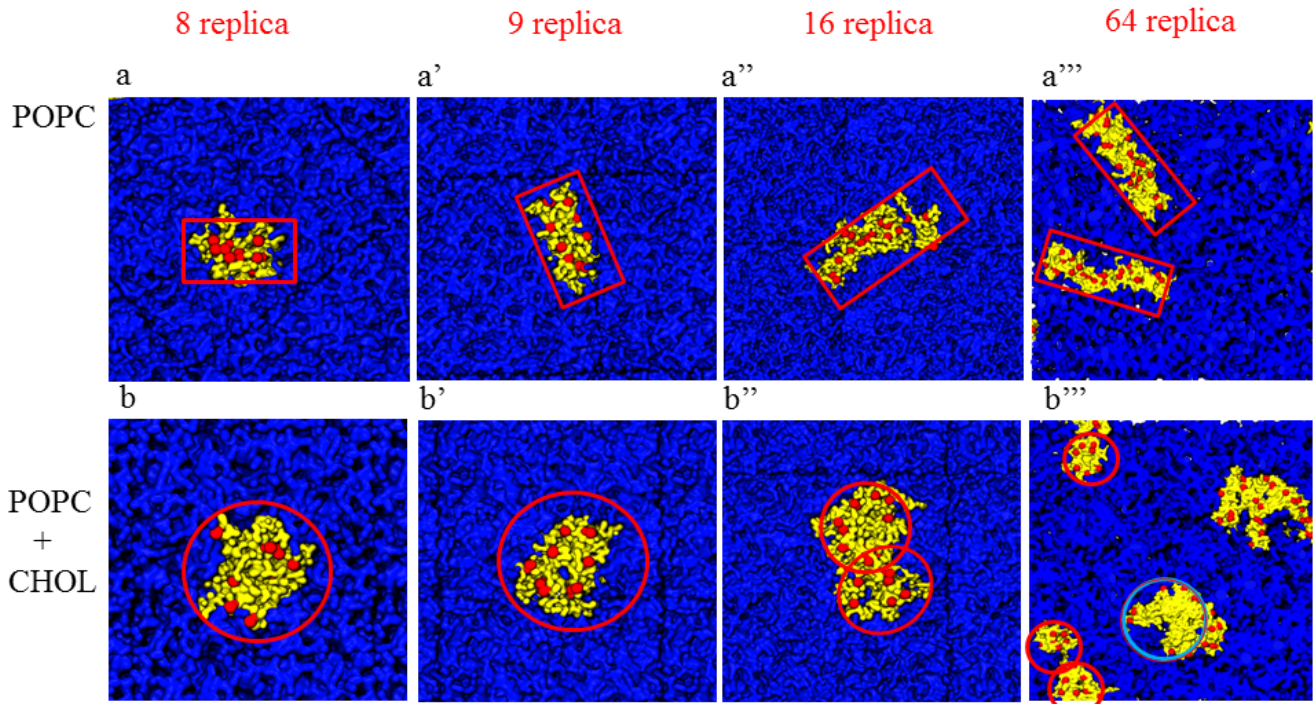

Fig 2. Self-assembly of amyloid peptides at different concentration, 8, 9, 16 and 64 peptides (in yellow and aspartic residues in red) from the left to the right respectively, after 15μs of CGMD simulation. In the upper panel peptides are embedded into a pure POPC membrane (blue), while in the bottom panel peptides are embedded into a POPC membrane where Chol is asymmetrically distributed (Chol not explicitly represented).

Liquid Ordered and Liquid Disordered States

In the absence of Chol, lipid bilayers can exist in one of two physical states, the gel (Lβ) state at lower temperatures and the liquid-crystalline (Lα) state at higher temperatures. In the Lβ state, the phospholipid hydrocarbon chains are in the fully extended all-trans conformation. The ordered chains can be packed more tightly together and a relatively small reduction in the volume is accompanied by huge lateral area condensation. Consequently, the more ordered straight chains determine an increase in membrane thickness. In the Lα state the entropy-favored gauche conformation of the hydrocarbon tails sharply increases in respect to the number of ordered trans-conformations.

In absence of Chol, lipid bilayer exists predominantly in the Lα phase, fundamental for normal membrane functions to occur.

The addition of increasing amounts of Chol progressively disrupts the lateral order of the gel phase (so), tends to order the liquid phase (Ld), and at a higher Chol content, stabilizes a new phase, the liquid-ordered phase (Lo), which has properties generally intermediate between those of solid-like (Lβ) and fluid-like (Lα) phospholipid bilayers. This Lo phase exhibits indeed both rapid transverse diffusion and translational disorder of the liquid disordered phase (Ld) and relatively orders lipid chains characteristic of the solid ordered phase (so).

Hence the 'liquid-crystalline ordered' phase is denoted as Lo, while the 'liquid- crystalline disordered' (Lα) phase is denoted as Ld.[41]

1.1 *Liquid disordered state (Ld)*

In a pure POPC membrane, or in presence of Chol symmetrically distributed, the peptide cluster size grows steadily over the time. In contrast, when Chol is asymmetrically distributed into the membrane, the cluster size reaches a plateau of about 7/8 peptides and then the membrane suddenly bends (Fig 3). What happens immediately afterwards (i.e. peptide rearrangements or further peptide aggregation) is not intentionally discussed and beyond the aim of this work, because all the following events result as an effect of the inhibited detaching of the vesicle due to the limited size of the system.

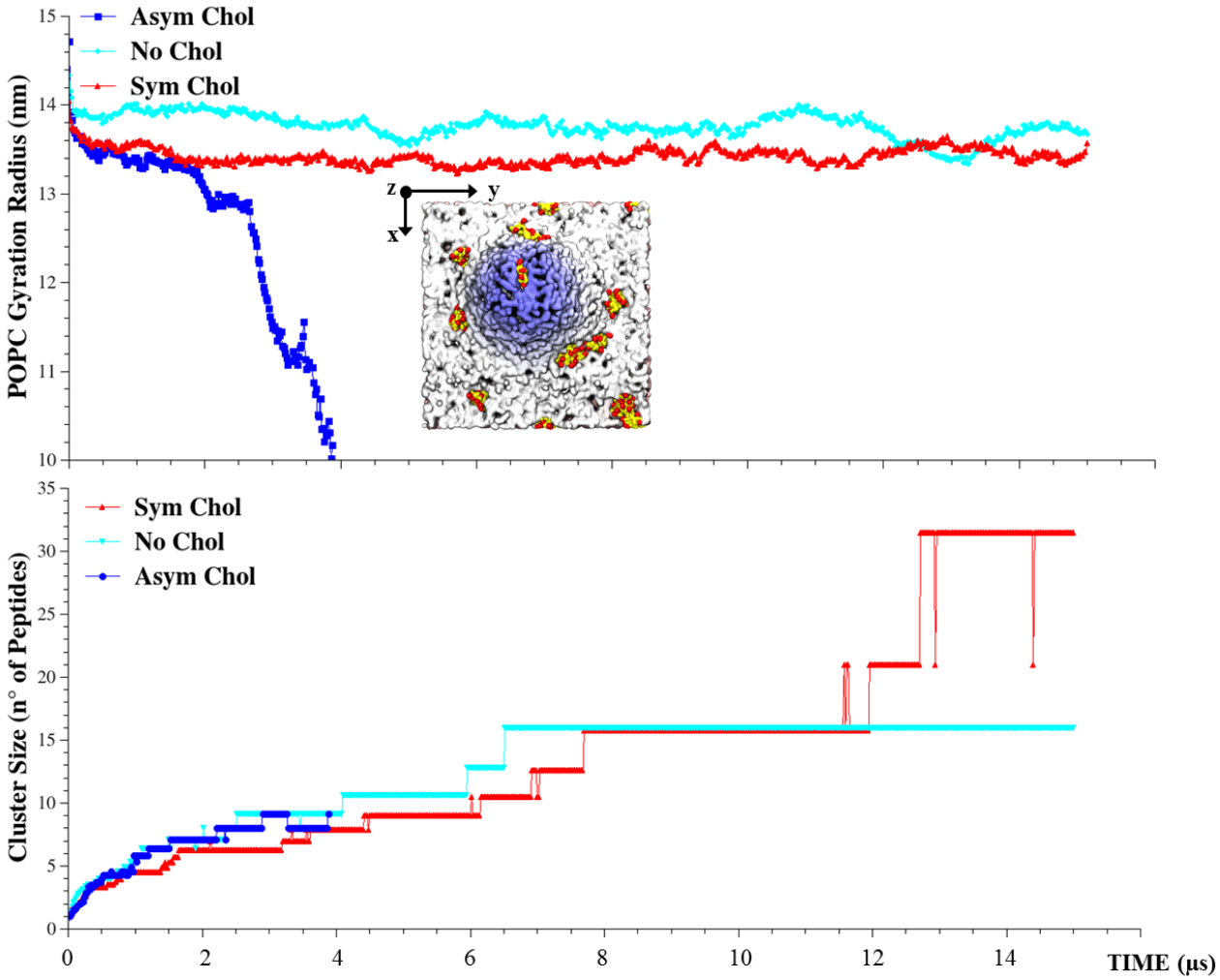

Fig 3. Top panel: Gyration radius of the membrane (POPC lipids) over 15 μs of simulation at 330K (membrane in the liquid-disordered state); Inset: Top view of a membrane (in white) with Chol asymmetrically distributed (not explicitly represented), which curvature is represented with a gradation of violet along z. Peptides are represented in yellow and Aspartic residues as red beads.

Bottom panel: Average size of clusters made up of Aβ peptides self-assembling over 15 μs at 330K.

Blue profiles refer to the system Ld-AC64, red profiles to the system Ld-SC64 and cyan profiles to the system Ld-NC64.

Lipid restraints

As said before, Aβ peptides have a strong tendency to stress the membrane curvature/vesiculation in concomitance to their self-assembling. In order to get a clear estimation of the average cluster size according the membrane composition, simulations have been performed also with position restraints

applied to lipids along the normal to the membrane. Positions restraints allow to inhibit eventual membrane deformation along *z* axis.

Interestingly, in comparison to free restraints systems described in the 1.1 and 1.2 sections, when position restraints are applied to lipids, the aggregation kinetics sensitively decreases, despite restraints are not applied along the plane xy, so that lipids and peptides can laterally freely diffuse. At the end of 45 μs, clusters reach average sizes of 7-9 peptides each, slightly smaller if Chol is asymmetrically distributed (Fig 4).

Note that in living systems, membranes are attached to the cytoskeleton, therefore, their mobility will result partially constricted. There is a multitude of functions the cytoskeleton can perform. Primarily, it gives the cell shape and mechanical resistance to deformation.[42] Plasma membrane-cytoskeleton interactions are disrupted in many human diseases.

Emerging evidence suggests that mutations contributing to AD are associated with dysfunction of cytoskeletal components that influence vesicular biogenesis, vesicle/organelle trafficking and synaptic signaling. Neuropathology of AD is defined by accumulation of another form of insoluble neurofibrillary tangles (NFT). NFT are fibrillar structures comprising largely tau, a microtubule binding protein that stabilizes the microtubule tracts necessary for vesicular trafficking, endo- and exo-cytosis and axonal polarity. Microtubules are a component of the cytoskeleton, found throughout the cytoplasm. Hyperphosphorylated forms of tau have lower binding affinities to microtubules and possibly destabilize them.[43] Interactions or modifications of microtubules may facilitate fibril formation, since proteins are more susceptible to aggregate when the membrane softness increases, as it emerges from this study (Fig 3). Moreover, aggregation could drive the concomitant deformation of the softer membrane as observed in section 1.1.

This aspect deserves future investigations.

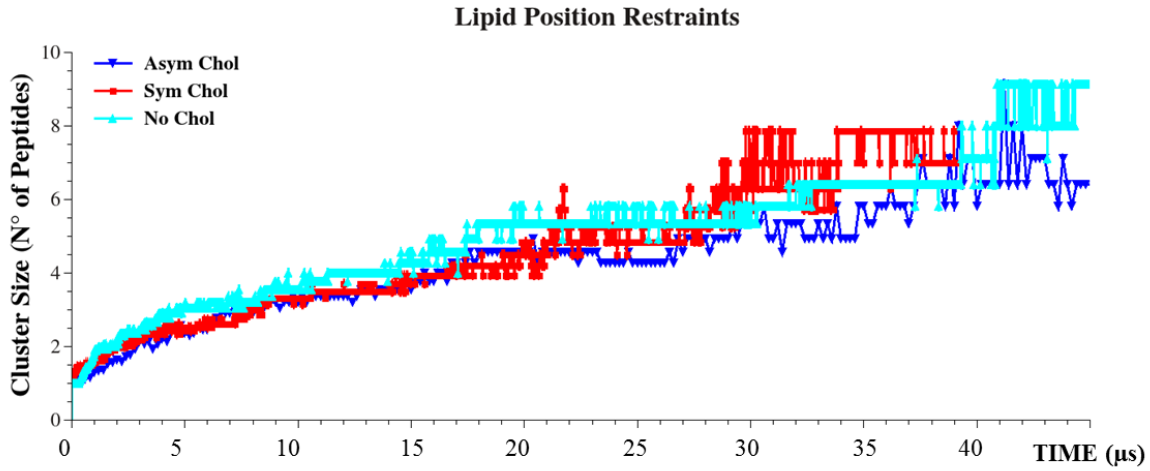

Fig 4. Cluster formation over the time of self-assembling Aβ peptides in the case of lipids **restrained** along the normal to the membrane. Blue profile refer to the system AC64, red profile to the system SC64 and cyan profile to the system NC64.

In general, Chol may favor the formation of round shaped clusters over linear assemblies, while in flat membrane the typical cluster size is 7-8 peptides. From the other side, the presence of Chol asymmetrically distributed (which is known to accumulate in high curvature regions[44]) amplifies membrane deformation, allowing consequent vesiculation (Fig 3). [45] Morita et al. have evidenced the capability of Aβ-42 oligomers to induce Lo domains budding from giant vesicles.[46]

The tendency of the membrane to vesiculate in presence of Aβ peptides reminds the experimentally observed vesicular uptake of extracellular Aβ by murine cortical neurons and neuroblastoma (SHSY5Y) cells.[47]

1.2 *Liquid ordered (Lo)*

In case of an asymmetrical distribution of Chol, in concomitance to the peptide aggregation (system Lo-AC64), the initial nod of the membrane bending leaves place now to irreversible displacement of peptides toward the water-membrane-interface (see Fig 5, bottom panel).

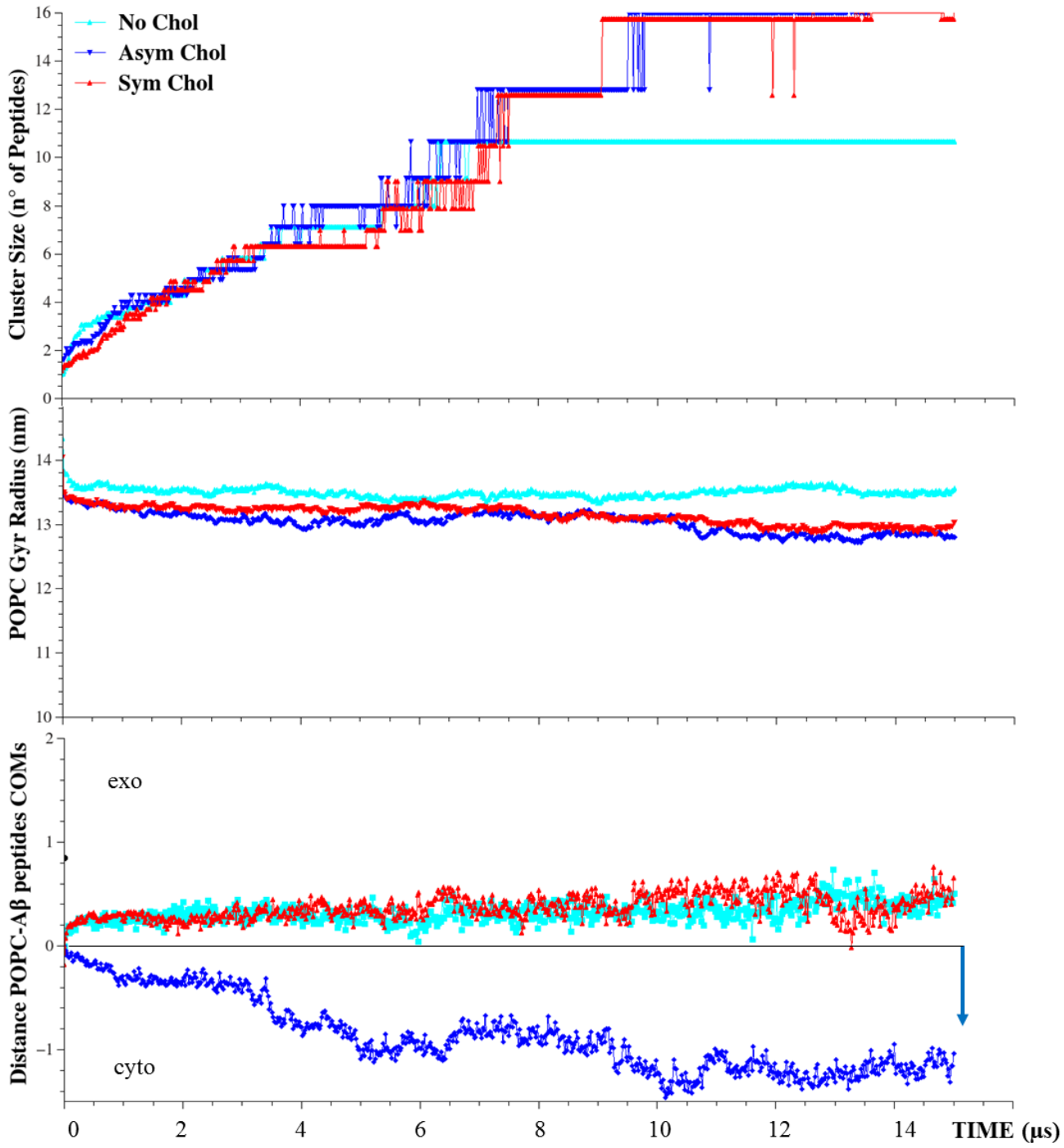

Fig 5. Top panel: Average size of clusters made up of Aβ peptides self-assembling over 15 μs at 280K. Middle panel: Gyration radius of the membrane (POPC lipids) over 15 μs at 280K (membrane in the liquid-ordered state); Bottom panel: Distance's profile along z between the POPC's and Peptide's Centers of Mass (COMs).
Blue profiles refer to the system Lo-AC64, red profiles to the system Lo-SC64 and cyan profiles to the system Lo-NC64.

This result suggests that stable clusters containing 8 or more peptides cannot survive inside a membrane. While several small clusters of 7-8 peptides can contemporaneously diffuse undisturbed inside a membrane, larger clusters probably induce an intolerable stress at membrane level. As a consequence, the membrane bends (low bending rigidity) or some of the clusters move out of the membrane (high bending rigidity).

2. Phenomena at short spatial range

2.1 *Liquid ordered state (Lo)*

Interestingly, by moving out at the water-lipid interface, peptides drag along the closest phospholipids headgroups (red beads, Fig 6) leaving space to a hydrophilic hole permeable to water and ions.

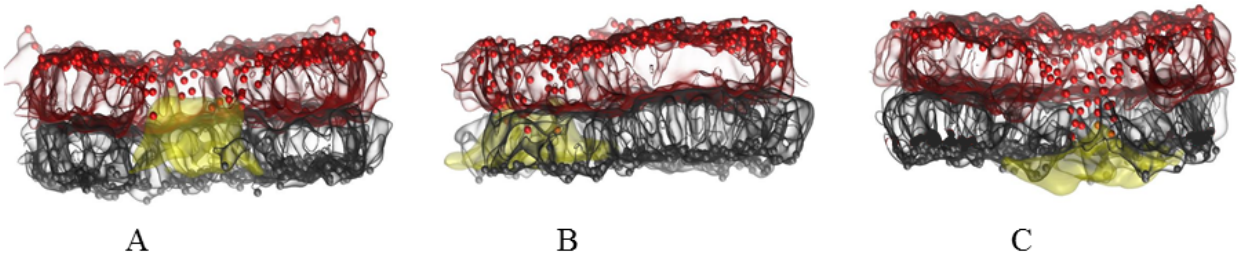

Fig 6. From the left to the right, snapshots at 0.2, 3, 5 μs respectively, of an octameric oligomer on the way out from a POPC membrane in presence of Chol asymmetrically distributed. The top (exo) and bottom (cyto) membrane leaflets have been represented in transparent red and gray, respectively. Lipid headgroups belonging to the exo layer are represented as red beads. Aβ peptides are represented in transparent yellow.

2.2 *Liquid disordered state (Ld)*

At short space scale (10 nm), if Chol is asymmetrically distributed, only in the presence of peptides the local membrane curvature increases (Fig 7). Curvature has important biological implications such as in

the budding of secretory vesicles or endocytosis, mechanisms adopted by cells to transport cargo from a donor compartment to the acceptor compartment.[48]

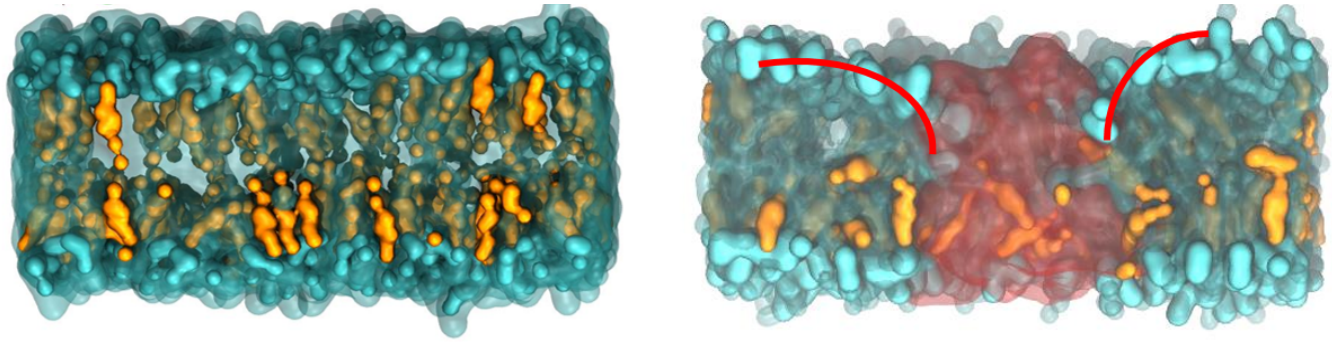

Fig 7. On the left, phospholipid bilayer (transparent cyan) with an asymmetrical distribution of Chol (in orange) in absence of proteins (AC-NP system); on the right, bilayer with an asymmetrical distribution of Chol (orange) in presence of proteins (transparent red) (AC8 system). Red lines mark the curved profile of the inner layer.

Atomistic simulations

It's experimentally known that amyloid peptides permeabilize membranes[49] and form pore-like structures[50].

Since the circularly shaped aggregates described before (Section 1), formed in the presence of asymmetrically or symmetrically distributed Chol, remind a typical pore or channel conformation, the evolution of the octameric assembly (SC8 and AC8 systems) has been further investigated for additional 200 ns for symmetrically and asymmetrically Chol distribution at atomistic level (AA), after backmapping[35] of the system from a coarse-grained to an atomistic representation.

The circular shape of octamers is preserved and the membrane permeability is increased in both cases (more when Chol is asymmetrically distributed), where a polar channel spanning the membrane allows free passage of water and cations (because of the negative net charge of the peptides) (Fig 8). Experiments have shown that, if overexpressed, Aβ peptides promote the contemporary increasing of the intracellular calcium load, that leads in turn to synaptic transmission failure.[51,52]

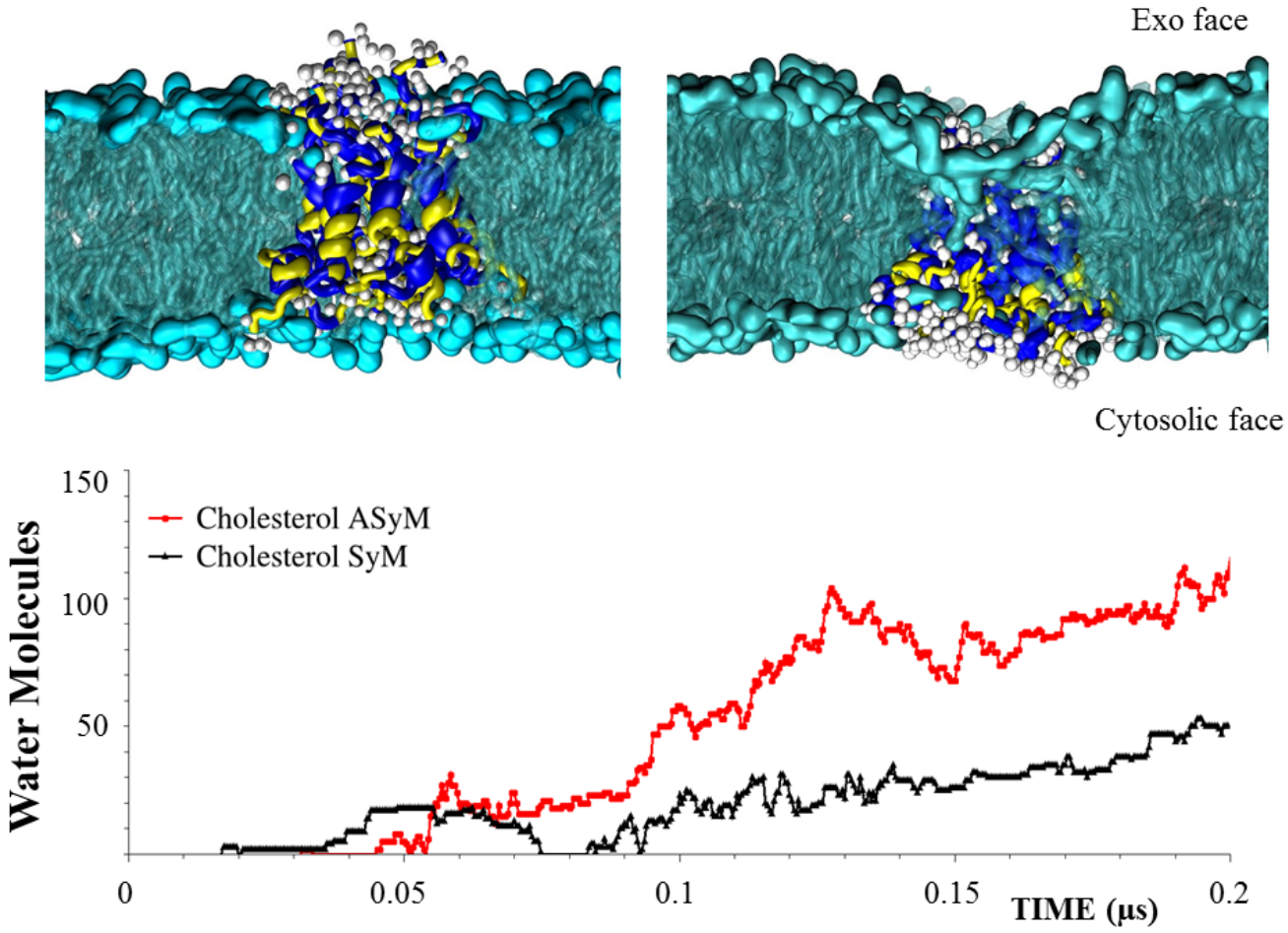

Fig 8. Top panel: On the left, water molecules (in white) passing through an oligomer (in yellow hydrophobic residues and in blue charged residues) embedded into a membrane (cyan) where Chol is symmetrically distributed (not represented). On the right side, water molecules (in white) passing through an oligomer (in yellow hydrophobic residues and in blue charged residues) embedded into a membrane (cyan) where Chol is asymmetrically distributed, with the higher concentration at level of the cytosolic layer (not represented).
Bottom panel: Water flux through the aggregates embedded into an asymmetric (red profile) or symmetric bilayer (black profile) calculated by the Gromacs tool g_flux[53] .

While in the case of a symmetrical distribution of Chol the system is stable inside the membrane over 200ns of simulation at atomistic level, uneven distribution of Chol makes the oligomer less stable, so the simulation has been prolonged up to 800ns.

During this long simulation time, as consequence of peptide's tilting, its charged residues (blue residues in Fig 8), mainly distributed at the N-terminal facing the exo layer, are forced toward the inner core of the membrane, dragging along the closest phospholipids headgroups belonging to the exo layer; the final effect is the enhancement of the local membrane curvature near the pore (Fig 7).

Due to computational limitations such as the positive external pressure[54], periodic boundaries conditions (PBC) and the limited size of the box[55], further membrane deformations or peptides or Chol rearrangements (such as flip flop events) could be hidden in the present study. It is worth of notice that, in presence of an asymmetric distribution of Chol, the hydrophobic C-terminal exposed at the cytosolic interface is unfolding and hairpins begin to form, while the small remaining portion inserted into the membrane keeps the helical configuration (Fig. 9 and Fig S3).

It is reasonable believe that the hydrophobic c-term could fold back again giving reason to the formation of a hairpin-like hook trait reminding the arrangement already hypothesized by Ma and Nussinov[56]. These authors have advanced several hypotheses on the possible arrangements of 17-42 Aβ fragments in solution, without considering the remaining part of the peptide.

From the present study an alternative point of view emerges. From one side this study confirms the Nussinov models for what concerns the hairpin-like hook structure of the fragment 17-42, but, on the other hand, a more complete picture is presented since the important role of the remaining part of the peptide is considered. The N-part of the peptides containing the polar residues keeps a helical configuration (Fig S3). These polar residues reside in the inner region of the pore allowing to maintain a hydrophilic interface useful for the passage of water and cations. The remaining hydrophobic part of the peptide folds back into the membrane as a hairpin that surrounds the polar core while interfacing nearby lipids. An important contribution to the stabilization of the lipid-membrane structure could be the interaction established between a polar residue at level of the hydrophobic loop and the lipid polar headgroups at level of the exo layer (Fig 9, right panel) which are forced inside the membrane by the peptide tilting.

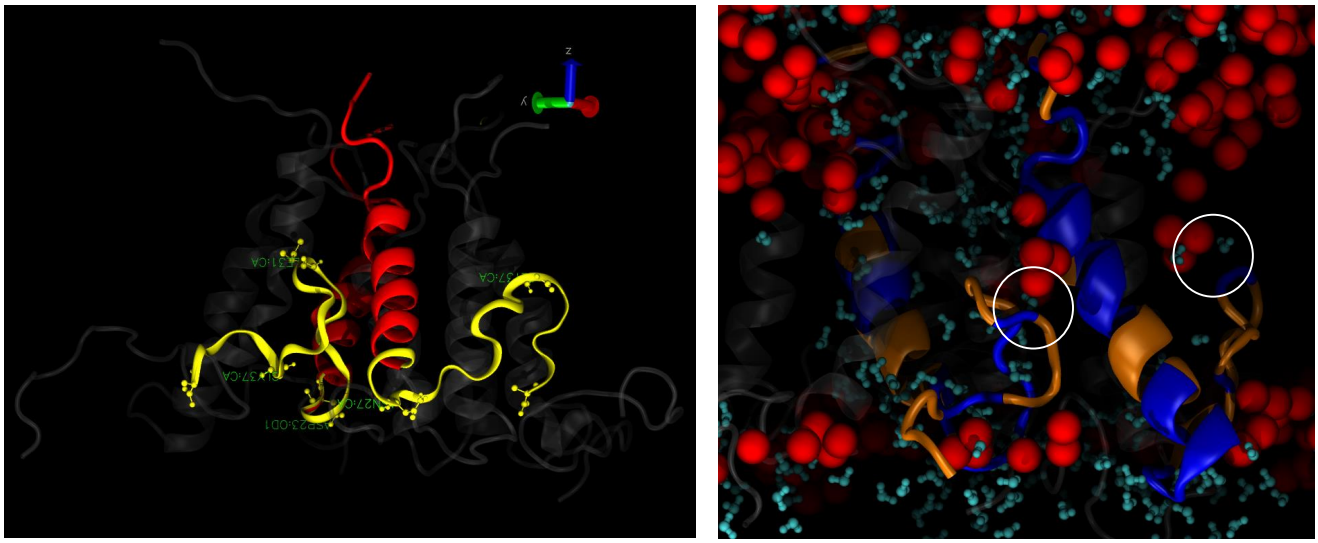

Fig 9. On the left, oligomer in transparent gray with two peptides differently colored to evidence in red the helical part residing into the inner core of the pore, and in yellow the remaining part interfacing the surrounding lipid matrix (not represented).

On the right, same system as previous with a different representation to evidence the hydrophilic headgroups (red) interacting with the polar residue at the level of the peptide loop, defining a polar continuity along the membrane.

At short spatial resolution, in the case of a symmetrical distribution of Chol, the helical peptides completely span the membrane in the usual symmetric fashion and the lipid-protein assembly appears to be stable over the whole simulation (Fig 8, left panel).

Unfortunately, the times' scale for sampling the conformational space are not approachable by a standard atomistic approach, despite in this study already a good compromise has been reached. A separate study is worth to be carried out to unveil potential evolution of the assembly.

CONCLUSIONS

From the present study several new important evidences are emerging:

1) Membrane composition affects the aggregate's shape: in presence of Chol, the aggregate's shape deviates from the linear arrangement observed and theorized in a pure POPC bilayer, preferring a circular shape.

2) In presence of Chol, small-sized assemblies (up to 7-8 replica) are the most represented building blocks, independently of protein concentration.

3) When the membrane is in the liquid-disordered state and Chol is asymmetrically distributed, the increased cluster size of Aβ aggregates (over the standard average 7-8 replica) induces membrane vesiculation. On the contrary, in the liquid-ordered state, membrane bending is aborted probably due to the increased rigidity[57], and oligomers, in turn, move toward the membrane-water interface.

4) Octamers embedded into a membrane allow flux of ions and water, at higher extent if Chol is asymmetrically arranged. If Chol is symmetrically distributed, peptides completely span the membrane, while in the case of an asymmetric Chol distribution, half pore is transversally composed by peptides hydrophilic residues and the other half by lipid headgroups folding inside (Fig 9). In any case, a few tilted cholesterol molecules appear distributed at the oligomer boundaries and are not actively involved in the flux through the pore. The last evidence has been previously proposed by Di Scala et al.[19]

The stability of small clusters in membrane has been investigated in previous studies where, however, a) only the short hydrophobic trait of the Aβ peptide has been considered, b) the oligomeric structure has been pre-assembled (in most of the cases with predominant beta-sheet conformation) or c) the peptide binding with Chol has been imposed. In this study there is no compulsion applied over the peptides' self-assembly and the complete sequence of the Aβ peptide (1-42) is taken into account. For the first time, it has been evidenced a straightforward stabilization by cholesterol of circular assemblies over linear assemblies. It emerges also that the peptide hydrophilic part, disregarded by many previous

studies[19,56], can not only be fundamental for the pore functionality and its formation but also in promoting membrane deformation, by forcing lipid headgroups of one of the two leaflets to fold inside. Moreover, it clearly emerges from this study that the stability of small oligomers into the membrane is sensitively correlated to the presence and arrangement of Chol, through a variation of the membrane softness.[58] Aβ self-assembling has been dynamically investigated by taking into account the subtle interplay with the concomitant induced membrane deformation, which can influence, in turn, either the stability and the final structure of channel-like assemblies.

Aβ could activate endocytic processes[59] by simply facilitating membrane vesiculation, which may promote Aβ internalization and its uptake by cells.[47] This suggests also a potential physiological involvement of Aβ at level of synaptic membranes. Several studies have evidenced a tight connection between Aβ, calcium ions and synaptic plasticity pointing out only at side effects of this correlation. Considering the central role of calcium as second messenger in neurological functions, it is hard to believe that its interaction with Aβ peptides occurs only accidentally. Below a critical concentration, Aβ peptides could assemble into small-sized clusters (less than 8 peptides, reminding pore structures) that bind selectively calcium ions, and, through concomitant induced vesiculation, drive their controlled internalization and storage inside the cell.

Many amyloid peptides were shown to form ions-channels in vitro. All of these channels spontaneously insert into membranes, are Ca^{2+} permeable and sensitive to the blockade by Zn^{2+}.

It is hypothesized that amyloid channels could be responsible of toxicity by promoting membrane depolarization or Ca^{2+} influx. However, the predominant evidence supporting the "channel hypothesis" is limited to in vitro systems, and comes from studies that have used exogenous amyloid peptides. These channels are proposed to have beta-sheet conformation, supported by the evidence that their formation can be prevented by Congo red.[60]

In this study, instead, it is assumed that Aβ peptides, with predominant *helical* configuration, embedded into the membrane at the *monomeric* state, form channels to absolve at their physiological function. A recent experimental study of Rondelli et al. evidenced how structured exogenous Aβ oligomers can only

insert into the outer layer of a raft-mime supported bilayer while early unstructured small sized oligomers, most probably monomers, can insert deeper into the membrane and form channel like structures.[15] Poration-like events observed in vitro, as suggested by Soklow et al., could be provoked by the *structured* Aβ oligomers absorption at the outer membrane leaflet, that inevitably reduces the normal dielectric barrier in lipid bilayers. [61]

Aβ monomers embedded into the membrane can form channels which may be either structurally and functionally unrelated to the "abnormal" ions channels proposed by the "channel hypothesis".

A dyshomeostasis of Chol could promote an increased production of Aβ peptides at toxic levels.

The stability of Aβ oligomers into the membrane could increase with aging and a proper membrane vesiculation can be affected, influencing in turn the intracellular trafficking. AD individuals' endosomes appear to be much larger (up to 32-fold) than the normal average.[62]

While vesiculation is impaired, Aβ assemblies of increasing size continue to accumulate, affecting in turn membrane stability. At this stage, part of the membrane stress could still be released through the expulsion of Aβ peptides. Once out of the membrane, Aβ peptides could now interact with GM1 headgroups because of their strong affinity (mechanism compatible with the finding of GAβ species in not yet mature plaques[10]) and the elongation phase can start. Cataldo et al. have shown that, in AD, endocytic pathway abnormalities precede extracellular deposition of Aβ.[62]

At advanced stages of Aβ deposition and fibril formation, membrane eventually breaks.[63]

In any case, the balance of calcium ions in or out the cell will result sensitively compromised.

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