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Aβ-AMYLOID FIBRILS ARE SELF-TRIGGERED BY THE INTERFACIAL LIPID ENVIRONMENT AND LOW PEPTIDE CONTENT

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ABSTRACT

We studied the surface properties of Aβ(1-40) amyloid peptide mixed with POPC (liquid state) or DSPC (solid state) phospholipids by using nano-structured lipid/peptide films (Langmuir monolayers). Pure Aβ(1-40) amyloid peptide forms insoluble monolayers without forming fibril-like structures. In a lipid environment (phospholipid/Aβ(1-40) peptide mixtures), we observed that both miscibility and stability of the films depend on peptide content. At low Aβ(1-40) amyloid peptide proportion (from 2.5 % to 10 % of peptide area proportion) we observed the formation of fibril-like structure when mixed only with POPC lipid. The stability acquired by these mixed films is within 20-35 mN.m^{-1} compatible with the equivalent surface pressure postulated for natural biomembranes. Fibrils are clearly evidenced directly from the monolayers by using Brewster Angle Microscopy. The so-called nano-structured fibrils are Thioflavin T positive when observed with fluorescence microscopy. The amyloid fibril network at the surface was also evidenced by Atomic Force Microscopy when the films are transferred onto mica support. Aβ(1-40) amyloid mixed with the solid DSPC lipid showed an immiscible behavior in all peptide proportions without fibrils formation. We postulated that the amyloid fibrillogenesis at the membrane can be dynamically nano-self-triggered at the surface by the quality of the interfacial environment, i.e. the physical state of the water-lipid interface and the relative content of amyloid protein present at the interface.

Abbreviations: Aβ(1-40), βeta amyloid 1-40 residue peptide; AD, Alzheimer Disease; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; DSPC, 1,2-disteraoyl-phosphatidylcholine; PAP, percentage of **P**eptide **A**rea **P**roportion in the lipid:peptide mixed surface; ThT, Thioflavin T.

INTRODUCTION

Alzheimer Disease (AD) is the most common neurodegenerative disease in older individuals. Although the major risk is the age, the pathogenesis and degenerative processes are not well known and it can begin undetectable a decade before the symptoms become noticeable.[1] However, in postmortem tissue samples, AD is characterized by two neuropathological lesions found in the brain, neuritic plaques accompanied of an extracellular insoluble core of amyloid peptides (Aβ peptides) and intracellular filamentous aggregates of phosphorylate tau proteins denominated neurofibrillary tangles.[1-2]

It is known that the Aβ peptides (of 1-40 and 1-42 residues length) are generated from the proteolytic (mis)processing of the Amyloid Precursor Protein (APP) by two different modes: the **non-amyloidogenic** and the **amyloidogenic** pathways.[1, 3] In the **non-amyloidogenic pathway**, APP is sequentially cleaved by α- and γ-secretases and endocytosed resulting an endosomal production of non-amyloidogenic P3 peptide. The **amyloidogenic pathway** is characterized by the (over)production of insoluble and neurotoxic Aβ fragments (1-40 and 1-42) from the cleavage of APP by β- and γ-secretase, resulting in the formation of Aβ oligomers which are the precursors of the aggregate plaques.[4] This vision is known as the ***consensus beta amyloid hypothesis.***[1]

The proteolytic process of APP to generate Aβ peptides is necessary associated to the membrane environment, however the implication of the membrane lipids in the early events of pathological aggregates have been neglected or rather, it has been given a more passive role. In this connection, membrane raft (or its more accurately definition as transient lipid nanodomains)[5] has been implicated in the Aβ accumulation and AD.[6-8]

POPC is a pivotal lipid in natural membranes due to its importance in the structure and function of cellular membranes.[7] POPC is the most abundant phospholipid in brain compared to the rest of phospholipids.[9] It has been reported that phospholipids level in brain decreases with age by almost 50 % between age 40 to 100 years old.[10] This apparent "physiological" change with age has induced to some authors to suggest that the decrease of phospholipid levels in brain could be related to AD,[7] specifically POPC.[11-12] Furthermore, from studies in CSF (cerebrospinal fluid), which is the most informative sample

for monitoring the pathological processes in brain, has been suggested that an increase in POPC hydrolysis could be associate with AD.[13]

APP is a transmembrane protein, so the proper interaction with membrane phospholipids or a non–leakage processing of Aβ peptides would contribute to the normal homeostasis in brain. According to Aβ amyloid hypothesis, the non-physiological accumulation of Aβ peptides would trigger early oligomerization and neuronal injury and further dissemination of AD.[1] So, taking into account that phospholipids provide an optimal environment to the membrane proteins function and contribute to the normal neuronal ionic homeostasis, many authors have focused their studies to the interaction of Aβ peptides and derivatives with lipids using membrane model systems. Particularly, it has been reported the electrostatic interaction of 25-35 segment of Aβ peptide with negatively charged phospholipid vesicles[14] and the complete Aβ(1-40) sequence with anionic lipid monolayers.[15]

Langmuir monolayers at the air-water interface is a proper technique to study the interfacial behavior of film forming peptides mixed with lipids. The potential of this approach to study the surface properties of Aβ amyloid peptides has been highlighted by Leblanc and coworkers.[16] Langmuir monolayers allow to manipulate the lipid/peptide mole ratio, the amount of the percentage of peptide area proportion, the physical state of the lipid and the degree of lateral compactness in a confined environment, mimicking a biological membrane interface. Aβ peptide (either 1-40 or 1-42) is part of the anchoring membrane domain of APP and is very hydrophobic. We have previously described that Aβ(1-42) forms insoluble monolayers at air-water interface with a lateral stability of around 30 mN.m^{-1} and a molecular area at maximal packing of around 2.5 nm^2/molecule.[17] In this previous report, using giant unilamellar vesicles (GUVs), we described the strong interaction of Aβ(1-42) with GUVs provoking both *i)* the reduction of lateral membrane cohesive forces and *ii)* the breaking of transbilayer gradient causing a time-dependent release of small and large solutes loaded in the liposomes.[17]

More recently, we reported that pure Aβ peptide, under the condition that had been proved to form stable and structured β-sheet monomolecular layers at air/water interface, presenting a peculiar high elastic shear modulus when compared to pure α-helix lytic melittin peptide.[18] A similar behavior was recently

reported for the adsorption of the intrinsically disordered C-terminal of the Hendra virus nucleoprotein (N_{TAIL}) to air/water interface.[19]

In the present work we show that Aβ(1-40) amyloid peptide forms ***a fibril like structure at a particular range of lipid/Aβ peptide proportion*** when the mixed surface is dominated by POPC. Certainly, between 2.5 and 10 % of Aβ amyloid **P**eptide **A**rea **Proportion** (PAP) the fibril-like structure can be evidenced by Thioflavin T (ThT) fluorescence, Brewster Angle Microscopy (BAM) and Atomic Force Microscopy (AFM). The surface peptide area contribution relative to lipid, PAP, is important when the film forming molecules have very dissimilar individual molecular areas.[20] Percentage of peptide area proportion can be calculated from the individual data according to: PAP = $100((n_P A_P/(n_L A_L + n_P A_P))$, where n_P and n_L are the moles of peptide and lipid in the mixture and A_P and A_L are the individual molecular areas of peptide and lipid, respectively. In addition, we also demonstrate that the fibrillogenesis effect induced by POPC at air-water interface is sensitive to physical state of phospholipid since the mixing behavior of Aβ peptide with DSPC, a lipid in solid state at room temperature (liquid condensed state in Langmuir monolayers words), behaves lateral immiscibility with no fibrils formation. The interfacial conditions in which the more natural POPC/Aβ amyloid peptide mixture induce fibril like structures strongly depends on lipid/peptide proportion and it was observable only at low Aβ amyloid proportion.

RESULTS

Surface properties of Aβ(1-40) in POPC or DSPC mixed films. π-Area isotherms and BAM analysis.

The formation of a insoluble monolayer of many amphiphilic molecules can occur at air-water interface.[21] By using a Langmuir trough, the compression of the insoluble monolayer is possible by imposing a movable barrier to generate surface pressure (π)-Area isotherms. Surface pressure defined as $π = γ_o - γ$ is the difference between the pure interfacial tension of water ($γ_o$) and the surface tension of amphiphilic covered surface (γ) depends on lateral packing, surface concentration and miscibility behavior of the components.[21-24] A modern view of the origin of the π value has been related to the changes of surface water activity at the interfacial region.[25]

According to our previous results, both Aβ(1-42) or the two residues shorter Aβ(1-40) have almost

identical behavior in monolayers at air water interface.[17-18] Pure Aβ peptides form insoluble monolayers at

air-water interface with a maximal lateral stability of around 30 mN.m^{-1} and a limiting molecular area at

maximal packing of around 2.5 nm^2/molecule (Fig. 1). The maximal stability of pure Aβ peptide

monolayers is in between the stability acquired by the lytic amphiphilic melittin peptide (~19 mN.m^{-1}) or

pure phospholipids in liquid-expanded state (~40 mN.m^{-1}).[26] Pure Aβ(1-40) peptide films behave as a

typical solid films with a very peculiar high rheological property.[18] The BAM imaging of pure Aβ(1-40)

film is homogeneous with neither observable fibril like structure (Fig 1), as we have observed previously,[18]

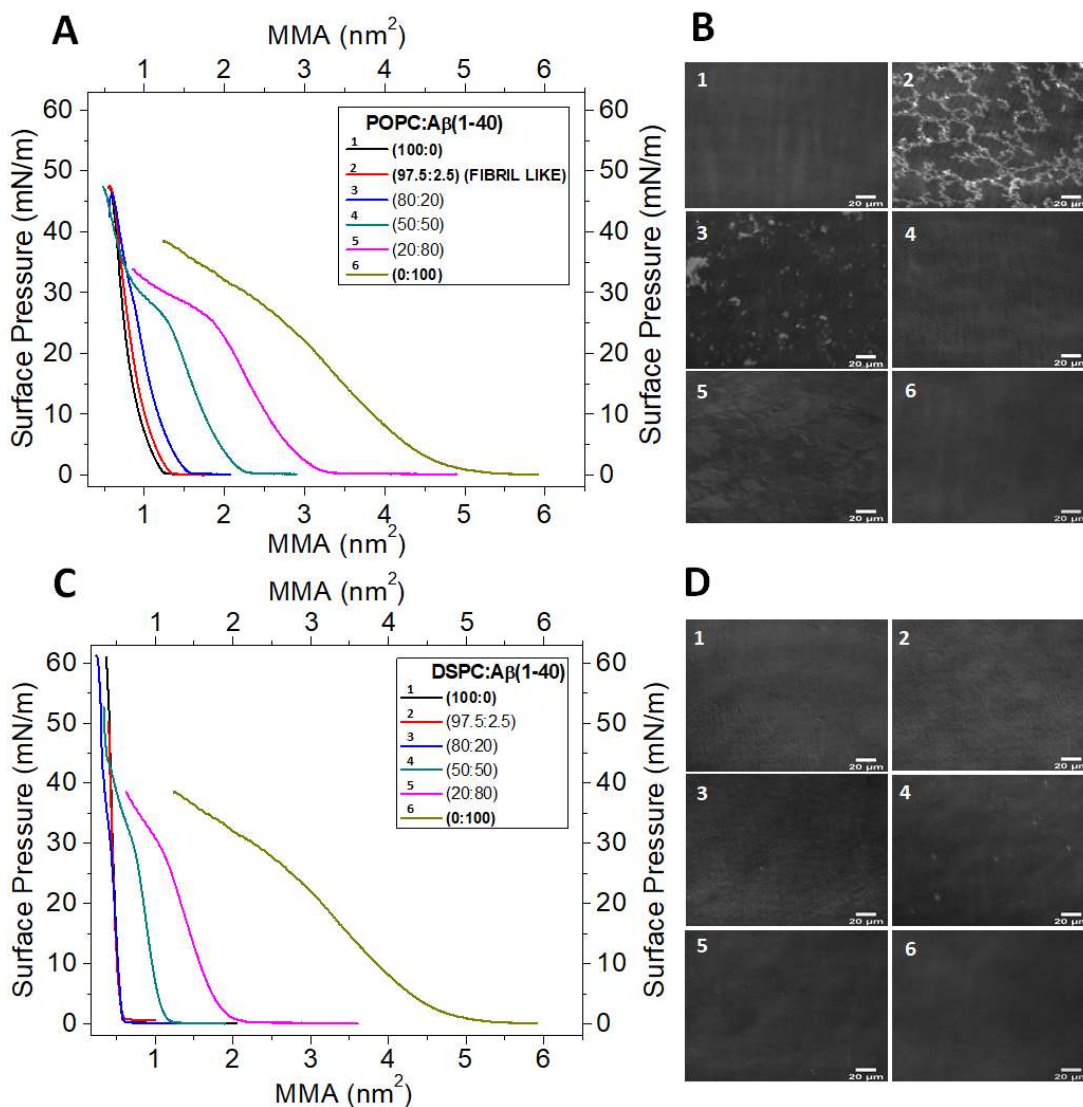nor sensitivity to ThT fluorescent probe (see below and Fig. 4).

Figure 1: Surface behaviour of Aβ(1-40) peptide in mixed films. π-Area isotherms for **(A)** POPC-peptide or **(C)** DSPC-peptide mixtures and their respective BAM images **(B)** and **(D)** obtained at 10 mN.m^{-1} in pure films or the mixture at the indicate lipid-amyloid percentage of peptide area proportion, PAP, (showed in the insert). The corresponding lipid:peptide mole fraction proportion for POPC:Aβ(1-40) peptide for each isotherm was 1:0; 0.995:0.005; 0.952:0.047; 0.835:0.164; 0.558:0.441; 0:1. The corresponding lipid:peptide mole fraction proportion for DSPC:Aβ(1-40) peptide for each isotherm was 1:0; 0.997:0.003; 0.971:0.029; 0.892:0.108; 0.674:0.326; 0:1. At the POPC:Aβ(1-40) mixture at 97.5:2.5 of PAP, the mixed film shows fibril-like structures (picture B-2) but the picture B-3 at 80:20 of PAP shows domains indicative of a different immiscibility behavior. Conversely, DSPC:Aβ(1-40) mixtures at the same PAP proportions showed a different behavior (solid-solid immiscibility at all proportions, see the text). MMA: mean molecular areas of the mixed film were calculated as indicated in Experimental Section. The solid bar in the pictures represents 20 μm. A greater detail of the profile of π-A isotherms at low Aβ(1-40) peptide content can be seen in Fig. S1.

We studied the surface mixing behavior of Aβ(1-40) peptide with two phospholipids differing in their physical state at room temperature (23 °C). POPC has a completed liquid-expanded isotherm (Fig. 1A and Fig. S1). For the contrary, DSPC behaves as completely liquid-condensed isotherm at air/NaCl 145 mM subphase (Fig. 1C). An expanded figure on the x-axis to appreciate the isotherms in greater detail can be seen in Fig. S1 of Supplemental Information. To observe the influence of Aβ peptide proportions in the mixture, we have used the following percent of Peptide Area Proportion (PAP): 97.5:2.5, 80:20, 50:50 and 20:80 of calculated as indicated above. To each value of PAP it corresponds a relative high mole fraction of POPC of 0.995; 0.952; 0.835; 0.558, respectively (see Fig 1), due to the dissimilar molecular areas of the phospholipid compared with Aβ(1-40) peptide. This is an important concept to take into account when mixing protein with lipids in Langmuir monolayers since they have great differences in the individual molecular areas; so a small mole fraction of any protein would correspond with a significant high total surface area occupied and, therefore, influencing the surface behavior of the mixture.[20, 26] Aβ(1-40) peptide mixed with the more solid DSPC phospholipid did not form fibril-like structure regardless of amyloid proportion. All the DSPC/amyloid mixtures behave as a solid-solid immiscibility with a first collapsing point similar to the value found for pure peptide (Fig, 1C, Fig 2 upper part and Fig. S1). The BAM pattern is compatible with solid phases without the formation of fibril-like structures. Fig 1D shows the π-A isotherms with the corresponding BAM images taken at 10 mN.m^{-1}. At this lateral packing (10 mN.m^{-1}) the molecular area of Aβ(1-40) peptide of 3.92 nm^2.molec^{-1} (see Fig. 1) is higher than POPC (0.94 nm^2) or DSPC (0.52 nm^2. molec^{-1}) thus, the mixed isotherms move towards greater molecular area values as the mole fraction of the amyloid peptide increases.

The miscibility behavior with POPC is completely different. Mixed films of POPC:amyloid at 97.5:2.5 of PAP form an observable fibril-like structure (see Fig 1B, picture 2). Is important to note that at this proportion of PAP corresponds to a mole fraction of amyloid of 0.005. We found this particular behavior with POPC at around this proportion. At this proportion the fibril-like structures are ThT positive (see and Fig 4). If the Aβ(1-40) peptide proportion is increased more than 10 % of PAP, corresponding to a peptide mole fraction of 0.021, the fibril-like structures disappear and the ThT analysis becomes negative.

In mixed interfaces, the profile and behavior of π-Area isotherms allow us to experimentally build the phase diagram,[27] see Fig. 2. For DSPC:Aβ peptide mixtures we found a typical solid-solid immiscibility. For this mixtures, the first collapsing point (first discontinuity in the π-Area isotherm, see Fig S2) is compatible with the protein phase and appears independent of the Aβ(1-40) proportion, indicating solid-solid immiscibility[21] at all peptide proportions (see Fig. 2 upper part).

For POPC:Aβ peptide mixtures the miscibility behavior is more complex. At low Aβ peptide mixtures (less than 10 % of PAP or less than 0.03 of peptide mole fraction), the surface miscibility analysis suggests that the mixed POPC:Aβ(1-40) films are miscible where the first collapsing point has an intermediate value in between the pure POPC or pure Aβ(1-40) peptide, with the concomitant formation of fibril-like structures observable by AFM (Fig. 1) and becomes ThT positive (Fig. 4). At greater Aβ peptide proportion the miscibility behavior is almost ideal (Fig. 2 lower part).

The complexity of POPC:Aβ peptide mixed behaviour can be appreciated from a more perusal BAM analysis. In other to ascertain the complexity of BAM analysis, we have determined the variation of BAM reflectivity upon film compression for POPC:Aβ(1-40) mixtures. From the analysis, we were able to discriminate into three well defined patterns of reflectivity (see Fig. 3A highlighted with different colours). The high reflectivity values corresponds to pure Aβ(1-40) peptide (pure peptide phase); low reflectivity values are similar to those found for pure POPC lipid (pure lipid phase) and, the third ones with intermediate reflectivity values attributable to a more protein enriched mixed phase including the fibril phase formation. POPC:Aβ(1-40) at PAP proportion of 97.5:2.5, 80:20 and 20:80, the mixed films

domains have intermediate reflectivity values (Fig. 3A). At both extremes, pure POPC or pure Aβ(1-40)

peptide films we have the limits of low and high reflectivity values, respectively. At 97.5:2.5 of PAP

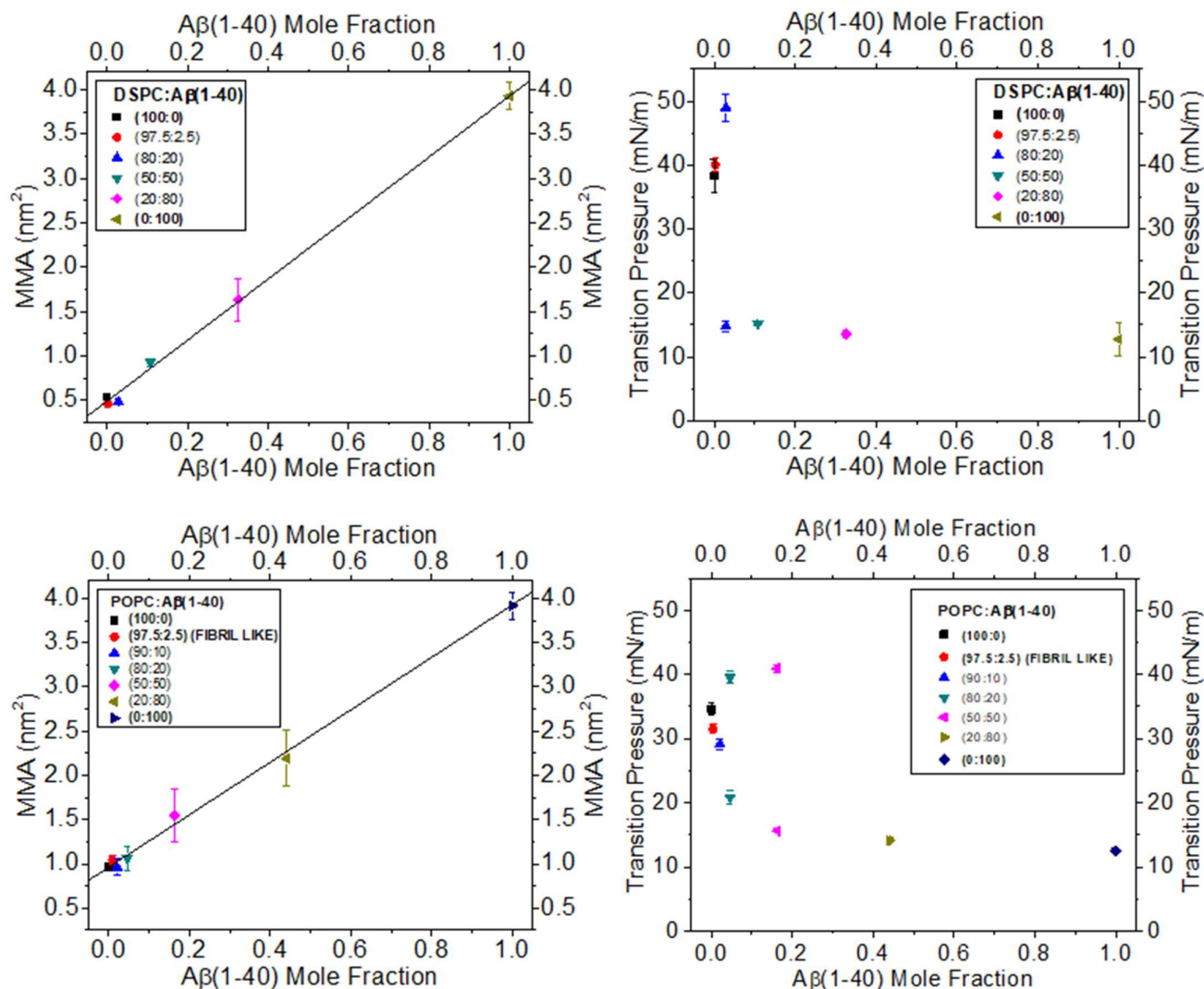(corresponding to a Aβ peptide mole fraction of 0.005) we found the typical fibril-like phase (Fig. 3B).

Figure 2: Phospholipid:Aβ(1-40) peptide miscibility phase diagram. Upper part: Phase diagram for DSPC:Aβ(1-40) mixtures showing lateral immiscibility; **left:** mean molecular area vs mole fraction taken at 10 mN.m^{-1}; **right:** first collapsing point in the π-Area isotherm. **Lower part:** Phase diagram for POPC:Aβ(1-40) mixtures. **left:** mean molecular area vs. mole fraction taken at 10 mN.m^{-1}; **right:** first collapsing point in the π-Area isotherm. The POPC:Aβ(1-40) mixture has a complex miscibility behavior. Here we included an additional mixture (90:10) for a more complete description of mixing analysis. At POPC:Aβ(1-40) mixed at (80:20) and (50:50) of PAP proportions behave lateral immiscibility. At lower Aβ peptide proportion, the mixture behaves partial miscibility with the first collapsing point corresponds to protein enriched phase at a value above of that observed for pure amyloid (see Fig. 1 and Fig S1for more details). Transition Pressure means the first discontinuity point calculated by second derivative of π-Area isotherm with respect to area. At intermediate peptide proportions we also observed a second transition pressure (collapsing point) related to the lipid-enriched phase (see Experimental section and Fig. S2). The straight black line in the left upper and lower part corresponds to the ideal molecular area (see Experimental section for more details).
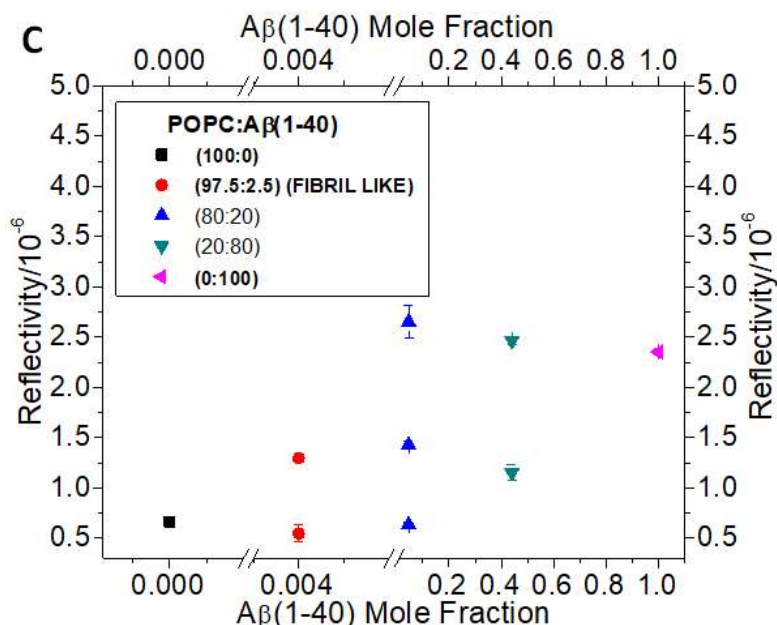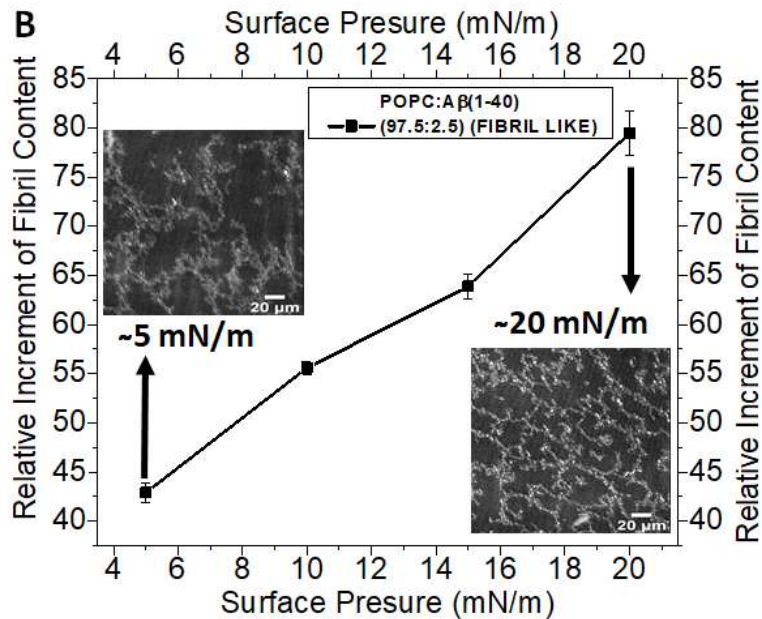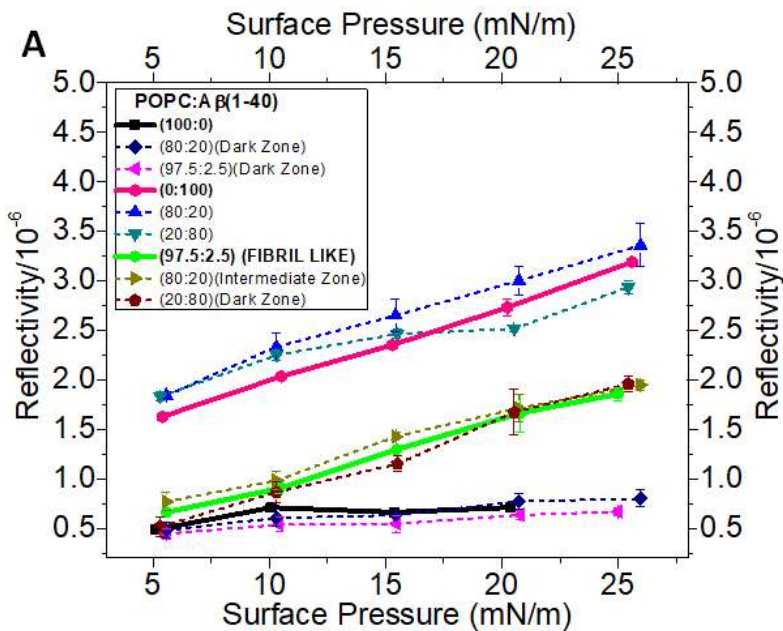
Figure 3: BAM reflectivity analysis of mixed POPC:Aβ(1-40) films. A: reflectivity values indicating the three distinguishable (and measurable) regions in BAM intensity from POPC:Aβ(1-40) mixtures indicated with three different colors. Low values are close to pure POPC lipid (black); high reflectivity range values were similar to pure Aβ(1-40) peptide (magenta) and intermediate reflectivity values correspond to fibril-like structures or protein enriched phases (light green). Insert describes the origin of the data indicating the PAP and the zone where the plotted values of reflectivity were taken. **B:** Relativity percentage increment of fibril-like formation as surface pressure increases at POPC:Aβ(1-40) at 97.5:2.5 of PAP proportion; left insert: picture of fibril-like structures taken at low surface pressure, right insert: picture of fibril-like structures taken at high surface pressure. The percentage of relative fibril content in B was calculated for each photograph in digital form between an all-black state (0 %) and all bright in the gray scale (100 %) by using Fiji Image J software. **C:** BAM reflectivity values of pure POPC, pure Aβ(1-40) and at different POPC:Aβ(1-40) proportions taken at the distinguishable domains at 15 mN.m^{-1}.

At this particular proportion, fibril structure growths as the surface pressure increases until almost duplicate the relative domains of fibril-like structures (see Fig. 3B). As Aβ peptide proportion increases up to a PAP greater than 20 % (above 0.1 of amyloid mole fraction), the mixed films appear with intermediate reflectivity values but no fibril-like structures were detected and, we also observed pure protein domains with high reflectivity. At above 50 % of PAP (0.047 of amyloid mole fraction), the mixed surface behaves as pure protein (Fig. 3C).

Thioflavin T and AFM analysis of mixed POPC:Aβ(1-40) films. It is well known the ThT probe has particular fluorescent properties when interact with fibril-like amyloids in solution [28-29] or at surfaces by using total internal reflection fluorescence microscopy (TIRFM).[30] We have adapted a method to transfer the mixed monolayer to mica support followed of an incubation with a drop of ThT for 10 minutes and the direct observation by FM (see Experimental section). We have routinely used this methodology to check if the fibril-like structures observed by BAM of POPC:Aβ(1-40) films at 97.5:2.5 of PAP are ThT positive. Transferred monolayers, in this proportion, show a fluorescence intensity pattern resembling fibril-like structure (Fig. 4B). We have checked that transferred monolayers of either pure Aβ(1-40) or POPC were ThT negative, (Fig. 4B). We have quantified the fluorescence intensity of ThT positive pattern of fibril-like structure induce by POPC in comparison with the using negative controls (pure lipid and pure peptide, see Fig 4A). We have used fibrils of α-synuclein formed in bulk condition as positive control to check the ThT interaction in a similar condition to that we used in transferred films (see Fig. 4B).
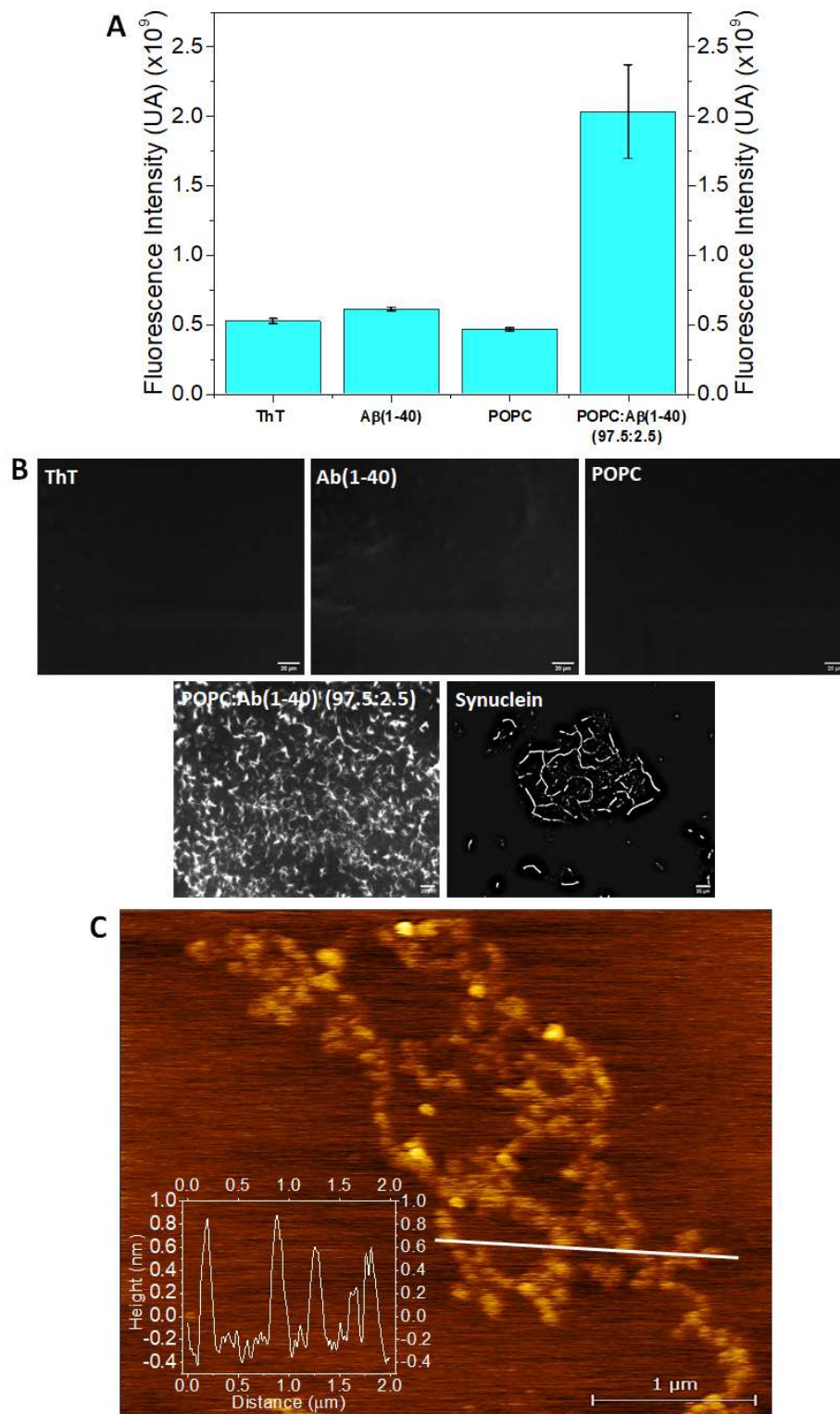
Figure 4: Thioflavin T confirmation and AFM observation of amyloid Aβ(1-40) fibrils induce by POPC at air-water interface. A: Fluorescence intensity values of transferred films from Langmuir trough (compressed to 20 mN.m^{-1}) to solid support after ThT incubation. ThT controls: transferred pure Aβ(1-40) peptide film, transferred pure POPC film; it is shown the average intensity of all fluorescence signal without imaging processing. **B:** Fluorescence images of negative controls and the fibril-like structure of POPC:Aβ(1-40) mixed at 97.5:2.5 of PAP compressed up to 20 mN.m^{-1} and then transferred to mica support. Positive control of α-synuclein formed in bulk condition and transferred to solid support and incubated with ThT for FM observation. In the lower right corner, the white bar represents 20 μm. **C:** AFM topography imaging from mixed POPC:Aβ(1-40) at 97.5:2.5 of PAP proportion, compressed up to 20 mN.m^{-1} then transferred to mica support; in the insert it is shown the periodicity of the fibril-like structure along of 2 μm length (see big white bar). It is worth to note that the image of AFM is 4x4 μm whereas the BAM images is 200x250 μm. (see Experimental Section for more details).

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DISCUSSION

Any hypothesis about the intimate molecular mechanism of amyloid formation in AD should be based on physiological conditions. Co-solvent effect, higher temperature or long-period of shaken, or combinations of some of them, are no normal constrains to trigger amyloid formation in brain. Most of authors converged that the (mis)processing and accumulation of Aβ peptides is initiated at the membrane environment.[1] Recently it has been highlighted the strong interrelationship between lipids and amyloid formation by either the membrane as promoter of amyloid aggregation or self-assembled forming co-aggregates with lipids that may disturb cell homeostasis.[31] Both Aβ peptides, (1-40) and (1-42) are strongly hydrophobic and they correspond to the transmembrane segment of the APP protein.[1] Both Aβ peptides are able to form insoluble and stable monomolecular layers at air/water interface as membrane lipids do, with a relative high lateral stability.[17,18] Previously, we also demonstrated that Aβ peptide is able to puncture bilayer membranes when the amyloid peptide is externally added to giant liposomes (GUVs).[17] However, in this previous report we advanced that the lipid/Aβ peptides miscibility was complex with a great tendency of lateral immiscibility behaviour.[17] It is recognized that Aβ amyloid peptides form β-sheet structure at the air/water interface.[32-34] Depending on the experimental system used, Jiang and co-workers suggested that the structuring in β-sheet conformation may obey a certain pathway with distinguishable intermediates either at air/water interface: unordered monomer$_{(bulk)}$→ α-helix$_{(sublayer)}$→ β-sheet$_{(interface)}$ [35] or in bulk conditions: amorphous monomer$_{(bulk)}$ → colloid-like aggregates$_{(bulk)}$ → ordered β-sheet oligomers$_{(bulk)}$ → fibrils$_{(bulk)}$.[36]

The Aβ peptide/lipid miscibility in Langmuir monolayers did not follow the rule similar to others very hydrophobic β-sheet peptides. Indeed, very hydrophobic signal sequences peptides mix quite well with lipids regardless of lipid phase.[37] Another characteristic of pure Aβ amyloid peptide monolayers is its high rheological shear stress at the surface behaving as a two-dimensional solid network with a very low interfacial diffusion coefficient ($D \sim 0.005\ \mu m^2.s^{-1}$) similar or lower than liquid-condensed lipids.[18] In the present report, we confirm our previous study that *pure* Aβ amyloid peptide monolayers in this solid network did not form fibril-like structures[18] nor they were sensitive to ThT probe (Fig 4). The presence of

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non-solvated liquid-condensed DSPC (a double saturated acyl chain phospholipid that at monolayers working temperature (23 °C) is below its critical transition temperature (54.9 °C)[38] do not promote fibrillation keeping the solid network of Aβ amyloid peptide and shows a solid-solid immiscible behaviour at all DSPC/Aβ peptide proportion (Fig. 1). POPC is the main phospholipid constituent of membrane lipids in brain[7, 9] with a fully liquid-expanded character at air/water interface[39] and a critical transition temperature of -3.7 °C.[40] The introduction of POPC, a more physiological fluid lipid partner at the interface, acts as an inductor of fibril-like structures (see Fig. 1B and Fig. 3B). This particular and well characterized behavior *is only observed* at 2.5-10 % of peptide area proportion (PAP), a small range of Aβ peptide proportion equivalent to 0.005-0.01 of Aβ(1-40) mole fraction in the mixture (Fig. 1B). The extent of fibrils formation was surface pressure dependent reaching an important and well defined non-covalent network at 20-30 mN.m^{-1} (Fig. 3B), a lateral pressure equivalent to bilayer and compatible with membranes. [41] It is important to note that, in our experimental conditions, the fibril-like structures formed as a consequence of the interfacial conditions of peptide proportion, type of lipid and packing are ThT positive, (see Fig. 4B). It is widely accepted the particular reactivity of the fluorescent probe ThT with fibrils is one of the requirements that an aggregate must meet to be considered amyloid.[29] In our experimental condition, the ThT positive amyloid fibrillogenesis only requires 2-5 minutes of spreading over a physiological saline subphase (NaCl 145 mM at 23 °C), mixed with a normal membrane phospholipid constituent and compressed to an equivalent membrane surface pressure. So, it was not necessary any extreme condition nor artificial co-solvent, high temperature, strong agitation or long period of incubation time for triggering irreversible amyloid fibrillation. It was previously reported that the presence of lipid free air/water interface accelerates the formation of amyloids either in Aβ or in α-synuclein peptides.[42,43] Thus, the required conditions of high bulk concentration, agitation or long period of incubation time are quite different to those assayed by us. Our approach introduces a more realistic membrane model system with only the presence of a common and natural phospholipid constituent of membranes.

From adsorption experiments, it has been reported that Aβ(1-40) peptide interacts with the negatively charged polar head groups of POPG/POPC monolayers throughout electrostatic interactions, but with no further penetration into the lipid interface. The interpretation was the charged surface is acting as local seeding and the beta sheet or α-helix content depends on the lipid/peptide mole ratio.[44] Furthermore, substances that inhibited the state of membrane charges inhibited the toxicity of Aβ(1-40) peptide.[45] However, the experimental conditions assayed were far to "mimic" a physiological membrane environment since a low ionic strength and a high POPG/POPC ratios were employed. However, these results are keeping with ours since the authors remarked that Aβ(1-40) peptide toxicity is mediated by nonspecific physicochemical interactions at the membrane interface. Therefore, all the data would converge to the concept of highlighting that it is possible that a given particular condition of the membrane has a more active and harmful role in inducing toxic beta amyloid fibrillogenesis.

In our hands, even that Aβ peptides forms insoluble and reproducible monolayers [17-18] they do not get along with phospholipids. As we have shown, mixed with the solid DSPC Aβ(1-40) peptide behaves immiscibility at all lipid/peptide proportions. This solid-solid lipid/peptide immiscibility in monolayers may be attributable to the strong β-sheet network of Aβ peptide at the interface keeping with a low interfacial solvation and high shear rheology.[18] Here, we demonstrated that this strong β-sheet network of *pure* Aβ peptide at the interface *is ThT negative* and does not form fibril-like structure searched either by BAM or AFM. The interfacial protein concentration in a monolayer at lateral packing compatible with biomembranes (20-30 mN.m^{-1}) is surprisingly high. Taken into account that the molecular area of Aβ(1-40) peptide at 25 mN.m^{-1} is about 3 nm^2 and, assuming an interfacial thickness of around 2 nm (taken into account the average thickness of AFM image, see Fig. 4) it gives a mean surface molecular volume of 6.000 nm^3, the *real protein surface concentration of a pure Aβ peptide monolayer* would reach the huge value of around **0.26 M**. This surface concentration corresponds to a protein density of around 1.15 gr.cm^{-3}, that is about the 80 % of the reported value for protein density (around 1.35 gr.cm^{-3}).[46] This is indicating that the *local low solvation and high protein concentration of Aβ(1-40) peptide at the interface is no enough to form amyloid fibrils*. To trigger amyloid formation at the interface it is necessary the joint action of a phospholipid in the liquid state. We obtained partial miscibility when we introduced POPC as

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lipid partner in the mixed interface. At POPC:Aβ(1-40) 97.5:2.5 of PAP (meaning that Aβ peptide

contributes with a 2.5 % of the total area available of the mixed lipid/protein interface), we clearly see the

ThT positive fibril-like structure. At this particular proportion, we observed a slight repulsive interactions

according to the miscibility analysis (mean molecular area above to ideal mixed area, Fig. 2 lower part).

This is keeping with the different BAM reflectivity found for fibril-like structure compared to pure protein,

indicating that a change in Aβ peptide conformation is induced by the liquid-expanded environment of

POPC. It is noteworthy that this effect occurred when Aβ peptide is surface diluted by the presence of a

higher hydrated polar head group of POPC lipid when compared to more solid DSPC lipid. The level of

interfacial hydration of phospholipids in different physical state in monolayers was already reported by

Brewer et al. by using the properties of fluorescence relaxation of LAURDAN.[41] For liquid expanded

interfaces (above phase transition of the phospholipid) the value of LAURDAN GP is around -0.3 whereas

for phospholipid in liquid condensed state is near to 0.5.[41] If we combine these values with the interfacial

water activity for liquid expanded phospholipid interfaces proposed by Damodaran,[25] the equivalent water

activity of our amyloid trigger condition would be around to 0.3, whereas it is expected to be close to 0 for

solid phospholipid interfaces. The highly non solvated interface of the liquid condensed phospholipid is not

enough to break off the solid arrangement of the Aβ peptide. A more hydrated phospholipid interface (in

liquid state) triggers the Aβ peptide conformation to form fibril-like structures with both the typical ThT

positive characteristic and a compatible AFM topography imaging. The hypothesis that the state of

interfacial water activity may modulate the formation of fibrils-like structure in a liquid-expanded lipid

environment is in keeping with the increment of fibrils structure upon film compression as it was shown in

Fig. 3B. Indeed, for this type of liquid-expanded phospholipid interface LAURDAN GP goes from -0.55 to

-0.3 with a change in the equivalent interfacial water activity from 0.9 to around 0.3 when the film is

compressed from 2 to 20 mN.m^{-1}.[25, 41] However, at this point it is necessary to be rigorous and it will be

necessary to measure the degree of LAURDAN fluorescence in mixed Aβ-amyloid:lipid Langmuir films,

that have not been done yet. We have obtained similar results with DMPC, a shorter saturated fatty acid

phospholipid with an equivalent liquid-expanded property than POPC (data no shown), indicating that the

physical state of the phospholipid is critic for Aβ peptide fibrillation. In keeping with these data, it was

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reported a greater binding affinity of Aβ(1-42) peptide for liquid disordered L_d membranes than for liquid

ordered L_o environment.[47] We try to model a 2D lipid/Aβ peptide organization model in its fibril-like state

in accordance with the obtained data (see Fig. 5). The fibril-like structures at the interface shown

intermediate values in BAM reflectivity which it is associate to the refractive index changes on the

monolayer. These intermediate values of BAM reflectivity are in between the pure POPC (low values) and

high values of the solid arrangement of pure Aβ peptide (see Fig 3).
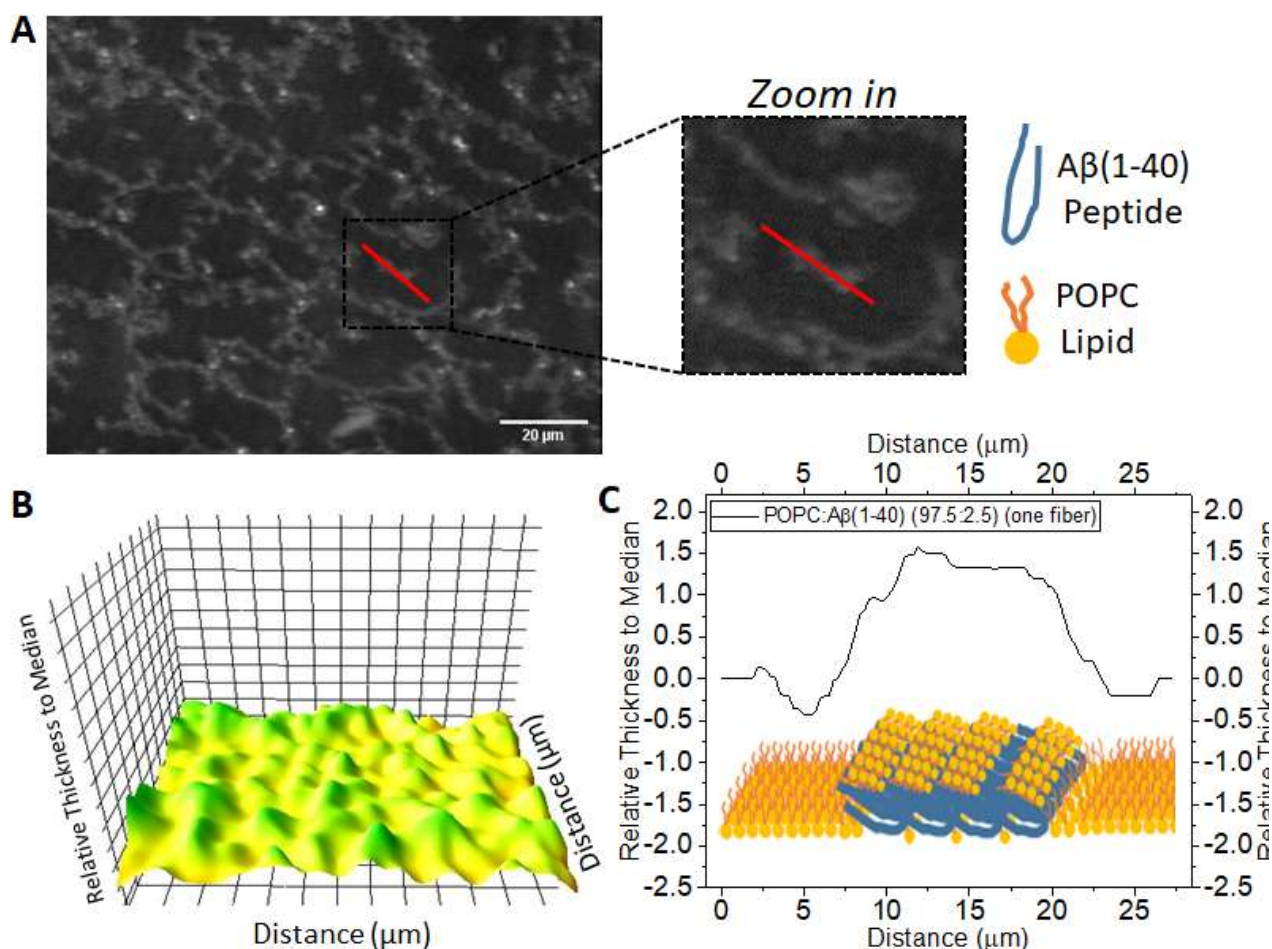
Figure 5: Modelling the fibril-like structure formed by Aβ(1-40) peptide imposed by POPC interfacial conditions.
A: (Left Panel) BAM imaging obtained at POPC:Aβ(1-40) (97.5:2.5 of PAP) showing the interconnected fibril-like structure (higher reflectivity) confining the more enriched POPC phase (lower reflectivity); (Right Panel) *zoom in* on the black dot-line square showing details, the transverse red line denotes the pass of the relative thickness plotted in 5C in black line. **B:** Three dimensional representation using FIJI program showing the relative thickness along the A imaging with false colours, the green colour is the maximum height whereas the yellow colour is the minimum. **C:** Cartoon view of a possible model of fibril-like structure formed by lipid-rich co-assemblies with Aβ(1-40) peptide in fibril structures keeping the relative thickness of the above zoom-in imaging (aprox.12 μm length x 0.2 μm height).

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Probably the interfacial conditions imposed by the liquid hydrated POPC gives an adequate environment to promote the formation of fibril-like structures of Aβ(1-40) peptide in which the lipid is taken part in the enriched fibril-like phase with a distinguishable intermediate collapsing point in the interface (see the value of collapsing point of this "intermediate phase" corresponding to the red point in Fig. 2 lower part right side, of 32 mN.m^{-1} and the BAM picture 2 of Fig. 1B). This fibril-like phase has a lateral stability lower than pure POPC of around 40 mN.m^{-1}.

Is well known that the Aβ amyloid peptides affect the membrane integrity,[17] however the intimate molecular mechanism of membrane disruption or fibril promotion at membranes is not clear yet.[31] The presence of membranes leading to amyloid peptide aggregation has been proposed by others authors either for Aβ peptides[48-49] or for α-synuclein.[50] By using Molecular Dynamics (MD) it was already postulated the importance of "surface quality" of membrane bilayers in Aβ amyloid binding and fibrillation.[47] The effective participation of phosphatidylcholine in the fibrillation process is supported by the fact that this lipid is found in amyloid fibril deposits in pathologic AD plaques or in Parkinson´s disease. [51-53,54] Taking into account the well probed observation about the active participation of phospholipids in amyloid fibrillogenesis, our experimental evidences are giving strong biophysical support to the active role of organized phospholipids in the formation of irreversible fibril-like structures in a membrane environment.

CONCLUSIONS

Nano-organized liquid-expanded POPC in lipid monolayers triggers the fibril-like formation structures of Aβ(1-40) peptide at the interface at low peptide area proportion. BAM imaging clearly showed protruding fibril structures that are ThT positive and were well visualized by AFM when the nano-arrangements is transferred to mica support. On the contrary, when the partner lipid of Aβ(1-40) peptide at the surface was the solid DSPC, amyloid peptide keeps its two-dimensional array and its immiscible lateral behaviour without forming amyloid fibrils. The amyloid fibrillation at the interface can be dynamically modulated by the quality of the lipid phase environment, the protein area proportion and the lateral packing.

EXPERIMENTAL SECTION

Reagents. Aβ(1-40) (D[1]AEFRHDSGYEVHHQKLVFFAEDVG[25]SNKGAIIGLM[35]VGGVV[40]), were

purchased from Bachem AG (www.bachem.com). The subphase was NaCl 145 mM. Water was obtained

from a MilliQ system (Millipore, USA) with a conductivity less than $0.085\ \mu S.cm^{-1}$ and a surface tension

of $72\ mN.m^{-1}$. NaCl from Merck (Gottingen, Germany) was roasted in an appropriate oven at 400-450 °C

for at least 4 hours to eliminate small amount surfactants impurities. POPC (1-pamitoyl-2-oleoyl-

phosphatidylcholine) and DSPC (1,2-disteraoyl-phosphatidylcholine) were purchased from Avanti Polar

Lipids, USA. DMSO and Chloroform were from Merck, Göttingen, Germany.

Peptide and lipid monolayers formation and compression π-Area isotherms. Langmuir monolayers

experiments were performed at 23±0.5 °C. The Langmuir monolayers room was kept at constant

temperature with the help of a continuous working individual roof-top air conditioning. Peptide monolayers

were formed using a 180 mL volume Langmuir trough. The subphase was unbuffered NaCl 145 mM, pH

5.6. All the settings were described previously.[17-18] Aβ peptide and lipids were dissolved in pure DMSO

and chloroform, respectively, into stock solutions of about 10 mM. Individual diluted working solutions

were freshly prepared to ~ 1 μM. Phospholipids were dissolved in pure chloroform and Aβ(1-40) in pure

DMSO. For mixed monolayers, peptide and lipids were premixed in solution at the desired proportion just

before to perform the experiment. 15-30 μl of solution was directly spread onto the subphase interface.

Surface pressure was determined with a Pt plate using the Wilhelmy method. The continuous symmetric

compression was through the automatic simultaneous movement of both barriers that confine the film. The

total film area were continuously recorded using trough and software provided by KSV Instruments® Ltd.

The usual compression rate used was adjusted at $10\ mm^2.min^{-1}$, lower barrier speeds were also checked

without altering the results. Monolayers formed by pure proteins of their mixtures with lipids usually have

slow collapsing transition process giving a non-abruptly discontinuity in the π-Area isotherms even at the

slower compression speed. For this reason, we shown as collapsing point or transition pressure of all

isotherms showed in Fig. 2 is the first discontinuity taken from the second derivative of the π-Area

isotherm (Fig. S2). These mathematically calculated values are lower than the first discontinuities

observable at first sight in π-Area isotherms in Fig. 1. Before compression the spread films were allowed to

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equilibrated by 2-3 minutes. Mean Molecular Area (*MMA*) or the average experimental area per molecule

is the available surface area divided by the amount of spread molecules at the interface, according to:

MMA= (A$_s$/ n$_L$+n$_P$), where A_s is the total available surface area of the trough and *n* is the amount of lipid n_L

or protein n_P molecules or their mixtures n_L+n_P spread onto A_s. A_s is automatically calculated by the

appropriate software that takes into account the barrier speed and the size of the available surface trough

area upon compression. A_{ideal} is proportional to mole fraction and individual molecular area of the pure

components of the mixed monolayer at a defined surface pressure π according to $A_{ideal}= (A_L X_L + A_P X_P)_\pi$,

where A_L and A_P are the individual molecular areas and X_L and X_P the mole fractions of pure lipid and

protein, respectively. *MMA* is the average of at least 4/5 independent determinations with its corresponding

standard deviation. The behavior of mixed lipid/peptide monolayers was analyzed by comparing the

experimental curves (*MMA vs.* π) with the theoretical isotherms (A_{ideal} vs. π) for the corresponding films in

which no interactions between the molecules are assumed. In a non-interacting behavior, *MMA* value tends

to be close to A_{ideal} (ideal mixing behavior).[20-21] Also, the miscible/immiscible of the mixed interface was

determined according to the surface phase rule.[21]

Brewster Angle Microscopy. Monolayers were spread over a Langmuir film balance, as described above,

and the films were directly observed along all π-Area isotherm using a Brewster Angle Microscope (BAM)

coupled to an EP3 Imaging Ellipsometer equipment (Accurion, Göttingen, Germany) with a 20x objective

(Nikon, Japan, NA 0.35). For each imaging acquisition, the monolayer was compressed by forward

compression steps of 5 mN.m^{-1}. During imaging acquisition (5 to 20 s) the lateral pressure did not exhibit

any detectable variation. Three to five images were sequentially taken for each surface pressure, in order to

have pictures of different regions of a same monolayer. Results obtained with BAM were compared with

the images obtained by using Atomic Force Microscopy (AFM) and Fluorescence Microscopy (FM).

Transferred experiments for AFM and FM. Compressed monolayers up to a desired surface pressure

were transferred to mica support (Nano-Tec V-1 grade Muscovite of 10 mm diameter and 0.15-0.21 mm

thickness, from Innovative Microscopy Suppliers, Argentina); the mica support is packed in several layers

which were used by removing one layer per experiment. The mica face was rinsed with distilled water

before transferring the surface material. Langmuir monolayers was then transferred to the support by

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dipping the mica, oriented perpendicular to the trough previously submerged, at the smallest speed (1 mm.min^{-1}) and keeping the surface pressure constant with a surface barostat couple to the barriers of the Langmuir trough. Previous to the transfer, the monolayer was allowed to stabilize (~2 min), before being transferred. Each monolayer of either pure components or their mixtures was first compressed up to 20 mN.m^{-1} before transferring to mica support.

Fluorescence Microscopy and imaging analysis. The fibril-like structures were visualized by FM by adding a drop of aqueous freshly prepared 10 μM ThT probe to the transferred monolayer onto mica support and leave to dry off for 5 minutes. Excitation wavelength was selected with a cutting filter of 450 nm and the emission taken at 482 nm. Negative controls were done: pure ThT, pure lipid and pure Aβ peptide were subjected to the same procedure (Fig. 4B). As positive control we tested preformed fibrils of α-synuclein formed in bulk condition according to Gallea and co-workers[55] and treated in the same way with ThT by using glass coverslip (Fig. 4B). The images were acquired with an Axioplan Carl Zeiss (Germany) fluorescence microscope equipped with an Olympus video camera (USA). We used a 20x and 40x objectives. The imaging processing and analysis was carried out using both Fiji and Pixinsight v. 1.8.6 software. Both programs allowed us to use some filters and 2D FFT to improve imaging resolution.

Atomic Force Microscopy. AFM measurements were performed on a multimode atomic force microscope SPM, Agilent Technology 5500, USA. We used the mica support as describe above. It was used in contact-mode scanning to measure the transferred Langmuir monolayers of Aβ(1-40) peptide/lipid mixed monolayers and their respective controls (pure POPC and pure Aβ(1-40) peptide). All the experiments were carried out at room temperature. Resolution images of 512 × 512 pixels were collected at a scanning rate in between 0.3 and 0.4 ln.sec^{-1}. The height and error-signal (vertical deflection), friction and topography images were taken simultaneously.

ASSOCIATED CONTENT

Supporting information

Supplementary figures are provided.

AUTHOR INFORMATION

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Notes

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TOC graphic

BAM

AFM

FM (ThT)

BAM

POPC:Aβ(1-40) (97.5:2.5)

POPC:Aβ(1-40) (97.5:2.5)

POPC:Aβ(1-40) (97.5:2.5)

DSPC:Aβ(1-40) (97.5:2.5)

Transferred monolayer