In Vitro Investigations of Interactions between Amyloid Beta Peptides (1-40, 1-42) Structures and Substrates with Different Natures

Jeffy Jimenez*, *Monica J. Escobar**, *David Morgan*[#], *and Alcantar Norma**.
**Dept. of Chemical Engineering, *[#] *Dept. of Pharmacology & Molecular Therapeutics*
University of South Florida, 4202 E. Fowler Ave. – ENB 118, Tampa, FL 33620

Abstract

Amyloid beta (Aß) peptides particularly $A\beta_{1-40}$ and $A\beta_{1-42}$ are the main components of neuritic plaques in Alzheimer's disease (AD) patients. Extracellular plaque formation has been recognized after incessant investigations along with the formation of intracellular tau protein tangles as the hallmarks of AD. From these two hallmarks, plaque formation has been linked mostly as a cause of the disease and the tangles mostly as a consequence. Our investigation is focused on studying the formation of AD plaques. The amyloid beta (Aß) is a physiological peptide secreted from neurons under normal conditions, along with other soluble forms cleaved from the amyloid precursor protein (APP). The soluble forms of APP have neuroprotective and neurotrophic functions, while the Aß is considered as an unwanted by-product of the APP processing. Under normal conditions there is an anabolic/catabolic equilibrium of the Aß peptide; therefore, it is believed that the formation of the plaque does not take place. On the other hand, the surface of the neurons may play an important role in the adhesion mechanisms of the Aß peptide. Our experiments show that the neuron surfaces along with the media conditions may be the most important causes for progressive formation of plaques. We have imaged on rigid (mica) and soft biomimetic (lipid bilayers on top of a PEG cushion layer drafted onto a silica surface) substrates the Aß peptide when incubated under three different media conditions, as a result we have identified three different structures that we have consistently with the literature called monomer, oligomer, and fibrils. We have studied the adhesion mechanisms associated with *in situ* plaque formation. The structure and topography characteristics of the Aß conformations have been followed with atomic force microscopy (AFM). The kinetics and rates of adhesion have been measured with attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy. Our results show the progress of the plaques' formation with time where simple monomers deposit on the substrates and allow the development of oligomeric species. The oligomers then grow into fibril-like structures leading finally to the plaques that eventually are seen to insulate real neurons and stop them from the synapse process. The ultimate outcome of this investigation will contribute to understand, prevent and determine possible mechanisms for removing AD plaques.

Introduction

The most common cause of senile dementia in our times is Alzheimer's disease (AD), which is an age-associated neurodegenerative disease, characterized by the loss of memory and language skills, damage cognitive function, and altered behavior. The first AD's clinical symptoms are typically seen after the age of 65 years old[1].

After decades of continuous research yet the pathological mechanisms of Alzheimer's disease are uncertain, however two hallmarks are well recognized: intracellular neurofibrillary tangles (NFTs) and extracellular senile plaques [2]. The NFTs are

structures composed of paired helical filaments mainly formed of abnormally hyperphosphorylated tau protein (P-TAU)[3], a neuronal microtubule-associated protein [4]. Some of the roles of the tau protein are: stabilization of axonal microtubules, signaling transduction, interaction with the actin cytoskeleton, and neurite outgrowing. It is believe that the formation of NFT's may be a consequence of the amyloid plaque formation [5]. The extracellular senile plaques are composed by amyloid peptide deposits of mainly 40- and 42-amino acids long ($A\beta_{1-40}$ and $A\beta_{1-42}$) [3]. Amyloid-β peptides do not appear to play a major physiological role. Indeed, based on what is known as the amyloid cascade hypothesis, Aβ plaque deposits or partially aggregated soluble Aβ, trigger a neurotoxic cascade, thereby causing neurodegeneration and finally pathology of AD[6]. Figure 1 shows both deposit of plaques and NFT.

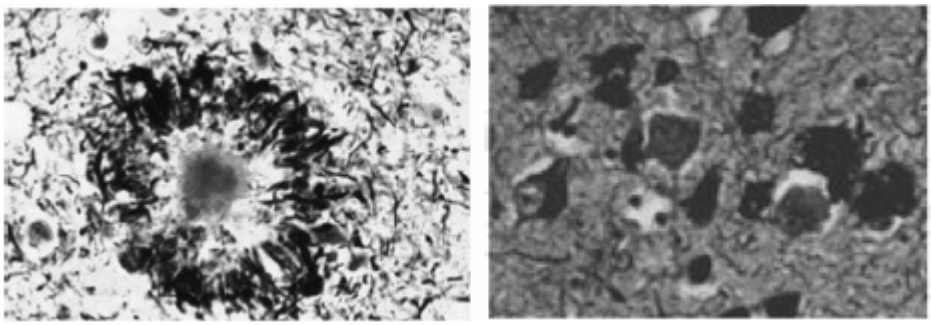

Figure 1. Two of the Alzheimer's Disease hallmarks: **(left)** A High-power photomicrograph of an amyloid plaque; **(right)** A photomicrograph of silverstained (black) neurofibrillary tangles in the cell bodies [6]

Amyloid-β is a physiological peptide secreted from the neurons under normal conditions among other soluble peptides cleaved from the transmembrane amyloid precursor protein (APP). APP is a large extracellular N-terminal domain with a smaller intracellular tail (Figure 2). Apparently the soluble forms of APP have neuroprotective and neurotrophic functions. However, Aβ is considered as an unwanted by-product of the APP processing [5].

The Aβ peptides are found as globular and non-fibrillar forms in small concentrations of pico- to nano-molar, and are found in the extracellular and cytoplasmic (inside the cell) regions in both normal as well as AD tissues. Nevertheless, significantly small oligomeric molecules, an non-fibrillar Aβ peptides, are sufficient to cause profound cytoskeletal degeneration and cell death through mechanisms of plaque's formation still not completely understood.

In this work, we conducted systematic investigations to elucidate the mechanisms between protein and membrane structures under different physiological conditions (i.e., varying the pH, temperature of the media, and the hypothetical cell membrane surface). The surface of the neurons plays an important role in the adhesion mechanisms of Aβ; mechanisms associated with a cascade of events that may begin with the aggregation of monomer (seed) as bigger molecules (oligomers) that are in solution and that start

accumulating on the neural cell membrane and that eventually result in larger structures (fibril) that form plaques, which block synaptic processes. Our approach is to mimic cell conditions changing the media and characterizing the structure formation and the interaction mechanisms of these forms with hypothetical cell surfaces.

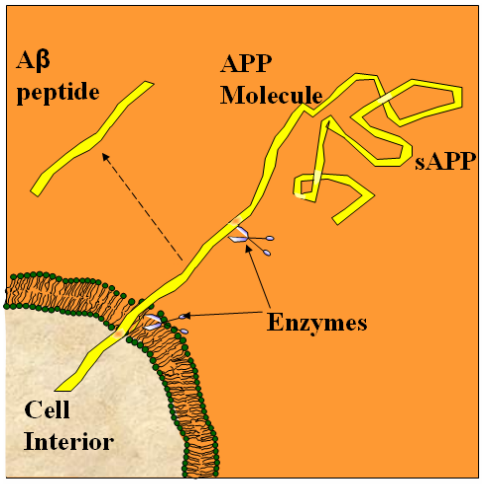

Figure 2. Synthesis of Aβ in the cell. The β-amyloid domain is partly embedded in the plasma membrane. To generate Aβ, APP is first cleaved by an enzyme (β-secretase), resulting in the release of β-secretase-cleaved soluble APP (sAPP). A second cleavage releases free β-amyloid

Our work is mainly focused in answering two questions: i) what are the intermolecular forces and kinetic mechanisms involved in the formation of the different amyloid-β ($A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$) peptide conformations (i.e., monomeric, oligomeric and fibril)?, and ii) what are the surface interactions between amyloid-β peptide conformations and cell membranes, and their role in plaque formation?

Therefore, this work will elucidate ways that may lead to plaque prevention and/or removal, based on the understanding of neurotoxic structures assemble, and surface energy features, in connection with the medium and the interacting substrate conditions.

Experimental methods and procedures

Conditioning of Aβ Peptides and Synthesis of Structures

$A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ Peptides: Lyophilized peptide (1mg) kept at -20°C was removed from the freezer and let equilibrated for 30 minutes at room temperature (RT). The peptide is then dissolved in HFIP following the procedure described by W. Blaine Stine et al.[7]. HFIP is used to break any residual tertiary and β-sheet secondary structure of the peptide. The peptide is composed by α-helix (~50-70%) and random coil (~30-50%) structures mainly [7]. The HFIP is then injected into the peptide flask with a gas-tight Hamilton 0.5ml glass syringe until a concentration of 3.33μg/μl is obtained. Peptide dissolution

takes not longer than five minutes. Such stock solution is aliquoted into 25 parts and storage in sterile micro centrifuge tubes of 12.5µl, containing 40µg of Aβ each approximately. Thus, each micro tube contains 9.238 nmol and 8.861 nmol for $A\beta_{1-40}$ and $A\beta_{1-42}$, respectively. Aliquoted samples are left under the laminar-flow hood to allow the HFIP to evaporate. The peptides are then placed for 12 hours in a desiccator at a vacuum of 27 in Hg. The final peptide samples have an even appearance (i.e., transparent film), which is appreciated at the bottom of the Eppendorf tubes. The tubes with the peptide are flushed and closed with nitrogen, wrapped with aluminum foil and parafilm, and enclosed in a jar at -20°C for later used.

Amyloid-β Conformations

Media conditions for monomeric precursors: The following procedures are the same for both $A\beta_{1-40}$ and $A\beta_{1-42}$. 40µg of pretreated Aβ peptide is removed from the freezer and let to equilibrate at RT. The pretreated peptide is dissolved in 2µL of DMSO, which is a polar, water-soluble organic solvent commonly used to solubilize hydrophobic peptides. After a minute of sonication with DMSO, 88µL of milli-Q water are added to the sample. Finally, it is incubated at RT [7].

Media conditions for oligomeric precursors: First, a tris/NaCl/HCl buffer solution is prepared. This buffer serves as incubation media for the oligomer's synthesis. The buffer is prepared in an Eppendorf of 50ml, where 0.3028g of tris and 0.2922g of NaCl are dissolved in DI water until the pH is fixed to 7.4. Sometimes a few drops of concentrated HCl need to be added. The pretreated peptide film kept at -20°C is then dissolved in 2µL of DMSO, sonicated for 1 min, and then incubated at 4°C in 88µL of Tris/NaCl/HCl. This procedure is described in detail by Kayed et al. [8].

Media conditions for fibrillar precursors: The first step to incubate Aβ fibril structures is to prepare a solution of ammonium hydroxide at pH=8.5. The next step is to prepare a PBS buffer. Fibrils are prepared by dissolving 40µg of the pretreated peptide (at RT) in 20µL of NH_3OH and later adding 20µL of PBS. The sample is gently vortexed and finally incubated at a concentration of 1µg/µL at 37°C. This procedure was suggested by Dr. David Morgan (PhD) from the Department of Pharmacology & Therapeutics in the College of Medicine at the University of South Florida (USF), and director of the Alzheimer Research Laboratory at USF.

Surface Characterization of the Amyloid Peptides Topology

The sample deposition procedure is conducted under laminar flow cabinet to avoid contamination by undesirable particles. The samples to be analyzed with the AFM are deposited on mica, which is an inert, smooth, and easily to clean mineral, and widely used for biological samples in AFM imaging. Mica sheets are freshly cleaved prior to each deposition. A small Teflon O-rings (external Φ 4.45mm, internal Φ 1mm) is used as an incubation chamber placed on top of the mica which ensure the maintenance of the desired location of fibril formation. 3µL of each incubated sample are dissolved with ultrapure water until a concentration of 20µM is reached. This concentration has been tested as the optimum to produce fibrils in a control manner. A small drop of such solution is then deposited on mica and incubated for 5 min, flushed with 3mL of ultrapure

water, dry with ultrapure nitrogen, and stored in a desiccator under vacuum (30 torr) for later AFM study.

Chemical Characterization of Amyloid Peptides

No fibrils were observed when $A\beta_{1-40}$ peptide was incubated. Therefore, the chemical characterization was done only for $A\beta_{1-42}$ peptide conformations. The chemical analysis is done using an ATR-FTIR spectrometer, and a zinc selenide ATR crystal. The ATR cell allows a maximum volume of 62μL solutions. The crystal was first cleaned with isopropanol, and then a fresh media solution was flushed before peptide contact. The background for the IR data was taken when the ATR crystal was in contact with non-peptide media. Spectra were taken at about 10 min intervals for about 3 hours for monomers, 10 hours for oligomers, and 12 hours for fibrils, which is the time that it took for the samples to reach saturation and steady state conditions.

Biomimetic Cell Membrane Construction and Studies of Interaction with Amyloid-β Peptide

Soft –Support Layer: Polyethylene glycol (PEG) is used to build the soft support layer. PEG is grafted to silica films deposited on silicon wafers creating a thin hydrophilic film. The silica deposition takes place reacting silane (SiH_4) and O_2 gases in a plasma enhanced chemical vapor deposition reactor. The samples are then exposed to a water plasma treatment in order to increase the hydroxyl group concentration on the surface controllably and reproducibly[9]. The surfaces are then rinsed with water and dried very well, since any traces of water could interfere with the PEG reaction. In the meantime, a solution of PEG (of wt ~ 400Da) is heated until it reaches 100°C maintaining constant agitation. The cleaned and activated surfaces are placed into the PEG for one hour. These parameters have been determined previously by Alcantar et. al. [9]. Once the PEG grafting reaction is done, the surfaces are retrieved from the hot PEG and gently rinsed with water (avoiding direct contact with the flux o water), and dried with ultrapure nitrogen. Sometimes the surfaces with PEG that are not used at the immediately after the reaction are storage in PEG media.

Lipid Deposition: The lipid bilayers are deposited onto the soft-support PEG layer using a Langmuir Blodgett (LB) trough. The lipids solution concentration used is 15 μg/μL. A total volume of 100 μL is gently spread on the air/water interface using a syringe (giving it about 5 minutes for the solvent to evaporate). During the deposition, the surface pressure is kept constant at 30 mN/m. The deposition takes place at constant pressure and using a barrier velocity of 50 cm^2/min.

Surface Topography and membrane/peptide interaction study: The soft-supported membranes constructed above have also been scanned by AFM in solution. They are kept in contact with saturated lipid solution at all times to avoid air exposed and consequently, damage to the sample. The biomimetic cell membranes were also scanned while in contact with Aβ fibrils (0.933 μM).

Results and Discussion

This work is based on the fact that real interactions of Aβ peptides, with both 40 and 42 amino acid sequences, could be controlled and studied by following a comprehensive

approach. The control on the structure of the Aβ peptides was done by optimizing the incubation conditions previously studied by Stine et al. [7] and Kayed et al.[8]. Depending on the conformation that is being pursued (i.e., monomeric, oligomeric and fibrillar), the peptide molecules will adopt diverse structures that defined their physical and chemical pathways of aggregation, which evolve with time. By tuning and promoting specific conformations, we were able to explicitly distinguish the response of the peptide in terms of their assembly, surface adhesion, chemical composition and kinetics.

Explicitly, our results are three-fold:

1) We resolved the differences of behavior depending on the number of amino acid residues contained in each peptide molecule (i.e., $A\beta_{1-40}$ and $A\beta_{1-42}$)
2) We promoted structural rearrangement depending on the incubation media and substrate character
3) We were able to correlate peptide structures (i.e., α-helix, β-sheet, β-turns, or random) with the adhesion dynamics and ultimately, to determine the role of molecular stability of the peptide that leads to the formation of the AD plaques.

Conclusions

- This physicochemical study provided a broad understanding of the intermolecular forces involved in the formation of Aß40 and Aß42 peptide conformations and their kinetics.
- We were able to control stable conformations of amyloid-ß peptides and to distinguish their aggregation by tuning the incubation media conditions. This could elucidate what media conditions are associated with the formation of AD plaques in the brain.
- We found that $A\beta_{1-40}$ affinity to rigid surfaces is definitely less compare to $A\beta_{1-42}$ peptides.
- The membrane surface rigidity plays an important role in AD plaque formation.

Final Remarks and Future Work

- This work has advanced our ability to master a systematic way to prepare the three different conformations of Aβ peptides that may play an important role in AD plaque formation.
- If these three conformations may influence plaque formation, we may be able to understand the molecular mechanisms associated with their adhesion, surface kinetics and assembly processes by using the Surface Forces Apparatus and the ATR-FTIR technique concurrently (IR-SFA).
- Further investigation developing essays with the two peptides together is needed to elucidate why only $A\beta_{1-42}$ segments cause adhesion, and how they influence $A\beta_{1-40}$ peptide dynamic processes.
- Additional studies to corroborate our initial findings on the effect of substrate mobility will be performed. For instance, we would like to vary systematically the rigidity of model membranes by introducing cholesterol in their structure.

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