



Amyloid Peptide Nucleation and Growth Using a Modeled Cell Membrane

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Abstract

Alzheimer's disease is an epidemic in the U.S. that affects 5.4 million Americans and is the 6th leading cause of death. Previous research has suggested that the accumulation of proteinaceous aggregates that are result of the enzymatic digestion of beta and gamma secretase, ultimately leads to the degeneration of a human brain. Various studies have also indicated that Alzheimer's disease is correlated with the interaction between the Amyloid peptide and the cell membrane. To further examine this relationship between the Amyloid peptide and the cell membrane, we analyzed the phase behavior of the accumulation of small Amyloid peptide oligomers at a 2D interface. We constructed an artificial model of the lipid monolayer using water, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissaminerhodamine B sulfonyl) (ammonium salt) [Rhod], and made various attempts to manipulate the phase behavior of the phospholipid bilayer. After examining the potentially prototypical phase behavior of the interface, we added various concentrations of Amyloid peptide in order to determine how self assembly at the interface influences phase behavior. This allowed us to evaluate the nucleation and growth stages of assembly in Alzheimer's disease within a model two-dimensional system. We hypothesized that the addition of Amyloid peptide to a model cell membrane containing DMPC and Rhod-DMPE would significantly change the stability of the condensed phase of the monolayer. Our results indicated that the Amyloid peptide partitions within our model membrane, and the accumulation of the peptide occurs rapidly (~milliseconds).

Introduction

One in 10 persons over 65 and nearly half of those over 85 develop Alzheimer's disease, which explains why there are at least four million Americans currently affected by Alzheimer's. It is presumed that as long as there is no cure or prevention, by the year 2050, the number will jump to 14 million. Alzheimer's disease (AD) is the most common form of dementia; loss of memory and other cognitive abilities serious enough to interfere with daily life. This progressive neurodegenerative disease accounts for 50 to 80 percent of dementia cases, affecting memory, thinking and behavior. Risk factors of the disease include: age and family history. As one becomes older, his/her risk of developing AD increases. Having a close blood relative, such as a brother, sister or parent who developed AD also increases one's risk. Likewise, having a specific combination of genes for proteins that appear to be abnormal also increases one's risk¹. Other risk factors that

are not as well proven include: longstanding high blood pressure, history of head trauma and simply being a female. Alzheimer's disease is of two types: early onset and late onset. The early onset form of the disease tends to progress rapidly, with its symptoms appearing before age 60, and may run in families. The late onset of the disease, the more common form, develops in people age 60 and older and may run in some families, but the role that genes play is less clear.

Diagnosis of Alzheimer's disease is typically made when certain symptoms are present and by making sure other causes of dementia are absent. Although one may be diagnosed through a history and physical exam by a skilled doctor or nurse, currently the only certain confirmation that someone has AD is to examine a sample of their brain tissue after death. During the autopsy, brain tissue of patients with Alzheimer's disease show (1) "Neurofibrillary Tangles" - twisted fragments of proteins within nerve cells that disrupt cellular function, (2) "Neuritic Plaques"- abnormal clusters of dead and dying nerve cells, other brain cells, and proteins, and (3) "Senile Plaques"- areas where products of dying nerve cells have accumulated. The dysfunction of the brain occurs as nerve cells (neurons) are destroyed, leading to decreased neurotransmitters which are necessary for nerve cells to send messages to one another.

Alzheimer's disease has been associated with many proteins. One particular protein research currently being done is the Beta Amyloid protein (β Amyloid). The β Amyloid peptide, 36-43 amino acids in length, is an isoform from the Amyloid Precursor Protein (APP). The APP is a transmembrane glycoprotein whose function is unknown. Normally, the APP protein is broken down and secreted out of the brain. However, when the APP is not broken down correctly, the cell begins to accumulate beta amyloid proteins. The peptides are created via enzymatic digestion of the APP by alpha, beta, or gamma secretases as shown in figure 1. Two particular peptides that are related to disease are A β 40 and A β 42. The A β 40 and A β 42 are both formed by the cleaving done in the trans-Golgi network. Between A β 40 and A β 42, A β 40 is more commonly created, but bulk studies have shown that A β 42 is the more fibrillogenic product and is most often associated with the final disease state.

The β Amyloid protein generates densely populated fibrous structures, also known as plaques. In addition to plaques, Neurofibrillary tangles structures, produced by the protein tau, can form in the brain. Tangles, when hyperphosphorylated, accumulate inside the cells themselves. Unlike tangles plaques accumulate on the outside of the cell. The plaques formed are insoluble fibrous aggregates. Insoluble fibrous aggregates are protein molecules whose sheet-like secondary structures becomes dominant. One proposed method for the formation of the



fibrous aggregates is the process of self-assembly. The proteins are first created and the individual proteins then accumulate to form colloidal sized spheres, which then assemble into linear chains. As the spheres grow larger, they grow a Coulomb repulsion force, resulting in the final product of this process; U-shaped loops.

Once fibrils attach together to form plaques, they can then insert themselves into the membrane of the neuron cell and cause substances outside the cell to leak in, ultimately resulting in dysfunction and eventually death of the neuron. Another case is one in which the plaques disrupt the calcium ion homeostasis. The calcium ion acts as a mediator, regulating neuronal survival and plasticity (ability to form synaptic connection) in the nervous system. Thus, damage to the homeostasis of the calcium ion channel ultimately prevents the ability of neurons to communicate with each other. Studies have also shown that the $A\beta$ peptide builds up selectively in the mitochondria. Another effect of the peptide is inhibiting certain enzymatic functions and the utilization of glucose by neurons.

Every cell in our body is composed of what is known as the cell membrane. Visually, the cell membrane is a wall or barrier that separates the interior from the exterior environment of the cell. The cell membrane is a flexible lipid bilayer composed mainly of phospholipids with imbedded proteins. The membrane consists of three major types of lipid molecules: phospholipids, cholesterol, and glycolipids². A phospholipid is a fat derivative which contains nitrogen molecules with a phosphate group attached to it. Cholesterol is a fatty substance manufactured by the liver. It has a rigid ring system and a short branched hydrocarbon tail; it is largely hydrophobic, but it has one polar group (hydroxyl). Cholesterol is important because it prevents the cell membrane from crystallizing. In the presence of cholesterol, during low temperatures, the bilayer is in a gel state in which molecules are tightly packed. In contrast, at higher temperatures, the bilayer is in a fluid phase in which the molecules are free to diffuse laterally³. Glycolipids, lipids that attach to a short carbohydrate chain, serve as markers for cell to cell communication and provide energy. Other essential components of the cell membrane are, but are not limited to, transmembrane proteins and signaling molecules.

Because the cell membrane is so complex in structure, experimenting with all its elements would only provide difficult results to analyze. In doing so, one would not be able to clearly define the dominant component in fibrillogenesis. Therefore, experimenting with a single aspect of the cell membrane will not only be easier to experiment with but will provide also a clearer understanding of the results. In our experiment, we focused particularly on the phospholipids of the cell membrane. The bilayer of the cell membrane is a chain of individual phospholipid molecules. The molecule itself is divided into two parts, a hydrophilic head and a hydrophobic tail. The head consists of one or two phosphate groups, while the tail is comprised of fatty acyl chains.

In our experiment, we are specifically observing and analyzing the change in phase behavior of phospholipids at an air-water interface in the presence of the beta amyloid peptide. Two dimensional phase behavior is defined by the temperature, composition and surface concentration of phospholipids packing at the interface. The characterization of condensed phases in two dimensions is analogous to three dimensions; namely, there is a significant increase in density. We apply fluorescent lipids to visualize these dense regions using fluorescence microscopy. Subsequently, we intend to study the correlation of the aggregated state of the Amyloid peptide partitioning to the monolayer and the development of Alzheimer's disease. We anticipate evaluating early state nucleation of peptide assemblies at interfaces and connecting this mechanism with plaque formation responsible for deteriorated brain cells in Alzheimer's disease. Our long term objective would be to explore these systems as a method for detecting small oligomeric peptide structures before the development of the more severe neurological symptoms described in the previous sections.

Materials and Methods

A three-welled Trough, Distilled water, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissaminerhodamine B sulfonyl) (ammonium salt) (RHOD), 1:40 Chloroform dilution (5ul Phosphorus with Fluorescent dye and 195ul of Chlorine), and Amyloid Peptide were used as reagents in the experiments. Since we are not experimenting on human extracted neuron cells, it becomes essential to observe the phase behavior of a modeled cell membrane with the beta amyloid peptide under the same conditions present inside of a living cell. The modeled cell membrane, containing only phospholipids, was created through a mixture of DMPC, Rhod-DMPE, and chloroform. After creating the model, we came across the issue of containing the cell membrane so that we may examine the phase behavior of the interface. We created several designs of a trough, focusing primarily on how we can effectively change concentration, attain a constant flat surface, and observe the phase behavior within a reasonable area. To create

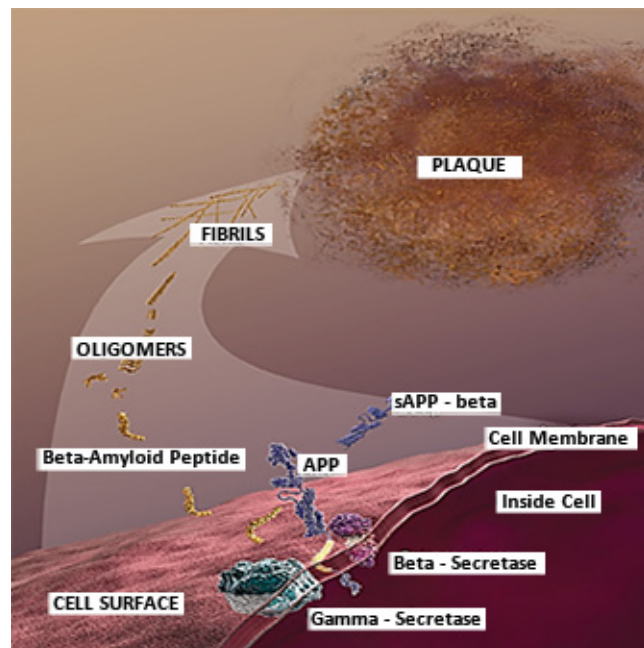


Figure 1. Formation of Amyloid Beta plaques (Adapted from: *Scientific Images*, National Institute on Aging. <<http://www.nia.nih.gov/alzheimers/scientific-images>>.)



the trough, we first determined the appropriate dimensions based on the needs such as flatness and concentration. After creating an ideal drawing, we constructed the design on Corel, a designing software. The design incorporated features that would allow us to perform multiple experiments during one trial. Using the laser associated with the Corel program, we cut a clear acrylic material into the design. The first design of the trough was replica of a rectangular 10 cm² trough surface. A trough of such area makes it easier to attain a flat surface. However, the problem with this design is that the given area for observing the phase is too large to be able to visualize all the phase changes on the surface at one time. The second design, similar to the previous one, contained a bath-like trough with a slider that can move across the surface of the trough, allowing us to change concentration. The problem with this design is that using the slider does not insure the exact amount of change in concentration. For instance, if the slider is pushed halfway across the trough it does not necessarily suggest that the concentration has increased twice as much for that new given area. This is because the slider will not ensure that all substances have traveled to the designated area. Our third and final design was a trough with three circular same sized wells as shown in figure 2. The wells were small enough to observe the majority of the changes on the surface. Because the wells are circular, the sides of the well can pin the water with an equal force all around. As a result, a flat water surface can be obtained. With the trough having three wells, we were able to experiment with several concentrations in one trial. Along with determining the most suitable design for the trough, we had to consider the size of the wells in the trough. After conducting a couple of experiments, we realized that a well too deep would cause the peptide aggregates to sink to the bottom, while a well too shallow would cause the peptide aggregates to accumulate on the surface and to form multiple layers of lipid. Both situations would yield results that would be difficult to analyze. After determining a reasonable depth through trial and error experimentation, we calculated the volume of water needed to obtain a flat surface. Obtaining a flat water surface is necessary because there can be an accumulation of the peptide in the center (concave) or on the sides of the well (convex). Both situations would create multiple layers of lipid. We determined this through two experiments: laser reflection and water evaporation. In the first experiment, we added a volume of water to each of the well and shined a laser on the diameter of the circular wells. This created a light reflection on a white backdrop. When the reflected light was measured to be the same length as the diameter of the circular well, we concluded that at that amount of water, the surface was flat. We came up with various amounts depending on the well, (ranging from 386-398ul). However, when we conducted experiments using the new calculations, problems still persisted. At times, we would get multiple layers of the lipids or be unable to observe an entire region because the image would be out of focus due to the evaporation. We then concluded that 400 ul, being close to those numbers, would almost always yield a flat surface. The water evaporates with the chloroform upon placing a cover slide; the convexity reduces and thus, the surface becomes flat. By knowing how long it takes the chloroform to evaporate, we estimated how

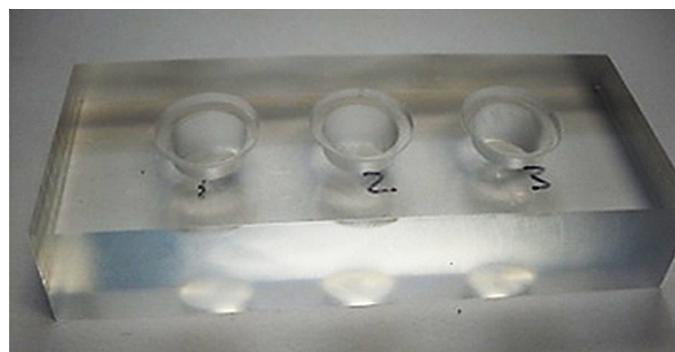


Figure 2. Three-well Langmuir trough.

long it will take for the water to evaporate until it's nearly flat. We set up the microscope by turning on the fluorescent lamp on, along with an attached camera and switched the focus to 20x magnifications. Then, using the 1,000 μ l micropipette we added 400 μ l of water into each well. We then immediately added 2 ul of the pink colored lipid mixture. We waited for 5 minutes, after which, we added the final 2 ul of the solution (a total of 4 μ l of the lipid solution). After waiting five minutes, we covered each of the wells with a cover slip to prevent water evaporation. We then examined the formation of a monolayer of a model lipid bilayer under a camera. We took 1 to 2 images of the bilayer before the addition of the amyloid peptide. Before adding the peptide we removed the cover slips and refocused the image on the computer. While one person stands near the computer holding the focus knob the other person immediately adds 1 μ l of the peptide. Upon adding the 1 μ l, the person holding the focus knob, immediately refocuses the image to capture the changes on video. When repeating these steps the peptide solution is diluted to various concentrations before being added with the lipids.

Results

When creating the modeled cell membrane, we concluded that the cell membrane is sensitive to minute changes in composition at the surface. We were thus able to visually explore the influence of the Amyloid peptide on the phase behavior at the modeled interface. Additionally, Nano- to pico-mole amounts of the Amyloid peptide were used to examine the accumulation of aggregates. A quantity of 20pMoles was used. The phase behavior shown in figures 4a and 4b, image 1 and 2 significantly differed from that in figure 3. Figures 4a and 4b show that the small circular structures in figure 3 were quickly buckling and aggregating. In addition, the results suggest a significant change in the shape of the lipid rafts. We noticed that the lipid rafts were quickly forming into fibrous strands, as suggested by the process of plaque formation. It was also determined that time played a key factor in the formation of these fibrous strands. When the peptide was added, instantaneous changes occurred, thus the phase behavior was visualized while the peptide was added to capture the complete process of plaque formation.

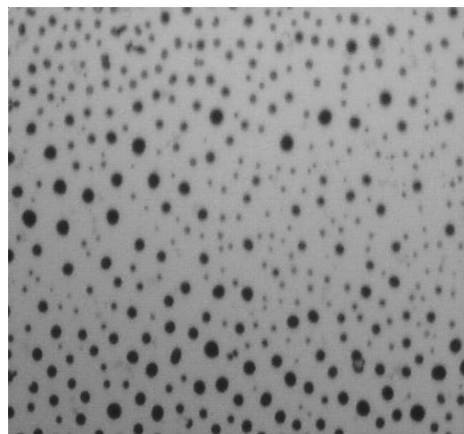


Figure 3. Before Addition of Amyloid Peptide. The dark circular regions represent lipids of the cell membrane, while the lighter regions represent fluid that flows in between the lipids. The size of the image is 150 microns across the diameter.

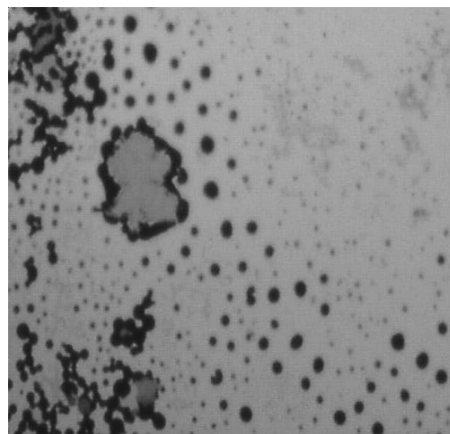


Figure 4a, Image 1. After Addition of Amyloid Peptide (Part1). The figure represents the phase behavior on the interface of the modeled cell membrane after the addition of .1ul of Amyloid peptide. Image 1 shows the accumulation of the Amyloid peptide immediately after it was added. In the image there is one region in which the lipids remain as their circular structure and another region in which fragmented lipids begin forming together. The image is presented under 20x magnification and a size of 150 microns across the diameter.



Figure 4b, Image2: After Addition of Amyloid Peptide (Part2). This figure as well represents the phase behavior on the interface of the modeled cell membrane after the addition of .1ul of Amyloid peptide. Image 2 specifically shows the accumulation of the Amyloid peptide seconds after Figure 3, Image 1 was captured. In image 2 there is a greater formation of fragmented lipids than in image 1. Furthermore, there is larger gas containment in various regions than that of image 1. The image is presented under 20x magnification. The size of the images is 150 microns across the diameter.

Discussion

We concluded that one cannot fully examine the accumulation of the Amyloid peptide. While we are able to see the final aggregation of the fibrous structures, we are not able to visualize the first stage of the transition of the phase behavior, which is how the peptides form into these fibrous structures. We presume that testing out various smaller concentrations of the Amyloid Peptide would allow us to visualize the dynamics of plaque formation. This experimental process would consist of several assays that have sequentially lower concentration until we can observe the formation of the plaques.

In addition to visually representing change in phase behavior of the cell membrane, the change was quantitatively represented with Perimeter analysis on the impact of the Amyloid Peptide. Perimeter analysis is a process of analyzing and calculating both the area and perimeter of a given shape. Together, comparing the area and perimeter, one can quantify the circularity of the shapes. When the peptide is added, the cell membrane rapidly changes its shape and so does the amount of energy present at the surface in the system. Thus, through perimeter analysis one can compare changes before and after the addition of the peptide and create a correlation between the changes in shape and the amount of energy present in the system. The perimeter analysis would be performed using Image J, an image analysis software. The results indicated number such as 6.4 for before the addition of the peptide and 1.2 after the addition. The difference in the two numbers suggests that not only the change in surface tension is larger, but also that a significant change has occurred within the cell itself.

Moreover, to explore the question of how can one detect the plaques before a patient's death, Congo red can be used to evaluate if the plaques are assembling in a fashion analogous to the deleterious neuronal plaques. Congo red is an organic soluble solvent, the sodium salt of benzidinediazo-bis-1-naphthylamine-4-sulfonic acid. The Congo red's molar extinction coefficient (a measurement of how strongly a chemical species absorbs light at a given wavelength) is 45000 L/mol, thus making it a suitable candidate to reflect the fluorescent activity when bound to amyloid fibrils. Studies have shown that an apple-green biofluorescence of Congo red under polarized light is indicative for the presence of harmful amyloid fibrils.

After conducting these experiments, we can then apply our findings as a sensing tool. Since we're using a modeled cell membrane as opposed to a real cell membrane, our findings are yet to be applied to an actual Alzheimer's patient. Although one may say that the data will not be significant because of the fact that we did not use an actual cell membrane, we have actually proven to produce the optimal conditions that are present in a real cell membrane, in the modeled version. Thus, while today, one cannot actually diagnose an Alzheimer's disease patient until after death, our data may be used to suggest that one in fact, can perhaps diagnose it. By extracting the spinal fluid of an individual, which contains fluid from the brain, one can search for aggregates and observe the formation of plaques of the Amyloid peptide if it is indeed present.

**References**

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