Detection of amyloid-β protein aggregate-induced membrane disruption
Research Paper
ABSTRACT

Alzheimer’s disease is an incurable and progressive neurodegenerative disease, which is characterized by extracellular amyloid-β protein plaques and intracellular tangles of hyperphosphorylated tau protein. The neurodegenerative disease affects the hippocampus and cerebral cortex of the brain related to memory and language. Currently, the only clinical diagnoses of this disease include neuron imaging techniques and tests to evaluate the patient’s abilities, yet confirmation of Alzheimer’s can only be done after death by an autopsy. The present problem we encounter is a knowledge deficit on the mechanism in the pathogenesis of Alzheimer’s disease, which makes it difficult to apply medical therapies accurately. The proposed hypothesis begins with the aggregation of amyloid-β protein in an insoluble conformation. The aggregates induce membrane disruption that results in deterioration of synaptic plasticity, dysfunction of calcium ion homeostasis, and apoptosis, leading to neurodegeneration. The purpose of this research is to contribute knowledge on the interaction of amyloid-β protein with the membrane. We will develop a dye leakage assay to determine whether amyloid-β disrupts the membrane. First, by developing a biomimetic model membrane system and evaluate its stability. The model membrane system will consist of porous silica microspheres (beads) loaded with fluorescein coated with a supported lipid bilayer. The stability of the system is evaluated using two controls. The first control is beads in Tris buffer, which does not disrupt the membrane. The second control is beads in 5% Triton X-100, which is a detergent that completely solubilizes the lipids destroying the membrane. Fluorescence readings of the supernatants of each control are taken every 30 minutes. Second, we will detect the effect of amyloid-β on this model membrane system. Amyloid-β will be added to the model membrane system and fluorescence readings will be taken of the supernatant every 30 minutes. Preliminary results have demonstrated a significant amount of dye leakage when using 5% Triton X-100, which gave fluorescence counts of about 2000. This system will then be adapted to detect Ca²⁺ leakage to create a better representation of the biological environment. Future work involves the development of a flow cytometry method for a high throughput screening of Ca²⁺ leakage.

A. BACKGROUND AND SIGNIFICANCE

Neurodegenerative diseases affect the neurons in the brain and cause impairment of a variety of cognitive, behavioral, and functional abilities. Alzheimer’s is one of the neurodegenerative diseases, which impacts the hippocampus and cerebral cortex of the human brain. This part of the brain is associated with language and memory. Figure 1 is diagram comparing a healthy brain next to an Alzheimer’s brain, where the structural damage can clearly be seen. The disease can be sporadic or inherited. It is characterized by intracellular tangles of tau protein and extracellular plaques of amyloid-β protein. According to the article, Alzheimer’s Disease, Alzheimer’s is the most common cause of dementia. It is considered the 6th leading cause of death overall ages and ranked 5th for those aged 65 years old and above, resulting from pneumonia, falls, or urinary tract infections (Dalvi, 2012). More than 25 million are affected and
Dementia care costs more than 300 billion, worldwide (Dalvi, 2012). These numbers are predicted to increase over time, unless new methods for treatment and diagnosis are developed.

Currently, there is no cure or direct diagnosis for Alzheimer’s disease. An autopsy is the only way to fully confirm Alzheimer’s. Doctors may use tests to evaluate a patient’s abilities, such as memory, and examine their medical records. Other times they will measure the protein content of the cerebrospinal fluid and investigate any aberrant increase (Dalvi, 2012). The author Dalvi describes the Mini-Mental Status Examination used to evaluate the severity of the disease depending on patient symptoms. According to Dalvi, there are several factors that can increase the chance of acquiring Alzheimer’s, which are: age, female sex, obesity, and gene mutations. Factors that reduce the risk are: exercise, educational level, good eating habits, and moderate alcohol consumption. Present day treatments are cholinesterase inhibitors and glutamate antagonists that are used to slow down the progression of the disease and prolong worsening of symptoms. Treatments under investigation are $\gamma$ and $\beta$ secretase inhibitors and amyloid-$\beta$ vaccinations. These treatments are focused on the proposed pathogenesis of Alzheimer’s disease.

Figure 1: Healthy brain and Alzheimer’s brain structure comparison. The size of an Alzheimer’s brain (right) patient shrinks due to tissue damage. The Hippocampus and cerebral cortex are associated with memory and language. [4]

Figure 2: Proposed mechanism involved in the pathogenesis of Alzheimer’s disease. Previous studies have shown that the mechanism involved in Alzheimer’s disease begins with the misfolding of amyloid-$\beta$ protein into insoluble $\beta$-sheet confirmation, which aggregate outside of neuron cells. These aggregates then cause dysfunction of Ca$^{2+}$ ion homeostasis leading to disregulation of signal pathways and apoptosis.
hypothesis that amyloid-\( \beta \) aggregation induces membrane disruption. The problem is that the mechanism in which this occurs has yet to be determined. Understanding this mechanism is crucial in the identification of new treatments and diagnostic methods because the disease could be handled at earlier stages before attaining any severe symptoms.

The proposed mechanism begins with the misfolding of amyloid-\( \beta \) protein into an insoluble \( \beta \)-sheet conformation, which then aggregates outside of the neurons. These aggregates induce membrane disruption that results in dysfunction of Ca\(^{2+} \) ion homeostasis, disregulation of signal pathways, and apoptosis, leading to Alzheimer\( \beta \) disease, shown in Figure 2. The author, of Unfolding the Role of Protein Misfolding in Neurodegenerative Diseases, introduces three hypotheses describing the mechanism for neuronal death. The first one is "the loss-of-function hypothesis (Soto, 2003)" where the loss of normal protein activity causes neurodegeneration. The second hypothesis, "gain-of-toxic-activity hypothesis (Soto, 2003)," states that the misfolding and aggregation of proteins results in toxicity inducing apoptosis. The third "brain inflammation hypothesis" suggests that protein aggregates can irritate the brain which causes chronic inflammatory reaction leading to apoptosis and synaptic changes (Soto, 2003). The proposed research will concentrate specifically on the ability of amyloid-\( \beta \) protein to disrupt the membrane by the development of a biosensor. This research will contribute insight into the pathogenesis mechanism of Alzheimer\( \beta \) disease, which will provide more knowledge to advance current therapies and diagnosis.

B. SPECIFIC AIMS

The goal of the proposed research is to develop a biomimetic model membrane system for the detection of amyloid-\( \beta \) protein aggregate-induced membrane disruption. Membrane disruption is one stage in the pathogenic mechanism of Alzheimer\( \beta \) disease that leads to dysfunction of Ca\(^{2+} \) ion homeostasis, disregulation of signal pathways, and apoptosis. The authors, of Neuronal calcium mishandling and the pathogenesis of Alzheimer’s disease, stated that the amyloid-\( \beta \) protein found in Alzheimer\( \beta \) patients can cause changes in the calcium signaling by increasing the calcium concentration inside the cell and exposes the neuron to excitotoxicity. This research will attempt to answer the question "Do amyloid-\( \beta \) aggregates cause dysfunction of Ca\(^{2+} \) ion homeostasis by inducing membrane disruption?" This biosensor will give us the capability to measure the permeability induced by amyloid-\( \beta \) protein by:

Specific Aim 1: develop a successful biomimetic model membrane system.

Specific Aim 2: detect membrane disruption and permeability by amyloid-\( \beta \) protein aggregates using fluorescence measurements.

C. EXPERIMENTAL DESIGN AND METHODS

C.1 Specific Aim 1: development of a successful biomimetic model membrane system.

To observe the interactions between amyloid-\( \beta \) protein and the membrane, we have to develop a biomimetic system. The process begins with the preparation of porous silica microspheres and a supported lipid bilayer. The article, Biosensors based on release of compounds upon disruption of lipid bilayers supported on porous microspheres, evaluates the
feasibility and stability of the model membrane system. The authors describe the various applications, for instance drug delivery or examination of biological interactions. They also provided information regarding the preparation of such a system. The model membrane system will consist of fluorescein loaded microspheres covered with a supported lipid bilayer.

First, fluorescein solution is prepared to have a 10 mM concentration. Fluorescein will be encapsulated inside the porous silica microspheres to measure the fluorescence when membrane disruption occurs. Tris buffer solution is prepared with Trizma base and sodium chloride. Hydrochloric acid is added to raise the pH to 7.4. Second, the preparation of the porous silica microspheres, we begin with cleaning the beads. The beads are cleaned with 10 mL solution of 4% ammonium hydroxide, 4% hydrogen peroxide, and the rest milliQ water. Ten samples are made with the microspheres solution in centrifuge tubes. The samples are centrifuged at 2000 rpm for 1 minute. The supernatant is decanted and replaced with milliQ water. This process is repeated until the supernatant is clear. Remove supernatant and add 10 mM fluorescein solution. The porous silica microspheres are suspended in the fluorescein solution for three days to assure that the beads are completely filled with dye. Preparation of the supported lipid bilayer begins with drying the Egg PC lipids in desiccator overnight. Use 21 μL of 25 mg/mL lipid/chloroform solution for every 25 mg of beads. They are then hydrated with 10 mM fluorescein and vortexed at 1450 for 30 minutes. Then, we sonicated for 30 minutes at 23 °C to make unilamellar vesicles. Lipid solution is then added to the centrifuge tubes containing the dye loaded beads. The samples are centrifuged and supernatant is decanted to remove fluorescein not encapsulated inside the microspheres by the lipid bilayer. This model system is a good representation of the cell membrane structure and curvature, shown in Figure 3.

The stability of the biomimetic system will be determined by using two controls. One control will simply be the fluorescein loaded silica microspheres coated by a supported lipid bilayer in Tris buffer solution. This control should experience a very low fluorescence. The second control will be the fluorescein loaded silica microspheres coated by a supported lipid bilayer in 5% Triton X-100, a detergent. This detergent has been shown to destroy the membrane by solubilizing the lipids. The fluorescence measurements of the supernatant are taken by the Spectramax M2 Platereader in 30 minute increments. The emission will be set to 520 nm and excitation to 488 nm. The samples are 100 μL each. Figure 4 visually depicts the two controls and sample. The sample being the fluorescein loaded lipid covered beads in a solution with amyloid-β protein.
C.2 Specific Aim 2: detect membrane disruption and permeability by amyloid-β protein aggregates using fluorescence measurements.

The sample will consist of fluorescein loaded porous silica microspheres coated by a supported lipid bilayer. It will be prepared with the same process as described in previous subsection. The difference is in preparation of the disrupting agent, in this case amyloid-β protein.

For the preparation of amyloid-β protein, we will follow Aβ purification protocol. The first step is to solubilize crude Aβ protein by passing it through an anti-static device and dissolving it in a solvent, containing 70% buffer with 0.1% TFA and 30% buffer with ACN with 0.1% TFA. Heat vial on hot plate at 70 °C and then make 1 mL aliquots of solution. Use HPLC for the purification of Aβ by running injection samples through size-exclusion chromatography column and collect sample at peak A. After collection of samples, HPLC is shut down and rotovap is used to evaporate the acetonitrile because lyophilizer cannot handle acetonitrile. The lyophilizer will be used to extract the water content in the Aβ protein solution. The sample should be allowed to dry for 24-48 hours. After lyophilization, the sample is weight and stored at -80 °C. The protein can now be added to the beads in buffer to test for membrane disruption by the same method used for the controls. After the addition of Aβ, we expect to see an increase in fluorescence, not as high as Triton X-100.

D. RESULTS AND DISCUSSION

The experiments for the first aim have been completed. We have successfully developed a stable model membrane system. We evaluated the feasibility of the system with two controls. The first control is the lipid bilayer covered fluorescein loaded beads in Tris buffer with a pH of 7.4 that displayed no membrane disruption. The second control consists of the lipid bilayer covered fluorescein loaded beads in 5% Triton X-100, which is a detergent, which demonstrated a significant amount of membrane disruption. Figure 5 clearly shows that control 1 is stable and control 2 has experienced complete membrane
disruption by examining the supernatant surrounding the beads. Also, the beads in control 1 have retained a bright orange color and in control 2 the beads are almost white. During the experiments fluorescence readings of the supernatant are taken every 30 minutes for two hours, shown on Figure 6. Control 1 (negative control) has fluorescence readings ranging between 40 – 140 counts. Control 2 (positive control) has fluorescence readings ranging from 1900 – 2500 counts, which is almost ten times as much as control 1. The 5% Triton X-100 has solubilized the lipids allowing the fluorescein dye to go into the supernatant giving it a light orange color. The fluorescein dye used has a 10mM concentration that alone gives about 2000 counts. We predict that in an environment containing amyloid-β protein the fluorescence readings will be between the two controls causing a significant amount of damage to the model membrane system.

E. CONCLUSIONS AND FUTURE WORK

Furthermore, the biomimetic model membrane system is a stable scaffold that will provide information for the evaluation of membrane disruption of amyloid-β protein by fluorescence measurements. This research will provide insight into the mechanism for the pathogenesis of Alzheimer’s disease to accurately treat the disease and diagnosis. The identification of this mechanism may also share similarities with other neurodegenerative diseases and abnormal compound associated diseases, such as diabetes. This system can then be used to model the effect of amyloid-β protein in Ca²⁺ ion homeostasis by encapsulating a calcium sensitive dye and measuring the permeability using fluorescence readings. This would be a more accurate

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Figure 6: Graph displaying the fluorescent profiles of the beads in the two control conditions. The baseline is Tris buffer with pH of 7.4. The negative control (control 1) consists of beads in Tris buffer, displays 0% dye leakage. The positive control (control 2) is beads in 5% Triton X-100, which demonstrates 100% dye leakage.
depiction of the cell environment because calcium is a primary molecule involved in signaling cascades. This system could also be used to examine the effect of different lipid compositions. After demonstrating amyloid-β aggregation induced membrane disruption, we can evolve the system into a flow cytometry method that will allow for the detecting of lower Aβ concentrations. This research will contribute to a variety of fields and technologies.
REFERENCES


