2006

Influence of surface chemistry on the aggregation of beta amyloid peptide

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INFLUENCE OF SURFACE CHEMISTRY ON THE AGGREGATION OF BETA AMYLOID PEPTIDE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Chemistry

by

Mariah Joan Wooldridge McMasters
B.S., Arkansas State University, 1999
August 2006
Dedication

This dissertation is dedicated to my dad

Hal Wooldridge

May 16, 1945-July 14, 2004
Acknowledgements

First, I must thank my husband, Anthony, for supporting me throughout our marriage and my graduate career. I know it has not been easy, especially when I was gone for weeks on end, but I appreciate you so much. I know that often other people thought we were crazy for doing some of the things we have done, but I am glad we did it. I don’t know if I would have finished if it weren’t for you prodding me every day, so even though I hated it at the time, thank you.

I also owe a great deal to my mom, Joan Wooldridge. Thank you for instilling in me a love of learning and for always encouraging me to do my best in whatever I tried. I know it was hard for you to let me go, but I appreciate the freedom you have given me to pursue my dreams.

I also thank my sister, Marcia Wooldridge. I appreciate all the love and support you have given me. You have always been there when I needed someone to vent to and when I needed a sounding board. I am grateful for all the cards and emails you have sent me.

I also want to thank my in-laws, Jim and Adena McMasters, and all the rest of the McMasters clan: Carmon, Shelli, Christina, Jon, Victoria, Tim, Tonya, Doug, Cody, Dave, and Randy. Thank you for supporting our move to Louisiana and for praying for us every step of the way.

I owe a great deal to my advisor, Dr. Robin McCarley. Without your guidance, none of this would be possible. Thank you for letting me do some nontraditional things in my studies. I appreciate your guidance and our discussions. Thank you especially for editing all my papers and my dissertation.
I would also like to thank Dr. Paul Russo for his vision in writing the IGERT grant. I greatly appreciate the opportunities I have had through IGERT. Thanks also go to Dr. Robert Hammer for allowing me to work on the Alzheimer’s project. I appreciate the useful discussions we have had. I also thank Dr. Yu-Tai Tao for opening his lab to me during my stay at Academia Sinica in Taiwan. Dr. Mark Delong, thank you for supervising me during my internship at GE Plastics. I learned a lot from you.

I am also grateful to the McCarley research group for the camaraderie and support you have all given me. Thanks go to Dr. Bikas Vaidya and Jed Aucoin for training me and helping me get started in my research. To Amy Morara, Becky Brauch, and Alison Smith, you were all special people during my stay at LSU. I am glad I had great friends to go to lunch with.

I want to thank my extended family in Arkansas, especially Aunt Brenda. Thank you for giving me a job throughout college and for writing recommendation letters for me. Wendy, Terry, Chelsea, Becky, Tommy, Conner, and Brooke, thank you for dropping everything to see me when I came home to visit. I wish I was there all the time.

Thanks to my Louisiana family, especially Aunt Esther, Lori, Gary, Amanda, Melissa Grace, Leah, Wes and Melissa. Thank you for adopting me into your family. You have made my time here a lot brighter and less lonely.

I also want to give thanks to those who guided me along my path to graduate school. To my high school chemistry teacher, Mr. Mike Woodside, thank you for introducing me to chemistry and giving me an excitement for it. Mrs. Leola Atchison, thank you for teaching me to write. Without you, this dissertation would not have chapters, much less paragraphs. Thanks to Dr. Michael Panigot for introducing me to
chemistry research, and to Dr. Gary Emmert for encouraging me to go to graduate school somewhere other than ASU. Even though it was hard to move, I am glad I broadened my horizons.

Finally I would like to thank my funding agencies, the National Institutes of Health National Institute on Aging (AG17983), the National Science Foundation (CHE-0108961), the National Science Foundation Division of Graduate Education (9987603) for an IGERT Fellowship and the National Science Foundation Office of International Science and Engineering (03-608) for an East Asia and Pacific Summer Institute Fellowship.
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List of Abbreviations

Acoustooptic modulator ................................................................. AOM
Alpha ......................................................................................................... $\alpha$
Amino Acid Analysis ........................................................................... AAA
Amplitude lifetime ............................................................................... $\tau_a$
Atomic force microscopy ................................................................. AFM
Beta ........................................................................................................ $\beta$
Beta amyloid ....................................................................................... $\alpha$\beta
Beta amyloid precursor protein ...................................................... APP
Boltzmann constant .......................................................................... $k_B$
Bovine serum albumin ........................................................................ BSA
Chi .......................................................................................................... $\chi$
Coherence lifetime .............................................................................. $\tau_c$
Diffusion coefficient ........................................................................... D
Effective lifetime .................................................................................. $\tau$
Fluorescence photobleaching recovery ............................................ FPR
Fourier self-deconvolution ............................................................... FSD
Fourier transform ................................................................................ FT
Frictional force ..................................................................................... F
Full width at half height line width value ........................................... FWHH
Gamma .................................................................................................... $\Gamma$
Grating constant ................................................................................ K
Hydrodynamic radius .......................................................................... $R_h$
Root mean squared................................................................. RMS
Scanning force microscopy.................................................... SFM
Self-assembled monolayer.................................................... SAM
Solvent viscosity ................................................................. \( \eta \)
Time .................................................................................... \( t \)
Voltage.................................................................................. \( V \)
Abstract

Alzheimer's disease is a progressive neurological ailment affecting 4.5 million aging Americans. The disease is characterized by the presence in the brain of self-assembled fibrils consisting of beta amyloid protein (Aβ). Soluble Aβ protein is present in normal human cerebrospinal fluid, but it is unclear what makes the protein aggregate into insoluble plaques. There is evidence that the Aβ fibril assembly is affected by interactions with biological surfaces, such as neuronal membranes. Here, surfaces consisting of self-assembled alkanethiol monolayers with different end groups were used to test the effect of surface chemistry on the structure and morphology of aggregates formed from the Aβ peptide. Reflection-absorption infrared spectroscopy and scanning force microscopy (SFM) were used to examine the interactions of the protein with the monolayers. It was found that the surfaces have a seeding effect on the Aβ protein in solution and can actually induce aggregation of the Aβ protein over time. The outcomes are important, because the work described here is the first attempt at relating the chemical makeup of supported, model monolayer surfaces and their propensity to interact with Aβ peptide in solution. Patterned surfaces consisting of fouling and non-fouling monolayers were constructed to determine how these different areas would affect the deposition and aggregation of the Aβ protein. It appeared that the Aβ protein adsorbed onto the ethylene glycol even though the ethylene glycol-terminated monolayers alone were previously seen to be non-fouling. This was important because it appears that the fouling monolayer can induce a change in conformation that allows the protein to stick to what are normally non-fouling surfaces.
Mica-supported lipid bilayers consisting of 1-Palmitoyl-2-Oleoyl-\textit{sn}-Glycero-3-Phosphocholine (POPC) and 1-Oleoyl-2-\textit{[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn}-Glycero-3-Phosphocholine (NBD-PC) were also used as a simple cell membrane model system. Fluorescence photobleaching recovery and SFM were used to determine the effects of association of the fibrils with the bilayers. It was found that the $\text{A}\beta$ peptide affected the fluidity of the lipid bilayers and inserted itself into the lipid bilayer. This experiment was important because it offers information about the insertion of the $\text{A}\beta$ peptide into cell membranes that could potentially be toxic to cells.
1.1 Research Goals and Aims

The goal of this research project is to understand the effects of surface chemistry on the aggregation of beta amyloid (Aβ) peptide in solution using self-assembled monolayers and lipid bilayers as models of biological surfaces. This is an important research area because the Aβ peptide is directly linked to Alzheimer’s disease. Surfaces of self-assembled, ω-substituted alkanethiol monolayers supported on Au were used to test the influence of different types of chemical moieties on Aβ peptide aggregation. Aβ peptide in solution was incubated with the monolayers to determine the possible impact of the monolayers on the aggregation of Aβ. Reflection-absorption infrared spectroscopy was used to provide information on the deposition of Aβ peptide onto the monolayers and the conformation of the protein deposits. Scanning force microscopy was used to obtain additional information about the morphology of Aβ aggregates. Patterned, self-assembled monolayers consisting of two different types of chemical end groups were used to determine the effects of the combination on the Aβ peptide aggregation. Lipid bilayer surfaces were also incubated with Aβ peptide solutions to determine any possible interactions between the two entities. Fluorescence photobleaching recovery and fluorescence microscopy were used to evaluate the effect of Aβ protein on the lipid bilayers. The specific aims of this work included:

- Determine which monolayers adsorb Aβ peptide
- Verify the conformation of the adsorbed Aβ peptide
- Determine the extent of Aβ aggregation and aggregate size
• Establish whether the monolayers have a seeding affect on the aggregation of Aβ peptide in solution
• Determine whether patterned monolayer surfaces consisting of two different alkanethiols promote deposition and aggregation of the Aβ peptide differently than from the single-constituent monolayers
• Ascertain the effect that Aβ protein has on the fluidity of the lipid bilayers.

1.2 Research Synopsis

The purpose of this research plan is to discover the effects of surface chemistry on the aggregation of beta amyloid (Aβ) peptide, the protein that is the hallmark of Alzheimer’s disease. It is not precisely known why Aβ peptide aggregates in vivo, but it is speculated that it coincides with the environment surrounding the Aβ peptide in the human body as shown in Figure 1.1. Surfaces of supported self-assembled monolayers with chemically different end groups were used to test this theory. It was determined that the deposition of Aβ peptide onto the monolayers and subsequent aggregation corresponded to the type of end groups on the monolayers and the

![Figure 1.1. Synopsis and motivation of research.](image-url)
properties, such as the hydrophobicity or hydrophilicity, of the end groups. It was also determined whether Aβ peptide has an affinity for certain functional groups on the surfaces and what the conformation of the adsorbed Aβ peptide was. The strategy to understanding this problem was to fabricate surfaces and let Aβ protein incubate on them. Initial analysis by reflection-absorption infrared spectroscopy was done to reveal possible deposition of Aβ peptide to the surface and the conformation of the peptide. Subsequent analysis by scanning force microscopy was done to determine the size and extent of aggregation. Combinations of different types of monolayers patterned on the same surface were also analyzed. Lipid bilayer surfaces were also used as a simplified cell membrane model. Aβ protein solution was incubated on the fluorescent-tagged lipid bilayers to determine the effect that the Aβ protein would have on the lipid bilayer fluidity, which was analyzed with fluorescence photobleaching recovery. This study gave information about possible insertion of the Aβ protein into the lipid bilayer, which could provide knowledge about the toxicity of the Aβ peptide. Fluorescence microscopy and scanning force microscopy were employed to image the Aβ peptide on the lipid bilayers.

1.3 Background

1.3.1 History of Alzheimer’s Disease

Alzheimer’s disease is a neurological ailment characterized by plaques and neurofibrillary tangles in the brains of humans. Alois Alzheimer first noted the disease in 1907.¹ Scientists estimate that around 4.5 million people in the US and 24 million people worldwide have AD.² The disease outwardly manifests itself with disorientation, memory loss, mental regression, and eventually death of the human. Autopsies on the brains of humans afflicted with Alzheimer’s disease reveal neurodegeneration, atrophy of the frontal, temporal, and parietal cortex, and extracellular deposition of fibrillar structures, called amyloid plaques, in the cerebral
vasculature. These amyloid plaques are thought to be a contributor to the pathogenesis of Alzheimer’s disease, and there is a direct correlation between the amount of amyloid plaque and the severity of the symptoms of the disease. The deposits were originally isolated and characterized by amino acid analysis in 1984. The major component of these ordered protein aggregates is the Aβ peptide.

1.3.2 Beta Amyloid Precursor Protein

The genesis of the amyloid deposits have been the focus of a large amount of research effort. The Aβ peptide originates from a group of 695-770 amino acid residues called the beta amyloid precursor protein (APP), a normal transmembrane glycoprotein that is generated by the endoplasmic reticulum of the cell. APP is normally found in brain cell membranes and the functions of APP include maintaining connections between brain cell, aiding in the growth and repair of brain cells and promoting cell adhesion. The soluble Aβ peptide is produced during normal cellular metabolism when the APP is cleaved. There are two different pathways by which the APP is cleaved to form the Aβ peptide. In the exocytotic processing path, the APP undergoes normal proteolytic cleavage by α-secretase as it is released from the cell membrane. In the endocytotic route, the APP is cleaved within the cell membrane by β- and γ-secretase. This proteolytic processing forms the Aβ peptide.

1.3.3 Beta Amyloid Peptide in the Body

Aβ peptide is an amphiphilic peptide made up of 39-43 amino acid residues with a hydrophilic N-terminus and a hydrophobic C-terminus. The Aβ protein has an assortment of physiological functions. In humans, these roles include modulating synaptic function, aiding in neuronal growth and survival, shielding against oxidative damage of cerebrospinal fluid and plasma lipoproteins, and surveillance against neuroactive compounds and toxins. The Aβ
peptide is commonly found in the cerebrospinal fluid and plasma of normal humans in the soluble form.\textsuperscript{14-16} Due to unknown causes, the $A\beta$ peptide folds into $\beta$-sheet structures in the brain of Alzheimer’s disease victims which lead to the formation of insoluble aggregates and fibrils\textsuperscript{17,18} that are toxic to neuronal cells.\textsuperscript{19,20}

1.3.4 Effects of Beta Amyloid Plaques

Insoluble $A\beta$ protein has a detrimental effect on the human brain. The $A\beta$ aggregates have been shown to alter the mitochondrion of brain cells, which, in turn, causes swelling and inflammation of the brain.\textsuperscript{21} It has been demonstrated that the organized $A\beta$ structures with $\beta$-sheet conformation disrupt cell membranes and cause homeostasis malfunctions and the interruption of cellular signals.\textsuperscript{7,22,23} $A\beta$ plaques also destroy synapse by inducing oxidative damage to the synapse membrane.\textsuperscript{24} Neurons are also damaged when the $A\beta$ structures form ion channels that allow for the uptake of calcium which causes instability in the normal cellular homeostasis.\textsuperscript{25} All of these processes combine and lead to degradation of the brain, mental dementia, and death of the human.

1.3.5 Surface-Induced Aggregation of Beta Amyloid Peptide

There is evidence that $A\beta$ fibril assembly is dependent on interactions with biological surfaces, such as membranes.\textsuperscript{26-28} It has been shown that the presence of negatively charged lipid vesicles in $A\beta$ solutions shifts the normal random coil conformation of $A\beta$ peptide to a $\beta$-sheet conformation under conditions of low ionic strength.\textsuperscript{26,27} This is due to the electrostatic binding of $A\beta$ peptide onto the lipid membrane surface and subsequent aggregation of the peptide.\textsuperscript{28} The use of solid state $^{31}$P NMR and circular dichroism in solution have demonstrated that the composition of lipid bilayers in vesicles (fraction of charged and uncharged lipids) has a profound impact on the ability of the vesicle-based lipid bilayers to extract the $A\beta$ peptide from
solution and increase is local concentration in the lipid bilayer and initiate random-coil to β-sheet conformational changes of monomeric Aβ₁₋₄₀ in aqueous solution. Consequently, it is possible that some type of moiety on the cell surface triggers the folding of Aβ peptide and subsequent plaque formation. Therefore, it is important to investigate the influence of surface chemistry on the aggregation of the Aβ peptide.

1.3.6 Self-Assembled Monolayers as Model Surfaces

A model system was chosen to determine the possible effects of surface chemistry on the aggregation of Aβ peptide. Self-assembled monolayers (SAMs) consisting of chemically modified alkanethiol chains on gold substrates were chosen to mimic these biological surfaces. These model surfaces were used to determine the effect of surface chemistry on the binding and nucleation of the Aβ peptide. The SAMs imitate small unilamellar lipid vesicles and form a stable, ordered surface in which the end groups of the monolayer can be modified. It is proposed that the different end groups of the alkanethiol chains will have an effect on the extent and speed of aggregation of the Aβ peptide. Studies of the deposition of the peptide onto the different monolayer surfaces have been conducted. Reflection-absorption infrared spectroscopy (RAIRS) and scanning force microscopy (SFM) were used to analyze deposition of the Aβ protein on the monolayer surfaces. The effects of the monolayers on the Aβ protein in solution were also analyzed.

1.3.7 Properties of Self-Assembled Monolayers

Several ω-substituted alkanethiol monolayers on Au were investigated, such as monolayers with methyl (HS-(CH₂)₁₇-CH₃), alcohol (HS-(CH₂)₁₁-OH), carboxylic acid (HS-(CH₂)₁₅-COOH), fluorine (HS-(CH₂)₂-(CF₂)₇-CF₃), and sulfonic acid (HS-(CH₂)₁₀-SO₃H) functionalities. Gold was chosen as the supporting substrate because it possesses a clean,
ordered surface that allows for the orderly formation of subsequent monolayers. The surface of the gold is arranged in a (111) cubic lattice formation. The packing order and angle of attachment of the monomer units of the monolayer depend on the structure of the monomer chain, the interactions between monomer nearest neighbors, and interactions between the monomer units and the lattice surface of the gold. The angle of attachment of the monolayers to the surface is approximately 30° with respect to the normal. The thickness of the monolayers ranges from 10-25 Å depending on the chain length and the angle of attachment. The chains that are more bulky, such as the carboxylic acid chains and the sulfonic acid chains, have higher angles of attachment with respect to the normal and are less thick when compared to unsubstituted alkanethiols with the same chain length. The fluorinated monolayer forms a hexagonal lattice with approximately 5.8 Å distance between each chain. The methyl monolayer is also in a hexagonal lattice but has a spacing of 5.0 Å between the chains because the unsubstituted alkanethiol chains are less bulky than the fluorinated alkanethiol monolayer. The carboxylic acid monolayer forms a double row configuration on the Au substrate.

1.3.8 Relationship of Self-Assembled Monolayers to Biological Surfaces

Self-assembled monolayers were chosen because of the wide varieties of surface functionalities available that correspond to different biological moieties found in the human brain. The carboxylic acid- and alcohol-terminated functionalities produce hydrophilic monolayers on Au that are highly polar and have high surface energies. They are used to model the phospholipid bilayers found in cell membranes with hydrogen bonding capabilities. The unsubstituted alkanethiol produces a hydrophobic, nonpolar monolayer with a low surface energy, which can also be used to model cell membranes. In addition, the hydrophilic sulfonic acid-terminated monolayer is similar to heparin from a charged functional group point of view,
because the heparin molecule has sulfonic acid functionalities along the protein backbone.\textsuperscript{35} It has been shown that heparin interacts with the Aβ peptide.\textsuperscript{36-38} Finally, the fluoro-methyl monolayer is a very hydrophobic, Teflon-like molecule that is analogous to the apolar side of the cell membrane; this is an important parallel because it is thought that when the amyloid precursor protein is cleaved within the membrane the toxic species of Aβ is produced.\textsuperscript{39, 40}

1.3.9 Patterned Self-assembled Monolayers

Microcontact printing of alkanethiols was developed by the Whitesides group at Harvard in 1993.\textsuperscript{41} The process is a type of soft lithography that involves using a stamp, usually polydimethyl siloxane (PDMS), to transfer an alkanethiol pattern to a metal surface followed by backfilling the bare areas by solution deposition of a different ω-terminated alkanethiol solution. This method is commonly used to study the interaction of proteins at interfaces and to immobilize proteins for various types of detection.

PDMS is a preferred polymer for stamps because it is a durable material that can be reused many times. It also offers a chemically inert, flexible surface with low surface free energy.\textsuperscript{42} The stamp is “inked” by applying a drop of alkanethiol solution to the stamp, allowing the alkanethiol to absorb into the PDMS for a brief amount of time and then drying the stamp with nitrogen. Drying the stamp prevents smearing of the pattern and preserves the arrangement of the microcontact printed alkanethiol by reducing the diffusion effects that occur after the imprinting is done. Using a dry stamp circumvents this process by printing the vapor phase molecules trapped in the stamp.\textsuperscript{42} Even though the stamp is dried, ethanol and alkanethiol are still present on and in the stamp after drying the stamp because the small amounts of the solution are absorbed into the bulk part of the poly siloxane matrix. The amount of alkanethiol present in
the stamp reflects the permeability of the PDMS stamp and the structure of the alkanethiol and solvent.\textsuperscript{43}

The inherent process of autophobic pinning also helps keep the pattern clean by reducing diffusion of the alkanethiol. Autophobic pinning occurs when a liquid in contact with a surface alters the chemistry of this surface and lowers its solid-vapor surface tension and solid-liquid surface tension, and the liquid edge retracts automatically back to the shape of the stamp.\textsuperscript{44} The formation of a self-assembled monolayer on gold by autophobic pinning is exothermic.\textsuperscript{45} Initially the alkanethiols are disordered on the gold surface, and as the alkanethiols find their places on the gold lattice the monolayer becomes ordered. Once the ordered monolayer forms, the drop edge of the monolayer spontaneously retracts to the original shape of the stamp.

### 1.3.10 Ethylene Glycol-Terminated Alkanethiols

Poly(ethylene glycol) has been used since the early 1980s.\textsuperscript{46} Poly(ethylene glycol) is well known to be protein resistant. The steric repulsion model can explain this protein resistance. As the protein moves toward the surface of the poly(ethylene glycol), the poly(ethylene glycol) chains collapse which causes steric repulsion with the protein.\textsuperscript{47} This process is applicable for long polyethylene glycol chains. For shorter chains of oligo(ethylene glycol) monolayers, protein resistance is contributed to the water at the protein-oligo(ethylene glycol) interface.\textsuperscript{48} This is known as the water barrier theory. The water molecules are tightly bound to the oligo(ethylene glycol) surface such that the protein cannot adsorb to the oligo(ethylene glycol). Self-assembled monolayers with two repeat units of ethylene glycol do adsorb protein due to a more densely packed monolayer, which prevents the water attachment to the ethylene glycol units.\textsuperscript{49} Oligo(ethylene glycol) chains with six monomer repeat units seem to be the most protein resistant in all assembly solvents because the oligo(ethylene glycol)
monomer has a lower packing density, which allows the water to fully surround the ethylene glycol chain, thereby preventing adsorption of hydrophilic proteins.

Although ethylene glycol end groups are usually known to be non-fouling, a few examples in the literature show protein to be attracted to ethylene glycol functionalities. Leckband et al. discovered that if streptavidin was forced into contact with a poly(ethylene glycol) chain, there existed reasonably strong attractive forces between the protein and the poly(ethylene glycol) moiety.\textsuperscript{50} It has also been shown that mucin, glycoproteins that line the gastrointestinal tract, adhere weakly to poly(ethylene glycol).\textsuperscript{51} Ethylene glycol surfaces that have fouling properties can arise by increasing the compression rates or temperature of the system, or by varying the polymer molecular weight.\textsuperscript{52}

1.3.11 Lipid Bilayers as Model Surfaces

Mica-supported lipid bilayers consisting of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC) (Figure 1.2) were used as a simplified cell membrane model system. Lipid bilayers are physiologically significant because phospholipids are principle components of cellular membranes and POPC is one of the main elements of mammalian cell membranes.\textsuperscript{53, 54} These model surfaces were used to determine the effect of the A\textsubscript{β} peptide on the fluidity of the lipid bilayers. Cell membranes in the body are fluid to allow for the exchange of vital substance, such as nutrients and waste, into and out of the cell. This experiment is important because it gives information about the insertion and binding of the A\textsubscript{β} peptide into cell membranes that could disrupt this cellular homeostasis, which would be potentially toxic to cells.
1.3.12 Interaction of Beta Amyloid Protein with Lipid Membranes

Previous studies have shown that Aβ protein interacts with lipid bilayers and cell membranes. Aβ peptide alters the fluidity of cell membranes such as synaptosomal plasma and mitochondrial membranes as shown by polarized fluorescence microscopy.\textsuperscript{55} It has also been shown that the presence of Aβ peptide aggregates causes a decrease in fluidity of POPC bilayers as inferred by changes in anisotropy of the bilayer.\textsuperscript{56} Solution NMR and circular dichroism have also been used to track the interaction of Aβ peptide in lipid environments. These studies demonstrated a conformation change from α-helical monomer to β-sheet fibrils that was induced by molecular structural characteristics of the membrane.\textsuperscript{57} Solid-state NMR confirmed that Aβ peptide binds to the lipid vesicles and X-ray spectroscopy has shown that Aβ peptide inserts into the lipid bilayers.\textsuperscript{58, 59}

There is evidence that the interaction of Aβ peptide with lipid vesicles is a result of electrostatic interactions. Aβ peptide inserts into normal phospholipid bilayers, but when they
are compressed the Aβ peptide is expelled completely from zwitterionic bilayers, such as 1,2-
dipalmitoylphosphatidylcholine (DPPC), while the Aβ peptide remains inserted in the negatively
charged bilayers, such as 1,2-dipalmitoylphosphatidylglycerol (DPPG). It was further
determined that the DPPG promotes seeding and aggregation of the Aβ peptide on the lipid
bilayer, while DPPC and dipalmitoyl trimethyl ammonium propane (DPTAP), a positively-
charged lipid, inhibit the aggregation of Aβ peptide.

It was first reported that cell membranes in the presence of Aβ peptide showed defects in
their surfaces. This led to further investigations regarding the interactions of cell membranes
and model lipid bilayers with Aβ peptide. It was next determined that the gangliosides-bound
Aβ peptide in the lipid bilayers experienced a conformation change from random coil structure to
β-sheet structure. It was later found that upon binding to cell membranes containing
gangliosides, the Aβ peptide showed seeding capabilities that promoted further aggregation of
the Aβ peptide. It was initially proposed that the interaction of the Aβ peptide with lipids did
not rely on charge-charge interactions, but were a result of the hydrophobic areas of the Aβ
peptide interacting with the hydrophobic core of the lipid bilayers. It was also found that the
presence of cholesterol in the lipid membranes attracts the Aβ peptide and helps immobilize the
Aβ peptide on the lipid membrane, which promotes the fibrillogensis of the Aβ peptide. The
use of 31P magic angle spinning NMR demonstrated that in addition to the initial hydrophobic
interaction of the Aβ peptide with the lipid bilayer there exists an electrostatic component to the
interaction. Solid state 31P NMR was used to further confirm that the adsorption of Aβ peptide
to lipid membrane is due to electrostatic mechanisms by demonstrating that the Aβ peptide fuses
with lipid membranes containing different charged headgroups. In addition to disrupting the
membrane fluidity, the Aβ peptide also generates the leakage of the contents of lipid vesicles;
which, in turn, could be correlated to the neurotoxicity of the Aβ peptide. Further studies comparing the type of Aβ peptide that interacts with the lipid bilayers found that the fibrillar Aβ peptide greatly reduced the fluidity of the lipid bilayers, while the monomeric, or unaggregated, Aβ peptide had a much smaller effect on the fluidity of the lipid bilayers. With the discovery that the soluble Aβ peptide oligomers were the most likely toxic species of protein, research shifted its focus towards analyzing the smaller Aβ peptide aggregates. It was determined that the oligomeric Aβ peptide changed the molecular structure of the lipid membranes which induced a conformational change of the Aβ peptide from the α-helix conformation to the toxic β-sheet conformation.

1.3.13 Techniques Used to Verify Protein Deposition and Aggregation

Reflection-absorption infrared spectroscopy (RAIRS) was chosen to analyze the interaction between the supported ω-substituted alkanethiol monolayers and the monomeric Aβ peptide. Infrared spectroscopy is a good tool for detecting proteins on surfaces because of the prominence of the amide I bands at roughly 1600 cm⁻¹ and the amide II bands at roughly 1500 cm⁻¹. The position of these bands also reveals information about the conformation of the peptide. The positions of the amide I and amide II bands for alpha helix conformation, random coil conformation, and beta structures of peptides are summarized in Table 1.1.

RAIRS is performed using p-polarized infrared radiation with the electric field oriented normal to the surface at a high (grazing) angle of incidence. At the high angle, the intensity of the infrared is optimized so the sub-micromolar quantities of chemical species can be detected. The transmission of the radiation into the Au layer creates a surface standing wave electric field that interacts with the vibrational modes of the adsorbates. The adsorbates must have a component of their dipole transition oriented normal to the surface, otherwise the infrared
radiation will not be absorbed. Curve-fitting deconvolution of the spectra was used to resolve overlapped peaks to determine the secondary structure of the protein. Quantification of the amounts of the various secondary structures cannot be performed due to the selection rules that apply for RAIRS.

**Table 1.1. Position of amide I and amide II IR bands for different protein conformations.**

<table>
<thead>
<tr>
<th>Conformation</th>
<th>Amide I peak position (cm⁻¹)</th>
<th>Amide II peak position (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta sheet conformation</td>
<td>1625-1640, 1665-1695</td>
<td>1530-1535</td>
</tr>
<tr>
<td>Random coil conformation</td>
<td>1650-1655</td>
<td>1545-1550</td>
</tr>
<tr>
<td>Alpha helix conformation</td>
<td>1645-1650</td>
<td>1548-1553</td>
</tr>
</tbody>
</table>

Scanning force microscopy (SFM) is a useful technique that can provide a variety of information regarding topography of surfaces. It was chosen to image alkanethiol monolayers and lipid bilayers upon exposure to Aβ protein to obtain information about the size of protein species on the different surfaces. SFM will also be used to understand the physical appearance of the aggregates. When analyzing the patterned monolayers, frictional SFM will be used to determine the position of the different chemical functionalities on the monolayers.

Fluorescence photobleaching recovery (FPR) was also chosen to evaluate the effect of Aβ protein on the lipid bilayers. FPR is a valuable technique that can be used to provide information regarding the diffusion coefficient and the fluidity of the lipid bilayers. Fluorescence microscopy and in situ SFM was used in conjunction with FPR to image the interaction of the Aβ protein with the lipid bilayers.

All of the aforementioned techniques are further described in Chapter 2.
1.4 References


Chapter 2

Materials and Methods

2.1 Chemicals and Products

Beta amyloid 10-35 [cat # 03-153, lot 0315302, BioSource, 79% peptide content], bovine serum albumin [CAS # 9048-46-8, Sigma, 96%], HS-(CH\(_2\))\(_{17}\)-CH\(_3\) [CAS # 2885-00-9, Fluka, 95%], HS-(CH\(_2\))\(_{11}\)-OH [CAS # 73768-94-2, Aldrich, 97%], HS-(CH\(_2\))\(_{11}\)-(OCH\(_2\)CH\(_2\))\(_3\)-OH [cat # TH002-02, lot A2709, ProChimia], 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine in chloroform [cat # 850457C, lot 160-181PC-138, Avanti Polar Lipids, Inc.], 1-oleoyl-2-[12-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl] -sn-glycero-3-phosphocholine in chloroform [cat # 810133C, lot F181-120NBD-24, Avanti Polar Lipids, Inc.], potassium hydroxide [CAS # 1310-58-3, Aldrich, 99.99%], phosphoric acid [CAS # 7664-38-2, Aldrich, 99.99%], and absolute ethanol [AAPER Alcohol & Chemical Company] were used as received. HS-(CH\(_2\))\(_{2}\)-(CF\(_2\))\(_7\)-CF\(_3\) was a gift from Marc Porter at Iowa State University. The HS-(CH\(_2\))\(_{15}\)-COOH and HS-(CH\(_2\))\(_{10}\)-SO\(_3\)H were synthesized previously.\(^1,2\) The poly(dimethyl siloxane) stamp was a gift from Yu-Tai Tao at Academia Sinicia in Taipei, Taiwan. Nanopure water was obtained by passing distilled water through a Barnstead reverse osmosis filter and then through a Nanopure water system.

2.2 Methodologies

2.2.1 Gold Substrate Formation

Gold substrates for infrared studies were prepared using 1” x 3” glass substrates. The slides were soaked in isopropanol for 30 minutes and then sonicated in isopropanol for 30 minutes. The slides were then rinsed with Nanopure water followed by an absolute ethanol rinse. The slides were dried with house nitrogen (liquid nitrogen boil-off) and placed in an
Edwards Auto 306 Vacuum Coater. Deposition of 15 nm chromium was performed at $3.0 \times 10^{-7}$ Torr followed by 200 nm gold at $1.0 \times 10^{-7}$ Torr. After removal from the evaporator, the substrates were immediately put in ethanol and purged with N$_2$. The gold substrates for scanning force microscopy studies were made using freshly cleaved mica with a deposition of 2000 Å gold. The gold on mica was then annealed at 400 °C for four hours under atmospheric conditions. The gold substrates were subsequently cleaned with UV light and then placed in ethanol.

2.2.2 Monolayer Formation

Au substrates were rinsed with ethanol and placed in 500 μM solutions of HS-(CH$_2$)$_{17}$-CH$_3$, HS-(CH$_2$)$_{11}$-OH, HS-(CH$_2$)$_2$-(CF$_2$)$_7$-CF$_3$, HS-(CH$_2$)$_{15}$-COOH, HS-(CH$_2$)$_{10}$-SO$_3$H, or HS-(CH$_2$)$_{11}$-(OCH$_2$CH$_2$)$_3$-OH for a minimum of one hour. After this self-assembly process, the monolayers on Au were rinsed with ethanol and dried under a stream of N$_2$. The monolayers on Au were prepared immediately before they were used in experiments.

2.2.3 Patterned Monolayer Formation

Patterning of monolayers was achieved via microcontact printing.$^3$ A poly(dimethyl siloxane), PDMS, stamp with relief lines ranging from 1μm to 10 μm was used. HS-(CH$_2$)$_{17}$-CH$_3$ solution (1 mM in ethanol) was applied to the stamp and allowed to absorb for 30 seconds. The stamp was then dried with N$_2$ and immediately placed on an Au substrate. Gentle pressure was initially applied to ensure complete contact. After three minutes, the stamp was removed from the Au, and the Au/patterned monolayer was rinsed with ethanol to remove unbound thiols from the surface. The sample was then placed in a 1 mM ethylene glycol-terminated solution to allow self-assembly of the ethylene glycol-terminated alkanethiol in the bare areas to completely fill in the pattern.
2.2.4 Lipid Bilayer Formation

Phospholipid vesicles were prepared via the Morrissey Lab Protocol, yielding a 2 mg/mL concentration. Briefly, 1.9 mg POPC and 0.1 mg NBD-PC in chloroform was dispensed into a 13 mm X 100 mm Pyrex test tube. The lipids were dried under N₂ until the chloroform was removed by evaporation. To remove any residual chloroform, the lipids were high vacuumed for 60 minutes. A 2-mL aliquot of pH 7.4, 500 mM phosphate buffer was added to the lipids and the resulting solution was allowed to sit at room temperature for 60 minutes after which the solution was vortexed to resuspend the lipids. Two methods of forming small, unilamellar vesicles were tested. In the first, the lipid solution was sonicated with a micro-tip Branson Sonifier 450 for five minutes to yield a suspension of small unilamellar vesicles. In the second method, an extruder was used to form the small unilamellar vesicles. The extrusion method was found to be superior for forming uniform vesicles.

Figure 2.1 Schematic of vesicle fusion on a hydrophilic surface.
Lipid bilayers were formed via spontaneous lipid fusion. Briefly, when lipid vesicles come in contact with a hydrophilic surface, they spontaneously reorder to form lipid bilayers as depicted in Figure 2.1.\textsuperscript{5} As the vesicle approaches the surface, electrostatic attractions between the hydrophilic surface and the vesicle cause the vesicle to spread out onto the surface until the vesicle burst and flattens out onto the surface to form a lipid bilayer. The vesicle solution was applied with a pipette onto freshly cleaved mica and allowed to incubate. After 30 minutes, the mica was rinsed with phosphate buffer. The resulting lipid bilayers were kept moist to prevent disassembly of the bilayers. The formation of the lipid bilayers was confirmed using height scanning force microscopy.

2.2.5 Beta Amyloid Solution Preparation

Beta amyloid (10-35) solutions were prepared using 15 mM phosphate buffer (no ionic strength) and 15 mM PBS/150 mM NaF for experiments at pH 7.4, and 10 mM KOH solution for experiments at pH 11.5. Lyophilized Aβ\textsubscript{10-35} peptide was weighed out into a polypropylene micro centrifuge tube [cat # RN2000-GMT, Dot Scientific]. The peptide was then dissolved in one of the above solutions by vortex to give a final peptide concentration of 100 \( \mu \)M. Peptide concentration was confirmed using amino acid analysis.

2.2.6 Amino Acid Analysis

Peptide concentration of the solutions applied to the supported alkanethiol monolayers and the supernatant solutions from the alkanethiol monolayers were determined by amino acid analysis of peptide solution hydrolysates. Amino acid content was determined by chromatographic separation with a Dionex 6550 utilizing an anion exchange column and NaOH gradient, with an electrochemical detector (Dionex ED50). The peptide solution was hydrolyzed and then injected (4 replicates), and the amino acid content of the individual residues was
compared to known amounts of standard, such as norleucine. The average value from the 4 replicates was recorded, and it was found that the standard deviation was ±0.01 μM.

2.2.7 Sample Incubation of Beta Amyloid Protein on Supported Alkanethiol Monolayers

For experiments involving the deposition of αβ₁₀⁻₃₅ peptide on alkanethiol monolayers, αβ₁₀⁻₃₅ peptide solution was applied onto the monolayers supported on Au to sufficiently cover the top of the sample, and then the solution-covered monolayers on Au were placed into a container under humidified N₂ (Figure 2.2). The container consisted of a polypropylene jar (Nalgene, cat # 2118-0016/500ml); the cover was drilled twice and septa were installed. An 18-gauge needle was placed in one of the two septa for exhaust; the other septum had a needle connected to a continuous humidified nitrogen purge. Evaporation of the solution was not noticed. Samples for RAIRS and SFM were prepared simultaneously and incubated in the same container. The Aβ solution was allowed to incubate at room temperature on the monolayers for 15 h. The Aβ solutions on the HS-(CH₂)₁₁-OH, HS-(CH₂)₂-(CF₂)₇-CF₃, and HS-(CH₂)₁₁-(OCH₂CH₂)₃-OH monolayers were allowed to incubate additionally for 7 days, 20 days, and 40 days. Controls consisted of the same solutions made without the Aβ peptide. After incubation,
the monolayers were rinsed with the protein-free KOH solution, dried under a stream of N₂, and subsequently evaluated with reflection-absorption infrared spectroscopy and scanning force microscopy. Two samples were analyzed for each monolayer to verify reproducibility.

2.2.8 Preparation of Beta Amyloid Samples for Analysis of Seeding Effects on Alkanethiol Monolayers

To analyze the HS-(CH₂)₁₁-OH, HS-(CH₂)₂-(CF₂)₇-CF₃, and HS-(CH₂)₁₁-(OCH₂CH₂)₃-OH monolayers for seeding effects, a separate approach was used. The 100 μM Aβ peptide solutions in ~10 mM KOH at pH 11.5 were prepared as described above. The high pH inhibits the aggregation of the protein in solution, so the possibility of surface-induced aggregation can be evaluated. In this scenario, the Aβ peptide solution was incubated on the monolayers for 15 h and 7 days. After the incubation period, the supernatant from the monolayers was removed and spotted onto a mica substrate to allow for adsorption of any solution-phase protein to the mica. After five minutes, the solution was rinsed away with Nanopure water, and the mica was then dried under a stream of N₂. The resulting sample was analyzed by tapping-mode scanning force microscopy under ambient conditions. The control sample, which consisted of the Aβ solution in a polypropylene microcentrifuge tube, was prepared and analyzed the same way.

2.3 Instrumentation and Theory

2.3.1 Reflection-Absorption Infrared Spectroscopy

All reflection-absorption infrared spectroscopy (RAIRS) was performed with a Thermo Nicolet Nexus FT-IR model 670 ESP with a MCT (mercury-cadmium-telluride) detector. The instrument was equipped with a Versatile Reflection Attachment with Retro-Mirror Accessory (Harrick), in order to perform external reflection measurements. RAIRS was carried out using p-polarized light at a grazing incident angle of 86° with respect to the surface normal of the sample. Samples were placed on a highly reflective gold substrate. Spectra were obtained using 512
scans at a resolution of 4 cm\(^{-1}\). The purge time, to remove possible contamination from water and carbon dioxide vapor, was approximately 9 minutes. Baseline corrections and subtractions for water and carbon dioxide were preformed using Omnic ESP Version 5.2a software. Deconvolution of spectra was performed using GRAMS/32 software that utilizes a Fourier deconvolution algorithm with a resolution enhancement of 1.4 and a Bessel smoothing factor of 85\%. All spectra were placed on a common scale for comparison. Bare gold, prepared by UV-ozone cleaning immediately before use, under the same experimental setup as the samples, was used as the reference. The monolayers on Au exposed to both the KOH solution and the \(A\beta_{10-35}\) in KOH were analyzed separately.

2.3.2 Theory of Reflection-Absorption Infrared Spectroscopy

RAIRS, which has become a useful tool for analysis of chemically modified surfaces\(^9\), was first described in 1966 by Greenler, but it was not widely used until the mid-1980s when the method was combined with commercial FTIR spectrometers\(^{10, 11}\). Infrared spectroscopy works by inducing a molecular vibration in a molecule. Energy is absorbed when the frequency of the radiation matches the frequency of the vibration and the absorb energy results in a peak in the infrared spectrum.

The instrumental setup for the spectrometer is as follows: an IR source produces an infrared beam that passes through a Michelson interferometer, which produces an interferogram that allows multiple vibrational frequencies to be monitored simultaneously. The Michelson interferometer uses a beamsplitter that divides the infrared beam into two beams. One beam is reflected off a stationary mirror, while the other beam is reflected off a moving mirror. Both beams are combined at the beamsplitter. Because one mirror is moving the signal that is produced when the two beams recombine has a constructive and destructive interference pattern.
This is called an interferogram and encodes all frequencies into one beam. A HeNe laser is employed as an internal wavelength calibration standard. The laser is aligned with the IR source and passes through the interferometer with the IR beam and monitors the position of the moving mirror. The wavenumber scale of an interferometer originates from this information. After leaving the interferometer, the IR beam is aimed at a sample on a highly reflective surface. The IR beam is positioned at a grazing angle to the surface normal where the infrared radiation interacts with the sample. The resulting signal is then reflected toward a detector where it is measured. A mathematical Fourier transform is then performed on the signal and an infrared spectrum is produced. The spectrum of the sample is compared with the peaks corresponding to the vibrational frequencies of the molecules in the sample to the spectrum of the same system with no absorption in the thin layer.

The intensity of the infrared radiation reflected from a metal substrate depends upon a combination of the angle of incidence of the infrared light and the state of polarization of the light. Incident infrared radiation (the light from the source) combines with the infrared radiation reflected from the substrate to create a standing wave electric field. The conditions that produce a standing wave with the highest amplitude are the most desirable. For light polarized perpendicular to the plane of incident light (s-polarized) the phase shift remains near to 180° for all angles of incidence. The addition of the incident vector and reflected vector for the s-polarized beam cancel each other out almost completely. Thus, the resulting standing wave electric field has a very low intensity and there should be little absorption of infrared light from a substrate with reflection of light s-polarized infrared light. For infrared radiation that is polarized parallel to the plane of incident light (p-polarized), the phase shift changes rapidly, especially at angles that are high relative to the normal. The incident and reflected standing
wave vectors at the surface of the substrate are oriented in the same direction and the addition of the vectors doubles the intensity of the standing wave. Therefore, the optimal conditions are that the infrared light should be $p$-polarized and the incident beam should be oriented at a high (grazing) angle to the normal of the surface. At the high angle, the intensity of the infrared is optimized so the sub-micromolar quantities of chemical species can be detected. The resultant standing wave electric field vector for $p$-polarized radiation is predominantly perpendicular to the metal surface, which means that those molecular vibrations whose change in electric dipole is parallel to the surface will be much less strongly excited.

Another factor affecting the interaction of the IR beam with the sample is the surface selection rule, which concerns how the molecules must be oriented in relation to the metal surface. The metal surface contains free electrons. When a molecule is adsorbed on a substrate, the molecule generates opposite image charges in the electrons in the metal substrate. If the molecule is oriented parallel to the surface, then the dipole moments of the molecule and the image charges of the substrate cancel each other out. When the molecule is adsorbed perpendicular to the surface, the dipole moment of the molecule and the resulting image charges of the substrate are oriented in the same direction, which gives rise to a dipole moment twice what would be expected. Therefore, only the dipole moments oriented perpendicular to the surface will be observed in the vibrational spectrum.\textsuperscript{9,12} Quantification of the amounts of the various secondary structures on a surface cannot be performed due to the selection rules that apply for RAIRS.\textsuperscript{9}

Infrared spectroscopy is especially helpful for analyzing proteins because of the prominence of the amide I bands at roughly 1600 cm\textsuperscript{-1} due to C=O and C-N stretching and the amide II bands at roughly 1500 cm\textsuperscript{-1} that occur because of N-H bending and C-N stretching.\textsuperscript{13}
The position of these spectral bands also reveals information about the conformation of the peptide. The characteristic amide I frequencies are due to distinctive electron densities in the amide C=O groups. These electron densities are produced by the differences in the length and direction of hydrogen bonds that give rise to differences in the strength of the hydrogen bond for various conformations of the protein. When the amide C=O group is engaged in a strong hydrogen bond, the electron density in the C=O group will be lower, and an amide I absorption band will appear lower. The strong intramolecular hydrogen bonding that stabilizes the β-sheet conformation is responsible for the low amide I frequencies for that type of conformation. The β-sheet conformation is also characterized by a split-pattern amide I band composed of a higher frequency component. This high frequency component occurs because of the sterically constrained non-hydrogen-bonded amide C=O groups within β-turns. The hydrogen bonds form between approximately every fourth amino acid in the α-helix conformation, which are longer and thus weaker than those formed in a normal β-sheet conformation, so the amide I frequencies will be increased. A protein with a random coil conformation has the weakest hydrogen bonding, so a higher amide I frequency will be present. The positions of peaks for alpha-helix conformation, random-coil conformation, and beta structures of peptides are summarized in Table 2.1.

Table 2.1. Position of IR peaks for secondary protein conformations.

<table>
<thead>
<tr>
<th></th>
<th>Amide I band position (cm⁻¹)</th>
<th>Amide II band position (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta sheet conformation</td>
<td>1625-1640, 1665-1695</td>
<td>1530-1535</td>
</tr>
<tr>
<td>Random coil conformation</td>
<td>1650-1655</td>
<td>1545-1550</td>
</tr>
<tr>
<td>Alpha helix conformation</td>
<td>1645-1650</td>
<td>1548-1553</td>
</tr>
</tbody>
</table>
2.3.3 Scanning Force Microscopy

A Digital Instruments Nanoscope III scanning probe microscope controller was used to perform all scanning force microscopy (SFM). A J-type piezoelectric scanner was used that is capable of acquiring a scan size of up to 125 μm x 125 μm. Noncontact silicon cantilevers [cat # NSC-15, MicroMasch] were used to obtain the images in tapping mode. These tips had a radius of curvature of 10 nm and a tip height of 15-20 μm, as stated by the manufacturer. In situ scanning force microscopy and lateral force microscopy (LFM) employed silicon nitride cantilevers [cat # NP-S, Veeco]. The silicon nitride tips had a radius of curvature of 10 nm and a tip height of 2.5-3.5 μm, as stated by the manufacturer.

Samples were attached to magnetic pucks using adhesive. Data for ex situ samples were taken at room temperature under ambient conditions. In situ experiments were performed under deionized water (18 MΩ cm) or phosphate buffer solution. Multiple scans from separate areas were taken for each sample, and the results shown are representative of the entire sample.

After capturing the images, an image flatten method was executed. In the height images shown, the brightest areas in the image are the tallest features. In the force images, the brightest areas are the areas that exert the greatest frictional force.

2.3.4 Theory of Scanning Force Microscopy

The scanning force microscope apparatus was developed in 1986 by G. Binnig, C. F. Quate, and C. Gerber. The instrument, also known as the atomic force microscope, is depicted in Figure 2.3. Briefly, the SFM operates by measuring attractive and repulsive forces between a cantilever tip and a sample. The sample is moved under the cantilever tip by a tube scanner operated by piezoelectric action. As the cantilever moves over the surface, a laser reflecting off the tip of the cantilever measures the vertical motion of the tip. The reflected laser
is detected by a photodiode which is divided into four quadrants. The photodiode converts the
signal into topographical information about the sample.

Three types of scanning force microscopy were used: tapping mode SFM, contact mode
SFM, and lateral force SFM, which is a variation of contact mode SFM. Tapping mode SFM
utilizes an oscillating cantilever that taps the sample surface during scanning. Constant
oscillation amplitude is maintained. As the cantilever comes in contact with the surface, the tip
is deflected, which subsequently shifts the position of the laser beam on the photodiode. The
photodiode translates the reflected laser beam into information regarding the vertical height of
the sample surface.

![Figure 2.3 Schematic of Scanning Force Microscope.](image-url)
Contact mode SFM operates by observing the change in cantilever deflection when scanning a cantilever across the sample surface. A constant deflection force is sustained between the cantilever and the sample by vertically moving the scanner, so that the force between the tip and sample stays steady. The photodiode determines the vertical distance that the scanner moves by measuring the difference of the laser position between the top two and bottom two quadrants of the photodiode.

Lateral force microscopy (LFM) measures the frictional force of the sample and is used in conjunction with contact mode SFM. As the sample is moved across the cantilever tip, an additional twisting motion of the cantilever caused by the differences in friction is measured. This twisting motion also changes the position of the laser on the photodiode between the right and left quadrants of the photodiode. As the cantilever scans across high-friction areas, the cantilever is torqued more than when scanning across low-friction areas. The frictional force can be described by the following equation:

\[ F = \alpha (V - V_0) \]  

Equation 2.1

where F is the frictional force, V is the voltage signal due to the deflection in the lateral direction, \( V_0 \) is the voltage signal of the undeflected tip with no frictional force, and \( \alpha \) conversion factor which is assumed to be constant.

The interaction of the probe tip and the chemical functionalities in each region of the patterned monolayer results in different voltage signals. The differences in the voltage signal are translated into variation in pixel brightness on the image of the surface. Areas with a higher friction produce brighter pixels in the LFM image. In the experiments involving the deposition of A\( \beta_{10-35} \) peptide on patterned alkanethiol monolayers, the probe tip experienced a greater frictional force in the areas terminated by the ethylene glycol than in the areas covered in the
unsubstituted alkanethiol monolayer. This occurred because the ethylene glycol-terminated monolayer has a higher surface energy than that of the unsubstituted alkanethiol monolayer.21

2.3.5 Fluorescence Photobleaching Recovery Spectroscopy

The Fluorescence Photobleaching Recovery (FPR) apparatus is shown in Figure 2.4.22 The main element of the FPR instrument is an Olympus BH2 epifluorescence microscope with the illuminator assembly adapted to allow illumination by a light source. A Lexel EXCEL 3000 argon ion laser capable of producing 2 W at 488 nm is used. The laser beam is passed through an acoustooptic modulator (AOM) [cat # 35085, Newport Research] driven by a modified radio-frequency source [cat # 31085-6DS, Newport Research]. The first-order diffracted beam, about 85% of the laser output, is used for photobleaching. A movable coarse diffraction grating (50, 100, 150, and 300 lines/inch), referred to as a Ronchi ruling, located at the rear focal plane of the

Figure 2.4 Schematic of Fluorescence Photobleaching Recovery Spectroscopy Apparatus.
microscope objective is used to produce a striped pattern in the beam. The patterned beam is
deflected by a dichroic mirror and focused by the microscope objective (4X, 7X, 10X, and 18X)
onto the stage, where it can bleach a fluorescently labeled sample. A photomultiplier tube
(PMT) [cat # 7265, RCA] receives the fluorescence emitted from the sample. An 846HP shutter
[cat # 846HP, Newport Research] remains closed during the photobleaching pulse to protect the
PMT. The PMT produces an intensity readout that is sent to a computer where it is translated
into information regarding the diffusion of the sample.

2.3.6 Theory of Fluorescence Photobleaching Recovery Spectroscopy

Fluorescence photobleaching recovery is a beneficial technique that can be used to
determine the physical characteristics of large molecules from their diffusion properties. In
brief, a laser is used to print a pattern onto a fluorescently labeled sample. The pattern begins to
fade and the fluorescence recovery is monitored as the bleached molecules diffuse into the
unbleached areas of the sample. The FPR apparatus is arranged such that either a spot or a
fringed pattern is bleached onto a sample. In the following experiments, a fringed pattern is
used. The fringe pattern is acquired by using a Ronchi ruling located at the rear focal plane of
the microscope objective lens. First, the Ronchi ruling is used as a photomask and the sample is
bleached with a laser. After bleaching with a laser, a striped image replicating the fringed
pattern of the Ronchi ruling is imprinted in the sample. The period of the striped pattern on the
sample is related to the Ronchi ruling constant, \( K = \frac{2\pi}{L} \), where \( L \) is the period of the repeat
pattern in the sample. During the fluorescence recovery phase, the Ronchi ruling is shifted at a
constant rate through the laser. This results in a period component in the fluorescence emission
of the sample as the periodic light field falls into and out of phase with the pattern that was
bleached into the sample.\(^{23}\) Fluorescence recovery is measured as a function of ac voltage,
which corresponds to the contrast of the pattern. The ac voltage decays exponentially at a rate proportional to the diffusion coefficient:

$$\text{ac volts} = \exp(-\Gamma t) \quad \text{Equation 2.2}$$

where $t$ is the time since the photobleach, $\Gamma=DK^2$ where $D$ is the tracer self-diffusion coefficient, and $K$ is the grating constant, $K=2\pi/L$, where $L$ is the period of the repeat pattern. The diffusion coefficient is obtained from the slope of $\Gamma$ versus $K^2$ plot. Size information can also be obtained via the Stokes-Einstein equation:

$$D_s=k_B\Gamma/(6\pi\eta R_h) \quad \text{Equation 2.3}$$

Where $k_B$ is the Boltzmann constant, $\eta$ is the solvent viscosity $R_h$ is the hydrodynamic radius. The shape of the molecule is assumed to be spherical.

2.4 References


Chapter 3

Surface-Induced Aggregation of Beta Amyloid Peptide

3.1 Introduction

Soluble beta amyloid (Aβ) protein is normally found in the human brain, but due to unknown causes, the Aβ peptide folds into β-sheet structures in the brain which, in turn, lead to the formation of insoluble aggregates and fibrils. These aggregates are toxic to neuronal cells. There is evidence that Aβ fibril assembly is effected by interactions with biological surfaces, such as membranes, therefore we propose that the physiological environment of the Aβ peptide may contribute to the formation of the amyloid plaques that are present in the brains of Alzheimer’s patients.

The goal of the work described here is a better understanding of the effects of surface chemistry on the aggregation of Aβ peptide. Surfaces composed of self-assembled, ω-substituted alkanethiol monolayers on Au were used to investigate their influence on the aggregation of Aβ peptide. Several different ω-substituted alkanethiol monolayers on Au were used, such as those terminated with methyl (HS-(CH₂)₁₇-CH₃), alcohol (HS-(CH₂)₁₁-OH), carboxylic acid (HS-(CH₂)₁₅-COOH), fluoro-methyl (HS-(CH₂)₂-(CF₂)₇-CF₃), sulfonic acid (HS-(CH₂)₁₀-SO₃H), and ethylene glycol (HS-(CH₂)₁₁-(OCH₂CH₂)₃-OH) functionalities. These model surfaces were used to determine the effect of surface chemistry on the deposition and aggregation of Aβ peptide.

Reflection-absorption infrared spectroscopy (RAIRS) and scanning force microscopy (SFM) were chosen to evaluate the conformation and topography of Aβ structures on the
monolayers. Infrared spectroscopy is a powerful tool for analyzing the conformation of proteins because the positions of the amide I and amide II bands are indicative of the environment of the amide carboxyl and N-H moieties. Scanning force microscopy allows for high-resolution imaging of the aggregation behavior of the Aβ protein on the monolayers, specifically the type and size of aggregates.

3.2 Surface-Induced Beta Amyloid Peptide Aggregation – Reflection-Absorption Infrared Spectroscopy Studies

To evaluate the ability of the monolayers to induce aggregation only on their surfaces, Aβ solutions with a high pH were used. The high pH inhibits the aggregation of Aβ peptide in solution so that the possible effects of the surface on aggregation can be evaluated. From RAIRS data of various alkanethiol monolayers on Au exposed to N₂-purged KOH solutions for times up to 40 days (with no protein present), there is no indication of any changes in monolayer structure.

RAIRS is a potent methodology for determining the secondary structural nature of proteins in various environments. The structure of proteins can be correlated with the positions of the amide I (C=O stretch, 1600-1700 cm⁻¹) and the amide II (C-N stretch and N-H deformation, 1530-1560 cm⁻¹) band frequencies. The β-sheet conformations are uniquely distinguished by a split-pattern amide I band composed of a lower frequency component, 1625-1640 cm⁻¹, and a less intense, higher frequency component at 1685-1695 cm⁻¹. In addition, the β-sheet conformation exhibits an amide II transition between 1530 and 1535 cm⁻¹. Another component of β-structure, namely β-turns, possess bands in the amide I region that are located between 1660 and 1680 cm⁻¹; the bands in the amide II region overlap with those of the β-sheet structure. It is common for β-structures to have more that one band present in each region. Random coil conformations are characterized by an amide I band in the 1650-1655 cm⁻¹ region.
and an amide II band in the 1545-1550 cm\(^{-1}\) region. \(\alpha\)-Helical conformations result in amide I transitions centered near 1645-1650 cm\(^{-1}\), as well as an amide II band between 1548 and 1553 cm\(^{-1}\). Low frequency amide I bands in the 1600-1625 cm\(^{-1}\) region can be attributed to side chain vibrations of the protein.\(^{20}\)

![Reflection-absorption infrared spectra of \(\alpha\)-substituted alkanethiol monolayers on Au exposed to aqueous solutions of 100 \(\mu\)M \(A\beta_{10-35}\) in 10 mM KOH (pH 11.5) for 15 h. RAIRS experiments clearly indicate that all of the monolayers on Au investigated here, except the \(\text{HS-(CH}_2\text{)}_{11}-(\text{OCH}_2\text{CH}_2\text{)}_3\text{-OH}\) monolayer, cause deposition of the \(A\beta\) peptide, as evidenced by observation of amide I and amide II bands for the surfaces studied within a 15-h incubation period. Results are displayed on a common scale in Figure 3.1 for the 15-h incubation period. Curve fitting band deconvolution was performed on each spectrum to resolve the overlapped peaks that sometimes occur when two protein conformations are present.\(^{21}\)

Figure 3.1. Reflection-absorption infrared spectra of \(\alpha\)-substituted alkanethiol monolayers on Au exposed to aqueous solutions of 100 \(\mu\)M \(A\beta_{10-35}\) in 10 mM KOH (pH 11.5) for 15 h.
results are displayed in Tables 3.1 and 3.2, with the reported values being for the center of each band. Curve fitted spectra are provided in Appendix A. Spectra of the various thiol monolayers on Au exposed to the KOH solutions for a 15-h incubation period (with no protein present) are provided in Appendix A.

Examination of the spectrum for the methyl-terminated monolayer exposed to Aβ solutions for 15 h revealed bands in the amide I and amide II regions that are attributed to the adsorption, not absorption, of Aβ peptide. Because no observable change in the peak maxima positions for the symmetric (ν_S, CH) and asymmetric (ν_A, CH) C-H stretches of the alkane moiety of the monolayer were noted, it appears that no absorption of the peptide occurred. If the protein were to penetrate the alkanethiol chains (absorption), disordering of the monolayer chains would be expected, leading to changes in the band positions of ν_S (CH) and ν_A (CH). It is concluded that adsorption is the most significant route in the deposition of Aβ on the monolayer-modified surfaces.

Amide I bands resolved by deconvolution of the original spectra were centered at 1632 cm\(^{-1}\), 1695 cm\(^{-1}\), and 1681 cm\(^{-1}\) (Table 3.1), which indicate a β-sheet conformation; at 1666 cm\(^{-1}\), which indicates a β-turn conformation; and at 1649 cm\(^{-1}\), which indicates an α-helix conformation. The presence of both β-structures and α-helix conformations is supported by the position of the amide II bands at 1536 cm\(^{-1}\) and 1553 cm\(^{-1}\) (Table 3.2) for the protein deposits. Quantification of the amounts of the various secondary structures cannot be performed due to the selection rules that apply for RAIRS.

Based on similar evaluations of the spectra, it is found that the sulfonic acid-, carboxylic acid-, alcohol-, and trifluoromethyl-terminated monolayers all cause adsorption/deposition of Aβ peptide on their surfaces. In addition, all of these surfaces are covered with protein possessing β-
sheet and \(\beta\)-turn, random coil, and \(\alpha\)-helical conformations. It is important to note that the ethylene glycol-terminated monolayers\textsuperscript{24, 25} do not exhibit IR spectra characteristic of A\(\beta\) peptide adsorption, for times up to 40 days, which was the limit of our study.

**Table 3.1. Amide I band positions for the deconvoluted RAIR spectra of monolayers exposed to 100 \(\mu\)M A\(\beta\)\textsubscript{10-35} in 10 mM KOH (pH 11.5) for 15 h.**

<table>
<thead>
<tr>
<th>Monolayer system</th>
<th>Band positions for deconvoluted amide I transitions in Figure 3.1 (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\beta)-sheet</td>
</tr>
<tr>
<td>HS-(CH(<em>2))(</em>{17})-CH(_3)</td>
<td>1632, 1695, 1681</td>
</tr>
<tr>
<td>HS-(CH(<em>2))(</em>{11})-OH</td>
<td>1636, 1687</td>
</tr>
<tr>
<td>HS-(CH(<em>2))(</em>{2})-(CF(_2))(_7)-CF(_3)</td>
<td>1634, 1698, 1690</td>
</tr>
<tr>
<td>HS-(CH(<em>2))(</em>{15})-COOH</td>
<td>1632, 1693</td>
</tr>
<tr>
<td>HS-(CH(<em>2))(</em>{10})-SO(_3)H</td>
<td>1638, 1689, 1681</td>
</tr>
</tbody>
</table>

**Table 3.2. Amide II band positions for the deconvoluted RAIR spectra of monolayers exposed to 100 \(\mu\)M A\(\beta\)\textsubscript{10-35} in 10 mM KOH (pH 11.5) for 15 h.**

<table>
<thead>
<tr>
<th>Monolayer system</th>
<th>Band position for deconvoluted amide II transitions in Figure 3.1 (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\beta)-structures</td>
</tr>
<tr>
<td>HS-(CH(<em>2))(</em>{17})-CH(_3)</td>
<td>1536</td>
</tr>
<tr>
<td>HS-(CH(<em>2))(</em>{11})-OH</td>
<td>1539</td>
</tr>
<tr>
<td>HS-(CH(<em>2))(</em>{2})-(CF(_2))(_7)-CF(_3)</td>
<td>1539</td>
</tr>
<tr>
<td>HS-(CH(<em>2))(</em>{15})-COOH</td>
<td>1537</td>
</tr>
<tr>
<td>HS-(CH(<em>2))(</em>{10})-SO(_3)H</td>
<td>1534</td>
</tr>
</tbody>
</table>

Further analysis of the fluorinated monolayer with longer incubation times revealed that the protein conformations present changed slightly over a period of 20 days (Figure 3.2), with a minor shift from \(\beta\)-structure towards \(\alpha\)-helical conformations. Curve fitting band deconvolution
was performed on each spectrum in Figure 3.2 to resolve overlapped peaks. These results are displayed in Table 3.3, with the values shown being for the center of each band. Curve fitted spectra are provided in Appendix 1.

![Reflection-absorption infrared spectra](image)

**Figure 3.2.** Reflection-absorption infrared spectra of CF$_3$-(CF$_2$)$_7$-(CH$_2$)$_2$-SH/Au exposed to aqueous solutions of 100 $\mu$M A$\beta_{10-35}$ in 10 mM KOH (pH 11.5) for 15 h, 7 days, and 20 days.

**Table 3.3.** Band positions for the deconvoluted RAIRS spectra of CF$_3$-(CF$_2$)$_7$-(CH$_2$)$_2$-SH/Au exposed to 100 $\mu$M A$\beta_{10-35}$ in 10 mM KOH (pH 11.5) for 15 h, 7 days, 20 days.

<table>
<thead>
<tr>
<th>Exposure time</th>
<th>Band positions for deconvoluted amide I transitions in Figure 3.2</th>
<th>Band positions for deconvoluted amide II transitions in Figure 3.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$-sheet</td>
<td>$\beta$-turn</td>
</tr>
<tr>
<td>15 h</td>
<td>1634, 1698, 1690</td>
<td>1682, 1674, 1660</td>
</tr>
<tr>
<td>7 days</td>
<td>1637, 1689, 1681</td>
<td>1670</td>
</tr>
<tr>
<td>20 days</td>
<td>1633, 1680</td>
<td>1662</td>
</tr>
</tbody>
</table>
The position of the amide I bands remained stable for the fluorinated monolayer, indicating the presence of β-structures for incubation times of 15 h, 7 days, and 20 days. Additionally, the Aβ peptide incubated on the fluorinated monolayer for 7 days had a random coil conformation indicated by the band centered at 1654 cm\(^{-1}\). The Aβ peptide incubated on the fluorinated monolayer for 20 days had an α-helix conformation in addition to the β-structure conformation. Intramolecular hydrogen bonding can explain the presence of the α-helix conformation. Intramolecular hydrogen bonding is promoted after a protein is adsorbed onto a hydrophobic surface because it is unlikely that the protein will form hydrogen bonds with the monolayer since the monolayer is terminated with fluorocarbon endgroups.\(^{26}\) When compared on a common scale, the intensities of the amide I and amide II bands increased over time, an observation that indicates that the monolayer had more Aβ peptide deposited on it as time progressed. Since the dipole moment of the C=O group (amide I band) is oriented orthogonally to that of the C-N group (amide II band) on the protein and both band intensities increase, it can be concluded that this increase is due to a growth in the amount of peptide on the surface and not just a change in orientation.

3.3 Surface-Induced Beta Amyloid Peptide Aggregation – Scanning Force Microscopy Studies

The fluorinated monolayer was chosen for in-depth SFM studies due to the observation in the RAIRS studies that the CF\(_3\)-layer accumulated more Aβ than the other surfaces as a function of time. SFM inspection of the fluorinated monolayer was made at 15 h, 7 days, and 20 days of exposure to the Aβ solution; the results are displayed in Figure 3.3. In Figure 3.3A is shown the SFM image of the CF\(_3\) monolayer on Au exposed to KOH for 15 h demonstrating the absence of any topographical features other than those associated with the monolayer-covered Au surface.
Figure 3.3. 2 μm x 2 μm tapping mode scanning force micrographs of CF₃-(CF₂)₇-(CH₂)₂-\text{SH}/Au exposed to: A. 10 mM KOH for 20 days; B. 100 μM \text{Aβ10-35} (in 10 mM KOH, pH 11.5) for 15 h, C. 7 days, and D. 20 days. Z-range=50 nm in A-D.
After 15 h of exposure to the Aβ solution, rod-like aggregates approximately 150 nm in length and 25 nm in height had formed (Figure 3.3B) on the fluorinated monolayer. By 7 days of exposure, the aggregates of Aβ peptide were observed to be roughly 300 nm in length and 50 nm in height (Figure 3.3C). Aggregation length and height for the largest particles had reached 450 nm and 50 nm by day 20 (Figure 3.3D). The length of individual aggregates was judged to be their greatest lateral dimension. SFM can be used to give only partial information about the length of the aggregates because tip-sample convolution can limit resolution.²⁷

The SFM results are in agreement with the findings of the time-dependent RAIRS work. Both studies indicate that the Aβ peptide continues to deposit on the monolayer with time, and the RAIRS data allow it to be concluded that the types of peptide conformations present do not change significantly with increased exposure time. SFM confirms that more Aβ peptide is adsorbed to the surface of the monolayer as exposure time is increased.

### 3.4 Seeding Effects of the Monolayers

An additional question to be addressed is whether Aβ aggregates that are adsorbed to the monolayer surfaces dissociate from the monolayer and enter solution. If the aggregates do dissociate from the monolayers, it is expected that they would act as seeds for initiating aggregation of Aβ protein in the solution.²⁸ A comparison between the supernatant from the fluorinated, alcohol, and ethylene glycol monolayers and a control, which consisted of the Aβ solution in a polypropylene microcentrifuge tube, was conducted using scanning force microscopy. A high-pH solution was used to retard aggregates from forming. Multiple SFM scans from various areas on the samples were performed, and the results shown are representative of the entire sample surfaces.
The results from the control, which consisted of Aβ peptide solution from a polypropylene vial that was subsequently spotted onto mica for analysis, are displayed in Figure 3.4A-B. After a 15-h incubation period, the solution had formed very few aggregates, Figure 3.4A. The aggregates that had formed were approximately 40 nm in length and 25 nm in height. Following an incubation of 7 days, the aggregates were larger, with lengths ranging from 75 to 100 nm and heights of approximately 40 nm, Figure 3.4B.

Figure 3.4. 10 μm x 10 μm tapping mode scanning force micrographs of 100 μM Aβ10-35 peptide in 10 mM KOH solution at pH 11.5 from a vial incubated at room temperature for A. 15 h and B. 7 days. For experiments, 50 μL of solution was spotted on mica, allowed to sit for 5 minutes, and then the resulting surface was rinsed with 18 MΩ-cm water and dried. Z-range=50 nm.

After a 15-h incubation of the Aβ solution with the CF3 monolayer, the Aβ protein had formed many aggregates in solution approximately 40 nm in length and 25 nm in height. Following a 7-day incubation with the CF3-layer, the Aβ aggregates were larger, with lengths ranging from 75 to 100 nm and heights of approximately 40 nm, Figure 3.5A.
In order to probe the effects of the monolayer on the production of the \( \text{A}\beta \) aggregates in solution, a study involving the alcohol monolayer was pursued. After a 15-h incubation of the Au-supported, alcohol-terminated alkanethiol with the \( \text{A}\beta \) solution, protein aggregates approximately 60 nm in length and 25 nm in height were observed (data not shown). After 7 days of incubation, the \( \text{A}\beta \) protein had formed larger aggregates approximately 50 nm in height and 100 to 135 nm in length (Figure 3.5B).

Figure 3.5. 10 \( \mu \text{m} \times 10 \mu\text{m} \) tapping mode scanning force micrographs of 100 \( \mu\text{M} \) \( \text{A}\beta_{10-35} \) peptide in 10 mM KOH solution at pH 11.5 from various experiments. A. Supernatant from the \( \text{CF}_3-(\text{CF}_2)_7-(\text{CH}_2)_2-\text{SH}/\text{Au} \) monolayer exposed to 100 \( \mu\text{M} \) \( \text{A}\beta_{10-35} \) peptide in 10 mM KOH for 7 days. B. Supernatant from the \( \text{HO}-(\text{CH}_2)_{11}-\text{SH}/\text{Au} \) monolayer exposed to 100 \( \mu\text{M} \) \( \text{A}\beta_{10-35} \) peptide in 10 mM KOH for 7 days. For experiments, 50 \( \mu\text{L} \) of supernatant was spotted on mica, allowed to sit for 5 minutes, and then the resulting surface was rinsed with 18 M\( \Omega \)-cm water and dried. Z-range=50 nm.

After analysis of the \( \text{A}\beta \) solutions, it is clear that the \( \text{A}\beta \) solution from the polypropylene vial, which was the control, exhibited very little protein aggregation. The \( \text{A}\beta \) solution from the fluorinated monolayer experiment had a much greater extent of peptide aggregation than the
control, while the alcohol monolayer had the most aggregation of Aβ protein, as judged by aggregation length and number. In comparison, the Aβ protein solution taken from the ethylene glycol-terminated monolayer exhibited very little protein aggregation (Figure 3.6A), which was comparable to the control from the vial. The ethylene glycol-terminated monolayer did not adsorb the Aβ protein (Figure 3.6B), which accounts for the fact that there was very little protein aggregation in solution. If the protein does not adsorb to the monolayer surface, there is not a nucleus present for seeding polymerization to begin. These results indicate that the Aβ aggregates do dissociate from the monolayers after their formation. However, in consideration of the SFM images of the exposed monolayer surfaces themselves (not shown) and of the RAIRS data (vide supra), it was evident that the fluorinated monolayer possessed more protein deposits.

Figure 3.6. 10 μm x 10 μm tapping mode scanning force micrographs of 100 μM Aβ10-35 peptide in 10 mM KOH solution at pH 11.5. A. Supernatant from the HS-(CH₂)₁₁-(OCH₂CH₂)₃-OH / Au monolayer exposed to 100 μM Aβ₁₀₋₃₅ peptide in 10 mM KOH for 40 days. B. HS-(CH₂)₁₁-(OCH₂CH₂)₃-OH / Au monolayer exposed to 100 μM Aβ₁₀₋₃₅ peptide in 10 mM KOH for 40 days. For the supernatant experiments, 50 μL of supernatant was spotted on mica, allowed to sit for 5 minutes, and then the resulting surface was rinsed with 18 MΩ-cm water and dried. Z-range=50 nm.
than did the alcohol monolayer. Therefore, for the fluorinated monolayer, there would be less protein available in solution from which aggregates would form.

Amyloid solutions before and after incubation with the alcohol- and fluoromethyl-terminated monolayers were evaluated to determine the peptide content (amino acid analysis) of the solution after incubation. Results confirmed the initial hypothesis regarding the difference between the adsorption propensities of the different monolayers, that is, the CF$_3$ monolayer had removed more Aβ peptide from the solution than had the alcohol-terminated monolayer. Amino acid analysis of the solutions after incubation revealed that the peptide content of the solution from the fluorinated monolayer was less than that of the alcohol monolayer by a factor of 10. Therefore, the reason the alcohol monolayer had more of a seeding effect was because there was more peptide in the solution for aggregates to form from the displaced seeds. In addition, by comparing the solutions from the monolayers with the solution from the polypropylene vial and the ethylene glycol-terminated monolayers on Au (controls), it can be concluded that the monolayers do induce aggregation of the Aβ peptide in solution, because the controls does not have the extent of aggregation that the solution from the monolayers have.

In Figure 3.7 is depicted a proposed model describing the interaction of the Aβ peptide with the monolayer surfaces. Initially, the Aβ peptide is adsorbed onto the monolayer surface (A to B), where it subsequently undergoes a change in conformation to a β-sheet (B to C). At this stage, the process can then follow two pathways. In one path (C to D), the surface accrues more peptide due in part to the confined β-sheet form acting as a template to yield larger surface-confined aggregates. In the second path (C to E), the β-sheet form of the peptide on the monolayer is moved to solution wherein the folded peptide (monomer or higher-order aggregates) interacts with monomeric Aβ peptide to form larger aggregates (E to F). These
larger, solution-phase aggregates can either continue to grow upon interaction with monomeric peptide or be deposited back onto the surface of the monolayer (F to D).

Figure 3.7. Model of the surface interaction and subsequent aggregation of Aβ_{10-35} peptide exposed to various ω-substituted alkanethiol monolayers.
The surface-induced aggregation of Aβ peptide as a route to the formation of solution-phase aggregates and surface-bound aggregates is an important issue in understanding the mechanism of action of Aβ peptide aggregates and their role in Alzheimer’s disease.\textsuperscript{6, 12} Although the impact of surface charge of lipid bilayer vesicles on the interaction of Aβ peptide with such lipid membranes has been demonstrated,\textsuperscript{6-9} little information exists surrounding the relationship between the deposition, folding and aggregation of solution-phase Aβ peptides and the chemical nature of non-ionic surfaces. The work described here is a first attempt at relating the chemical makeup of supported, model monolayer surfaces and their propensity to interact with Aβ peptide in solution. The outcomes are important, for it is clear that the relatively “non-specific” interactions of Aβ peptides with certain surfaces may be similar to that observed for ganglioside-terminated lipid vesicles, the latter which have been shown to cause formation of Aβ peptide β-sheets.\textsuperscript{29}

3.5 Conclusions

Reflection-absorption infrared spectroscopy analysis revealed that deposition of Aβ\textsubscript{10-35} occurred onto both hydrophilic and hydrophobic surfaces under conditions that do not lead to aggregation in solution. In addition, the RAIRS studies demonstrated that the conformation of the adsorbed Aβ\textsubscript{10-35} does not change over a period of 40 days of exposure, and the amount of Aβ deposited increases with exposure time. Scanning force microscopy confirmed that the amount of aggregated deposits increased with exposure time. Studies of the solutions exposed to monolayers revealed that the monolayers have a seeding effect on Aβ peptide in solution, leading to the conclusion that monolayers actually induce aggregation of Aβ peptide in solution.
3.6 References


25. P. Harder, M. Grunze, R. Dahint, G. M. Whitesides and P. E. Laibinis. Molecular Conformation in Oligo(ethylene glycol)-Terminated Self-Assembled Monolayers on Gold and


Chapter 4

Effect of Patterned Monolayers with Different Chemical Functionalities on the Surface-Induced Aggregation of the Beta Amyloid Peptide

4.1 Introduction

It has been found that Au surfaces consisting of self-assembled monolayers (SAMs) terminated with methyl (HS-(CH$_2$)$_{17}$-CH$_3$), alcohol (HS-(CH$_2$)$_{11}$-OH), carboxylic acid (HS-(CH$_2$)$_{15}$-COOH), fluoro-methyl (HS-(CH$_2$)$_2$-(CF$_2$)$_7$-CF$_3$), sulfonic acid (HS-(CH$_2$)$_{10}$-SO$_3$H) chemical functionalities, adsorbed A$eta$ protein (fouling) and could induce the aggregation of A$eta$ protein in solution, and that SAM surfaces terminated with ethylene glycol (HS-(CH$_2$)$_{11}$-(OCH$_2$CH$_2$)$_3$-OH) chemical functionalities did not adsorb A$eta$ protein (non-fouling) or cause aggregation of the A$eta$ protein in solution. The next step in this series of experiments was to form patterned surfaces consisting of monolayers with fouling and non-fouling surface properties to determine if areas of fouling monolayers embedded in non-fouling monolayers affected the deposition of the A$eta$ protein on both of these surfaces or the aggregation of the A$eta$ protein in solution. This system was used to model a cell membrane that has a particular type of isolated chemical functionality which could act as a nucleation site for inducing aggregation of A$eta$ protein on a surface that would not usually affect the A$eta$ protein. This is an important model because it has been shown that A$eta$ protein will bind to gangliosides implanted in lipid vesicles, and this immobilized protein can act as a nucleation seed to form the A$eta$ protein aggregates.$^{1-3}$ The formation of aggregated A$eta$ protein from soluble A$eta$ protein by seeded polymerization is regarded as a crucial step in the progress of Alzheimer’s disease.$^4$

Microcontact printing was chosen to construct the patterned monolayer surfaces. This technique is generally used to control topographical features and spatial presentation of surface molecules. Microcontact printing has become a useful tool in the manufacture of microanalytical
devices, such as chemical and biological sensing devices. Patterned monolayers are also commonly used as model system for studying protein deposition at interfaces.\textsuperscript{5-8}

Ethylene glycol functionalities are commonly used as a surface coating to prevent non-specific protein deposition. Several literature studies have revealed that an ethylene glycol-terminated SAM, HS(CH\textsubscript{2})\textsubscript{11}(OCH\textsubscript{2}CH\textsubscript{2})\textsubscript{3}OH on Au, is resistant to protein deposition.\textsuperscript{9-11} Preliminary reflection-absorption infrared spectroscopy (RAIRS) and scanning force microscopy (SFM) studies showed that the ethylene glycol-terminated SAM was non-fouling for the A\textbeta protein for up to a forty-day incubation period (Figures 3.1 and 3.6). Therefore, it appeared that this SAM was suitable to be used as a non-fouling model surface in the A\textbeta protein on patterned monolayer experiments. The methyl-terminated alkanethiol monolayer, HS(CH\textsubscript{2})\textsubscript{17}CH\textsubscript{3} on Au, was shown to adsorb the A\textbeta protein in the previous studies (Figure 3.1), so it was a suitable coating to be used for the fouling areas in the patterned monolayer surfaces. The methyl-terminated alkanethiol was chosen over the other fouling alkanethiol monolayers because it resists degradation and has a chemical composition similar to cell membranes.\textsuperscript{5, 12-15}

4.2 Motivation

The aim of this project was to use a patterned monolayer surface constructed via microcontact printing of a fouling SAM, HS(CH\textsubscript{2})\textsubscript{17}CH\textsubscript{3} on Au, surrounded by a non-fouling SAM, HS(CH\textsubscript{2})\textsubscript{11}(OCH\textsubscript{2}CH\textsubscript{2})\textsubscript{3}OH on Au, to determine the effect that the small, fouling areas have on the deposition and aggregation of the protein in the presence of a non-fouling monolayer. This experiment was used to gain a better understanding of how surfaces, specifically the combination of fouling and non-fouling areas on the same surface, influence the aggregation of A\textbeta peptide. SFM and lateral force microscopy (LFM) were used to detect and monitor the protein on the patterned surfaces. Because of the tribological variances of the two
types of monolayers, LFM was used to distinguish between different types of chemical functionalities present in the pattern. Height-mode SFM was used to determine where the Aβ protein had adsorbed to the monolayer surface.

There are several questions to be answered by this experiment. It will be informative to observe how the combination of fouling and non-fouling areas affects the adsorptive properties of each other. It could be determined whether the protein will still adhere to the fouling monolayer in the presence of a non-fouling monolayer, and if the non-fouling monolayer resists all protein deposition in the presence of a fouling surface. If the protein does adsorb onto the fouling monolayer, it could further be determined if the protein remains confined to that fouling monolayer. It is possible that the seeding effect of the fouling monolayer has the capacity to cause the protein to grow onto the non-fouling surface, which could possibly give insight to how Aβ plaques form in the brain. Previous studies indicate that the protein can absorb on a fouling monolayer, form larger aggregates and then desorb from the surface into solution where it acts as a nucleus for further aggregation in solution, which is called the seeding effect of the surface.

In the previous studies analyzing the seeding effect of the monolayers, the alkanethiol monolayer induced aggregation of the protein in solution, while the ethylene glycol-terminated monolayer seemed to prevent aggregation of the protein in solution. Therefore, the effect of the combination of the surface fouling and non-fouling moieties on the aggregation of the protein in solution will also be determined. If the protein does desorb from the surface, it will also be verified what happens to the protein after desorption. It is also possible that the aggregated protein could desorb from the fouling surface and drop back down on the non-fouling surface. It will be determined if the protein would settle on the non-fouling surface and continue to aggregate on that surface.
4.3 Experimental

The patterning of the monolayer was constructed via microcontact printing. Poly(dimethyl siloxane), PDMS, stamps with different size patterns were used. Two different sizes of patterns were used to determine if the dimensions of the fouling and non-fouling areas affected the deposition of the aggregates of Aβ protein to the surfaces. Methyl-terminated alkanethiol, HS(CH₂)₁₇CH₃, in ethanol was applied to the stamp and allowed to absorb for 30 seconds. The stamp was then dried with N₂ and immediately applied to a freshly cleaned Au substrate. After three minutes, the stamp was removed from the Au, and the Au/patterned methyl-terminated alkanethiol monolayer was placed in an ethylene glycol-terminated alkanethiol solution, HS(CH₂)₁₁(OCH₂CH₂)₃OH, in ethanol. The ethylene glycol-terminated alkanethiol then self-assembled in the bare areas to completely fill in the pattern. A patterned monolayer is shown in Figure 4.1. The height image (left side) was relatively smooth displaying the Au (111) crystallite features associated with the monolayer-covered Au surface while the friction image (right side) exhibited the differences in the surface energy of the separate chemical functionalities. The light color in the Z-range friction scale of the LFM images represents a higher surface friction. Therefore, in this and all of the following figures, the ethylene glycol-terminated monolayer correlate to the lighter areas of the LFM images, while the methyl-terminated alkanethiol monolayer were the darker areas of the LFM images.

After the patterned monolayers were formed, they were incubated with Aβ protein for various amounts of time. Analysis was done using in situ SFM while incubating the Aβ protein with the patterned monolayer so that the formation of the Aβ aggregates could be analyzed in real time. Analysis was done using SFM and LFM to simultaneously measure the height and friction of the samples.
4.4 Deposition of Beta Amyloid on a Small-Scale Patterned Monolayer

In situ SFM was performed to monitor possible deposition and aggregation of \( \alpha \beta \) protein on the patterned monolayer. A small scale patterned was originally chosen. The stamp consisted of small areas with repeat pattern widths of 1 \( \mu \)m. Approximately 15% of the resulting patterned monolayer consisted of the fouling \( \text{HS}(\text{CH}_2)_{17}\text{CH}_3 \) monolayer. Initial analysis was done with a patterned monolayer that had been exposed to 100 \( \mu \)M beta amyloid in 10 mM KOH solution at pH 11 for 15 hours (Figure 4.2). The spacing of the line pattern was approximately one micrometer apart. The height SFM image is located on the left, while the right side contains the frictional LFM image. The brighter color in the Z-range scale of the LFM images represents a higher surface friction. Therefore, in Figures 4.2 and 4.3, the ethylene glycol-terminated monolayer are depicted by the lighter areas of the LFM image, while the alkanethiol monolayer...
are the darker areas of the LFM image. This difference in friction occurred because the ethylene glycol functionality had a higher surface energy than the unsubstituted alkanethiol functionality.\textsuperscript{17} When the A\textbeta protein was incubated on a small-scale pattern of the fouling and non-fouling monolayers, it appeared that the A\textbeta protein adsorbed onto the ethylene glycol domains even though the ethylene glycol-terminated monolayers were supposed to be non-fouling. After the A\textbeta protein had been incubated on the patterned monolayer for 40 hours, the A\textbeta protein spread significantly onto the ethylene glycol-terminated portion of the monolayer (Figure 4.3). The aggregates were approximately 100 nm in length and 25 nm in height. This was unexpected because previous studies (Chapter 3) showed that the ethylene glycol-terminated

![Figure 4.2](image_url)

**Figure 4.2.** 5 \( \mu \text{m} \) x 5 \( \mu \text{m} \) in situ contact mode scanning force micrographs of a patterned monolayer exposed to 100 \( \mu \text{M} \) beta amyloid in 10 mM KOH solution at pH 11 for 15 hours. The height image is on the left and the friction image is on the right. In the friction image the HS(CH\textsubscript{2})\textsubscript{11}(OCH\textsubscript{2}CH\textsubscript{2})\textsubscript{3}OH monolayer is shown in the lighter areas. The Z-range is 50 nm for the height image and 0.1 V for the friction image.
Figure 4.3. 5 μm x 5 μm in situ contact mode scanning force micrographs of a patterned monolayer exposed to 100 μM beta amyloid in 10 mM KOH solution at pH 11 for 40 hours. The height image is on the left and the friction image is on the right. In the friction image the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer is shown in the lighter areas. The Z-range is 50 nm for the height image and 0.1 V for the friction image.

The patterned monolayer exhibited negligible Aβ protein deposition. In this case, boundary failure of the patterned monolayer was found, which is defined as the invasion of the protein onto the non-fouling regions. The adsorptive properties of the methyl-terminated monolayer appear to be stronger than the protein-resistant characteristics of the ethylene glycol-terminated monolayer because the Aβ protein was present on the ethylene glycol-terminated portion of the monolayer. In this situation, it appears that the Aβ protein did not grow onto the non-fouling monolayer from the fouling monolayers, but rather nucleated and aggregated on the fouling monolayer, then desorbed from the fouling monolayer and readsorbed on the non-fouling monolayer surface. The spacing of the two monolayers could be too close together allowing for mixing of the monolayers to occur since the monolayers are slightly fluid. If the monolayers are mixed,
the Aβ protein would adsorb onto the few HS(CH$_2$)$_{17}$CH$_3$ chains present in the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH areas.

### 4.5 Deposition of Beta Amyloid on a Large-Scale Patterned Monolayer

A larger-scale pattern was chosen next to determine if the closeness of the two monolayers could be the reason the Aβ protein was able to adsorb onto the ethylene glycol monolayers. The PDMS stamp used in these studies was constructed using a semiconductor wafer as a negative relief pattern. This stamp provided a variety of patterns on the same stamp with repeat pattern widths ranging from 10 μm to 20 μm. Approximately 12% of the resulting patterned monolayer consisted of the fouling HS(CH$_2$)$_{17}$CH$_3$ monolayer.

![Figure 4.4. 50 μm x 50 μm scanning force micrographs of a patterned monolayer exposed to 100 μM beta amyloid in 10 mM KOH solution at pH 11 for 12 hours. The height image is on the left and the friction image is on the right. In the friction image the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH monolayer is shown in the lighter areas. The Z-range is 100 nm for the height image and 1.0 V for the friction image.](image-url)
The patterned monolayers were analyzed with ex situ SFM at 12- and 48-hour intervals. After a 12-hour incubation period (Figure 4.4), the Aβ protein had begun to adsorb onto the alkanethiol-terminated monolayer. Although the majority of the ethylene glycol-terminated monolayer remained protein free, several large Aβ protein aggregates (~500 nm) were present on the ethylene glycol-terminated monolayer. When examining a close up height image, the edges of the pattern were sharply defined, as shown in Figure 4.5, indicating that the Aβ protein was mainly confined to the methyl-terminated monolayer. At this point in time, the adsorptive properties for the two monolayers were what would be expected.

After the Aβ protein was exposed to the patterned monolayer for 48 hours, the protein had completely covered the methyl-terminated monolayer (Figure 4.6). There were some large structures present on the surface that are attributed to defects in the gold. The interface between the two monolayers was still clearly defined, but there was visible protein deposition on the
ethylene glycol-terminated monolayer. Once again, when comparing the height SFM and friction LFM for the 48-hour incubation period, the ethylene glycol-terminated areas were lighter. Under these experimental conditions, it appeared that while the Aβ protein adsorbed mainly on the HS(CH₂)₁₁CH₃, it also migrated to the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer as was seen on the small scale pattern. These results indicated that the spacing of the monolayers was not the culprit of the protein deposition onto the ethylene glycol-terminated monolayer on the smaller pattern.

Figure 4.6. 20 μm x 20 μm scanning force micrographs of a patterned monolayer exposed to 100 μM beta amyloid in 10 mM KOH solution at pH 11 for 48 hours. The height image is on the left and the friction image is on the right. In the friction image the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer is shown in the lighter areas. The Z-range is 50 nm for the height image and 1.0 V for the friction image.

4.6 Deposition of Bovine Serum Albumin on a Patterned Monolayer

In order to determine the effect of the patterned monolayer on another protein, experiments were repeated using bovine serum albumin (BSA) as a control. BSA is a
homogenous, sticky protein commonly used to test surfaces for fouling properties.\textsuperscript{19-21} The small- and large-scale patterned substrates were constructed as described previously and the BSA protein solution (100 μM in 15 mM PBS) was allowed to incubate on the patterned monolayers for 12- and 48-hour intervals.

The results of incubating the BSA protein on a small-scale patterned monolayer (5 μm and 2 μm stripes) for 48 hours are shown in Figure 4.7. On this surface, the BSA protein remained confined to the fouling HS(CH\textsubscript{2})\textsubscript{17}CH\textsubscript{3} monolayer surface, unlike the Aβ protein experiments under the same conditions. There was a minute amount (~ 0.6 aggregates per μm\textsuperscript{2}) of BSA aggregates present on the HS(CH\textsubscript{2})\textsubscript{11}(OCH\textsubscript{2}CH\textsubscript{2})\textsubscript{3}OH surface.

\textbf{Figure 4.7.} 10 μm x 10 μm scanning force micrographs of a patterned monolayer exposed to 100 μM bovine serum albumin in 15 mM PBS solution at pH 11 for 48 hours. The height image is on the left and the friction image is on the right. In the friction image the HS(CH\textsubscript{2})\textsubscript{11}(OCH\textsubscript{2}CH\textsubscript{2})\textsubscript{3}OH monolayer is shown in the lighter areas. The Z-range is 50 nm for the height image and 0.5 V for the friction image.

The results of incubating the BSA protein on the large-scale substrate for 12 hours are shown in Figure 4.8. The BSA protein appeared to only adsorb on the methyl-terminated areas
of the substrate. After 48 hours (Figure 4.10), the deposition of the BSA protein was mostly limited to the methyl-terminated areas of the substrate although the deposition of BSA protein had branched onto the ethylene glycol-terminated areas. The patterned edges were still easily discernable, which indicates that the BSA protein adsorbed mainly on the methyl-terminated surface. In this case, the BSA protein behaved as expected in regards to fouling on the monolayers, even on the small scale pattern. This indicates that mixing of the monolayers did not occur because the BSA protein would have adsorbed on the mixed monolayers.\textsuperscript{22, 23} Therefore, it was assumed that mixing of the monolayers also did not occur on the patterned monolayer substrate used in the Aβ protein and that the Aβ protein actually attached on the surfaces with small-scale pattern.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.8.png}
\caption{81 \textmu m x 81 \textmu m scanning force micrographs of a patterned monolayer exposed to 100 \textmu M bovine serum albumin in 15 mM PBS solution at pH 11 for 12 hours. The height image is on the left and the friction image is on the right. In the friction image the HS(CH\textsubscript{2})\textsubscript{11}(OCH\textsubscript{2}CH\textsubscript{2})\textsubscript{3}OH monolayer is shown in the lighter areas. The Z-range is 50 nm for the height image and 1.0 V for the friction image.}
\end{figure}
4.7 Incubation of Beta Amyloid Supernatant from the HS-(CH$_2$)$_{17}$-CH$_3$ Monolayer on the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH Monolayer

Because the deposition of A$\beta$ protein on the patterned monolayers was probably not due to mixing of the monolayers, it was thought that the HS(CH$_2$)$_{11}$CH$_3$ monolayer induced a change in protein conformation that allowed A$\beta$ protein to adsorb to the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH monolayers. In order to test this hypothesis, A$\beta$ protein in 10 mM KOH at pH 11.5 was first incubated on the HS(CH$_2$)$_{17}$CH$_3$ monolayer for 12 hours. The A$\beta$ protein supernatant was removed from the HS(CH$_2$)$_{17}$CH$_3$ monolayer on Au and applied to the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH/Au substrate. The A$\beta$ protein solution was then incubated on the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH monolayer for 12 hours, then the monolayer was rinsed and analyzed with RAIRS. Surprisingly, after incubation on the HS(CH$_2$)$_{17}$CH$_3$ monolayer, the A$\beta$ protein adsorbed to the surface of the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$-OH monolayer (Figure 4.10). After curve-fitting deconvolution was performed on the spectrum, it was confirmed that A$\beta$ protein
was present only in a $\beta$-sheet conformation. The resolved peaks were located at 1612, 1629, 1639, 1673, and 1695 cm$^{-1}$. The peak at 1612 cm$^{-1}$ is attributed to side chain interactions, while the remaining peaks are indicative of $\beta$-sheet structures. Therefore, the HS(CH$_2$)$_{17}$CH$_3$ monolayer had the ability to induce a conformation that was able to adsorb to the previously non-fouling HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH monolayer. Taken in relation to the patterned monolayer experiments, it can be concluded that the small areas of fouling HS(CH$_2$)$_{17}$CH$_3$ monolayer had the ability to produce a class of $A\beta$ protein that was folded in a way that allowed it to attach to the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH monolayer.

**Figure 4.10.** Curve-fitting deconvolution of RAIR spectrum of supernatant of 100 $\mu$M $A\beta$ in 10 mM KOH (pH 11.5) that was first incubated on HS-(CH$_2$)$_{17}$-CH$_3$/Au for 12 h then removed and incubated on the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH monolayer for 12 hours. Original spectra (solid), fitted curve (dash-dot), and individual Gaussian components (dot) with a reduced $\chi^2$ value of 3.09.
Although ethylene glycol end groups are usually seen to be non-fouling, there are a few examples in literature that show protein to be attracted to ethylene glycol functionalities. Leckband et al. discovered that if streptavidin was forced into contact with a poly(ethylene glycol) chain, there existed reasonably strong attractive forces between the protein and the poly(ethylene glycol) moiety. It has also been shown that mucin, glycoproteins that line the gastrointestinal tract, adhere weakly to poly(ethylene glycol). Therefore, it is not unreasonable that the Aβ protein would adsorb to the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer.

4.8 Effects of a Patterned Monolayer on the Aggregation of Beta Amyloid Protein in Solution

Previous studies (Chapter 3) indicated that the Aβ protein can adsorb on a fouling monolayer, form larger aggregates and then desorb from the surface into solution where it acts as a nucleus for further aggregation in solution. In the earlier studies analyzing the seeding effect of the monolayers, the alkanethiol monolayer induced the aggregation of the protein in solution, while the ethylene glycol-terminated monolayer did not affect the aggregation of the protein in solution. Therefore, the effect of the combination of the surface fouling and non-fouling moieties on the aggregation of the protein in solution was also analyzed. A comparison between the Aβ supernatant from the HS(CH₂)₁₁CH₃ and the ethylene glycol-terminated monolayers and the patterned monolayers was conducted using scanning force microscopy. The Aβ protein solution was taken off the monolayer surfaces, applied to mica, allowed to adsorb for five minutes, then rinsed and analyzed with SFM. A high pH solution was used to impede the aggregates from forming in the solution.

The results of the Aβ peptide solution taken from the ethylene glycol-terminated monolayer are displayed in Chapter 3, Figure 3.6. Following incubation on the monolayer surface for 7 days, the solution had very few aggregates (approximately five aggregates per 100
μm²), with lengths ranging from 25 to 30 nm and heights of approximately 10 nm. After a 7-day incubation of the Aβ solution with the HS(CH₂)₁₇CH₃ monolayer, the Aβ protein had formed many more aggregates (approximately 55 aggregates per 100 μm²) in solution than did the sample from the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer. The aggregates were approximately 100 to 120 nm long and had heights of approximately 50 nm (Figure 4.11A).

The solution from the patterned monolayers was analyzed in order to probe the effects of the patterned monolayer on the production of the Aβ aggregates in solution. After 7 days of incubation, the Aβ protein had formed larger aggregates approximately 35 nm in height and 75 to 90 nm in length (Figure 4.11B) with a population of approximately 50 aggregates per 100 μm².

![Image](image-url)

**Figure 4.11.** 10 μm x 10 μm scanning force micrographs of 100 μM Aβ₁₀⁻₃₅ peptide in 10 mM KOH solution at pH 11.5. A. Supernatant from the HS-(CH₂)₁₇CH₃ /Au monolayer exposed to 100 μM Aβ₁₀⁻₃₅ peptide in 10 mM KOH for 7 days. B. Supernatant from the HS-(CH₂)₁₁(OCH₂CH₂)₃OH patterned monolayer exposed to 100 μM Aβ₁₀⁻₃₅ peptide in 10 mM KOH for 7 days. For the supernatant experiments, 50 μL of supernatant was spotted on mica, allowed to sit for 5 minutes, and then the resulting surface was rinsed with 18 MΩ-cm water and dried. Z-range=50 nm.

After comparing the analysis of the Aβ solutions from all three surfaces, it was clear that the Aβ solution from the patterned monolayers favored the aggregation activities of the solution
from the HS(CH$_2$)$_{17}$CH$_3$ monolayer rather than the solution from the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH monolayer. The A$\beta$ solution from the HS(CH$_2$)$_{17}$CH$_3$ monolayer experiment had a much greater extent of peptide aggregation than the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH monolayer, as judged by aggregation length and number. In comparison, the A$\beta$ protein solution taken from the patterned monolayer made up of both the HS(CH$_2$)$_{17}$CH$_3$ and the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH monolayers exhibited protein aggregation that was comparable to the HS(CH$_2$)$_{17}$CH$_3$ monolayer. It can be concluded that even small amounts of the HS(CH$_2$)$_{17}$CH$_3$ in the patterned monolayers do induce aggregation of the A$\beta$ peptide in solution to the same extent that the monolayer surface made entirely of HS(CH$_2$)$_{17}$CH$_3$ did.

4.9 Conclusions

When the A$\beta$ protein was incubated on a small-scale pattern of the fouling and non-fouling monolayers, it appeared that the A$\beta$ protein adsorbed onto the ethylene glycol even though the ethylene glycol-terminated monolayers alone were previously seen to be non-fouling. Since the A$\beta$ protein was present on the ethylene glycol-terminated portion of the monolayer, it appeared that the adsorptive characteristics of the methyl-terminated monolayer were greater than the protein-resistant properties of the ethylene glycol-terminated monolayer. It seemed that the A$\beta$ protein nucleated on the fouling monolayer, then desorbed from the fouling monolayer and readsoresorbed on the non-fouling monolayer surface. On the large-scale patterned monolayer, it appeared that the A$\beta$ protein did adsorb on the HS(CH$_2$)$_{17}$CH$_3$, and migrated to the HS(CH$_2$)$_{11}$(OCH$_2$CH$_2$)$_3$OH monolayer although not to the extent that was seen on the small scale pattern. A possible reason that there was less protein present on the larger-scale pattern than the small-scale pattern could be that after the A$\beta$ protein desorbed from the surface, the protein did not travel far before it readsoresorbed on the surface; therefore, the length of diffusion of the protein
was not great. On the large-scale pattern monolayer configuration, the domain constraints of the surface have more of an effect on the deposition of the protein on surfaces. When the Aβ protein was first incubated on the HS(CH$_2$)$_{17}$CH$_3$ monolayer alone, removed and then incubated on the HS(CH$_2$)$_{11}$((OCH$_2$CH$_2$)$_3$OH monolayer, the protein adsorbed on the HS(CH$_2$)$_{11}$- (OCH$_2$CH$_2$)$_3$OH monolayer. This was surprising because when the freshly-made Aβ protein was incubated on the HS(CH$_2$)$_{11}$((OCH$_2$CH$_2$)$_3$OH monolayer, the HS(CH$_2$)$_{11}$((OCH$_2$CH$_2$)$_3$OH monolayer was non-fouling for up to 40 days. This was important because it appears that the HS(CH$_2$)$_{17}$CH$_3$ monolayer can induce a change in conformation that allows the protein to attach to what are normally non-fouling surfaces. When analyzing the BSA protein on the patterned surface, the protein behaved as expected in regards to fouling on the monolayers, even on the small-scale pattern.

The effect of the patterned monolayer on the aggregation of the Aβ protein in solution was investigated. It was clear that the Aβ solution from the patterned monolayers favored the aggregation behavior of the Aβ solution from the HS(CH$_2$)$_{17}$CH$_3$ monolayers rather than the Aβ solution from the HS(CH$_2$)$_{11}$((OCH$_2$CH$_2$)$_3$OH monolayer. The Aβ solution from the HS(CH$_2$)$_{17}$CH$_3$ monolayer experiment had a much greater extent of peptide aggregation than the HS(CH$_2$)$_{11}$((OCH$_2$CH$_2$)$_3$OH monolayer, as judged by aggregation length and number. In comparison, the Aβ protein solution taken from the patterned monolayer made up of both the HS(CH$_2$)$_{17}$CH$_3$ and the HS(CH$_2$)$_{11}$((OCH$_2$CH$_2$)$_3$OH monolayers exhibited protein aggregation that was comparable to the HS-(CH$_2$)$_{17}$-CH$_3$ monolayer. It can be concluded that even small areas of fouling monolayer embedded in a non-fouling monolayer can induce aggregation of the Aβ peptide in solution. This is important because it establishes that in the brains of humans it is possible that a particular type of isolated chemical functionality on the cell membrane can act as
a nucleation site for inducing aggregation of Aβ protein on a surface that would not usually affect the Aβ protein.

4.10 References


Chapter 5
Interaction of Beta Amyloid Protein with Lipid Bilayers

5.1 Introduction

There is evidence that Aβ fibril assembly is effected by interactions with biological surfaces by inducing β sheet aggregation. In addition, it has been shown that the presence of negatively charged lipid vesicles in Aβ solutions shifts the random coil conformation of Aβ peptide to a β–sheet conformation under conditions of low ionic strength. This is due to the electrostatic binding of Aβ peptide onto the lipid membrane surface and subsequent aggregation of the peptide. Consequently, it is possible that some type of moiety on the cell surface triggers the folding of Aβ peptide and subsequent plaque formation.

It has also been proposed that the toxicity of the Aβ protein is due to the interactions it has with the cell membranes. The toxicity is possibly caused by the formation of ion channels when the Aβ protein inserts into the cell membrane, which would disrupt the homeostasis of the cell, leading to cell death. Since the Aβ peptide is cleaved from the Aβ precursor protein within the cell membrane, it is possible that the Aβ peptide inserts itself back into the cell membrane after cleavage. The Aβ peptide would then be attached to the cell membrane, providing a nucleus for subsequent protein aggregation. Therefore, it is critical to investigate the interaction of Aβ peptide and model membranes with each other. Lipid bilayers were chosen to represent the cell membranes.

The goal of the work described here is an understanding of the effects of lipid membranes on the aggregation of beta amyloid (Aβ) peptide and the effect of the Aβ peptide on the lipid bilayer. Mica-supported lipid bilayers consisting of 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-
Phosphocholine (POPC) and 1-Oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phosphocholine (NBD-PC) were used as a model cell membrane system. Lipid bilayers are physiologically significant because phospholipids are the principle components of cellular membranes and POPC is one of the main elements of mammalian cell membranes.\textsuperscript{7,8} These model surfaces were used to determine the effect of the lipid bilayers on the binding, deposition, and aggregation of the Aβ peptide. Fluorescence photobleaching recovery (FPR), fluorescence microscopy, and in situ scanning force microscopy (SFM) were chosen to evaluate the effect of Aβ protein on the lipid bilayers.

5.2 Composition of Fluorescently Labeled Lipid Bilayers

Small, unilamellar lipid vesicles were constructed using the Morrissey protocol and were subsequently adsorbed onto mica substrates using vesicle fusion to form the lipid bilayers.\textsuperscript{9,10} The lipid bilayers were first analyzed using fluorescence microscopy (Figure 5.1). The image shows a surface that has some wavelike patterns with higher fluorescence intensity that is attributed to the fact that the lipid bilayer was moving even when the image was being obtained. The camera captured the movement and produced these patterns. This image indicated that a lipid bilayer was formed using this method.

![Figure 5.1. Fluorescence microscopy image of 5% NBD-PC / 95% POPC lipid bilayer on mica.](image.png)
Initial analysis was done using lipid bilayers made with different proportions of POPC and NBD-PC to determine the correct ratios of the two lipids and the correct method for making the lipid bilayers. Preliminary analysis was performed using 100% NBD-PC vesicles in phosphate buffer solution to determine the diffusion coefficient of the lipid bilayers. In the first experiment, the lipid bilayers were fused onto the mica surface and analyzed without rinsing the surface after the bilayer had formed (Figure 5.2). The diffusion coefficient was calculated to be $6.63 \times 10^{-8} \pm 3.08 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$. Next, the bilayers were formed and they were then rinsed with phosphate buffer solution after the bilayer had formed in order to remove any unadsorbed lipid vesicles (Figure 5.3). After the lipid bilayers were analyzed, the diffusion coefficient was
calculated to be $6.65 \times 10^{-8} \pm 7.17 \times 10^{-9}$ cm$^2$s$^{-1}$, which was comparable to the unrinse lipid bilayers.

Since the NBD-PC lipids do not exist in nature and a high concentration of a fluorescent label can cause quenching that leads to errors in the diffusion coefficient, a mixture of POPC and NBD-PC was used. Initial concentrations were 20% NBD-PC and 80% POPC. This mixture produced a totally quenched sample that had no fluorescence signal. Next, a 10% NBD-PC and 90% POPC mixture was used that also produced a quenched sample.

Finally, a 5% NBD-PC and 95% POPC sample was made that produced a viable fluorescence signal. After the lipid bilayer was formed on mica, the sample was rinsed with phosphate buffer solution to eliminate any nonfused lipid vesicles. Analysis was done using FPR to obtain the diffusion coefficient (Figure 5.4). The diffusion coefficient from these

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.3.png}
\caption{Fluorescence photobleaching recovery of 100% NBD-PC lipid bilayer on mica that was rinsed after bilayer formation. The diffusion coefficient was found to be $6.65 \times 10^{-8} \pm 7.17 \times 10^{-9}$ cm$^2$s$^{-1}$.}
\end{figure}
experiments was $5.99 \times 10^{-8} \pm 5.51 \times 10^{-9}$ cm$^2$s$^{-1}$, which was comparable to the 100% NBD-PC lipid bilayer sample.

Figure 5.4. Fluorescence photobleaching recovery of 5% NBD-PC / 95% POPC lipid bilayer on mica that was rinsed after bilayer formation. The diffusion coefficient was $5.99 \times 10^{-8} \pm 5.51 \times 10^{-9}$ cm$^2$s$^{-1}$.

5.3 Analysis of the Formation of Fluorescently Labeled Lipid Bilayers with In Situ Scanning Force Microscopy

The formation of the lipid bilayers from lipid vesicles was analyzed using in situ SFM. Figure 5.5 depicts the topography of the 5% NBD-PC / 95% POPC lipid bilayer surfaces during their formation on a mica support. Initially, the surface had a lamellar structure with completely planar layers. The thickness was considerably higher (~ 40 nm) than that of a typical POPC lipid bilayer (Figure 5.2A). After approximately 12 minutes, (Figure 5.2B) the lipid bilayer had formed with a few defects. Before the lipid bilayer was rinsed, there were a few nonfused
vesicles present on the surface (Figure 5.2C). After the lipid bilayer surface was rinsed with phosphate buffer solution, the lipid bilayer had very few surface defects.

Figure 5.5. In situ scanning force microscopy images of lipid vesicles (5% NBD-PC / 95% POPC in phosphate buffer solution) forming a lipid bilayer on a mica surface. A. Initial image of lipid vesicles on mica. Z-range = 50 nm. B. Lipid bilayer begins to form after 4 minutes deposition time. Z-range = 100 nm. C. Nonfused lipid vesicles on the lipid bilayer surface before rinsing. Z-range = 50 nm. D. Lipid bilayer after rinsing with phosphate buffer solution. Z-range = 20 nm.
Following the formation of the supported lipid bilayer, analysis was done to determine the thickness of the bilayer. A high tip force (deflection setpoint = 10 V) was used to scrape away the bilayer while scanning in a 3 μm x 3 μm area. After four scanning cycles, the tip force was reduced to a normal force (deflection setpoint = 2 V) and the scanning area was enlarged to 10 μm x 10 μm, which allowed for imaging of the bare area in the lipid bilayer so that a depth profile analysis could be performed (Figure 5.6A). Section analysis was performed to create a height profile of the lipid bilayer (Figure 5.6B). The thickness of the bilayer was approximately six nm, which corresponded to literature values which were six nm.\textsuperscript{11}

**Figure 5.6.** A. In situ scanning force microscopy image of 5% NBD-PC / 95% POPC lipid bilayer on mica. A high tip scanning force was used to scrape away the bilayer and then the scanning area was enlarged using a normal tip scanning force for a depth profile analysis. B. Height profile of the lipid bilayer; the thickness of the bilayer is approximately six nm. Z-range = 10 nm.
5.4 Interaction of Beta Amyloid Peptide with Lipid Bilayers

To determine if the Aβ peptide interacted with the lipid bilayers, thioflavin-T-labeled 100 μM Aβ10-35 peptide fibrils were incubated for one hour on a 100% POPC lipid bilayer supported on mica. Thioflavin-T is a fluorescent dye that binds to the hydrophobic core of the Aβ protein and allows the Aβ peptide to be imaged with a fluorescence microscope. The preformed Aβ fibrils were approximately 25 μm in length. After the exposure time was complete, the lipid bilayer was rinsed with phosphate buffer solution. Initial analysis was done using fluorescence microscopy (Figure 5.7). The Aβ peptide fibrils appeared to have a strong interaction with the lipid bilayers since the fibrils were still present on the surface even after rinsing.

![Figure 5.7. Fluorescence microscopy image of 100% POPC lipid bilayer on mica exposed to preformed, thioflavin-T labeled 100 μM Aβ10-35 peptide fibrils for one hour.](image)

FPR analysis was done to determine the effect of the Aβ protein fibrils on the fluidity of the lipid bilayers. Preformed Aβ1-40 protein fibrils were incubated on the 5% NBD-PC / 95% POPC lipid bilayers over a period of five hours (Figure 5.8). During this time, the diffusion coefficient of the lipid bilayers decreased from $5.99 \times 10^{-8}$ cm$^2$ s$^{-1}$ to $8.3 \times 10^{-9}$ cm$^2$ s$^{-1}$, indicating that the Aβ protein interacted with the lipid bilayers. It is thought that the Aβ protein fibrils interacted with the lipid bilayers because the hydrophobic areas of the Aβ protein were attracted to the interior hydrophobic tail regions of the lipid bilayer.
The interaction of the Aβ peptide with the lipid bilayers was also monitored with in situ scanning force microscopy. The 5% NBD-PC / 95% POPC lipid bilayer was formed as described previously and imaged to ensure that a defect-free surface had formed. The freshly-made Aβ_{10-35} peptide was then added to the lipid bilayer. Perturbations began to form in the lipid bilayer after approximately ten minutes. After approximately one hour, the Aβ peptide began to insert into the lipid bilayer causing further defects in the surface (Figure 5.9). This is illustrated by the presence of a Aβ peptide aggregate in the middle of a hole in the lipid bilayer. The Aβ peptide appears to have caused pore-like defects in the lipid bilayer surface. It should be noted that when the lipid bilayer was imaged with a Aβ peptide-free phosphate buffer solution (control) no defects formed in the surface for comparable incubation times.

![Figure 5.8](image)

**Figure 5.8.** Diffusion coefficient of preformed beta amyloid 1-40 fibrils incubated on 5% NBD-PC/95% POPC lipid bilayer on mica over time.
Figure 5.9. In situ scanning force microscopy image of 5% NBD-PC / 95% POPC lipid bilayer on mica exposed to: A. 15 mM phosphate buffer solution for one hour; B. 100 μM Aβ_{10-35} peptide (in phosphate buffer solution) for one hour; C. Zoom image of 100 μM Aβ_{10-35} peptide (in phosphate buffer solution) for one hour; D. Height profile of the lipid bilayer and beta amyloid protein aggregate. Z-range = 50 nm.
5.5 Interaction of Nystatin with Lipid Bilayers

For comparison purposes, the interaction of nystatin, an antimicrobial agent, with the lipid bilayer system was examined. Nystatin, which is used as an antifungal medicine, is known to insert into lipid bilayers. The nystatin (10 μM in 15 mM phosphate buffer solution) was applied to a 5% NBD-PC / 95% POPC lipid bilayer and allowed to incubate for 24 hours. The surface was rinsed with phosphate buffer and imaged using in situ contact mode SFM. It appeared that the nystatin attached to the lipid surface and disrupted the lipid membrane. The nystatin aggregates were 500 nm in length and 25 nm in height. When compared to the results of the Aβ peptide, it appeared that the Aβ peptide disrupted the lipid bilayer more than the nystatin did, giving evidence that the Aβ peptide can form ion pores.

Figure 5.10. In situ scanning force microscopy image of 5% NBD-PC / 95% POPC lipid bilayer on mica exposed to 10 μM nystatin (in 15 mM phosphate buffer solution) for 24 h. B. Zoom view of Figure 5.10A. Z-range = 50 nm.
5.6 Effect of Lipid Bilayers on the Aggregation of Beta Amyloid Peptide

The effect of the lipid bilayers on the aggregation of the monomeric \( A\beta_{10-35} \) peptide was analyzed. The \( A\beta \) peptide was dissolved in KOH solution with a high pH (pH ~ 11.5) to prevent aggregation. The \( A\beta \) peptide was incubated on the 5% NBD-PC / 95% POPC lipid bilayers. The \( A\beta \) peptide solution was removed from the lipid bilayer surface and applied to mica, which allows for the solution phase deposition of protein. The samples were then analyzed using SFM. After a 48-hour incubation time (Figure 5.10A) large, amorphous protein aggregates had formed.

![Image A](image1.png)  ![Image B](image2.png)

**Figure 5.11.** In situ scanning force microscopy images of supernatant from lipid bilayer from the 5% NBD-PC / 95% POPC lipid bilayer on mica exposed to 100 \( \mu \)M \( A\beta_{10-35} \) peptide in 10 mM KOH solution at pH 11.5 for 48 hours; Z-range = 100 nm (A). Supernatant from lipid bilayer from the 5% NBD-PC / 95% POPC lipid bilayer on mica exposed to 10 mM KOH solution at pH 11.5 for 48 hours; Z-range = 10 nm (B). 50 \( \mu \)L of supernatant was spotted on mica, allowed to sit for 5 minutes, and then the resulting surface was rinsed with 18 M\( \Omega \)-cm water and dried.

in solution. A control consisting of a \( A\beta \) peptide-free KOH solution incubated on a lipid bilayer for the same amount of time and the applied to mica was used for comparison (Figure 5.10B). This sample had none of the large structures characteristic of the \( A\beta \) peptide solution. Therefore,
it can be concluded that the large, amorphous structures that resulted when the Aβ peptide solution was incubated on the lipid bilayer are truly Aβ peptide and not portions of the lipid bilayer that has disassembled because of the KOH (control) solution.

5.7 Conclusions

The interaction of the Aβ peptide with the lipid bilayers was analyzed using FPR and in situ SFM. FPR was used to monitor the fluidity of the bilayers. The baseline fluidity of the lipid bilayers was established, and then the Aβ protein was incubated on the bilayers. It was demonstrated that the fluidity of the lipid bilayers was reduced when incubated with Aβ peptide, indicating that there was an interaction with the lipid bilayers and the protein. The interaction of the protein with the bilayer was further analyzed with in situ SFM. The in situ SFM revealed that the Aβ peptide actually physically disrupted the lipid bilayers causing pores to form in the lipid bilayers. It is thought that the hydrophobic groups of the Aβ peptide interact with the hydrophobic inner areas of the lipid bilayer. This is a useful model of the cytotoxicity of the protein, because if pores formed, they would act as ion channels. These ion channels would disrupt the cellular homeostasis, which would lead to cell death.4

The Aβ protein solution that was incubated on the lipid bilayers was also examined. The solution showed evidence of extensive Aβ peptide aggregation, indicating that the lipid bilayers induced aggregation of the peptide in solution. This information is important because it provides evidence about the formation of amyloid plaques in the brains of humans. It is possible that the Aβ protein inserts into the lipid bilayer and is immobilized where it can act as a seeding nucleus for further aggregation of the protein to form.
5.8 **References**


Chapter 6
Outcomes, Conclusions and Future Direction

6.1 Summary of Outcomes and Conclusions

6.1.1 Surfaces Can Induce Aggregation of Beta Amyloid Peptide in Solution

Reflection-absorption infrared spectroscopy (RAIRS) analysis revealed that adsorption of Aβ occurred onto both hydrophilic and hydrophobic surfaces under conditions that normally do not lead to aggregation in solution. In addition, the RAIRS studies demonstrated that the conformation of the adsorbed Aβ\textsubscript{10-35} does not change over a period of 40 days of exposure, and the amount of Aβ deposited increases with exposure time. Scanning force microscopy (SFM) confirmed that the amount of aggregated deposits increased with exposure time. Studies of the solutions exposed to monolayers revealed that the monolayers have a seeding effect on Aβ peptide in solution, leading to the conclusion that monolayers actually induce aggregation of Aβ peptide in solution.

6.1.2 Adsorptive Behavior and Aggregation Properties of Beta Amyloid Peptide on Patterned Surfaces

Patterned surfaces made up of fouling (HS(CH\textsubscript{2})\textsubscript{17}CH\textsubscript{3}) and non-fouling (HS(CH\textsubscript{2})\textsubscript{11}(OCH\textsubscript{2}CH\textsubscript{2})\textsubscript{3}OH) monolayers were constructed to determine how these different areas would affect the aggregation and adsorption of the Aβ protein. When the Aβ protein was incubated on a small-scale pattern (~ 1 μm repeat pattern) of the fouling and non-fouling monolayers, it appeared that the Aβ protein adsorbed onto the ethylene glycol even though the ethylene glycol-terminated monolayers alone were previously seen to be non-fouling. Because the Aβ protein was present on the ethylene glycol-terminated portion of the monolayer, it seemed that the adsorptive characteristics of the methyl-terminated monolayer were greater than the
protein-resistant properties of the ethylene glycol-terminated monolayer. It appeared that the Aβ protein nucleated on the fouling monolayer, then desorbed from the fouling monolayer and readsorbed on the non-fouling monolayer surface. On the large-scale patterned monolayer (~10-30 μm repeat pattern), it appeared that the Aβ protein adsorbed mainly on the HS(CH₂)₁₇CH₃, but also adsorbed on the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer although not to the extent that was seen on the small scale pattern. It was determined that the HS(CH₂)₁₇CH₃ monolayer must alter the Aβ protein in such a way that allows it to adsorb to the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer. In order to test this theory, the Aβ protein was first incubated on the HS(CH₂)₁₇CH₃ monolayer alone, removed and then incubated on the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer alone. Subsequently, the Aβ protein adsorbed on the HS(CH₂)₁₁-(OCH₂CH₂)₃OH monolayer. This was surprising because when freshly-made Aβ protein was incubated on the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer, the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer was non-fouling for up to 40 days. This was important because it appears that the HS(CH₂)₁₇CH₃ monolayer can induce a change in conformation that allows the protein to stick to what are normally non-fouling surfaces.

The effect of the HS(CH₂)₁₇CH₃ / HS(CH₂)₁₁-(OCH₂CH₂)₃OH patterned monolayer on Au on the aggregation of the Aβ protein in solution was investigated. It was clear that the Aβ solution from the patterned monolayers favored the aggregation behavior of the solution from the HS(CH₂)₁₇CH₃ monolayers rather than the solution from the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer. The Aβ solution from the HS(CH₂)₁₇CH₃ monolayer experiment had a much greater extent of peptide aggregation than the HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayer, as judged by aggregation length and number gain from the SFM experiments. In comparison, the Aβ protein solution taken from the patterned monolayer made up of both the HS(CH₂)₁₇CH₃ and the
HS(CH₂)₁₁(OCH₂CH₂)₃OH monolayers exhibited protein aggregation that was comparable to the HS-(CH₂)₁₇-CH₃ monolayer. It can be concluded that even small areas of fouling monolayer embedded in a non-fouling monolayer can induce aggregation of the Aβ peptide in solution. This is important when a parallel is made between this study and physiological circumstances. If there are small areas of chemically different of damaged cell membranes in the human brain, normally insoluble Aβ peptide may be conformationally changed to a type of protein that can attach to normal areas of the brain and form plaques.

6.1.3 Interaction of Beta Amyloid Peptide with Lipid Bilayers

The interaction of the Aβ peptide with lipid bilayers was analyzed using fluorescence photobleaching recovery (FPR) and in situ SFM. FPR was used to monitor the fluidity of the bilayers by photobleaching areas of the fluorescent bilayer sample and recording the lateral motion as the bleached areas mix with the unbleached areas. The baseline fluidity of the lipid bilayers was established, and then the Aβ protein was incubated on the bilayers. It was demonstrated that the diffusion coefficient of the lipid bilayers was reduced when incubated with Aβ peptide, which indicated that there was an interaction between the lipid bilayers and the protein. The interaction of the protein with the bilayers was further analyzed with in situ SFM and revealed that the Aβ peptide actually physically disrupted the lipid bilayers and caused pores to form in the lipid bilayers. It is probable that the hydrophobic groups of the Aβ peptide interact with the hydrophobic inner areas of the lipid bilayer, which causes the Aβ peptide to insert itself into the lipid bilayer. This is a useful model of the cytotoxicity of the protein, because if pores formed in a cell membrane, they would act as ion channels. These ion channels would disrupt the cellular homeostasis, which could lead to cell death.¹
The Aβ protein solution that was incubated on the lipid bilayers was also examined. The solution showed evidence of extensive Aβ peptide aggregation, indicating that the lipid bilayers induced aggregation of the peptide in solution. This information is important because it provides evidence about the formation of amyloid plaques in the brains of humans. It is possible that the Aβ protein inserts into the lipid bilayer and is immobilized where it can act as a seeding nucleus for further aggregation of the protein to form.

6.2  Future Directions

6.2.1 Interaction of Lipids with Known Inserting Proteins

It was seen that the Aβ protein inserted into lipid bilayers under certain experimental conditions. For comparison purposes, the interaction of other proteins with the lipid bilayer system described here should be examined. Several antimicrobial proteins, such as protegrin-1, are known to insert into lipid bilayers. It has been well documented that this protein inserts into lipid bilayers and membranes, and it would be helpful to examine protegrin-1 under the same conditions as the Aβ protein was studied for comparison purposes. It would also be useful to inspect the interaction of the amylin protein with the lipid bilayers. Amylin is a protein associated with Type 2 Diabetes and is similar in structure to the Aβ protein. It has been shown that the amylin protein inserts into lipid bilayers and vesicles, therefore it could be compared with the insertion of the Aβ protein into lipid bilayers.

It would also be helpful to study the interaction of Aβ protein with various other types of lipids and different compositions of lipid membranes. Membranes with composed of mixtures of anionic lipids, such as dipalmitoylphosphatidylglycerol, cationic lipids, such as dipalmitoyltrimethylammonium propane, and zwitterionic lipids, such as dipalmitoylphosphatidylcholine, should be analyzed.
6.2.2 Interaction of Aggregation-Inhibitor Peptides with Lipid Bilayers

It has been confirmed that the inhibitor peptides synthesized by the Hammer Group (AMY-X) can interact with the Aβ protein assembly.\textsuperscript{17-19} It is proposed that these inhibitors will have an effect on the interaction of the Aβ protein with the lipid bilayers. It was seen previously that the interaction of the Aβ protein with lipid bilayers affected the lipid bilayer fluidity and physically disrupted the bilayers. It is possible that the AMY-X inhibitors will be able to curb the interaction of Aβ protein with lipid membranes. This hypothesis should be studied using FPR and in situ SFM. The fluidity of lipid membranes exposed to Aβ protein in the presence of AMY-X will be examined using by FPR and the assembly will be imaged using in situ SFM.

6.2.3 Detection of Biomarkers for Alzheimer’s Disease

Microcontact printing has become a useful tool in the manufacture of microanalytical devices, such as chemical and biological sensing devices. The evolution of cell and protein arrays depends largely on developing techniques that control topographical features and spatial presentation of surface molecules, such as microcontact printing. These arrays can be used for drug discovery, diagnostic assays and biosensors. With the discovery of a biological marker for the detection of Alzheimer’s disease,\textsuperscript{20} microanalytical devices can now be developed to detect Alzheimer’s disease. Microcontact printing is a viable option for the construction of such detection devices.

Patterning and immobilization of biologically active moieties with micrometer and nanometer scale control has proven integral to a range of applications in basic research, diagnostics and drug discovery. Future goals could be to capture Aβ protein in a specific orientation using microcontact printing and then use for other applications such as high throughput detection systems for drug development and for detection of Alzheimer’s disease. It
is possible to orient the Aβ protein specifically so that a β-sheet conformation is produced while maintaining the biomolecular behavior of the patterned Aβ protein. Detection systems that employ the Aβ-specific antibody could also be developed using patterned monolayers.

6.3 References


Appendix A

Deconvolution of Infrared Spectra

When infrared spectroscopy is performed on proteins that are oriented in different secondary structures, the spectroscopic bands sometimes overlap, as is seen in the amide bands of the Aβ peptide. When this happens, curve-fitting band deconvolution must be performed to resolve the overlapped peaks that occur when two or more protein conformations are present.

Infrared spectroscopy works by inducing a molecular vibration in a molecule. Energy is absorbed when the frequency of the radiation matches the frequency of the vibration of the molecule and causes a transition where the molecule goes from the ground vibronic state to an excited vibronic state; this results in the vibrational spectrum. After the molecules are excited, they quickly return to the ground state. This relaxation is called the amplitude lifetime, $\tau_a$. When the transition initially occurs, all of the excited molecules vibrate simultaneously, but the molecules begin to vibrate out of sequence with each other as difference in motion and vibrational frequencies take over. This randomization is called the coherence lifetime, $\tau_c$. As the vibrations become chaotic, the components begins to interfere with each other and dephase, giving rise to the effective lifetime, $\tau$, which is a combination of the $\tau_a$ and $\tau_c$ components.$^1$

Several factors must be determined when performing curve fitting. One of these is the type of peak function to use. The type of function depends on the relationship between $\tau_a$ and $\tau_c$. When $\tau_c >> \tau_a$, the excited molecule relaxes before incoherence becomes significant and the IR peak has a Gaussian shape.$^1$ In this situation, a Gaussian function will resolve the overlapped peaks the best. This function is given by the equation:

$$f(x) = \frac{1}{\sqrt{2\pi}w} e^{-\left(\frac{x-x_0}{w}\right)^2} \quad (4 \ln(2))$$

Equation A.1
where \( x_0 \) is the position (frequency) of the peak, \( H \) is the peak height (intensity), and \( w \) is the line width which is given as the full width at half height value.\(^2\)

When \( \tau_c \ll \tau_a \), the incoherence happens quickly, and dephasing of the molecular vibrations is prominent.\(^1\) The shape of these overlapped peaks is sharp in the center with long wings sloping down from the center. A Lorentzian function will help resolve overlapped peaks the best in this situation. This function is given by the equation:

\[
f(x) = \frac{H}{4\left(\frac{x - x_0}{w}\right)^2 + 1}
\]

Equation A.2

The GRAMS32 program chooses the best fit based on the experimental relationship between \( \tau_a \) and \( \tau_c \). In the work described here, the Gaussian function was used to resolve the peaks since \( \tau_c \gg \tau_a \).

Another parameter affecting the correctness of fit is the number of peaks present. Fit is described by \( \chi^2 \), which is given by the following equation:

\[
\chi^2 = \sum_{i=0}^{n} \left( \frac{\text{Actual}_i - \text{Calculated}_i}{\text{RMS Noise}} \right)^2
\]

Equation A.3

where the \text{Actual} and \text{Calculated} variables are the measured and calculated intensity data, respectively, and the \text{RMS Noise} value is the estimated root mean squared noise in the actual data.\(^2\) The value \( n-f \) is the number of degrees of freedom in the data set, where \( n \) is the number of data points in the fitted region and \( f \) is the total number of variables from all the peak and baseline functions.\(^2\) If too many peaks are chosen a better fit will be produced, but some of the resolved peaks may be invented by the program to produce the fit. If too few peaks are chosen, a peak that is actually present will not be resolved, and this will result in a poor fit. Therefore, a
Fourier self-deconvolution (FSD) must be performed before the curve fitting process to determine how many unresolved peaks are actually present. A Fourier transform (FT) is first applied to the data:

\[
F(x) = \int_{0}^{\infty} A_i(\nu) \cos 2\pi \nu x \, d\nu
\]

Equation A.4

\[
= 0.5A_i^{0} \gamma_i \cos(2\pi \nu_i^{0}x) \exp(-2\pi \gamma_i x)
\]

where the coefficient \(0.5A_i^{0} \gamma_i\) is directly proportional to the area of the peak, the cosine term depends on the center wavenumber of the peak, and the exponential decay term is the deconvolution filter. An inverse FT function is then applied to the data to fully resolve the spectrum. The inverse FT function is achieved by multiplying \(F(x)\) by \(\exp(2\pi \gamma' x)\) to give the new function, \(F'(x)\):

\[
F'(x) = F(x) \exp(-2\pi \gamma' x)
\]

Equation A.5

\[
= 0.5A_i^{0} \gamma_i \cos(2\pi \nu_i^{0}x) \exp[-2\pi(\gamma_i - \gamma')x]
\]

After the peaks are resolved, a goodness of fit parameter, \(\chi^2\), is calculated. Typically, a \(\chi^2\) value less than 10 is deemed acceptable.

To determine if the program worked accurately under the parameters that were set, a test was done. Three peaks were made up using the Excel program. These peaks were added together to produce a mock overlapped peak, as is shown in Figure A.1. The resulting peak was imported into GRAMS/32 where the FSD and curve fitting functions were performed using the Gaussian function to resolve the peaks. The results are depicted in Figure A.2. When the starting data and the processed data are plotted in the same graph, the curve fitted peak match up almost exactly \((\chi^2 = 1.2)\) with the original peaks (Figure A.3). This proves the accuracy of the GRAMS/32 program in resolving the overlapped peaks, provided the correct parameters are used.
Figure A.1. Peak created in Excel and added together to produce an imaginary overlapped peak.

Figure A.2. Peaks resulting from curve fitting and deconvolution of the imaginary overlapped peak.
Figure A.3. Peaks created in Excel and added together to produce an imaginary overlapped peak combined the peaks resulting from curve fitting and deconvolution of the imaginary overlapped peaks.
Figure A.4. Deconvoluted RAIR spectra of HS-(CH$_2$)$_{17}$-CH$_3$/Au exposed to aqueous solutions of 100 μM Aβ in 10 mM KOH (pH 11.5) for 15 h. Original spectra (dash), fitted curve (dash-dot), and individual Gaussian components (dot) with a reduced $\chi^2$ value of 5.2.
Figure A.5. Deconvoluted RAIR spectra of HS-(CH$_2$)$_{15}$-COOH/Au exposed to aqueous solutions of 100 μM Aβ in 10 mM KOH (pH 11.5) for 15 h. Original spectra (dash), fitted curve (dash-dot), and individual Gaussian components (dot) with a reduced χ$^2$ value of 10.0.
Figure A.6. Deconvoluted RAIR spectra of HS-(CH$_2$)$_{10}$-SO$_3$H/Au exposed to aqueous solutions of 100 $\mu$M A$\beta$ in 10 mM KOH (pH 11.5) for 15 h. Original spectra (dash), fitted curve (dash-dot), and individual Gaussian components (dot) with a reduced $\chi^2$ value of 2.0.
Figure A.7. Deconvoluted RAIR spectra of HS-(CH$_2$)$_{11}$-OH/Au exposed to aqueous solutions of 100 $\mu$M A$\beta$ in 10 mM KOH (pH 11.5) for 15 h. Original spectra (dash), fitted curve (dash-dot), and individual Gaussian components (dot) with a reduced $\chi^2$ value of 9.1.
Figure A.8. Deconvoluted RAIR spectra of CF$_3$-(CF$_2$)$_7$-(CH$_2$)$_2$-SH/Au exposed to aqueous solutions of 100 μM Aβ in 10 mM KOH (pH 11.5) for 15 h. Original spectra (dash), fitted curve (dash-dot), and individual Gaussian components (dot) with a reduced $\chi^2$ value of 5.3.
Figure A.9. Deconvoluted RAIR spectra of CF_3-(CF_2)_7-(CH_2)_2-SH/Au exposed to aqueous solutions of 100 μM Aβ in 10 mM KOH (pH 11.5) for 7 days. Original spectra (dash), fitted curve (dash-dot), and individual Gaussian components (dot) with a reduced χ^2 value of 4.3.
Figure A.10. Deconvoluted RAIR spectra of CF$_3$-(CF$_2$)$_7$-(CH$_2$)$_2$-SH/Au exposed to aqueous solutions of 100 μM Aβ in 10 mM KOH (pH 11.5) for 20 days. Original spectra (dash), fitted curve (dash-dot), and individual Gaussian components (dot) with a reduced $\chi^2$ value of 5.4.
References


Appendix B

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Vita

Mariah Joan Wooldridge McMasters was born December 10, 1976, in Paragould, Arkansas, to Hal and Joan Wooldridge. She attended Greene County Tech School District and graduated in 1995. She then attended college at Arkansas State University. While at ASU, she conducted research on beta deuterated tryptophan under the direction of Dr. Michael Panigot. She graduated in 1999 with Bachelor of Science degrees in chemistry and mathematics. The same year she married Anthony McMasters. The following year they moved to Louisiana, and Mariah started graduate school at Louisiana State University. She joined the research group of Dr. Robin McCarley on the Inhibiting Alzheimer’s Disease project, where she investigated the relationship between the aggregation of beta amyloid protein and chemically modified surfaces. While at LSU, she served as a National Science Foundation East Asia and Pacific Summer Institute Fellow to Taiwan and completed an internship at GE Advanced Materials Plastics Division. Mariah is currently completing the degree requirements for the degree of Doctor of Philosophy in chemistry.