Systematic Study of Amyloid Beta Peptide Conformations: Implications for Alzheimer’s Disease

Jeffy Pilar Jimenez

University of South Florida

Follow this and additional works at: https://digitalcommons.usf.edu/etd

Part of the American Studies Commons

Scholar Commons Citation
Systematic Study of Amyloid Beta Peptide Conformations: Implications for Alzheimer’s Disease

by

Jeffy Pilar Jiménez

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Department of Chemical Engineering
College of Engineering
University of South Florida

Major Professor: Norma Alcantar, Ph.D.
David Morgan, Ph.D.
Ryan Toomey, Ph.D.

Date of Approval:
July 27, 2005

Keywords: atr-ftir, biomimetic membrane, fibril, lipid bilayer, oligomer, sfa, soft supported

© Copyright 2005, Jeffy P. Jiménez
Dedication

I entirely dedicate this new achievement to my beloved: mom, dad, sister and brothers, for their support, love, advice, and words of encouragement.
Acknowledgments

Completion of this thesis involved guidance, dedication, and patience for which I am in debt to my major professor, Dr. Norma Alcantar. I would like to thank my committee members, Dr. David Morgan and Dr. Ryan Toomey for their words of encouragement and support. My sincere gratitude goes to:

- The Chemical Engineering Faculty and Staff for their help during the completion of my master
- The National Science Foundation - Integrative Graduate Education and Research Training (NSF-IGERT) program for their support and their concern on my educational and professional enhancement, and my special gratitude to Mr. Bernard Batson, Coordinator of the USF-IGERT program
- My peer graduate students
- Dr. Alessandro Anzalone, Mr. Jose I. Rey, and Mrs. Zoe Seda for their advice and support
Table of Contents

List of Tables v
List of Figures vi
Abstract viii

Chapter One: Introduction 1

1.1. Alzheimer’s Disease 1
1.2. Significance of this Work 5
1.3. Research Aims 6

1.3.1. Aim 1 6
1.3.2. Aim 2 7
1.3.3. Aim 3 7
1.4. Structure of this Thesis 7

Chapter Two: Fundamentals in Biochemistry and Cell Biology 9

2.1. Amino Acids 9
2.2. Peptide 10
2.3. The Peptide Bond 10
2.4. Proteins 11

2.5. Architecture of Protein Molecules 12

2.5.1. Protein Shape 12
2.5.2. Levels of Protein Structure 13
2.6. Amyloid-β Peptide and Protein Denature Process 15

2.7. Amphiphilic Molecules 16

2.8. Biomolecular Interactions 18
   2.8.1. Van der Waals Attractive Forces 18
   2.8.2. Hydrogen Bonds 18
   2.8.3. Ionic Bonds 18
   2.8.4. Hydrophobic Interactions 19

2.9. The Neuron Cell Membrane 20

Chapter Three: Chemistry and Conformations: Amyloid Peptide and Cell Membrane 22

3.1. Amyloid-β Peptide Conformations 22
   3.1.1. Monomers 22
   3.1.2. Oligomers 23
   3.1.3. Fibrils 23

3.2. Common Pathways of Aggregation Amyloid-β Peptide 24

3.3. Biomimetic Cell Membrane 26

Chapter Four: Experimental and Characterization Techniques 29

4.1. Surface Deposition Techniques 29
   4.1.1. Plasma Enhanced Chemical Vapor Deposition 29
   4.1.2. Langmuir-Blodgett Deposition 31

4.2. Physical and Chemical Characterization Techniques 32
   4.2.1. Atomic Force Microscopy (AFM) 32
   4.2.2. Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) Spectroscopy 33
Chapter Five: Experimental Design and Procedures  

5.1. Reagents and Materials  

5.2. Experimental Procedures  

5.2.1. Conditioning of Aβ Peptides and Synthesis of Structures  

5.2.1.1. Aβ1-40 and Aβ1-42 Peptides  

5.2.1.2. Amyloid-β Conformations  

5.2.2. Surface Characterization of the Amyloid Peptides Topology  

5.2.3. Chemical Characterization of the Amyloid Peptides  

5.2.4. Biomimetic Cell Membrane Construction and Studies of Interaction with Amyloid-β Peptides  

5.2.4.1. Soft-Support Layer  

5.2.4.2. Lipid Deposition  

5.2.4.3. Surface Topology  

Chapter Six: Results and Discussion  

6.1. Reaching the Overall Goal  

6.2. Aβ (1-40) and Aβ (1-42): Study and Comparison of their Structure and Kinetics  

6.2.1 Aβ (1-40) vs. Aβ (1-42): Comparison of their Structure  

6.3. Chemical Characterization of Aβ (1-42) Peptide Conformations  

6.3.1. Monomeric Conformation  

6.3.2. Oligomeric Conformations  

6.3.3. Fibril Conformation  

6.4. Membrane Construction
List of Tables

Table 1. Comparison of Biomolecular Interactions 20
Table 2. Primary Reagents Used 38
Table 3. Instrument Description and Miscellaneous Materials 39
Table 4. Amyloid β Peptides 40 and 42, Monomeric Stability Comparison 50
Table 5. Amyloid β (1-40) and (1-42), Oligomers Formation and Stability 51
Table 6. Amyloid β (1-40) and (1-42), Fibrils Formation and Stability 52
Table 7. Amide I and II IR Regions for Peptide Molecules 53
Table 8. Evolution of Individual Conformations for Monomeric Precursors 58
Table 9. Evolution of Individual Conformations for Oligomeric Precursors 60
Table 10. Evolution of Individual Conformations for Fibrillar Precursors 62
List of Figures

Figure 1. Two of the Alzheimer’s Disease Hallmarks 2
Figure 2. Regions in the Brain Affected by AD 3
Figure 3. Synthesis of Aβ in the Cell 4
Figure 4. L and D Isomers of Amino Acids 9
Figure 5. The Peptide Bond 11
Figure 6. Amyloid β (1-42) Peptide 13
Figure 7. Two Structural Motifs Arrange 14
Figure 8. Representation of an Amphiphilic Molecule 16
Figure 9. Micelle Formation by Amphiphilic Molecules in Aqueous Solution 17
Figure 10. Schematic Representation of a Neuron Picturing the Cell Membrane 21
Figure 11. Alzheimer’s Disease Amyloid-β Peptide Monomer 22
Figure 12. Pathway of Aβ Aggregation and Fibril Formation 24
Figure 13. Model of a Prototypical Cell Membrane 27
Figure 14. General Schematic Diagram of a PECVD Reactor 30
Figure 15. Water Plasma is Induced over the Silica Surface 30
Figure 16. Types of Deposition using LB Based on the Order of Layers 31
Figure 17. Atomic Force Microscope 32
Figure 18. IR Spectrum 35
Figure 19. Schematic Diagram ATR - FTIR Spectrometer 36
Figure 20. Langmuir-Blodgett Trough 45
Figure 21. Schematic of Lipid Bilayer Construction 46
Figure 22. Picture of the Wafer Contented in the Petri Dish 47
Figure 23. Average Structural Ratio (Height vs. Width) Comparison 54
Figure 24. Example of the Deconvolution for Fibrils at Initial Conditions 56
Figure 25. FTIR Spectra of Amyloid β (1-42) Peptide Monomeric Precursors 57
Figure 26. Structures Evolution with Time of Oligomeric Precursors 59
Figure 27. Time Evolution Spectra of Fibril Structures of Aβ (1-42) 61
Figure 28. Comparison of Surface Dynamics for the Three Peptide Structures 63
Figure 29. Surface Tension of DSPE as a Function of Lipid Concentration 64
Figure 30. Loading/Unloading Cycle for DSPE at 2.45mg/mL and 30mN/m 65
Figure 31. Deposition and Transfer of the Lipid DSPE Bilayer 66
Figure 32. Interacting Mechanisms Between Lipid Bilayers and Aβ (1-42) 66
Figure 33. Section Profile of Biomimetic Membrane in Contact with Fibrils 67
The amyloid beta peptide particularly the 40 and 42 amino acid residues are the responsible for plaque formation in Alzheimer's disease (AD) patients. Extra cellular plaque formation has been recognized after incessant investigations along with the formation of intracellular tau protein tangles as the hallmarks of AD. Furthermore, the plaque formation has been linked mostly as a cause of the disease and the tangles mostly as a consequence. Our investigation is focused on studying the formation of AD plaques. The amyloid beta (Aβ) is a physiological peptide secreted from neurons under normal conditions, along with other soluble forms cleaved from the amyloid precursor protein (APP). These soluble forms of APP have neuroprotective and neurotrophic functions, while the Aβ is considered an unwanted by-product of the APP processing. Under normal conditions there is an anabolic/catabolic equilibrium of the Aβ peptide; therefore, it is believed that the formation of the plaque does not take place. On the other hand, the neurons' surface may play an important role in the adhesion mechanisms of the Aβ peptide. Our experiments show that the neuron surfaces along with the media conditions may be the most important causes for progressive formation of plaques. We have
incubated rigid supports (mica) and soft biomimetic substrates (lipid bilayers on top of a PEG cushion layer drafted onto a silica surface) with the three different conformations of the Aβ peptide (monomeric, oligomeric and fibrils structures) to determine the adhesion mechanisms associated with in situ plaque formation. The soft biomimetic substrates have been assembled first by depositing and activating a thin film of silica (i.e., to create surface silanol groups). This film is then reacted with polyethylene glycol (PEG), which is a biocompatible polymer, to create a cushion-like layer that supports and allows the lipid bilayer to have high mobility. A lipid bilayer is then deposited on this soft support to reproduce a cell membrane using the Langmuir Blodgett deposition technique. The characterization of such biomimetic membranes has been studied by using Atomic Force Microscopy (AFM) in liquid environments. Our results show that these lipid bilayers are highly mobile. Additionally the structure and topography characteristics of the Aβ conformations have been followed with atomic force microscopy (AFM). The kinetics and rates of adhesion have been measured with attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy. Our results show the progress of the plaques' formation with time where simple monomers deposit on the substrates and allow the development of oligomeric species. The oligomers then grow into fibril-like structures leading finally to the plaques that eventually are seen to insulate real neurons and stop them from the synapse process. The ultimate outcome of this investigation will contribute to understand, prevent and determine possible mechanisms for removing AD plaques.
Chapter One
Introduction

1.1. Alzheimer’s Disease

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

After the age of 85, one out of every two people is affected either by AD or by what is called a mild cognitive impairment (MCI) condition [2]. MCI is recognized by a significantly reduced memory with cognition being within a normal range and is considered as a symptom indicating the onset of dementing disorders primarily represented by AD [2]. More than 15% of the cases of MCI turn into Alzheimer’s disease [3]. The estimated number of AD’s cases is over 20 million worldwide and is expected to keep growing as the world population ages [2].

The pathologic mechanisms of Alzheimer’s disease are still uncertain, however two hallmarks are recognized: intracellular neurofibrillary tangles (NFTs) and extracellular senile plaques [4]. The NFTs are structures composed of paired helical filaments mainly formed of abnormally hyperphosphorylated tau protein (P-TAU) [5] which is mainly but not exclusively neuronal microtubule-associated protein (MAP) [6].
Some of the roles of the tau protein are: stabilization of axonal microtubules, signaling transduction, interaction with the actin cytoskeleton, and neurite outgrowing. The formation of this NFT is believed to be a consequence of the amyloid plaque formation [2]. Figure 1 shows both deposit of plaques and NFT. The extracellular senile plaques are composed by amyloid peptide deposits of mainly 40- and 42-amino acids long (Aβ_{1-40} and Aβ_{1-42}) [5]. Amyloid-β peptides do not appear to play a major physiological role indeed the based on the original amyloid cascade hypothesis amyloid plaque depositions or partially aggregated soluble Aβ trigger a neurotoxic cascade, thereby causing neurodegeneration and finally pathology of AD [7].

![Figure 1. Two of the Alzheimer’s Disease Hallmarks: (left) A High-power photomicrograph of an amyloid plaque; (right) A photomicrograph of silver stained (black) neurofibrillary tangles in the cell bodies [7]](image)

The plaques and tangles are accumulated in neurons throughout the cortical and limbic brain regions [5] (regions shown in Figure 2). The cortical region is responsible for the abilities and activities related to our thinking, some examples are language and abstract thinking, it is also involved in basic aspects of perception, movement, and adaptive response to the outside world [8]. The limbic region is primarily responsible for our emotional life and the formation of memories [8].
Based on the studies over the years a hypothesis involving plaque formation has been proposed and studied. The amyloid-β hypothesis sustains that the main cause of AD is the presence of this protein as the main component of the plaques that block the synapses in the neuronal cells [9]. A historical report about the discoveries that relate AD with amyloid-β peptides is shown in Appendix A.

**Figure 2. Regions in the Brain Affected by AD**

The amyloid-β is a physiological peptide is secreted from the neurons under normal conditions in addition with other soluble peptides that are cleavaged from the transmembrane amyloid precursor protein (APP). APP consists of a large extracellular N-terminal domain and a smaller intracellular tail (Figure 3). Apparently the soluble forms of APP have neuroprotective and neurotrophic functions. However, Aβ is considered as an unwanted by-product of the APP processing [2].

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

**Figure 3.** Synthesis of Aβ in the Cell. The β-amyloid domain is partly embedded in the plasma membrane. To generate Aβ, APP is first cleaved by β-secretase, resulting in the release of β-secretase-cleaved soluble APP (β-sAPP). In the second step, the 99 amino acid C-terminal fragment (C99 CTF) is cleaved by the γ-secretase complex releasing free β-amyloid[3]

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

Under normal conditions, in the brain there is an anabolic/catabolic equilibrium of the amyloid-β peptide; that prevents the formation of AD plaques [2]. However, once this
equilibrium is broken, the protein progressively aggregates causing the plaque formation [2]. There are more than 13,000 publications [10] concerning Aβ peptide have shown different approaches to treat and prevent AD. These studies involve genetics and molecular biology, among other approaches. However, among all these publications little has been done about the interacting mechanisms of the proteins structures with the cell membrane. In this thesis, we conduct systematic investigations to elucidate the mechanisms between protein and membrane structures under different physiological conditions (i.e., varying the pH, temperature of the media, and the composition of the membrane). The surface of the neurons plays an important role in the adhesion mechanisms of Aβ; mechanisms associated with a cascade of events that may begin with the aggregation of monomer (seed) as bigger molecules (oligomers) that eventually result in larger structures (fibril) that accumulates on the cell membrane forming plaques blocking synaptic processes. It is believed that this surface coating leads to intracellular tangles formation and finally to neuron death. Our approach is to mimic cell conditions changing the media and characterizing the structure formation and the interaction mechanisms of these forms using a biomimetic cell membrane.

1.2. Significance of this Work

This work represents a contribution to the “amyloid hypothesis” investigation, that not only states that AD is characterized by the reduction and increase levels of sAPPα and Aβ respectively when the APP metabolism is altered, but also views other abnormalities in AD as either leading to Aβ plaque formation/deposition [11]. We are mainly focused in answering two questions:
a. What are the intermolecular forces and kinetic mechanisms involved in the formation of the different amyloid-β (Aβ40 and Aβ42) peptide conformations (i.e., monomeric, oligomeric and fibril)?

b. What are the surface interactions between amyloid-β peptide conformations and cell membranes, and their role in plaque formation?

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

1.3. Research Aims

This work has been divided in three main aims:

1.3.1. First Aim

To study and compare conformation assembly for Aβ (1-40) and Aβ (1-42): This will be done by looking at the stability of the monomeric state and the formation and stability of oligomers and fibrils. Many studies have proven the medium influence on the amyloid peptide conformations [12-16]. Therefore, we need to find a reproducible conformational assembly scheme based on the medium conditions employed, these are controlling pH, temperature, and incubation time. This study is done for the two characteristic peptides found in the Alzheimer’s disease patients, Aβ (1-40) and Aβ (1-42). We are using the Atomic Force Microscopy (AFM), which allows one to visualize the structures with a resolution within nanometers, giving topological details of the structures.
1.3.2. Second Aim

To study and understanding of oligomeric and fibrillar kinetics: In order to properly understand the adhesion mechanisms of Aβ conformations to the cell membrane that drive to plaque formation, it is needed to understand first their kinetics of its formation. This study is conducted using the Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy. This technique allows one to recognize the structural chemistry of the molecules under study in real time.

1.3.3. Third Aim

To study the adhesion mechanisms: This aim is subdivided in two stages:

a. The study of adhesion behavior of monomeric, oligomeric and fibrillar forms with variable substrates looking at the substrate rigidity or softness dependency. This study is done by coupling AFM and ATR-FTIR analysis.

b. To analyze the dominant processes during the aggregation of the three Aβ conformations by deconvolution of the ATR-FTIR spectra and the relationship of the topography ratios of the AFM scans. For this specific aim different substrates have been used such as mica, and biomimetic cell membranes constructed by assembling lipid bilayers.

1.3. Structure of this Thesis

For a systematic study of the interactions between Aβ and membrane surfaces it is needed to know some basic concepts concerning molecular biology, biological
interaction forces, and cell membrane (interface properties), which will be described in Chapter two among other basic concepts.

A brief description of the systems that have been studied are presented in Chapter three. The interest on looking at the monomeric, oligomeric and fibrillar conformations and their relationship to AD are also explained. Additionally, a concise explanation involving biomimetic cell membrane structural design is described.

Chapter five illustrates the technical details of this investigation and all the protocols followed in the preparation and characterization of the systems.

The results and discussion of this work are presented in chapter six, as well as a comprehensive analysis of the data.

The conclusions for this work along with the final suggestion and future work are presented in Chapter seven.
Chapter Two

Fundamentals in Biochemistry and Cell Biology

2.1. Amino Acids

Amino acids are the building blocks of proteins. A so-called $\alpha$-amino acid consists of a central carbon atom, called the $\alpha$ carbon, linked to an amino group, a carboxylic acid group, a hydrogen atom, and a distinctive $R$ group. The $R$ group is often referred to as the side chain. Because of the four different groups connected to the tetrahedral $\alpha$-carbon atom, $\alpha$-amino acids are chiral; the two isomers are called the $L$ isomer and the $D$ isomer (Figure 4). Only $L$ amino acids are constituents of proteins [17]. Essentially, 20 amino acids are components of protein in living organisms. The properties displayed by proteins, which in one way or another mark the path of protein aggregation, come from amino acid features such as the capacity to polymerize, novel acid-based properties, varied structure and chemical functionality in the amino acid side chains, and chirality [18].

![Figure 4. L and D Isomers of Amino Acids (R refers to the side chain). The L and D isomers are mirror images of each other](image-url)
2.2. Peptide

Peptide is the name assigned to short chains of amino acids with less than fifty amino acid residues [17]. A common classification for peptides uses the number of amino acid units in the chain. That is, each unit in the protein chain is called an amino acid residue, whereas a chain of amino acids is called a sequence. The word residue comes from the act of residing, after an amino acid forms a peptide linkage upon joining a peptide chain. For instance, a peptide is a short chain with two amino acid residues, tripeptides are formed by three residues and so forth. Since the terminology becomes burdensome after 12 residues, the peptide chains with more than 12 and less than about 20 amino acid sequences are referred to as oligopeptides. According to this terminology, only when the chain exceeds 20 amino acids in length, the term polypeptide is then used [18].

2.3. The Peptide Bond

Peptides and proteins are sequential and unbranched polymers of amino acids linked head to tail. In every instance, a carboxyl group is linked to an amino group through a type of covalent amide linkage referred to as peptide bonds [18]. When a peptide bond is formed, the reaction releases water molecules.

The peptide “backbone” of a protein follows a repeated sequence –N-C\(\alpha\)-C-, where the N represents the amide nitrogen, and the C\(\alpha\) is the \(\alpha\)-carbon atom of an amino acid in the polymer chain. A schematic of the peptide backbone is shown in Figure 5 [18].
The geometry of the protein backbone is somewhat responsible for the unique qualities that proteins attain. For instance, the peptide bond is essentially planar, and has what it is considered a double-bond strength (although it is not a double-bond in essence) that prevents free rotation around it. Also, the peptide bond is uncharged, the absence of charge allows tightly packing of amino acid chains, linked by peptide bonds, into globular structures [17]. Even though peptide bonds only lost or gain protons at extreme pH conditions, the overall charge of a protein can be induced by changing the pH of the media [18].

In contrast with the peptide bond, the bonds between the amino group and the α-carbon atom, and between the α-carbon atom and the carbonyl group are mainly single. This causes two adjacent rigid peptide units to rotate about these bonds only and allows the residues to take various orientations with respect to each other in the chain. Consequently, the proteins can fold in scores of different ways owing to the free rotation between two each amino acid groups in the chain [17].

**Figure 5.** The Peptide Bond Shown in its Usual Trans-Formation of Carbonyl O and Amide H. The Cα atoms are the α-carbon of two adjacent amino acids joined in a peptide linkage. The dimensions and angles are average values observed by crystallography analysis of amino acids and small peptides. The peptide bond is stippled.
2.4. Proteins

In brief, the term protein defines molecules composed of one or more polypeptide chains. Proteins having only one polypeptide chain are monomeric proteins. Proteins composed of more than one polypeptide chain are multimeric proteins or oligomeric. Amyloid-β and other fibril-like proteins are called homomultimeric proteins, since they only contain one kind of polypeptide. Heteromultimeric proteins are composed of several different kinds of polypeptide chains. Multimeric proteins are usually designated by Greek letters and subscripts to denote their polypeptide composition such as in the case of β-amyloid [18].

2.5. Architecture of Protein Molecules

The architecture of proteins is usually arranged as follows:

2.5.1. Protein Shape

Proteins can be organized according to their basic of shape. That is, they can be fibrous or globular. Fibrous proteins tend to have relatively simple, regular linear structures, such as β-amyloid fibrils. These proteins often have structural roles in cells (e.g., collagen and fibrinogen). Typically, they are insoluble in water or in dilute salt solutions. In contrast, globular proteins are roughly spherical. The polypeptide chain is compactly folded in globular proteins, so that hydrophobic amino acid side chains are in the interior of the molecule and the hydrophilic side chains are at the outside of the molecule. Hence, globular proteins are very soluble in aqueous solutions.[18]
2.5.2. Levels of Protein Structure

To cope with the complex architecture of protein molecules, they have been classified in several levels depending on their structural conformation.

a. Primary Structure

The amino acid sequence is the primary structure of a protein. A schematic representation of this conformation is shown in Figure 6 [18].

![Figure 6. Amyloid β (1-42) Peptide Visualized as a Simple Amino Acid Sequence of 42 Residues](image)

b. Secondary Structure

Secondary structures are one level higher than primary structures and represent a three-dimensional arrangement of the polypeptide in space [18]. Polypeptide chains can arrange themselves into characteristic patterns by means of hydrogen bonding interactions between adjacent amino acid residues. These patterns can appear as helical or pleated segments as shown in Figure 7. These segments constitute regular structures that extend along one dimension, like the coils of a spring. Most proteins exhibit this type of structures, and depending on the medium conditions they arranged themselves as α-helix or β-sheet structures. Amyloid peptide structures are usually described in this classification.
Figure 7. Two Structural Motifs Arrange the Amino Acid Sequence into a Higher Level of Organization Predominantly: The α-helix and the β-pleated strand. Atomic representations of these secondary structures are seeing. The flat, helical ribbon is used for the α-helix and the flat, wide arrow is used for β-structures. Both of these structures own their stability to the formation of hydrogen bonds between the N-H groups and the O=C functions within the polypeptide backbone.

c. Tertiary Structure

A higher level of structure is generated when polypeptide chains assume a more compact three-dimensional shape by bending and folding. For instance, ternary structure allows proteins to adopt a globular. Ternary structures are usually seen in those proteins commonly existing in cells. The globular conformation promotes a lower surface-to-volume ratio and shields the protein from interacting with the solvent [18].
d. Quaternary Structure

Many proteins consist of two or more tertiary structures. Each of these tertiary structures is commonly referred to as a subunit of a protein. This subunit organization in proteins constitutes yet another level in the hierarchy of protein structure [18].

2.6. Amyloid-β Peptide and Protein Denature Process

The term amyloid was introduced in 1854 by the German physician scientist Rudolph Virchow. In his studies, he analyzed cerebral corpora amyloacea that had an unusual and abnormal appearance when seen under the microscope. Virchow concluded that the substance responsible for such macroscopic abnormality was cellulose and named it “amyloid”, derived from the Latin amylum and the Greek amylon that means “starch”. Later on, Friedreich and Kekule demonstrated the presence of protein in a “mass” of amyloid and pointed the high nitrogen content and absence of carbohydrates. Several events have led to the study of amyloid from a single type of protein to a class of proteins with propensity to undergo distinct changes in conformation (denature), hence resulting in fibril formation.[19]

For instance amyloidoses are fundamentally diseases related to denatured proteins. The propensity to denature of such proteins may be enhanced by mutation, environmental factors or posttranslational modifications. Additionally, some proteins are “natively denatured” or conformationally mutable. Cells contain elaborate machinery to ensure that proteins fold properly, such machinery includes proteins such as chaperones and processes such as selective degradation systems. The proteasome is a degradation system that disposes of misfolded proteins. A characteristic that distinguishes amyloids
from other misfolded or denatured proteins is their ability to aggregate into higher order oligomeric and fibrillar structures that allows them to evade quality control systems such as the proteasome. Inhibition of the proteasome or lysosomal\(^1\) degradation systems can lead to the accumulation of misfolded amyloidogenic proteins and peptides. Amyloid aggregates also inhibit the proteasome\(^2\), most likely by adopting stably folded structures that cannot be unfolded and therefore cannot be transported into the central catalytic pore of the proteasome enzyme complex. It is thought that the critical initiation or nucleating event in the formation of amyloid is the aggregation of stable \(\beta\)-sheet structures that may form a seed. This system grows and propagates owing to the amyloid structure’s relatively resistance to degradation \textit{in vivo} and the concentration of aggregation of misfolded proteins. Thus, a small increase in the concentration of misfolded proteins can dramatically accelerate the onset of AD. [20]

2.7. Amphiphilic Molecules

The concept of amphiphilic character is very important because the amyloid-\(\beta\) peptide and the lipids forming the cell membrane show this behavior. Amphiphilic molecules are compounds that consist of both strongly polar and strongly nonpolar groups. The name amphiphilic comes from the Greek \textit{amphi} meaning “both”, and \textit{philos} meaning “loving. They are also referred to as amphipathic molecules from the Greek

\[\text{Nonpolar tail} \quad \text{Polar head}\]

\textbf{Figure 8.} Representation of an Amphiphilic Molecule: Sodium Palmitate

---

\(^1\) Lysosomal: a membrane-bound cavity in living cells that contains enzymes that are responsible for degrading and recycling molecules.

\(^2\) Proteasome: a cluster of proteins found in the cytoplasm of living cells that degrades damaged or redundant proteins.
pathos meaning “passion, suffering”. Salts of fatty acids are typical examples of amphiphilic molecules that have special biological relevance. They have a nonpolar hydrocarbon tail and a strongly polar carboxyl head group, such as sodium salt palmitic acid (Figure 8). Their behavior in aqueous solution reflects the combination of the polar and nonpolar nature of these substances. The ionic carboxylate component hydrates readily, whereas the long hydrophobic tail is intrinsically insoluble. Because of this, sodium palmitate, a soap, shows little tendency to form a true ionic solution in water.

Nevertheless, sodium palmitate and other amphiphilic molecules readily disperse in water because the hydrocarbon tails of these substances are joined together in hydrophobic interactions as their polar carboxylate functions are hydrated in typical hydrophilic fashion.

The term micelle refers to a cluster of amphiphatic molecules (Figure 9). The amphipathic behavior of biomolecules has an enormous biological significance. The polar ends express their hydrophilicity by means of ionic interactions with the solvent, whereas the nonpolar ends are excluded from water and clustered into a hydrophobic domain constituted by the hydrocarbon tails of many of these molecules. Membranes are structures that define the limits and comportments of cells. [18]
2.8. Biomolecular Interactions

Molecules are formed by covalent bonds that allow atoms to hold together and thus to form molecules. Weak chemical forces work mainly in intramolecular or intermolecular attractions between atoms, such as hydrogen bonds, Van der Waals forces, ionic bonds, and hydrophobic interactions [18]. Each force is described below.

2.8.1. Van der Waals Attractive Forces are the result of induced electrical interactions between closely approaching atoms or molecules as their negatively charged electron clouds fluctuate instantaneously in time. These fluctuations allow attractions to occur between positively charged nuclei and the electron density of the incoming atom [18].

2.8.2. Hydrogen Bonds are formed between a hydrogen atom covalently bonded to an electronegative atom (such as oxygen or nitrogen) and a second electronegative atom that serves as the hydrogen bond acceptor. Hydrogen bonds are stronger than Van der Waals forces (as Table 1 shows) and have an additional property. That is, H bonds tend to be highly directional, forming straight bonds between donor, hydrogen, and acceptor atoms[18]. Hydrogen bonds are also more specific than van der Waals interactions because they require the presence of complementary hydrogen donor and acceptor groups. This type of bond has a remarkable influence in the possible structures that a molecule can adopt.

2.8.3. Ionic Bonds are the result of attractive forces between oppositely charged polar molecules, such as negative carboxyl groups and positive amino
groups. Since the opposite charges are restricted to sterically defined positions, ionic bonds can impart a high degree of structural specificity. [18]

2.8.4. **Hydrophobic Interactions** are due to the strong tendency of water to exclude nonpolar groups or molecules. Hydrophobic interactions arise from water molecules which prefer and share stronger interactions with one another. Since the strongest chemical interaction that is possible between two molecules actually determines its properties, the preferential hydrogen bonding interactions between polar water molecules excludes nonpolar groups. It is this exclusion of nonpolar groups that drives their tendency to cluster in aqueous solution. Thus, nonpolar regions of biological macromolecules are often in the molecule’s interior of the system, so that they are excluded from the aqueous environment. [18]

One of the reasons why living systems are restricted to a narrow range of environmental conditions is due to the fact that weak forces are present in biomolecular interactions. Biomacromolecules are functionally active only within a narrow range of media conditions, such as temperature, ionic strength, and alkalinity. Extremes situations of these conditions disrupt these weak forces, which are essential to maintaining the intricate structure of macromolecules. The loss of structural order in these complex macromolecules is called denaturation, and it is inevitably accompanied by loss of functionality. [18]
Table 1. Comparison of Biomolecular Interactions

<table>
<thead>
<tr>
<th>Force</th>
<th>Strength (kJ/mol)</th>
<th>Distance (nm)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van der Waals interactions</td>
<td>0.4 - 4.0</td>
<td>0.2</td>
<td>Strength depends on the relative size of the atoms or molecules and the distance between them. The size factor determines the area of contact between two molecules. That is, the greater the area, the stronger the interaction. Attractive VDW forces are inversely proportional to the sixth power of the distance, $r$, separating two atoms or molecules: $F \approx \frac{1}{r^6}$.</td>
</tr>
<tr>
<td>Hydrogen bonds</td>
<td>12 – 30</td>
<td>0.3</td>
<td>Relative strength is proportional to the polarity of the H bond donor and H bond acceptor. More polar atoms form stronger H bonds.</td>
</tr>
<tr>
<td>Ionic bonds</td>
<td>20</td>
<td>0.25</td>
<td>Strength also depends on the relative polarity of the interacting charged species. Some ionic bonds are also H bonds: $\text{HN}^+ \ldots \text{OOC}$</td>
</tr>
<tr>
<td>Hydrophobic interactions</td>
<td>&lt; 40</td>
<td>-</td>
<td>Force is a complex phenomenon determined by the degree to which the structure of water is disordered as discrete hydrophobic molecules or molecular regions coalesce.</td>
</tr>
</tbody>
</table>

2.9. The Neuron Cell Membrane

As it has been explained so far, the Aβ plaques are found on the surface of the neurons, hence it is important to identify what else is present on their surface. Therefore, the neuron cell membrane is the outer most surface facing the plaques and defines the boundaries that separates the inside and outside of the neuron cell. A lipid bilayer makes the backbone of cell membranes.
Membranes resemble supramolecular complexes in their construction since they are complexes of proteins and lipids maintained by covalent forces (Figure 8).

Some proteins traverse the lipid layer and influence the cell membrane structure. Some are for structural support, whereas others are receptors, channels or tunnels that allow passage of certain molecules into and out of the cell. Thousands of these channels populate the cell membrane and allow passage of the ions. For instance, sodium is a major player to the neuron cell because of its positive charge. This ion flows across the membrane in different ways depending on the exact status of excitation of the neuron. Additionally, in the case of cell membranes, the hydrophobic interactions are particularly important in maintaining the membrane structure. These interactions reflect the tendency of nonpolar molecules to come together as they are excluded by a polar solvent. [18]
Chapter Three

Chemistry and Conformations: Amyloid Peptide and Cell Membrane

3.1. Amyloid-β Peptide Conformations

3.1.1. Monomers

Monomers are about 1.0 ± 0.3 nm in size [16], with a molecular weight of: 4329.9 Da for Aβ (1-40) and 4514.1 Da for Aβ (1-42) (Appendix E). Monomers present mostly random coils and α-helix secondary structures (Figure 7).

The conformation of Aβ (1-40) and Aβ (1-42) single monomers can be visualized in Figure 11. These models have been generated using a comparative modeling online resource called 3D-JIGSAW (version 2.0). For this program, the source protein sequence is entered and then a mathematical model is developed based upon the potentials data that its bank holds assuming that there are not internal or solvent interactions whatsoever. The complete sequences for the Aβ (1-40) and Aβ (1-42) are provided in appendix E.

Figure 11. Alzheimer’s Disease Amyloid-β Peptide Monomer Theoretical Configuration
Monomers are highly stable in fluorinated alcohols including hexafluoroisopropanol (HFIP) and trifluoroethanol (TFE). These alcohols disrupt hydrophobic forces in aggregated amyloid preparations, and promote $\alpha$-helical secondary structures [16]. By dissolving $\text{A}\beta$ peptides in 100% HFIP, the resulting structures for A$\beta$ (1-40) and A$\beta$ (1-42) solutions are mostly $\alpha$-helical (~50-70%) and random coil (~30-50%), with a little $\beta$-sheet secondary structures (<1%) [16].

### 3.1.2. Oligomers

The oligomers also known as protofibrils are considered by several authors [21-23] as intermediate configurations in the fibrillogenic process. Oligomers are soluble spherical aggregates of 2-20 nm and have been study for many different types of amyloids with electron and atomic force microscopy [16, 20]. These spherical conformations appear at intermediate times of monomeric incubation and disappear as fibrils appear, suggesting that they are an intermediate step in the pathway of fibril formation. Their sizes vary depending on the number of monomer aggregates. The size of this quasi-stable intermediate aggregate ranges from dimmers up to particles of a million Da or greater and have been observed by a several techniques including SDS gel electrophoresis, fluorescence resonance energy transfer, light scattering, and atomic force microscopy [16, 20].

### 3.1.2. Fibrils

Many proteins assemble into amyloid fibrils, even those that are not disease related. Amyloid fibrils are approximately 6-10nm in diameter. The unbranched fibrils are characterized by a “crossed $\beta$-sheet” structure (Figure 7) in which the polypeptide...
chain is oriented perpendicularly to the fiber axis with the hydrogen bond parallel to the axis. The ability to form amyloids is not limited to disease related proteins. Rather, it appears to be a fundamental polymer motif of the polypeptide backbone, although it is not commonly observed in native proteins. Specific sequences or side chain interactions are not required for amyloid formation, since many different polyamino acids form crossed β-sheet amyloid fibers. This also suggests that the fundamental tendency to form amyloids is overcome by the fact that specific sequences tend to adopt stable structures with time.

3.2. Common Pathways of Aggregation amyloid-β peptide

One particular hypothetical pathway for Aβ fibril assembly is depicted in Figure 12. The earliest event in the process of aggregation is the formation of dimmers. Aβ derived from a segment of APP is believed to be predominantly α-helical in its native state. On the other hand, monomeric Aβ is largely conformed as random coil in solution.

Figure 12. Pathway of Aβ Aggregation and Fibril Formation.
The formation of Aβ dimmers coincides with the adoption of partial β structure. The Aβ dimmers assemble into higher order aggregates or oligomers, since there is a remarkable increase in the amounts of higher order aggregates and a decrease in the amount of dimmers.

These higher order Aβ aggregates are protein micelles with a hydrophobic core and a polar exterior that appear as 3 nm spherical particles determined by atomic force or electron microscopy [20].

At later times, these spherical shape particles appear to form curvilinear strings or “protofibrils”. The protofibrils undergo a conformation change to form the straight, unbranched mature fibrils. Once the amyloid fibril lattice has been established, the fibril can grow by the addition of Aβ monomer or dimer at the ends of the fibril. The higher order aggregates have molecular weights ranging from approximately $10^5$ to $10^6$ Da and with an average size corresponding to approximately 24 monomers. Aβ oligomers that appear as spherical particles are approximately 3 nm in diameter and have the characteristics of a protein amphipathic, since it lowers the surface tension of water. Formation of high molecular weight aggregates displays a critical concentration of approximately 25µM [20] and it is correlated with the formation of a new hydrophobic environment. At longer incubation times, the oligomers also appear to co-aggregate to form curvilinear fibrils with a characteristic beaded appearance. The spherical oligomers and protofibrils appear to be intermediates in the pathway of fiber formation because they disappear as mature fibrils accumulate and the rate of monomer dissociation from them is too slow to account for fibril growth. The transition appears to involve a major
conformational change, because fluorescence quenching analysis indicates that the carboxyl terminus is highly shielded from the aqueous solvent in the soluble oligomeric state, whereas it is exposed to the solvent in the fibrillar state. Once the amyloid fibril lattice has been established, it can grow by the addition of monomers onto the ends of the fibrils. [20]

Additionally, numerous *in vitro* studies have indicated that Aβ oligomers are toxic to cells or interfere with the normal function of neurons [21-23]. Mounting evidence indicates that soluble amyloid oligomers are generally toxic for a wide variety of disease related amyloids. Moreover, soluble oligomers are intrinsically toxic even though they are formed by proteins that are not disease related. The idea that soluble Aβ oligomers may play a primary role in AD pathogens is attractive, however, the evidence to support it is largely correlative and derived from *in vitro* toxicity studies. In order to evaluate the role of soluble Aβ oligomers in diseased human brain, there needs to be a way to distinguish them from soluble Aβ monomer, APP and Aβ fibrils. All of these species contain the same amino acid sequence, but differ in conformation [20]. These conformations are analyzed separately in this work.

### 3.3. Biomimetic Cell Membrane

Biomimetic cell membranes are used to represent the neuronal surface. This cell membrane model interacts *in vitro* with the three conformations of the amyloid-β explained above and is built with features of real cell membranes.

A double layer of lipids molecules makes up the main substance of the cell membrane. Lipids containing phosphorus in their head group are called phospholipids. In
the cell membrane, some molecules such as cholesterol (there are mainly three types of cholesterol lipids: phosphoglycerides, sphingolipids, and sterols), and proteins are located between the phospholipids. Cholesterol is known to strengthen the membrane and promotes rigidity. A variety of different proteins float within the phospholipids. Some proteins act as receptors in the cell, that is, as points of attachment for materials coming to the cell in the blood or tissue fluid. Additionally, enzymes are also participating in reactions occurring at the plasma membrane. Some are transporters, shuttling materials into or out of the cell, and some form channels through which only selected substances can pass. Carbohydrates are present in small amounts, combined either with proteins or with lipids. These carbohydrates cells help to recognize each other and to adhere to one-

![Figure 13. Model of a Prototypical Cell Membrane.](image)

another. A representation of a model cell membrane is shown in Figure 13 [24]. Lipid bilayers are represented by outer, hydrophilic (exposed to an aqueous environment) and inner, hydrophobic (maintains a barrier to solute movement) domains. Inserted through the membrane or in either inner or outer planes are various proteins that permit transport,
cell-to-cell and signal molecule interactions, and linkage of the membrane to the intracellular cytoskeleton along with cholesterol and carbohydrate molecules.

Additionally, most biological membrane lipids are composed by double-chained phospholipids or glycolipids with 16 to 18 carbons per chain, one of which is unsaturated or branched. These properties are not accidentally but carefully design by nature to ensure:

a. Biological lipids will self-assemble into thin bilayer membranes that can compartmentalize different regions within a cell, as well as protect the inside of the cell from the outside.

b. Because of their extremely low critical micelle concentration (CMC) the membranes remain intact even when the bathing medium is grossly depleted of lipids.

c. Due to unsaturation or branching, the membranes are in the fluid state at physiological temperatures which let them deliver various molecules through them.

The construction of the membrane for this work will be done on a substrate such that it allows the lipid bilayer to be in a mobile state, supported by a soft cushion. Such conditions simulate the real conditions of cell membranes. Preliminary studies involving just a simple lipid bilayer were performed. However, future work involves the addition of a new component to the bilayer to tune its mobility and surface conformation. We will also study the response of these model membranes the three different conformation of the amyloid β peptide (i.e., monomers, oligomers and fibrils).
4. Experimental Methodology and Characterization Techniques

4.1. Surface Deposition Techniques

Two deposition techniques are mainly used in this work, one is a chemical vapor deposition based on oxidation reaction and the other is a physical deposition based on interacting forces. Both deposition techniques are described below.

4.1.1. Plasma Enhanced Chemical Vapor Deposition

Plasma enhanced chemical vapor deposition (PECVD) is a thin film deposition method that is used to deposit thin film on metals and other materials that cannot sustain high temperature. It takes place at relatively low temperatures (< 300 °C) due to the use of a radio frequencies (RF) source that induces a plasma discharge to transfer energy into the reactant gases. The ionized gas (plasma) breaks down the gases and the activation energy barrier is lowered forming radicals (decomposition). These radicals produce many instable and stable species in the vapor phase, which then react with each other to form heavy stable molecules and deposit into a film.

In this work, PECVD is utilized to deposit a thin layer of silica (i.e., silicon dioxide (SiO₂)) that is later use as the substrate for a reaction with polyethylene glycol (PEG) that ultimately serves as the cushion support for the biomimetic model membranes.
The silica films are produced by reacting SiH₄ and O₂ gases in this reactor (Figure 14). The plasma is produced by a helical resonator discharge source using RF powered copper coil at 13.56 MHz [25].

![General Schematic Diagram of a Plasma Enhanced Chemical Vapor Deposition Reactor](image)

**Figure 14.** General Schematic Diagram of a Plasma Enhanced Chemical Vapor Deposition Reactor

PECVD is also used to produce water plasma (see Figure 15). Water plasma treatments are used to increase the hydroxyl group concentration on the silica surface controllably and reproducibly. The conditions for this process were established previously, so that the silica surface is completely saturated with hydroxyl groups, without roughening the surface [25].

![Water Plasma is Induced over the Silica Surface to Create Active SiOH Species that React with PEG to Produce a Soft Polymeric Support for the Model Membranes](image)

**Figure 15.** Water Plasma is Induced over the Silica Surface to Create Active SiOH Species that React with PEG to Produce a Soft Polymeric Support for the Model Membranes
4.1.2. Langmuir-Blodgett Deposition

The Langmuir Blodgett deposition technique is used to deposit compact and fine organized monolayers. The molecules that are deposited can be organic or inorganic, but with amphiphilic nature. The monolayer is formed on the surface of the subphase (water) by dissolving the amphiphilic substance in a suitable solvent. In this investigation chloroform is used to dissolve the lipids that form the monolayer on the water surface.

Once the monolayer is formed the deposition takes place upward or downward depending on the nature of the substrate and on the type of deposition that is been pursued (See Figure 16). The deposition is explained in more detail in the next Chapter.

Bilayers or multilayer can be deposited with this sophisticated technique but simple. The deposition of the biomimetic cell membrane is constructed as the type Y (Figure 16). However, we have a PEG monolayer instead of an initial monolayer of lipids.

Figure 16. Types of Deposition Using LB Based on the Order of Layers.
4.2. Physical and Chemical Characterization techniques

4.2.1. Atomic Force Microscopy (AFM)

The Atomic Force Microscope (AFM) also known as Scanning Force Microscope (SFM) or Scanning Probe Microscope (SPM). This technique allows one to visualize topographies of samples in very small ranges with resolution of about 10 pm [26]. The AFM technique operates by measuring attractive or repulsive forces between a tip and the sample. As the tip is drag through the sample surface a profile of heights is constructed as function of the cantilever deflection lectures that are translate into a real image of the surface under study [26]. There are two types of operational modes: contact and non contact. In its repulsive "contact" mode, the instrument lightly touches a tip at the end of a leaf spring or "cantilever" to the sample, moreover in contact mode the AFM measures hard-sphere repulsion forces between the tip and sample; this mode is mostly use when working in solid-liquid interfaces. In non contact mode, the AFM derives topographic images from measurements of attractive forces; but the tip does not touch the sample. This is also referred as tapping mode and it is mostly used for solid-gas interfaces. In this investigation where used both modes, due to the characteristic of the

![Figure 17. Atomic Force Microscope](image-url)
medium as will be explained in the following chapter. Figure 17 shows a schematic representation of a common AFM. [26]

4.2.2. Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) Spectroscopy

Infrared spectroscopy is being widely used for the analysis of peptides and proteins due to its reliability of probing the universally available amide (peptide) bonds, which show a distinct IR signals for differently folded peptides and proteins [27].

Fourier transform infrared (FTIR) spectroscopy has been predominantly utilized to study the secondary structure (α-helix and β-sheets) of proteins in recent years. Proteins or polypeptides have a continuous chain of amino acids connected via amide bonds also known as the “peptide bond”. The frequency at which amide bond vibrations occur can be attributed to different secondary structures in which the amide bonds are present. The differences in vibration of the amide bonds are due to different hydrogen bonding among amino acid residues (see Appendix D.2). For instance, α-helix and β-sheet folding have ordered hydrogen bonding, although differing in their patterns. The differential pattern in H-bonding, along with geometric orientations of amide bonds in α-helix, β-sheet, and random coil structures allows the different vibration frequencies associated with individual secondary structural folding. Amide vibrations (see Appendix D.1) in-plane mode involve C=O stretching, C-N stretching, N-H stretching and O-C-N bending, while an out-of-plane mode is due to C-N torsion [28]. These vibrations result in characteristic spectral features of proteins. Three major spectral regions (amide I, amide II, and amide III) have been identified based on theoretical and experimental studies [27].
The amide I vibration region, (1700-1600 cm\(^{-1}\)), has been widely used due to its strong signal exhibited by proteins in this region. However, there are several difficulties encountered in this region when analyzing protein spectra. The first difficulty encountered in this region is the OH vibrations caused by liquid water and water vapor that need to be subtracted out of the protein solution spectrum. Another problem is the difficulty of peak assignments. Since there is a greater deal of overlapping of peaks representative of the different secondary structures, it is difficult to assign bands to their correct structure. For example, bands at 1650-1655 cm\(^{-1}\) could be assigned either to \(\alpha\)-helix or random coil. Using the amide III region (1220-1320 cm\(^{-1}\)), many of these problems are resolved. In the amide III region, OH vibrations due to water do not interfere with spectrum in the amide III region as much as in the amide I region. The overlapping of bands arising from different secondary structure of a protein is not significantly encountered in this region. In the amide I region, the frequencies at which the different amide bond vibrations occur are not as localized as they are in amide III. In the amide III region, various spectral bands are more resolved in the original protein spectrum than they are in the amide I spectra. This fact allows a greater ease in peak refinement, as well as in peak assignment. The only drawback in using amide III is that the protein signal is significantly weaker than the signal obtained in the amide I region [27] as is shown in Figure 18, where two proteins has been superimposed: \(\alpha\)-chymotrypsin, and fibrils of A\(\beta\)1-42 (this was a sample taking after 10 hours of preparation).
Because IR spectroscopy does not affect protein and peptide samples either in solid or liquid form, it can be used for data collection. Two types of sample accessories are typically used: Windows such as calcium fluoride, and attenuated total reflectance (ATR) accessory, generally made of zinc selenide or germanium. For protein absorption studies, or for studies with solid powder and thin films, ATR accessory is most commonly used for sampling. One of the advantages of ATR technique in recording protein spectrum is the avoidance of solvent interference in IR spectra, because it limits the effective sample thickness to a thin layer near the surface of an internal reflection crystal (Figure 19) [27].

![Figure 18. IR Spectrum of α-Chymotrypsin (black line), and Fibrils of Aβ1-42 (in blue). The amide I region (1600-1700cm⁻¹) corresponds to the C=O stretch weakly coupled with C-N stretch and N-H bending. The amide II region (1500-1600 cm⁻¹) represents C-N stretch strongly coupled with N-H bending. The amide III region (1200-1350cm⁻¹) is N-H in-plane bending coupled with C-N stretching and also includes C-N and N-H deformation vibrations.](image)
Once the spectra are obtained, protein band positions are identified after spectral processing by Fourier self-deconvolution and/or second-order derivatization. A variety of software packages are available commercially.

**Figure 19.** Schematic Diagram of Light Undergoing Multiple Reflections in an ATR - FTIR Spectrometer

The data obtained from the deconvolved and second-derivative spectra are used to determine the number of bands and their positions in order to resolve the protein spectrum into their components.

This is accomplished with a curve-fitting process employing computer software commercially available from the manufacturers of IR instruments. The software resolves the original protein spectrum to individual bands that fit the spectrum. Two main parameters control the fitting process: (1) the individual bands, and (2) the baseline
position. Each individual band in turn is controlled by three parameters:

a. The height of the band.

b. The position of the band (wavenumber).

c. The bandwidth at half-height.

The program iterates the curve-fitting process, and each iteration flows (increases or decreases) each parameter (height, bandwidth, position and baseline) to determine individual parameters in order to achieve the best Gaussian, Lorentzian, or a mixture of Gaussian/Lorentzian-shaped curves that fit the original protein spectrum. A best fit is determined by the root mean square (rms) of differences between the original protein spectrum and the strength of individual bands. In order to estimate the strength of individual band, the band area or intensity is used to calculate the relative contribution of each band(s) to a particular secondary structure of the protein.

Additionally the fractional areas of the fitted component bands are directly proportional to the relative proportions of structure that they represent. The percentage of helices, β-structures and turns may be estimated by addition of the areas of all of the components bands assigned (after deconvolution) to each of these structure (based on the specific wavelengths this structures are) and expressing the sum as a fraction of the total area [28].
Chapter Five

Experimental Design and Procedures

Regarding the reagents used in the experiments, section 5.1 describes most of these in Table 2. Details about peptide composition can be found in Appendix E. Table 3 describes the instrumentation and related materials.

5.1. Reagents and Materials

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Acquired From</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyloid-β peptide (1-40)</td>
<td>Aβ(1-40)</td>
<td>White powder</td>
</tr>
<tr>
<td>Amyloid-β peptide (1-42)</td>
<td>Aβ(1-42)</td>
<td>White powder</td>
</tr>
<tr>
<td>1,1,3,3,3-Hexafluoro-2-Propanol (p:\textsuperscript{a} &gt;99%)</td>
<td>HFIP</td>
<td>Fisher Sci. ACROS Org.</td>
</tr>
<tr>
<td>Methyl Sulfoxide Anhydrous (p:\textsuperscript{a}:&gt;99%)</td>
<td>Me\textsubscript{2}SO or DMSO</td>
<td>Fisher Sci. ACROS Org.</td>
</tr>
<tr>
<td>Dulbecco's Phosphate Buffered Saline powder</td>
<td>PBS</td>
<td>MP.Biomedicals</td>
</tr>
<tr>
<td>Tris (Crystallized) p:\textsuperscript{a} &gt;99%</td>
<td>Tris</td>
<td>Fisher Sci. ACROS Org.</td>
</tr>
<tr>
<td>Polyethylene glycol (400Da)</td>
<td>PEG</td>
<td>Sigma</td>
</tr>
<tr>
<td>1,2-Dipalmitoyl-sn-Glycero-3-Phospho-ethanolamine-N-</td>
<td>DSPE (lipid)</td>
<td>Avanti Polar Lipids, INC.</td>
</tr>
<tr>
<td>Ammonium hydroxide</td>
<td>NH\textsubscript{3}OH</td>
<td>Fisher Sci. ACROS Org.</td>
</tr>
<tr>
<td>Hydrochloric acid (HPLC)</td>
<td>HCl</td>
<td>Fisher Sci. ACROS Org.</td>
</tr>
</tbody>
</table>

\textsuperscript{a} purity

38
The water used for all the experiments is ultra purified and obtained from a Millipore Water (Milli-Q) station with a resistance <18MΩ, and organics content <5ppb.

### Table 3. Instrument Description and Miscellaneous Materials

<table>
<thead>
<tr>
<th>Material / instrument</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifuge</td>
<td>Fisher Sci., model AccuSpin-400</td>
</tr>
<tr>
<td>Laminar flood cabinet</td>
<td>Forma Sci., 95 fpm &amp; 98 fpm</td>
</tr>
<tr>
<td>Vortex</td>
<td>Fisher Sci., Volts 120 Vac, Watts 150, 50/60 Hz</td>
</tr>
<tr>
<td>Atomic Force Microscope (AFM) for gas/solid Int.</td>
<td>DI Dimension 3000. Tapping Mode.</td>
</tr>
<tr>
<td>Cantilevers</td>
<td>Silicon. Nanosensors INC. $R=0.01-0.025$ Ohm/cm, $L=132$ μm, diam.=15nm, $f_0=281-339$ kHz, $k=28-54$ N/m.</td>
</tr>
<tr>
<td>AFM liquid/solid Int.</td>
<td>Asylum Res. Corp. Contact mode. The cantilever used is special for contact mode, and is made of</td>
</tr>
<tr>
<td>Cantilevers</td>
<td>SiN. $L=60$μm, diam.=&gt;80nm, $f_0=37-13$ kHz, $k=0.027-0.006$ N/m</td>
</tr>
<tr>
<td>FTIR Spectrometer</td>
<td>Nicolett, FT-IR 850 Bench</td>
</tr>
<tr>
<td>PECVD Reactor</td>
<td>Six-way stainless steel cross vacuum chamber with a 16-in long, 2-in-diam Pyrex cylinder connected to the feedthrough port at the top. With a helical resonator discharge source using a radio-frequency (rf) powered copper coil at 13.56 MHz, and surrounded by a grounded cylindrical copper shield enclosing the Pyrex tube. The rf power, provided by a RF Plasma products model RF5S power supply</td>
</tr>
<tr>
<td>L-B Trough</td>
<td>Nima Technology, Model 611D</td>
</tr>
<tr>
<td>Sonicator</td>
<td>Fisher Sci. Model FS30</td>
</tr>
<tr>
<td>pH meter</td>
<td>Fisher Sci., Accumet 1003</td>
</tr>
<tr>
<td>Water Bath</td>
<td>Precision 180 series water bath, T range: RT + 5 to 100°C</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>All gas tight Hamilton syringes, Pipettes, Adjustable micro Ependorf pipetters; Autoclaved Pipette tips, Ependorf Micro Tubes (1000μL). Dessicator. Mica. Silicon/Silica wafer.</td>
</tr>
</tbody>
</table>
5.2. Experimental Procedures

5.2.1. Conditioning of Aβ Peptides and Synthesis of Structures

5.2.1.1. Aβ1-40 and Aβ1-42 Peptides

Lyophilized peptide (1mg) kept at -20°C was removed from the freezer and let equilibrated for 30 minutes at room temperature (RT). The peptide is then dissolved in HFIP following the procedure described by W. Blaine Stine et al. [29]. HFIP is used to break any residual tertiary and β-sheet secondary structure of the peptide. The peptide is composed by α-helix (~50-70%) and random coil (~30-50%) structures mainly [29]. The HFIP is then injected into the peptide flask with a gas-tight Hamilton 0.5ml glass syringe until a concentration of 3.33µg/µl is obtained. Peptide dissolution takes not longer than five minutes. Such stock solution is aliquoted into 25 parts and storage in sterile micro centrifuge tubes of 12.5µl, containing 40µg of Aβ each approximately. Thus, each micro tube contains 9.238 nmol and 8.861 nmol for Aβ (1-40) and Aβ (1-42), respectively. Aliquoted samples are left under the laminar-flow hood to allow the HFIP to evaporate. The peptides are then placed for 12 hours in a desiccator at a vacuum of 27 in Hg. The final peptide samples have an even appearance (i.e., transparent film), which is appreciated at the bottom of the Eppendorf tubes. The tubes with the peptide are flushed and closed with nitrogen, wrapped with aluminum foil and parafilm, and enclosed in a jar at -20°C for later used.

5.2.1.2. Amyloid-β Conformations

a. Monomers

The following procedures are the same for both 1-40 and 1-42. 40µg of pretreated
Aβ peptide is removed from the freezer and let to equilibrate at RT. The pretreated peptide is dissolved in 2µL of DMSO, which is a polar, water-soluble organic solvent commonly used to solubilize hydrophobic peptides. After a minute of sonication with DMSO, 88µL of milli-Q water are added to the sample. Finally, it is incubated at RT [29].

b. Oligomers

First, a tris/NaCl/HCl buffer solution is prepared. This buffer serves as incubation media for the oligomer’s synthesis. The buffer is prepared in an Eppendorf of 50ml, where 0.3028g of tris and 0.2922g of NaCl are dissolved in DI water until the pH is fixed to 7.4. Sometimes a few drops of concentrated HCl need to be added. The pretreated peptide film kept at -20°C is then dissolved in 2µL of DMSO, sonicated for 1 min, and then incubated at 4°C in 88µL of Tris/NaCl/HCl. This procedure is described in detail by Kayed et al. [30].

c. Fibrils

The first step to incubate Aβ fibril structures is to prepare a solution of ammonium hydroxide at pH=8.5. The next step is to prepare a PBS buffer. Fibrils are prepared by dissolving 40µg of the pretreated peptide (at RT) in 20µL of NH₃OH and later adding 20µL of PBS. The sample is gently vortexed and finally incubated at a concentration of 1µg/µL at 37°C. This procedure was suggested by Dr. David Morgan (PhD) from the Department of Pharmacology & Therapeutics in the College of Medicine at the University of South Florida (USF), and director of the Alzheimer Research Laboratory at USF.
5.2.2. Surface Characterization of the Amyloid Peptides Topology

The sample deposition procedure is conducted under laminar flow cabinet to avoid contamination by undesirable particles. The samples to be analyzed with the AFM are deposited on mica, which is an inert, smooth, and easily to clean mineral, and widely used for biological samples in AFM imaging. Mica sheets are freshly cleaved prior to each deposition. A small Teflon O-rings (external \( \Phi 4.45 \text{mm} \), internal \( \Phi 1 \text{mm} \)) is used as an incubation chamber placed on top of the mica which ensure the maintenance of the desired location of fibril formation. 3µL of each incubated sample are dissolved with ultrapure water until a concentration of 20µM is reached. This concentration has been tested as the optimum to produce fibrils in a control manner. A small drop of such solution is then deposited on mica and incubated for 5 min, flushed with 3mL of ultrapure water, dry with ultrapure nitrogen, and stored in a desiccator under vacuum (30 torr) for later AFM study.

5.2.3. Chemical Characterization of Amyloid Peptides

No fibrils were observed when A\( \beta \)40 peptide was incubated. Therefore, the chemical characterization was done only for A\( \beta \)42 peptide conformations. The chemical analysis is done using an ATR-FTIR spectrometer, and a zinc selenide ATR crystal. The ATR cell allows a maximum volume of 62µL solutions (Figure 19). The crystal was first cleaned with isopropanol, and then a fresh media solution was flushed before peptide contact. The background for the IR data was taken when the ATR crystal was in contact with non-peptide media. Spectra were taken at about 10 min intervals for about 3 hours for monomers, 10 hours for oligomers, and 12 hours for fibrils, which is the time that it
took for the samples to reach saturation and steady state conditions. This part of the study was conducted in the Materials Research Laboratory at the University of California-Santa Barbara (UCSB).

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

5.2.4.1. Soft –Support Layer

Polyethylene glycol (PEG) is used to build the soft support layer. PEG is a linear or branched neutral polyether soluble in water and most organic solvents. Its chemical formula is: HO-(CH₂CH₂O)ₙ-CH₂CHOH. PEG is utilized in this study because its biotechnical applications. In fact, Norma Alcantar and collaborator have studied this polymer widely [25], including its use as cushion substrate or coating polymer. PEG is unusually effective at excluding other polymers from its presence in aqueous solution, such as lipids. This property is directly related to the fact that PEG rejects proteins and forms two-phase systems with other polymers; it is nontoxic; it has immunogenicity and nonantigenicity; it does not harm active proteins or cells even if it interacts with cell membranes; it attaches to other surfaces with very little effect on their chemistry; and it increases the solubility of large molecules despite their size. In addition, PEG coated surfaces become hydrophilic and protein rejecting. Therefore in our study we are insuring there is no absorption of the Aβ to the silica surface, if there is any adhesion, it is due to the interaction of the lipids themselves with this peptide. The inert character of PEG is based on its molecular conformation in aqueous solution, where PEG exposes uncharged hydrophilic groups and shows very high surface mobility (steric exclusion). It is worth
mentioning that in order to maintain its biological and biocompatible properties, the PEG has to be chemically attached to the surface. PEG is grafted to silica films deposited on silicon wafers creating a thin hydrophilic film. The silica deposition takes place reacting silane (SiH₄) and O₂ gases in a PECVD reactor (Figure 14). The samples are then exposed to a water plasma treatment in order to increase the hydroxyl group concentration on the surface controllably and reproducibly [25]. Finally, the surfaces are storage on a dry and free of particles container.

Concentrated sulfuric acid is used for cleaning the surfaces by submerging them overnight, and then are rinsed with DI water, dried with ultrapure nitrogen, and exposed to UV-light for about 5 minutes. The reactivation is done by submerging the surfaces in a NaOH solution at 10 %w for about 5 minutes. The surfaces are then rinsed with water and dried very well, since any traces of water could interfere with the PEG reaction. In the meantime, a solution of PEG (of wt ~ 400Da) is heated until it reaches 100°C maintaining constant agitation. The cleaned and activated surfaces are placed into the PEG for one hour. These parameters have been determined previously by Alcantar et. al. [25]. Once the PEG grafting reaction is done, the surfaces are retrieved from the hot PEG and gently rinsed with water (avoiding direct contact with the flux of water), and dried with ultrapure nitrogen. Sometimes the surfaces with PEG that are not used at the immediately after the reaction are storage in PEG media.

5.2.4.2. Lipid Deposition

The lipid bilayers are deposited onto the soft-support PEG layer using a Langmuir Blodgett (LB) trough (Figure 20). The first step is to ensure that the LB trough is
impeccable, since it is very important to avoid particles clinging to the surface. These particles can contaminate and perturb the deposition quality. To clean the trough, we use DI water and sometimes organic solvents. We look at the quality of the water by watching for bubble formation. Bubbles are a sign of contamination. A suction tube is used to eliminate these bubbles out of the water without touching the surface of the trough avoiding damage to the trough surface. The water is suctioned several times until non-bubbles are observed.

**Figure 20.** Langmuir-Blodgett Trough. The principal components the trough are highlighted in this photo

The LB trough is plugged to a computer that allows one to control parameters such as surface area, superficial tension, and substrate deepness necessary for the actual deposition. The trough is located inside of a laminar flow cabinet, which turn on when the trough is being cleaned. However, the air flow is off, while the lipids are being compressed or deposited. Once the water surface is clean, the surface pressure sensor is calibrated to 0 N/m. An isotherm is recorded using a velocity of 50 cm²/min. The trough
is clean and ready to used, if the isotherm is a straight line. The target surface is submerged for the deposition in the back socket of the trough. The trough is cleaned once more to avoid any possible particles introduced by the surface. Another isotherm is run to ensure cleanliness. The pressure is set to zero and the surface area is set to 300 cm$^2$. The area, deposition pressure, and lipid concentration used for the depositions were chosen based on previous experiments. Typical lipid concentrations are 15 µg/µL. A total volume of 100 µL is gently spread on the air/water interface using a syringe (giving it about 5 minutes for the solvent to evaporate). During the deposition, the surface pressure is kept constant at 30 mN/m. The deposition takes place at constant pressure and using a barrier velocity of 50 cm$^2$/min (Figure 21).

**Figure 21.** Schematic of Lipid Bilayer Construction. *(upper left)* PEG grafting reaction, *(upper right)* bilayer deposition, and *(bottom)* soft supported bilayer

### 5.2.4.3. Surface Topography

The soft-supported membranes constructed above have also been scanned by AFM in solution. They are kept in contact with saturated lipid solution at all times to
avoid air exposed and consequently, damage to the sample (Figure 22). The biomimetic cell membranes were also scanned while in contact with Aβ fibrils (0.933 µM). Scans are shown and explained in the next chapter.

**Figure 22.** Picture of the Wafer Contended in the Petri Dish. This is were the biomimetic membrane has been deposited
Chapter Six

Results and Discussion

This work is based on the fact that real interactions of Aβ peptides, with both 40 and 42 amino acid sequences, could be controlled and studied by following a comprehensive approach. The control on the structure of the Aβ peptides was done by optimizing the incubation conditions previously studied by Stine et al. [29] and Kayed et al. [30]. Depending on the conformation that is being pursued (i.e., monomeric, oligomeric and fibrillar), the peptide molecules will adopt diverse structures that defined their physical and chemical pathways of aggregation, which evolve with time. By tuning and promoting specific conformations, we were able to explicitly distinguish the response of the peptide in terms of their assembly, surface adhesion, chemical composition and kinetics. Explicitly, our results are three-fold:

a. We resolved the differences of behavior depending on the number of amino acid residues contained in each peptide molecule (i.e., Aβ-40 and Aβ-42)
b. We promoted structural rearrangement depending on the incubation media and substrate character
c. We were able to correlate peptide structures (i.e., α-helix, β-sheet, β-turns, or random) with the adhesion dynamics and ultimately, to determine the role of molecular stability of the peptide that leads to the formation of the AD plaques.
6.1 Reaching the Overall Goal

According to the three aims that we set from the beginning:

a. -- To study and compare conformation assembly for Aβ (1-40) and Aβ (1-42).

b. -- To study the adhesion mechanisms:

c. -- To study and understanding of fibrillar kinetics with model membranes:

We have been able to achieve our goal of elucidating, from the physicochemical point of view, the implications of the stability and conformations of Aβ in the formation of AD plaques.

6.2 Aβ (1-40) and Aβ (1-42): Study and Comparison of their Structure and Kinetics

The three Aβ peptide conformations that were considered in this work are: monomeric, oligomeric and fibrillar. From the AFM data, we were not only able to directly measure the height and width that corresponds to each of these structures on rigid (mica) and soft supported (model membrane) substrates, but also, to understand how their assembly occur with time and how their height/width ratio relates to their kinetic mechanisms for both peptides.

6.2.1 Aβ (1-40) vs. Aβ (1-42): Comparison of their Structure

The Aβ conformations were prepared on mica and analyzed by AFM. The samples were incubated as a function of time to observe the following processes: solubility, stability and dynamics of formation. The evolution of the monomers is shown below from their initial preparation until 72 hours of incubation in solution. The Aβ40 compared to Aβ42 shows very little absorption with time. In addition, the Aβ40 aggregates slowly into large clusters, with no indication of fibrillar structures being ever
formed (Table 4). On the contrary, the Aβ42 evolve in two different stages where an initial effect took place dominated by dissolution of the molecule, and the formation of thin large fibrils resulted between 9 and 20 hours of incubation.

**Table 4. Amyloid-β Peptides 40 and 42, Monomeric Stability Comparison**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EVOLUTION TIME [hours]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Aβ40</td>
<td><img src="image1" alt="Image" /></td>
</tr>
<tr>
<td>Aβ42</td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Aβ40</td>
<td><img src="image7" alt="Image" /></td>
</tr>
<tr>
<td>Aβ42</td>
<td><img src="image10" alt="Image" /></td>
</tr>
</tbody>
</table>

The scan size for all the images is 2 µm and the height scale is indicated by the bar at the right hand side of each picture.
Likewise, oligomeric structures show similar results, where the Aβ40 did not form fibrillar conformations but clusters. Again, the Aβ42 formed fibrillar structures after 9 hours of incubation (Table 5).

Table 5. Amyloid-β (1-40) and (1-42), Oligomers Formation and Stability

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EVOLUTION TIME [hours]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>9</td>
</tr>
<tr>
<td>40</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>&gt; 25</td>
</tr>
</tbody>
</table>

They did not form fibrils

More and longer fibrillar conformations were observed

Similarly, scan size for all the images is 2 µm and the height scale varies from each picture and is indicated by the bar at the right hand side of each picture.
In the case of fibrillar structures, the Aβ40 behaves in the same way as for monomers and oligomers. It was very surprising that almost no fibrils were detected, although the incubation conditions should have produced them. On the contrary, fibrils were observed on the Aβ42 after 10 hours of incubation (Table 6).

**Table 6.** Amyloid-β (1-40) and (1-42), Fibrils Formation and Stability

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EVOLUTION TIME [hours]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;5</td>
</tr>
<tr>
<td>Aβ</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td><img src="image1.png" alt="Image" /></td>
</tr>
<tr>
<td>42</td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>40</td>
<td><img src="image7.png" alt="Image" /></td>
</tr>
<tr>
<td>42</td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Scan size is 2 µm and the variable height scale as indicated on each picture.
A very interesting result display by the Aβ42 fibrils is that they are intrinsically different for each structure. In other words, if we look at the scans after 20 hours of incubation time, the fibrils formed by the oligomers are thicker than that formed by fibrils or monomeric precursors. In addition, the fibrils formed at longer times seem to be similar in thickness and shape. This indicates that the preferential structure independently of the precursor molecules for the Aβ42 is fibril conformation.

In order to compare the evolution of the topography of Aβ structures quantitatively, we have measured and compared the average ratios between height and width for each scan, which relates to the affinity of adhesion and surface density. Details about how these values were calculated are shown in the Appendix F section.

This analysis indicates that two of the three structures undergo a conformation change at 11 hrs for monomers and 18 hrs of incubation for oligomers for both Aβ40 and Aβ41, which corresponds to taller fibrils while the thickness remains low. This conformation change (maximum point) determines a saturation point, where the fibrils seem to pile up instead of spreading on the substrate. After that, both molecules show that the fibrils continue spreading onto the surface again. However, for fibril structures the behavior is very different. The Aβ40 and Aβ42 show opposite performance. In addition, we observed three interesting stages, one at 4, one at 10 and one at ~48 hours. The one at 4 and 50 hours correspond to a maximum in the Ratio value for Aβ40, but a minimum values for Aβ42. This is also related to the saturation of the surface as explained above. At 10 hours both peptides show similar surface density and topography.
6.3. Chemical Characterization of Aβ (1-42) Peptide Conformations

By using the ATR-FTIR technique, we are able to recognize the type of conformation that each precursor molecule has as a function of time. This study shows the evolution and dynamics of each chemical structure as a function of incubation conditions. We were able to distinguish specific structures depending on the IR vibration modes of the peptides. These structures show well defined processes indicating its dilution, molecular arrangement, and a steady state (equilibrium).

Figure 23. Average Structural Ratio (Height vs. Width) Comparison as a Function of Time Between Aβ40 and Aβ42 Conformations as Scanned by AFM
(top left) Monomeric precursors
(bottom left) Oligomeric precursors
(bottom right) Fibrillar precursors
Prior to present this analysis; let’s define the ranges of wavenumber used to classify the existence of determined structure (Table 7).

**Table 7. Amide I and II IR Regions for Peptide Molecules**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Wavenumber (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amide I region(^3)</strong></td>
<td></td>
</tr>
<tr>
<td>Beta turn</td>
<td>1695-1670 Intermolecular β-structure</td>
</tr>
<tr>
<td></td>
<td>1690-1680 Intramolecular β-structure</td>
</tr>
<tr>
<td>Random coil</td>
<td>1645-1648</td>
</tr>
<tr>
<td>Alpha helix</td>
<td>1657-1648</td>
</tr>
<tr>
<td>Beta sheet</td>
<td>1640-1630 Intramolecular β-structure</td>
</tr>
<tr>
<td></td>
<td>1625-1610 Intermolecular β-structure</td>
</tr>
<tr>
<td><strong>Amide II region(^4)</strong></td>
<td></td>
</tr>
<tr>
<td>NH(_2) deformations</td>
<td>1567 N-H bending (60%); C-N stretching (40%)</td>
</tr>
<tr>
<td>Related to proteins absorption/desorption</td>
<td>centered at 1550</td>
</tr>
</tbody>
</table>

From the FTIR spectra, a deconvolution of the absorbance data was performed to differentiate from all the structures present in each conformation. The deconvolution analysis is done with Origin version 6.1. A sample of how this analysis was done is presented in Figure 24. This corresponds to a sample of fibrillar structures at initial conditions.

---

\(^3\) Amide I region is between 1600 and 1700 cm\(^{-1}\)

\(^4\) Amide I region is between 1600 and 1700 cm\(^{-1}\)
Figure 24. Example of the Deconvolution for Fibrils at Initial Conditions. The black line represents original data; the red line one represents the outcome of the deconvolution fitting. The resulting peaks are shown in green.

6.3.1. Monomeric Conformation

Figure 25, shows the time evolution spectra for monomeric conformation of Aβ (1-42) peptides. Spectra were taken from its initial preparation until about 150min of incubation time. This time interval was chosen based on the AFM analysis. It was observed that fibrils did not form during this time (Table 4). Two particular regions are observed corresponding to the Amide I and II. The amide I band, between 1600 and 1700 cm\(^{-1}\), is associated with the backbone conformation of the peptide (-C\(\alpha\)=O). Amide II results from the N-H bending vibration (40-60%) and from the C-N stretching vibration (18-40%), and is sensitive to the structural configurations.
Figure 25. FTIR Spectra of Amyloid-β (1-42) Peptide Monomeric Precursors

The deconvolution analysis of these peaks based in the assignment from Table 6 appears in the next Table, which the evolution of the various species from initial conditions until 150 min of incubation time.

The N-H bending and C-N stretching (Not-shown), progressively decrease with time until they equilibrate after the transition process (>100 min).
Table 8. Evolution of Individual Conformations for Monomeric Precursors

**Intermolecular β-turns:** Three stages can be seen: 1) dilution process, from 0-30 min., 2) peptide rearrangement, after 45 minutes there is a steady increase of this structure, and 3) equilibrium process, from 100 min to 150

**Intramolecular β-turns:** Notice a decrease in the amount of this type of structure from 0 to 30min, which is associated with the dilution zone. >45 min, the protein steadily reaches a stable concentration. The amount of β turns decays during monomeric evolution

**α-helix structure:** After dissolution stage, these structures are suddenly visible. They appear in the transition process, and disappear again coinciding with the formation of β sheets

**β sheets structures:** Increase continuously until they reaches an equilibrium state (>105 min). The sudden shift corresponds to the lost of α-helix structure
6.3.2. Oligomeric Conformations

The oligomeric precursors were studied from initial conditions until after 9 hours (Figure 26). Remember that between 10 to 15 hours, the oligomers started producing fibrillar conformations as shown Table 5. Overall, the IR spectra clearly demonstrated that the oligomeric species are centered in the Amide I (1685-1670 cm$^{-1}$). This is region was deconvoluted into two regions that correspond to $\beta$ turns both inter- and intra-molecular (Table 9). In addition, there is a well defined region that can be assigned to Amide II (1567-1500 cm$^{-1}$), and corresponds to the absorption process of oligomeric structures onto a substrate. Similarly to the monomeric species, these molecules undergo a dissolution process initially (0-3 hours). The absorption process accelerates with time until it reaches equilibrium from about 3 hours until after 9 hours of incubation.

**Figure 26.** Structures Evolution with Time of Oligomeric Precursors of Aβ (1-42) Peptides
Table 9. Evolution of Individual Conformations for Oligomeric Precursors

**Intermolecular β-turns:** Two stages can be seen: 1) dilution process, which also includes the rearrangement of the peptide molecules and, 2) steady state equilibrium

**Intramolecular β turns:** Similar behavior as for intermolecular β turns. It is amazing how both structures follow parallel paths

**NH₂ deformations:** Taken from the Amide II region, two stages are seen as function of time, where they follow similar formation and transition paths

**Absorption/desorption process:** It is clear that oligomeric species deposit on the substrate surface. Very good agreement with AFM data (Table 5).
6.3.3. Fibril Conformation

The time evolution of fibrillar species is a little bit more complicated than for that for other configurations, because they show a longer dissolution processes, a transition process, an a state where some chemical conformation reach equilibrium but other molecules keep changing and do not reach a constant dynamics. The IR spectra show a very interesting behavior. First, the regions assigned to β-sheet decays with time, where as the Amide II region decreases and reaches an equilibrium state. This indicates two processes, one, the β sheets are evolving and are been redistributed or transformed into other chemical conformations. Second, the fibrils form and absorbs onto the surface in the first 3 hours (Table 10). After that, they rearrange or chemical alter while being attached to the substrate. This is a very important finding since indicates that the fibrils may form in solution, but they form, react and transform even after being absorb on a surface (Figure 27).

Figure 27. Time Evolution Spectra of Fibril Structures of Aβ (1-42)
Table 10. Evolution of Individual Conformations for Fibrillar Precursors

**Intermolecular β-turns:** Three stages can be seen: 1) dilution process & peptide rearrangement where they decreased (<0-210), 2) steady state formation (270-610 min), and >620 disappearance.

**α-helix:** Similarly, 3 stages are observed but they inversely proportional to the β turns.

**β sheets:** Two types of behavior are visible, 1) during the dissolution, the conformations are mainly intramolecular structures (1640-30 cm⁻¹), and 2) steady-state existence of intermolecular conformations (1625-10 cm⁻¹)

**Absorption/desorption process:** As explained before, the fibrils absorb during the first 3 hours and after that, they stop absorbing but they change conformation while being adhered to the substrate.

---

**Intermolecular β-turns**

- Transition to Steady State
- Peptide rearrangement
- Dilution

**α-helix**

- Transition to Steady State
- Peptide rearrangement
- Dilution

**β sheets**

- Steady state
- Intermolecular beta sheet structures
- Dilution and rearrangement
- Intramolecular beta sheet structures

**Absorption/desorption process**

- Transition to Steady State
- Peptide rearrangement
- Dilution
The N-H bending and C-N stretching behavior shows a constant dynamics that varies with respect to the other species. The interactions come and go according to the dissolution and transition processes.

We found an astonishing behavior when we determine the integrated absorbance for each structure in time. The fibrils and oligomers go from a maximum energetic level, when they just started dissolving, to a lower region when formation/absorption mechanisms are competing with each other. This change of energy takes place in the first three hours of formation. On the other hand, monomeric precursors do not change with time but after 3 hours, they start degenerating.

**Figure 28.** Comparison of Surface Dynamics for the Three Peptide Structures: Integrated Absorbance Evolution with Time
6.4. Membrane Construction

One of the goals of this research is to study the interactions of fibril structures with model membranes. The membranes are prepared by using L-B deposition techniques on rigid and soft supported surfaces. The lipid membranes were constructed with DSPE and its surface stability was studied as explained next.

At the air/water interface, we observed the behavior of DSPE layers as a function of lipid concentration. We found that the optimum concentration for the deposition of the layers was 2.45mg/mL, and the optimum surface pressure should be between 20-40 mN/m. Other concentrations resulted on weaker layers or limited the areas to perform the transfer of the lipid layers to a substrate (Figure 29).

![Figure 29. Surface Tension of DSPE as a Function of Lipid Concentration. Low concentrations resulted in low ordering, and high concentrations resulted in rigid and fragile layers](image-url)
Once the optimum concentration was determined, we performed loading/unloading cycle to test the response of the layer to dynamic changes. We observed that this concentration also promote stable layers (Figure 30).

![Diagram of pressure-area isotherm](image)

**Figure 30.** Loading/Unloading Cycle for DSPE at 2.45mg/mL and 30mN/m

The layers were transferred to the PEG/silica substrates with a transferred ration of 99.94%. The pressure-area ($\pi$-A) isotherm is shown in Figure 31 for our system. Once the layers were deposited we look at them using AFM in lipid rich-aqueous solutions. The images of the membranes show a layer that has high mobility (Figure 32a). This layer was then exposed to a very high concentration of fibrillar solution and then scanned in situ. We found that initially, some fibril structures deposited on the surface of the membrane (Figure 32b). However, they did not stay absorbed for very long. We believe
that the mobility of the layers influence the absorption mechanism to which this fibrils attach to surfaces, since the fibrils did not appear in further scans of the model membrane surface (Figure 32c).

**Figure 31.** Deposition and Transfer of the Lipid DSPE Bilayer onto the PEG/silica Substrates to Create the Model Membranes

**Figure 32.** Interacting Mechanisms Between Lipid Bilayers and Aβ (1-42) Peptide Fibrils Using AFM Liquid Imaging. (a) 10 by 10 µm scan of the model membrane surface as deposited, (b) Scan after fibrils have been added to the membrane, and (c) Scan soon after fibril exposure. The fibril structures refused to adhere on the surface possibly owing to the surface mobility of the soft-supported membranes.
One of the questions is if the fibrils really are influenced by the mobility of the membrane. Let’s look at the section scan from Figure 32b (Figure 33) to analyze such behavior. The height of the fibrils corresponds to 6nm, which happens to match the height of the fibers adhered to rigid supports also detected by AFM (Table 4, 5, and 6). Therefore, it is reasonable to assume that the fibers indeed adhere to cell membranes, but the surface mobility makes such adhesion very weak. This is an important finding since the implications of finding the molecular mechanism of Aβ peptides to membranes may lead us to determine the processes that control fibril adhesion and plaque formation. That is, these findings might lead us in a direction to find release mechanisms that will prevent or stop adhesion all together.

**Figure 33.** Section Profile of Biomimetic Membrane Surfaces in Contact with Fibrils. The height of the fibrils average 6nm, which corresponds to the values report elsewhere and reported early in this chapter (See Table 6) [23, 26]
Chapter Seven

Conclusions and Future Work

7.1 General Findings and Conclusions

This project had a very well defined goal from the beginning, which was to study, control and determine the implications of having three different structures of the two peptides that are most influential in AD brain’s dysfunction, the Aβ-40 and Aβ-42. We were able to establish the procedures to prepare stable and reproducible configurations of Aβ peptides, so that we could study their interactions with biomimetic membranes.

The most important findings in terms of amyloid beta peptide adhesion to surfaces are as follow:

a. There is a distinct trend between the Aβ1-40 and Aβ1-42 in terms of adhesion to rigid surfaces. Aβ1-40 exhibits a poor adhesion to mica, whereas Aβ1-42 shows a strong adhesion to rigid supports. This difference in adhesion behavior may be correlated to the toxicity and propensity to form longer aggregates that ultimately form fibrils and plaques in normal conditions, since our ATR data shows that fibrillar configurations have a very complex adhesion mechanism to surfaces.

b. The Aβ1-40 show to be more stable in terms of aggregation. In most cases, this peptide did not form fibrillar structures even though the conditions to form them were heavily induced.
c. There is a strong correlation between data from the two surface analytical techniques used in this project. AFM and FTIR data show the effect of a dilution process. This mechanism takes place in the first 30 min. of precursor incubation. However, it has a great impact in the evolving time to achieve equilibrium state. There was a transition zone where the structures of the peptides rearrange and most of the conformations decrease or increase at this time (between 1-3 hours).

d. In the case of the Aβ1-42, the formation of bigger aggregates into fibrillar configurations started to take place > 9 hours of incubation for most cases.

e. We were able to correlate the individual configurations of these molecules with kinetics and dynamics of adhesion.

f. We were able to control fibril formation or fibrilogenesis, since it was shown that nonfibrillar assemblies were maintained and studied in vitro.

g. The adhesion process of fibrillar structures and aggregates is very complex and depends on the preparation conditions and the affinity of the peptide to surface structure.

h. We were able to distinguish between intermolecular β turn structures (1695-1670 cm⁻¹), intramolecular β turn structures (1690-1680 cm⁻¹). Typically, these structures are not seen in protein conformation studies.

i. AFM studies illustrate how oligomeric and monomeric configurations of Aβ-40 and 42 follow a general trend in terms of their ratio of height and width. That is, they absorbed onto themselves instead of the substrate until saturation is reached. Only after that the peptide will continue absorbing onto the substrate.
j. For fibril configurations, the ratio of height/width of Aβ-40 and Aβ-42 follows an inversely proportional trend.

k. Biomimetic membranes can be built onto PEG layers to mimic real surface mobility.

l. The interactions between biomimetic membranes and fibrils show that the fibrils initially absorb, but that the absorption is very weak. This may be a function of surface flexibility and softness created by the PEG underneath layer.

m. By comparing the adhesion behavior of the fibril to the mica with the adhesion behavior to biomimetic membranes, we concluded that the rigidity of the substrate indeed is plays an important role in the adhesion mechanisms of the fibrils.

n. These findings led us to hypothesize that normal fibrils attach to real cell membranes because they have lost mobility somehow. It has been discussed that cholesterol may have an important role in AD plaque formation. This hypothesis agrees with the literature studies were Statins have helped to reduce the effects of AD symptoms [31-33].

7.2 Final Remarks and Future Work

a. This work has advanced our ability to master a systematic way to prepare the three different conformations of Aβ peptides that may play an important role in AD plaque formation.

b. If these three conformations may influence plaque formation, we may be able to understand the molecular mechanisms associated with their adhesion, surface kinetics and assembly processes by using the Surface Forces Apparatus and the
ATR-FTIR technique concurrently (IR-SFA).

c. Further investigation developing essays with the two peptides together is needed to elucidate why only Aβ42 segments cause adhesion, and how they influence Aβ40 peptide dynamic processes.

d. Additional studies to corroborate our initial findings on the effect of substrate mobility will be performed. For instance, we would like to vary systematically the rigidity of model membranes by introducing cholesterol in their structure.
References


Appendices
## Appendix A. Milestones Toward Formulating and Testing the Aβ Hypothesis of AD[9]

<table>
<thead>
<tr>
<th>Date</th>
<th>Milestones</th>
</tr>
</thead>
<tbody>
<tr>
<td>1892-1907</td>
<td>Lesions associated with Alzheimer’s neurodegeneration described and categorized (Alzheimer 1907; Bloq and Marinesco 1892; Redlich 1898)</td>
</tr>
<tr>
<td>1920-40</td>
<td>Amyloid plaques and perivascular deposits described and proposed to adversely affect cerebral function (Divry 1927; Scholz 1938)</td>
</tr>
<tr>
<td>1970-86</td>
<td>Isolation (Nikaido et al. 1971), amino acid composition (Allsop et al. 1983; Roher et al. 1986, Selkoe et al. 1986) and N-terminal sequences of perivascular (Glenner and Wong 1984) and parenchymal plaque Aβ (Masters et al. 1985)</td>
</tr>
<tr>
<td>1987-88</td>
<td>Amyloid precursor protein (APP) gene cloned, located on chromosome 21 and Aβ recognized as putative proteolytic product (Kang et al. 1987; Tanzi et al. 1987, 1988; Goldgaber et al. 1987; Robakis et al. 1987; Kitaguchi et al. 1988; Ponte et al. 1988)</td>
</tr>
<tr>
<td>1990-92</td>
<td>Mutations in proximity to Aβ secretase sites cause AD (Levy et al. 1990; Chartier- Harlin et al. 1991; Goate et al. 1991; Murrell et al. 1991; Citron et al. 1992; Hendriks et al. 1992) and confirm centrality of Aβ hypothesis</td>
</tr>
<tr>
<td>1992-93</td>
<td>Aβ and p3 identified as definite proteolytic products from APP (Seubert et al. 1992, 1993; Shoji et al. 1992; Busciglio et al. 1993)</td>
</tr>
<tr>
<td>1992-93</td>
<td>ApoE identified as an Aβ-interacting protein (Wisniewski and Frangione 1992; Strittmatter et al. 1993) and its alleles identified as genetic risk factors in sporadic forms of AD (Coder et al. 1993)</td>
</tr>
<tr>
<td>1999-99</td>
<td>α-secretases identified (Buxbaum et al. 1998; Lammich et al. 1999)</td>
</tr>
<tr>
<td>1999-2000</td>
<td>Major Aβ degradative and clearance pathway identified (Iwata et al. 2000; Qiu et al. 1999; Vekrellis et al. 2000) and improved clearance of Aβ from the brain by immunization with Aβ (Schenk et al. 1999)</td>
</tr>
<tr>
<td>1995-2000</td>
<td>First rational anti-amyloid therapeutic strategies (Higaki et al. 1995) and commencement of early phase human trials (Bristol Myers Squibb, Elan Pharmaceuticals, Prana Biotechnology)</td>
</tr>
</tbody>
</table>
Appendix B. The 20 Protein Amino Acids
### Appendix C. Chemical Forces and their Relative Strengths and Distances

<table>
<thead>
<tr>
<th>Force</th>
<th>Strength (kJ/mol)</th>
<th>Distance (nm)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van der Waals interactions</td>
<td>0.4 - 4.0</td>
<td>0.2</td>
<td>Strength depends on the relative size of the atoms or molecules and the distance between them. The size factor determines the area of contact between two molecules: the greater the area, the stronger the interaction. Attractive force is inversely proportional to the sixth power of the distance, ( r ), separating two atoms or molecules: ( F \approx \frac{1}{r^6} ).</td>
</tr>
<tr>
<td>Hydrogen bonds</td>
<td>12 – 30</td>
<td>0.3</td>
<td>Relative strength is proportional to the polarity of the H bond donor and H bond acceptor. More polar atoms form stronger H bonds.</td>
</tr>
<tr>
<td>Ionic bonds</td>
<td>20</td>
<td>0.25</td>
<td>Strength also depends on the relative polarity of the interacting charged species. Some ionic bonds are also H bonds: ( \text{\text{---OH}} \ldots \text{\text{CH}_3} )</td>
</tr>
<tr>
<td>Hydrophobic interactions</td>
<td>&lt; 40</td>
<td>-</td>
<td>Force is a complex phenomenon determined by the degree to which the structure of water is disordered as discrete hydrophobic molecules or molecular regions coalesce.</td>
</tr>
</tbody>
</table>
Appendix D. Infrared Spectra

Appendix D.1. Characteristic Infrared Bands of Proteins [28]

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Wavenumber (cm(^{-1}))</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3300</td>
<td>N-H stretching in resonance with overtone</td>
</tr>
<tr>
<td>B</td>
<td>3110</td>
<td>(2 x amide II)</td>
</tr>
<tr>
<td>I</td>
<td>1653</td>
<td>80% C=O stretching; 10% C-N stretching; 10% N-H bending</td>
</tr>
<tr>
<td>II</td>
<td>1567</td>
<td>60% N-H bending; 40% C-N stretching</td>
</tr>
<tr>
<td>III</td>
<td>1299</td>
<td>30% C-N stretching; 30% N-H bending; 10% C=O stretching; 10% O=C-N bending; 20% other</td>
</tr>
<tr>
<td>IV</td>
<td>627</td>
<td>40% O=C bending; 60% other</td>
</tr>
<tr>
<td>V</td>
<td>725</td>
<td>N-H bending</td>
</tr>
<tr>
<td>VI</td>
<td>600</td>
<td>C=O bending</td>
</tr>
<tr>
<td>VII</td>
<td>200</td>
<td>C-N torsion</td>
</tr>
</tbody>
</table>

Appendix D.2. Characteristic Infrared Bands of Amino Acid Side Chains [28]

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Wavenumber (cm(^{-1}))</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>1465</td>
<td>CH(_2) bending</td>
</tr>
<tr>
<td>Valine</td>
<td>1450</td>
<td>CH(_3) asymmetric bending</td>
</tr>
<tr>
<td>Leucine</td>
<td>1375</td>
<td>CH(_3) symmetric bending</td>
</tr>
<tr>
<td>Serine</td>
<td>1350-1250</td>
<td>OH bending</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1720</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1560, 1415</td>
<td>CO(_2) asymmetric stretching</td>
</tr>
<tr>
<td></td>
<td>1415</td>
<td>CO(_2) symmetric stretching</td>
</tr>
<tr>
<td>Asparagine</td>
<td>1650</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1615</td>
<td>NH(_2) bending</td>
</tr>
<tr>
<td>Lysine</td>
<td>1640-1610, 1550-1485, 1160, 1100</td>
<td>NH(_3)(^+) bending</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1602, 1450, 760, 700</td>
<td>Benzene ring vibrations</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1600, 1450</td>
<td>Benzene ring vibrations</td>
</tr>
<tr>
<td>Arginine</td>
<td>1608, 1586</td>
<td>Benzene ring vibrations</td>
</tr>
</tbody>
</table>
Appendix E. Material Safety Data Sheets.

Appendix E.1. Aβ1-40

Certificate of Analysis

Product Name: Beta Amyloid 1-40
Product #: 620-78

Molecular Weight: 4329.9 amu
Mass Spectral Analysis: Electrospray "Exhibits correct MW" (see attached MS spectrogram)
HPLC Analysis: Peptide Purity: 97.2% (see attached RP-HPLC chromatogram)
Solubility: 1 mg/ml in water
Appearance: White lyophilized powder
Peptide Content: 83.4%
Counter Ion: Trifluoroacetate
Date of Mfg: 09/08/04

Amino Acid Analysis:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Calc.</th>
<th>Found</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>3.00</td>
<td>3.02</td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>1.00</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>Asx</td>
<td>4.00</td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td>Glx</td>
<td>6.00</td>
<td>6.21</td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>3.60</td>
<td>2.81</td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>2.50</td>
<td>6.80</td>
<td>*1</td>
</tr>
<tr>
<td>Leu</td>
<td>2.50</td>
<td>2.13</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>2.50</td>
<td>2.20</td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>1.90</td>
<td>1.08</td>
<td></td>
</tr>
</tbody>
</table>

Remarks: Not for human use, research purposes only.
*1 Ile-Ile & Val-Val bond was only partially hydrolyzed during hydrolysis.

IMPORTANT
Store in Freezer (-20°C) Immediately Upon Arrival

Quality Assurance By: [Signature]

AMERICAN PEPTIDE COMPANY, INC.

Headquarters
777 E. Ontario Avenue
Sunnyvale, CA 94086 USA
Tel: 408-243-7800 Fax: 408-243-7801

1271 Avenue Chelsea
Vista, CA 92081 USA
Tel: 760-757-6800 Fax: 760-757-8811

1-0-1 Kugawakigaoka
Motomi, Ishikawa 352-0191

81
Appendix E.1. (Continued)

American Peptide Company, Inc.
HPLC Analysis Report

Product Number/Lot Number: 62-0-78 / S09018T1

<table>
<thead>
<tr>
<th>Data File</th>
<th>c:\star\data\62-0-78\62-0-78001.run</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel</td>
<td>1 = UV 215 RESULTS</td>
</tr>
<tr>
<td>Sample ID</td>
<td>Manual Sample</td>
</tr>
<tr>
<td>Instrument (Inj):</td>
<td>Waters 2690</td>
</tr>
<tr>
<td>Injection Date:</td>
<td>09/07/2004 02:07:14</td>
</tr>
<tr>
<td>Injection Method:</td>
<td>c:\star\method\left side method</td>
</tr>
<tr>
<td>Run Time (min):</td>
<td>20.000</td>
</tr>
<tr>
<td>Calc Date:</td>
<td>09/07/2004 02:31:48</td>
</tr>
<tr>
<td>Times Calculated:</td>
<td>4</td>
</tr>
<tr>
<td>Calculation Method:</td>
<td>c:\docume<del>1\ape\locals</del>Analysis</td>
</tr>
<tr>
<td>Run Mode:</td>
<td>Peak Area</td>
</tr>
<tr>
<td>Peak Measurement:</td>
<td>Calculation Type: Percent</td>
</tr>
<tr>
<td>Wavelength: 215nm;</td>
<td>Flowrate: 1.5ml/min; Buffer A: 0.05M Tris in Water; Buffer B: 0.05M Tris in 80% Aceto</td>
</tr>
<tr>
<td>Column: PLRP-S 300A, C18 (4.6 mm x 250mm) 3u; Gradient(Linear): 15% - 35% Buffer B in 20 min; Inj Vol.: 2</td>
<td>Analyst: Shilpa Patel;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Result (%)</th>
<th>Ret Time (min)</th>
<th>Peak Area (counts)</th>
<th>Width 1/2 (sec)</th>
<th>Peak Height (counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0294</td>
<td>5.837</td>
<td>249490</td>
<td>22.8</td>
<td>8641</td>
</tr>
<tr>
<td>2</td>
<td>0.4222</td>
<td>10.483</td>
<td>102319</td>
<td>30.9</td>
<td>3477</td>
</tr>
<tr>
<td>3</td>
<td>97.2408</td>
<td>11.919</td>
<td>23568784</td>
<td>48.4</td>
<td>451780</td>
</tr>
<tr>
<td>4</td>
<td>1.3077</td>
<td>18.952</td>
<td>316956</td>
<td>19.2</td>
<td>14561</td>
</tr>
<tr>
<td></td>
<td>100.0001</td>
<td></td>
<td>24237548</td>
<td></td>
<td>478450</td>
</tr>
</tbody>
</table>
Appendix E.2. Aβ1-42

Certificate of Analysis

Product Name: Beta-Amyloid (1-42)  
Lot #: R07010T1


Molecular Weight: 4514.1 amu

Mass Spectral Analysis: Electrospray "Exhibits correct MW"  
(see attached MS spectrogram)

HPLC Analysis:  Peptide Purity: 99.0%  
(see attached RP-HPLC chromatogram)

Solvency: 1mg/ml in 0.05M Tris buffer

Appearance: White lyophilized powder

Peptide Content: 82.1%

Counter Ion: Trifluoroacetate

Date of Mfg: 07/08/03

Cert. of Analysis Remarks: Peptide Content calculated by % Nitrogen.

Amino Acid Analysis:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Calc.</th>
<th>Found</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>4.00</td>
<td>3.08</td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>1.00</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>Asx</td>
<td>4.00</td>
<td>3.94</td>
<td></td>
</tr>
<tr>
<td>Gln</td>
<td>4.00</td>
<td>3.96</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>6.00</td>
<td>6.04</td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>3.00</td>
<td>3.01</td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>3.00</td>
<td>1.96</td>
<td>*1</td>
</tr>
<tr>
<td>Leu</td>
<td>2.00</td>
<td>2.06</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>2.00</td>
<td>2.04</td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>1.00</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>3.06</td>
<td>3.06</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>2.00</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>1.96</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>6.00</td>
<td>4.27</td>
<td>*1</td>
</tr>
</tbody>
</table>

Remarks: Not for human use, research purposes only.

*Ile-Ile, Val-Val & Val-Ile bonds were only partially hydrolyzed during hydrolysis.

IMPORTANT
Store in Freezer (-20° C)
Immediately Upon Arrival

Quality Assurance By: [Signature]  
Quality Control Department  
Date: 07/08/03

AMERICAN PEPTIDE COMPANY, INC.

Headquarters  
777 E. Evelyn Avenue  
Sunnyvale, CA 94086 USA  
Tel: 408.770.7024  
Fax: 408.770.7003

1271 Avenida Chula Vista  
Vista, CA 92081 USA  
Tel: 760.593.8090  
Fax: 760.593.8016

1-2-1 Kubogawa  
Monya, Ibaraki 302-0104 Jap  
Tel: 81.029.26.2311  
Fax: 81.029
Appendix E.2. (Continued)

American Peptide Company, Inc.
HPLC Analysis Report

Product Number/Lot Number: 62-0-80 / R07010T1

Data File: c:\star\data\62-0-80\62-0-80.run
Channel: 2 = UV 440 RESULTS
Sample ID: 62-0-80
Instrument (Inj): Waters 2690
Injection Date: 07/08/2003 09:54:39
Injection Method: c:\star\methods\right side method
Run Time (min): 20.000
Calc Date: 07/08/2003 10:17:09
Times Calculated: 3
Calculation Method: c:\docume~1\apc\locales~1\tem
Run Mode: Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Wavelength: 215 nm; Flowrate: 1.5 ml/min; Buffer A: 0.05M Tris in H2O; Buffer B: 0.05M Tris in 80% Acetonitril
Column: Phenomenex C18 (4.6 mm x 250 mm 5 micron); Gradient (Linear): 25% - 45% buffer B in 20 min; Inj Vol.: 20
Analyst: Shilpa Patel

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Result (%)</th>
<th>Ret Time (min)</th>
<th>Peak Area (counts)</th>
<th>Width 1/2 (sec)</th>
<th>Peak Height (counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3186</td>
<td>8.496</td>
<td>40147</td>
<td>0.0</td>
<td>2832</td>
</tr>
<tr>
<td>2</td>
<td>99.6814</td>
<td>9.476</td>
<td>12560566</td>
<td>29.2</td>
<td>402815</td>
</tr>
<tr>
<td></td>
<td>100.0000</td>
<td></td>
<td>12600713</td>
<td></td>
<td>405647</td>
</tr>
</tbody>
</table>
Appendix E.2. (Continued)
Appendix F. One Example on AFM Data Processing

I. Amyloid β (1-40) Monomer 0 hours (File: ab40-monomer-0h-2um)

a. Group A:
b. Group B:

![Graph showing H1 and W1, H2 and W2 measurements.]

c. Group C:

![Graph showing H1 and W1 measurements.]

88
Appendix G. Transferred Area Calculation

Transferred Rate Area ($A_{TR}$) :

$$A_{TR} = \frac{\text{total transferred area to the wafers and tweezer}(\text{calculated})}{\text{area transferred (read from data})} \times 100\%$$

From Figure 31, one can see the transferred area, this is

Transferred area = (the initial area at which 30mN/m is reach –

final area after deposition)

Hence,

Transferred area = 230.37 cm$^2$ – 177.32 cm$^2$ = 53.05 cm$^2$

Now the wafer’s dimension were

1.912 cm

with a thickness of .05 cm

The total area for the wafer is

$$A_{\text{wafer}} = 2 \times (1.912\text{cm})^2 + (0.05\text{cm} \times 1.912\text{cm}) \times 4 = 7.7\text{cm}^2$$

The deposition was done to two wafers at the same time with the same dimension, therefore the total area transfer to the wafer twice because we are building a bilayer, is;

$$A_{\text{transfer to the wafers (bilayer)}} = 7.7\text{cm}^2 \times 4 = 30.8\text{cm}^2$$
Appendix G. (Continued)

Next we need to calculate the area transferred to the tweezers used to hold the wafers, and the dimensions were:

\[
\begin{array}{c}
1.31 \text{ cm} \\
1.896 \text{ cm} \\
\end{array}
\begin{array}{c}
1.31 \text{ cm} \\
1.189 \text{ cm} \\
\end{array}
\]

Thickness = 0.5 \quad \text{Thickness} = 0.2

Area of 1:

\[
A_1 = (1.31 \text{ cm} \times 1.896 \text{ cm}) \times 2 = 4.97 \text{ cm}^2 \\
+ (0.5 \text{ cm} \times 1.31 \text{ cm}) \times 2 = 1.31 \text{ cm}^2 \\
+ (0.5 \text{ cm} \times 1.896) = 0.948 \text{ cm}^2
\]

Total area 1: \( A_1 = 7.23 \text{ cm}^2 \)

Area of 2:

\[
A_2 = (1.31 \text{ cm} \times 1.189 \text{ cm}) \times 2 = 3.12 \text{ cm}^2 \\
+ (0.2 \text{ cm} \times 1.31 \text{ cm}) \times 2 = 0.524 \text{ cm}^2 \\
+ (0.2 \text{ cm} \times 1.189) = 0.2378 \text{ cm}^2
\]

Total area 2: \( A_2 = 3.88 \text{ cm}^2 \)
Appendix G. (Continued)

Total area transferred to the tweezers= \(2 \times A_1 + A_2 = 22.22 \text{cm}^2\)

Total transferred area is:

\[\text{Area transferred to the wafers} + \text{area transferred to the tweezers} = \]

\[A_{\text{total transferred}} = 30.8 \text{ cm}^2 + 22.22 \text{ cm}^2 = 53.02 \text{ cm}^2\]

Hence the transferred rate is:

\[A_{TR} = \frac{52.02 \text{ cm}^2}{52.03 \text{ cm}^2} \times 100\% = 99.94\%\]