Effects of Anti-Aβ Monoclonal Antibodies on the Amyloid Beta Peptide

Fibrillogenesis and their Involvement in the Clearance of

Alzheimer’s Disease Plaques

by

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DEDICATION

I entirely dedicate this new achievement to my beloved: mother, father, sister and brothers, for their support, love, advice and words of encouragement no matter the distance.
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Alzheimer’s disease (AD) is the most common cause of senile dementia worldwide. AD is a neurodegenerative disorder characterized by the loss of memory and language skill, collapse of the cognitive function, and distortion of social behavior. As of today, the onset mechanisms of AD and cure are unknown; however, three hallmarks are commonly encountered: extra and intracellular accumulation of amyloid beta (Aβ) peptide plaques, formation of intracellular neurofibrillary tangles, and inevitable neuronal death. Hypothetically, a possible scenario provoking or involved in the onset of AD is a cascade effect that starts with an imbalance in the production and clearance of Aβ peptide that consequently leads to its accumulation, formation of tau protein tangles and neuronal death. This work studied and characterized the mechanisms governing Aβ peptide aggregation and the effects of using anti-Aβ monoclonal antibodies to modify this process. These mechanisms play an important role in the formation
of AD plaques and are critical in the search for therapies involving Aβ peptide plaque clearance. Yet, antibody-based therapies for plaque clearance are not well understood, adding to the existing concerns about side effects in humans, hence there is a necessity of knowledge in this matter. In this work different N-terminus, C-terminus, and Mid-domain antibodies were used against Aβ peptide species (monomers, oligomers, and fibrils) to probe peptide aggregates modification and disruption. Additionally, construction of a soft supported lipid bilayer membrane was proposed to study the adhesion mechanisms of Aβ peptide and interactions with antibodies, mimicking the neuronal cell surface. The main characterization techniques used in this work were: atomic force microscopy (AFM) and transmission electron microscopy that allowed the physical exploration and visualization of the different processes of aggregation in terms of adhesion, size evolution, and distribution of the peptide; and attenuated total reflectance Fourier spectroscopy (ATR/FTIR) which allowed monitoring the change of secondary structures for the peptide during the processes studied. It is endeavored that this work will help to elucidate the effects attributed to the molecular interactions between Aβ peptide species and antibodies to target Aβ plaque's clearance in the brain of AD patients. Ultimately, this study provides novel information critical for the formulation of effective therapies to prevent and treat AD with less collateral effects. It also represents a contribution to the basic scientific knowledge regarding peptide-antibody interactions with application to other diseases related to protein misfolding.
CHAPTER 1
INTRODUCTION AND PREMISES

1.1. Alzheimer’s Disease Facts and Current Therapeutics

Alzheimer is an irreversible neurodegenerative disease characterized by the progressive loss of memory, coordination, social skills, and reasoning. In 2006 Alzheimer’s disease (AD) was reported as the sixth leading cause of death across all ages in the United States [1, 2]. Furthermore, AD significantly reduces the life expectancy and quality of those afflicted by it [3]. Statistics show that before a person’s 65th birthday 1 person in every 1000 is diagnosed with AD, and after this age this rate increases to 1 person in every 8. The chance raises to 1 person in every 5 after the age of 80 [4].

As of today, nearly five million Americans live with AD [5]. This number is expected to quadruple in the next four decades [5]. Worldwide AD cases have reached 26 million people, and it is expected to increase to 106 million by 2050. In addition, reports show that in the United States alone the government and affected families spend approximately 148 billion dollars annually to treat patients with AD [1, 5].
The onset mechanisms of AD and the cure are still unknown; however, three pathological hallmarks are commonly observed among patients with AD: the presence of extracellular deposits of amyloid beta (Aß) plaques, intracellular neurofibrillary tangles (NFTs), and neuronal apoptosis. Figure 1 (a) and (b) shows the first two major microscopic hallmarks mentioned above [6].

In this work special attention is rendered to understanding the process of Aß plaque formation and clearance.

Aß peptide deposits (also known as senile plaques) are mainly composed of amyloid beta peptides containing 1-40 and 1-42 amino acids residues (Aß1-40 and Aß1-42 respectively) [7-9].

**Figure 1.** Alzheimer’s Disease Hallmarks. (a) Left: a normal neuron. Right: depicting the three hallmarks of AD (Aß plaques, NTF’s, and neuron’s apoptosis) [10]. (b) Classical histopathology of AD’s brain showing plaques and neurofibrillary tangles (arrows), taken from Cordell, 1994 [11]
Despite the fact that Aβ peptide does not have a known function in the brain, it is present in healthy brains. Studies show that insoluble Aβ peptides along with a soluble sub-product are proteolytically cleaved from a larger transmembrane protein known as amyloid precursor protein (APP) [12, 13].

Soluble forms of APP are believed to have neuroprotective and neurotrophic functions. However, Aβ is considered an unwanted by-product of the APP processing [4]. The Aβ peptides are found as globular and non-fibrillar forms in small concentrations of pico- to nano-molar and can be traced in the extracellular and cytoplasmic (inside the cell) regions in both normal and AD brain tissues. Nevertheless, significantly small oligomeric and protofibril species of Aβ peptides are enough to cause profound cytoskeletal degeneration and cell death by plaque formation through underlying mechanisms still not understood [14-20].

It is also known that plaques and tangles are accumulated in neurons, mainly throughout the cortical and limbic brain regions [21]. The cortical region is responsible for the abilities and activities related to thinking, basic aspects of perception, movement, and adaptive response to the outside world. The limbic region is primarily responsible for emotional behavior and the formation of memories [22].

In the past decade there have been significant advances in the field of AD; however, an effective treatment for its cure is not available yet [23]. Currently, some common drugs containing active ingredients such as cholinesterase
inhibitors\(^1\) or glutamine regulators\(^2\) are not able to cure the disease. Rather, these drugs only serve to “delay the progression of some of AD’s symptoms” [24].

There is a tremendous need to find an effective treatment that radically stops and prevents the brain’s cognitive degeneration and the irreversible loss of memory. A feasible and promising treatment is immunotherapy. In the past, active immunization (vaccination) was tested in a phase II clinical trial [25, 26], which was suspended after observing severe undesired immune response to the treatment (7% of the patients out of 312 subjects presented encephalitis). Nevertheless, patients monitored afterward showed less cognitive function degeneration in comparison to the control individuals, and this study gave important insights into immunotherapeutic treatments [26, 27].

\(^1\) Razadyne® (galantamine) also known as Reminyl®, Aricept® (donepezil), Cognex® (tacrine, with safety concerns), and Exelon® (rivastigmine); these are usually prescribed to patients with mild to moderate AD, to prevent the cholinesterase’s breakdown.

\(^2\) Namenda® (memantine), an N-methyl D-aspartate (NMDA) antagonist; prescribe to patients with moderate to severe AD, to regulate production of glutamate (in large amounts leads to brain cell death).
A possible therapy currently under study is passive immunization, which consists of the administration of monoclonal antibodies as a means to assist the dissolution and clearance of Aβ peptide plaques from the brain in patients with AD.

1.2. **Hypothesis**

It is possible to assess the effects of different monoclonal anti-Aβ antibodies directed against aggregates of Aβ peptides *in vitro*.

1.3. **Specific Aims and Objectives**

With the purpose of proving the current hypothesis, the work was divided into four specific aims, explained below.

1.3.1. **In vitro Assessment of Physicochemical Changes Endured by Aβ-Peptide during Aggregation**

The aggregation process of Aβ peptides was first monitored and characterized chemically by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR); this technique allowed the identification of the peptide’s secondary structures involved in such event. The chemical characterization results were then checked by the physical characterization of the species encountered at different collection times using atomic force microscopy (AFM).
Two isoforms of Aß peptide, Aß\textsubscript{1-40} (with 40 amino acid sequences) and Aß\textsubscript{1-42} (with 42 amino acid sequences), were studied individually and in combination (1:1 Aß\textsubscript{1-40} to Aß\textsubscript{1-42} equimolar mixture, Aß\textsubscript{40/42}). The main objective was to create a baseline understanding of the kinetic and structural processes during fibrillation and to adapt an analytical method for posterior studies.

1.3.2. Analysis and Evaluation of Effects of Monoclonal Antibodies on Aß Peptide Fibrils

The purpose was to determine the effects of antibodies on mature fibrils. The antibodies used for this study had different affinity and specificity. The substoichiometric molar ratio of antibody to peptide used in this study was 1:1000.

1.3.3. Influence of Monoclonal Antibody on the Initial Phase of Fibrillization

Physical, chemical, and biochemical methods were used to analyze the action of different antibodies on Aß peptide species. This aim focused on understanding the extent to which the antibodies were influencing the aggregation process and to evaluate the possible effects on plaque clearance, by targeting monomers + oligomers, being this the initial phase of aggregation.
1.3.4. Construction and Evaluation of a Biomimetic Cell Membrane as a Soft Substrate for Future Studies Involving Peptide-Antibody Interactions

The main objective was to produce a stable, soft support that could serve to mimic the role played by the cell membrane as a substrate for the AD plaques and to test the action of monoclonal antibodies on Aβ peptide aggregates.

1.4. Contribution of this Work

The principal contribution of this work was to provide an in vitro method to evaluate the effects of antibodies on amyloid beta peptide species, probing their effectiveness to destabilize neurotoxic forms of Aβ and disrupt their natural aggregation pathway.

This work provided basic scientific knowledge to help understand and improve antibody-based therapies for AD, with extension to other protein misfolding related diseases.

1.5. Broader Impacts

The methods and findings from this work opened paths to evaluate critical biomolecular interactions to understand protein misfolding related diseases and possible therapies.
CHAPTER 2
BACKGROUND

This chapter gives a general review of concepts and principles that provide the bases to later discuss our results and explain the phenomena observed during the different studies performed in this work.

2.1. Amyloid Beta Peptide, Aggregation Process, and Plaque Formation

The name amyloid comes from the Latin “amylum” and Greek “amylon”, meaning starch in both languages. Rudolf Virchow in 1854 used this name to refer to the material deposited in the brain as macroscopic abnormalities, due to its reactivity to iodine-sulfuric acid, a reagent used to identify starch and cellulose derivatives [28]. It was later in 1859 when Friedreich and Kekule demonstrated that the material identified as cellulose was protein [29].

Amyloid peptides are not exclusive to Alzheimer's disease (AD). They are also associated with diseases in other organs such as the liver, spleen, and kidney [29]. The amyloid peptide found in neuritic plaques in AD patients is known as amyloid beta (Aβ) peptide due to its natural tendency to form β-sheet secondary structures [30].
As mentioned in Chapter 1, AD’s neuritic plaques are mainly composed of two isoforms of Aβ peptide, Aβ1-40 and Aβ1-42 [31]. These peptides are fragments produced by the proteolytic cleavage of a larger transmembrane molecule (APP), a process briefly sketched in Figure 2. The products of this proteolytic cleavage are soluble neuroprotective secreted ectodomain sAPPα (via α-secretase cleavage) and extracellular Aβ40/42 peptides (via consequent β and γ-secretase cleavage) [32, 33]. It is worth mentioning that even though the concentration of secreted Aβ1-42 is near 10% of the Aβ1-40 concentration, Aβ1-42 peptide is the main component of neuritic plaques deposits [13, 34].

**Figure 2.** Amyloid Beta Peptide Enzymatic Cleavage. Schematic of Aβ1-40 and Aβ1-42 production by cleavage of the APP proteins by the intervention of specialized enzymes
The mechanisms of neuritic plaque formation are still unclear. However, it has been suggested that soluble species of A\(\beta\) peptide undergo a series of molecular rearrangements (misfolding) triggered by environmental stress that lead to the formation of fibrillar deposits.

Under normal conditions, cells with elaborate quality control machinery guarantee that proteins folded improperly are eliminated. This machinery includes proteins, specialized organelles and cells that act as chaperones and activate processes to selectively eradicate misfolded proteins. In the case of the central nervous system (CNS) the specialized quality control cells are the microglial cells. Proteasomes and lysosomes structures also contribute to the degradation and elimination of misfolded proteins inside cells [35].

However, a particular characteristic that distinguishes amyloid and a few other misfolded proteins (such as alpha synuclein, cystatin c and tau among others) is the ability to aggregate into higher order species such as oligomers, protofibrils, and fibrils. These species cannot be transported into the central catalytic pore of the proteasome enzyme complex for degradation. This characteristic allows amyloid peptides to evade proteasome and lysosome quality control machineries, which leads to accumulation of misfolded amyloidogenic peptide. This system grows and propagates, offering resistance to degradation for the amyloid toxic [6]. In addition, a small increase in the concentration of misfolded protein can dramatically accelerate the process of plaque formation [36].
In vitro amyloid beta peptide fibrillization can be recreated and studied at different levels of aggregation by manipulating the peptide’s concentration, temperature, pH, and ionic strength of the media [37, 38]. Similarly, it is possible to alter and destabilize production of Aβ species (oligomers, and protofibrils) critical during fibrillogenesis by introducing an external agent (i.e. beta sheet breakers). Although active and passive immunotherapy have shed light into the possible mechanism involved in the activation of the immune system and clearance of Aβ from the brain, little is known about the impact on protein folding produced by monoclonal antibodies binding to Aβ peptide. This work evaluates the anti-Aβ monoclonal antibodies molecules as external agents used to alter peptide’s aggregation and promote peptide solubility.

2.2. Amino Acids, Peptide Bond, Peptides, and Levels of Structure

Amino acids are the building blocks of peptides and polypeptides. They are formed by two main functional groups: an amino and a carboxyl group, which are both linked through a chiral central carbon atom (Cα) that also holds hydrogen bonding and a distinctive R group (side chain) as shown in Figure 3. The arrangement of these constituents around the central carbon atom makes them chiral, meaning they have two isomers, L-isomer and D-isomer. However, the L-isomer is found only in peptides and polypeptides. Naturally, the amino acid residues present in a peptide or polypeptide molecule influence the properties and adopted conformations.
Peptides and polypeptides are sequential and unbranched polymers of amino acids linked from head to tail, with less than 50 and more than 50 amino acid residues, respectively [39].

The union between a carboxyl group and an amino group through a covalent amide linkage is referred to as a peptide bond [35], which is shown in Figure 4. The peptide bond is essentially planar, and it has what is considered a double-bond strength that prevents free rotation around it. However, vibration and translation are possible. In this study, though, only vibration was quantifiable with ATR-FTIR spectroscopy; as it will be explained in Chapter 3.

It is important to mention that the peptide bond is uncharged, and this absence of charge allows the amino acid chains to tightly pack together, linked by peptide bonds into globular structures [39]. The main event responsible for the change in conformation will be the loss or gain of protons depending on the media’s pH. The molecular interactions among peptides are also affected by temperature changes and the media’s ionic strength.
A final point pertinent to this section is the presentation of the four different levels of structure adopted by peptides and polypeptides, which are explained as follows.

### 2.2.1. Primary Structure

They are simply formed by an amino acid sequence in a linear fashion and without taking into account three-dimensional rearrangements of the residues.

### 2.2.2. Secondary Structure

They represent a three-dimensional arrangement of the polypeptide in the space. This is conceived by the disposition of the hydrogen bonds between
adjacent amino acid residues in a molecule and between molecules, which allows the peptide molecules to arrange themselves into characteristic patterns. The following were the main secondary structures or patterns investigated in this work: parallel β-sheet, antiparallel β-sheet, α-helix, unordered (or random coils), and β-turns structures, shown as part of the short peptide in Figure 5.

**Figure 5.** Peptide’s Secondary Structures

The most important and known secondary structures are the helical and pleated segments (formed by parallel or antiparallel β-strands). These segments constitute regular structures that extend along one dimension. Most proteins exhibit these types of structures, which allow peptides to play specific roles in the body.
2.2.3. **Tertiary Structure**

This structure is present when polypeptide chains assume a tight three-dimensional shape, adopting a globular form. This type of structure gives to the polypeptides a lower surface-to-volume ratio allowing the molecules to shield from the solvent [35].

2.2.4. **Quaternary Structure**

This structure is composed of several folded peptides or polypeptides that are gathered in a more complex molecule, as in the case of enzymes.

2.3. **Antibodies**

Antibodies, also known as immunoglobulins, are natural bioengineered proteins with high binding specificity to a wide range of molecules including peptides, carbohydrates, and nucleic acids [40]. Figure 6 shows the characteristic parts of an antibody molecule. Antibodies have a particular “Y” shape, which is formed by two heavy chains and two light chains. The type of heavy chain determines the antibody isotype (Figure 7) to which they belong.

The Fab (fragment antigen binding) region of the antibody is composed of domains from both heavy and light chains of the antibody, and plays an important role in the binding and specificity to a particular site of the antigen known as the epitope. In the case of this work, the antibodies used were specific to Aβ peptides, either 1-40, 1-42 or both, with epitopes variable depending on the antibody.
Figure 6. Sketch of an IgG Monoclonal Antibody Molecule. The region of light chains is generally recognized as Fab and promotes specific binding characteristics.

The distinction between the different antibody isotypes is based on their heavy chain, which in turn is related to the source where they are produced; for instance, IgA is found in the mucous secretions, while the IgG is mainly found in the blood and in tissue liquids. A functional difference between the various isotypes is based on the part of the immunoresponse’s cascade of which they are part. Between the 5 main isotypes shown in Figure 6 (IgG, IgA, IgE, IgD, and IgM), the most common isotype in the human body is the IgG, which was also used in this work.
Figure 7. Heavy Chain Isotypes of Mammalian Antibodies. IgG provides the majority of antibody-based immunity against invading pathogens.

In general, antibodies that are synthetically produced can be classified in two large groups: monoclonal and polyclonal. Polyclonal antibodies are obtained from the sera of immunized animals, and they are a mixture of different immunoglobulin types binding to multiple sites on the antigen used for immunization [41]. On the other hand, monoclonal antibodies have a single immunoglobulin type binding to a single specific site of a molecule (antigen, protein, carbohydrate or nucleic acid) used for immunization.
Monoclonal antibodies are synthetically produced by the fusion of antibody-producing mouse B cells, which have a finite life span, with "immortalized" cells of a mouse myeloma (cancer cells). These fused cells are cultivated and successful immortalized antibody producing cells are selected \[41\]. This process is pictured in Figure 8. Each cell or clone obtained by this method is capable of producing large amounts of a single type of antibody, which binds to specific epitopes of the antigen molecule (Aβ_{1-40}, Aβ_{1-42} or both).
2.4. Immunotherapy Preceding Work

Immunotherapy for treating AD is a subject that has been investigated since the late 1990’s. Beka Solomon (1996) and her group were the first who reported the effectiveness of N-terminus antibodies to block fibril formation in vitro [42]. After this publication, many other studies involving immunotherapy for AD were published from Solomon’s group and from other scientists all over the world [43].

In 2003, preliminary results were published of the first clinical trial (Elan-Wyeth clinical trial) [44] in which patients with AD where vaccinated with AN-1792 (purified Aß_{1-42}), and after advancing to a Phase II (372 patients with mild to moderated AD), the study was stopped after finding 7% of the patients suffering from encephalitis. Nevertheless, the immunized patients were monitored after the treatment was stopped and a reduction in cognitive decline in comparison to the controls was found. This has been the basis to continue in the search for an effective but safe immunotherapy treatment for patients with AD [45].

It is important to note that immunotherapy can be classified in two types: active and passive immunization (Figure 9). Briefly, active immunization can take place either by injecting into the individual the whole antigen or part of it, whichever way provokes an immune response resulting in the production of specific antibodies to the injected antigen. The other type of immunization consists of the injection of specific antibodies directed to the antigen of interest (either monoclonal or polyclonal).
**Figure 9.** Active vs. Passive Immunization. Adapted from Schenk, 2002 [27]

An ideal scenario for using immunotherapy to target a specific event (clearance of Aβ deposits) would be decreasing the body’s immune response to a mild level, so that the external agent (antibody in the case of passive immunization) could mediate the desired event in a controlled manner. From here derives the idea of using monoclonal antibodies to trigger the clearance of Aβ deposits.

Indeed, this idea has been tested in mice, finding activation of the clearing plaque mechanisms and cognitive recovery to a certain level [46-54]. But still the key questions remain: What is the mechanism involved in this clearing event? Is it possible to determine what antibody will activate and help the body to clear these deposits in the most effective way? What about the quantities?
The present study provides insights that help to understand, from a molecular interaction point of view, the effect of the antibody on the plaques and help to identify the species at different stages of the plaques formation and clearance.

2.5. Antibodies - Peptides Interactions Theories

For the purpose of this study it is important to consider the affinity between the antibody and the Aβ peptide from a thermodynamic point of view. The affinity is related to the type of interactions that occur at the interface between the two molecules. In terms of energy, the affinity between two molecules will depend on two thermodynamic factors, the enthalpy and entropy at the interface [55].

When antibodies and Aβ peptide molecules are free in aqueous solution, they have a considerable configurational (translation and rotational energy, measurable with ATR-FTIR spectroscopy) entropy that is lost when the association (binding) occurs. This effect is observed in the analysis to be presented in Chapters 4 to 6.

Moreover, the noncovalent interactions (H-bonds, salt-bridges and van der Waals forces) between each molecule’s surface residues with the water are lost when the antibody and Aβ peptide enter in contact. Nevertheless, when the bound water is released from the two interacting interfaces, it leads to an increase in entropy of the solvent. Hydrophobic residues that become buried at the interface cause an increase in entropy of the solvent in a similar way.
However, at the same time, new hydrogen bonds, salt bridges, and van der Waals interactions are formed between the antibody and the Aβ peptide [40, 56].

Energy losses and gains due to molecular interactions driving aggregation can be indirectly detected as a function of integrated absorbance with ATR-FTIR spectrometric analysis.

In addition, there are other factors that might also affect the molecular interactions between the antibody and the Aβ peptide such as the presence of salts (ions in solution) and the posttranslational modifications involving morphological changes, as is the case of monomers, oligomers and fibrils species adopted by the Aβ peptide [57].
CHAPTER 3

ANALYTICAL TECHNIQUES AND MATERIALS

In this study, different analytical methods are combined to provide a comprehensive physicochemical characterization and explanation of the processes of peptide aggregation, as well as the effects produced by different anti-Aβ monoclonal antibodies when they are tested against Aβ peptide aggregates. This chapter explains the techniques used in a systematic way by dividing them into physical, biochemical and chemical characterization. The techniques explained here are brought into play in the subsequent chapters.

3.1. Physical Characterization

These techniques served to evaluate the morphology of the samples by studying their dimensions as well as dispersion and distribution of the different peptide aggregates across the substrate’s deposition area.

Two physical characterization techniques were utilized: transmission electron microscopy (TEM) and atomic force microscopy (AFM). The primary aggregates of interest for the study were monomers, oligomers or protofibrils, and fibrils.
3.1.1. Transmission Electron Microscopy (TEM)

This is a microscopy technique that consists of a focused beam of primary electrons that is directed through the sample under study. The electron beam can be generated by thermionic emission from a filament, commonly tungsten. However, the electron’s beam can also be produced by a lanthanum hexaboride (LaB₆) source. The electrons are accelerated using electrostatic and electromagnetic fields to focus the beam and deflect it to a constant angle [58].

The instrument used was a Morgagni-268D transmission electron microscope from FEI Corporation (NE, US), which is shown in Figure 10. The source of primary electrons in this instrument comes from the thermionic emission of a tungsten filament. The instrument provides a point-to-point resolution of 0.45 nm and a resolution between lines of 0.34 nm. It has a Soft Imaging Mega 3 digital camera with an ultimate resolution of 1280x1024 pixels per image. All processes of the specimen’s imaging takes place in a vacuum chamber (10⁻⁶ Torr).
The accelerating voltage chosen for the analysis was either 100kV or 60kV depending on the thickness of the sample, and it was adjusted to get the best contrast.

The sample preparation depends on the specimen to be studied. For instance, in the case of amyloid peptide, a drop (5 μL) of the sample is simply placed on a special grid (rinsed with 5 μL of Millipore water) and dried with ultrapure nitrogen.

In this study two different types of grids were used: Formvar coated grid stabilized with evaporated carbon film (150 mesh, FCF150-Cu) and inert silicon nitride grids (DuraSiN™). The last one used to characterize the sample not only with TEM but also with AFM. Both grids were purchased from Electron Microscopy Science (PA, US), and they are shown in Figure 11 below.

![Figure 11. TEM Grids Used in this Work. (a) Carbon grid (150 mesh) coated with Formvar film, a transparent and inert polymer. (b) DuraSiN™, showing window covered with silicon nitrate](image-url)
3.1.2. Atomic Force Microscopy (AFM)

This technique is also known as scanning force microscopy (SFM) or scanning probe microscopy (SPM). It allows for the visualization of the topographical structure of conductive and non-conductive materials. Commonly, an AFM consists of a piezoelectric scanner, on which the sample rests; this scanner allows movements in a precise fashion in the x, y, and z directions. However, having the three-dimensional movement freedom in the same scanner makes the instrument very sensitive to disturbances, resulting in long collection times.

In this work, the AFM used (XE-100, Park Systems Inc., CA, US) consists of two piezoelectric scanners; one scanner accounts for the x-y movements only, and another piezoelectric scanner, located on the head where the cantilever rests (Figure 12(a)), separately allows movement on the z direction. This new design increases imaging flexibility and reduces the collection time. Another important component in the AFM is a photodiode, which senses the movements of the scanning tip when a laser positioned on it deflects due to its interaction with the sample. This deflection is then translated by the photodiode into either a current or a voltage, and ultimately into a digital image (Figure 12(b)).

With AFM, it is possible to image and characterize samples on a solid-gas interface as well as solid-liquid interface. In this work images were collected in both edia; however, primarily images were collected on solid-gas interfaces.

Atomic force microscopy allows characterization of the sample's surface by three different modes, as explained below.
3.1.2.1. Contact Mode

In which the tip of the cantilever gets in touch with the sample (hard materials).

3.1.2.2. Non-Contact Mode

Where the tip does not contact the sample. The non-contact mode is used in characterization of soft materials (biological, i.e. cells).

Figure 12. AFM and Scanning Process. The sample’s topology can be studied by dragging a micro-scale cantilever across its surface. (a) Shows a full diagram of the instrument; (b) illustrates a detail of the cantilever and detection system on Aβ fibrils

3.1.2.3. Intermittent or Tapping Mode

This mode can be used to prevent the cantilever from being trapped by capillary forces caused by the presence of an extremely thin film of water
surrounding the sample in air. Also this mode is use to preserve the sample, especially when biological samples are analyzed. Tapping mode was used to analyze the Aβ peptides deposited on mica.

Therefore, in this study tapping was the mode of preference for the samples’ characterization. During the tapping mode, the cantilever is deliberately excited by an electrical oscillator to amplitudes of up to approximately 100 nm, creating an effective tapping modality as the cantilever travels over the sample (Figure 12(b)). In addition, because the contact time of the tip with the sample is relatively short, it is possible to reduce lateral force (that could cause distortion of the image) and also preserve the sample’s morphology [59].

There are a wide variety of cantilevers commercially available; the selection depends on the application and media. In this work, the cantilever used was the type Tap300Al (Budget Sensors, Bulgaria). These cantilevers were purchased with an aluminum coating to increase the laser’s reflectivity. This type of cantilever has a resonant frequency of 300 ± 100 kHz and a force constant of 40 N/m.

The substrate material used to image the peptide’s samples was mica. This is a preferred material when working with biological specimens [59]. Mica was selected because it is atomically smooth (0.2 to 0.6 Å), and at the same time, an easy to clean (sheets cleavage) and inert material. In this study natural mica of the type ASTM V-1, purchased from Axim Mica (NY, US), was used.

The physical characterization methods used in this study facilitated checking the physical size of the aggregates during time and determining the
kind of secondary structures dominating at each stage of the aggregation process of samples with anti-Aβ monoclonal antibodies and alone.

3.1.2.4. General Protocol of Sample Deposition and Data Analysis

In this work all the samples were deposited under a laminar flow cabinet (Class 100) to avoid contamination by settling of undesirable particles.

Each aliquot collected at specific times was diluted with Millipore water to 20 μM. Then, 5 μL of the diluted solution was deposited on freshly cleaved mica and incubated for 5 min. A small Teflon O-ring (external Φ 7mm, internal Φ 5mm) placed on the mica served to confine the peptide solutions. The excess of peptide solution was flushed with 1 ml of Millipore water, dried with ultrapure nitrogen, and stored in a vacuum dessicator at 30 in Hg for later AFM imaging.

As mentioned in Section 3.1.2, the images were taken with a XE-100 atomic force microscope from Park Systems Corp. (Santa Clara, CA) at 25°C. The scans were performed with TAP300Al cantilevers (Budget Sensors, Bulgaria) using tapping mode, to preserve the sample. The image resolution was 512x512 pixels, and the scan rate was 0.75 Hz, the set point of the cantilever was variably moved to satisfy the image quality but never lowered beyond -0.260 μm. Images were collected at 5 random points on the mica surface, and each point was imaged using three different scan sizes, first 35x35-μm x-y, then zooming to 10x10-μm x-y, and finally to 2x2-μm x-y; this was done to assure a fair exploration of the sample distribution across the mica.
The processing and analysis of the images was performed with WSxM software (version 5.0) [60].

3.2. Biochemical Characterization

3.2.1. Aß Soluble Species Detection: SDS-PAGE and Immunoblotting

The combination of these two methods, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and Western Immunoblotting allow for discriminating the different soluble peptide species in the incubation solution when separated by their difference in molecular weight.

In this sense, the sample, after being diluted in water and centrifuged, is first analyzed with SDS-PAGE, in which the peptide is loaded on a 4-12% Bis-Tris gel, and then exposed to an electrical current that allows the diverse aggregates species to migrate and distribute on the gel at different speeds, depending on their molecular weight. Normally, the gel used is polyacrylamide. This is an inert material, and serves only as a medium for the peptides to move (under buffered conditions).

The peptide’s species recognition takes place once the peptide (distributed on the gel) is carefully transferred to a polyvinylidene difluoride membrane where it is likely to bind. A step prior to recognition is the “blocking” step, in which either Bovine serum albumin (BSA) or non-fat dry milk are used to prevent the interaction of the antibody with the unbounded sites of the membrane, and therefore creates “noise.”
The membrane is incubated with a primary antibody specific to N-terminal of the peptide (Aβ) by gentle agitation for 12 hours. Then, the gel is rinsed to remove any unbounded primary antibody, and at this point, a second antibody that targets the primary antibody is added to enhance recognition.

Finally, the immunostained peptides can be visualized using chemiluminescence techniques. For the effect of this work, this step was performed in accordance with the manufacturer’s recommendations (ECL, Amersham, Denmark). Western blots images were captured with a Fujifilm Lass 300 Intelligent dark box scanner and quantified with a linear range of detection using Alpha Ease software (AlphaEaseFC, Alpha Innotech Corporation, San Leandro, CA). This analysis allows one to quantify the intensity of the bands corresponding to the following: monomers species (4.5 KDa), Low Molecular Weight (LMW) oligomers or protofibrils (14 to 21 KDa), High Molecular Weight (HMW) oligomers or protofibrils (38 to 98 KDa), and larger soluble aggregates (188 to 250 KDa). Figure 13 shows the schematic representation of the analysis explained above.
Figure 13. SDS-PAGE and Western Blot Schematic. It shows the steps involved in the biochemical characterization of Aβ aggregates. The sample is diluted and centrifuged, then exposed to an electrical current through a gel separating aggregates by molecular weight, then the aggregates are transferred to a membrane and immunotagged. The membrane is digitized and the results are quantified with computer software.

It is important to mention that during the peptide aggregation, monomers associate as a paranuclei species (pseudostable) at the initial times of incubation [61]. This paranuclei can be detected from the monomeric through the LMW region. In the next chapter, this technique is linked to the physical view of the different species and is also investigated with atomic force microscopy.
3.3. Chemical Characterization

Vibrational spectroscopy was used in this work to analyze and monitor the chemical configuration changes of amyloid peptides. This technique also allowed to determine and understand the effects of antibody molecules targeting Aβ peptide. While the experimental setup is relatively simple, the analysis of the resulting data is rather complex. Hence, this section explains the most important details to be considered for the analysis and further understanding of the results.

3.3.1. Attenuated Total Reflection - Fourier Transform Infrared Spectroscopy (ATR-FTIR)

3.3.1.1. Principles

This technique is based on the combination of two main principles: the first one is the detection of the vibrational energy emitted by the molecules when excited by an infrared (IR) light; and the second principle is based on enhancing the interaction between the molecules analyzed and the IR beam, therefore improving the detection sensitivity of the method. The last principle, occurs by using a crystal with high refractive index that allows the IR beam to undergo multiple internal bouncing when entering the crystal in a 45° angle of incidence, the beam creates an evanescent field at each point of contact with the sample’s interface (Figure 14). The evanescent field created by the beam, approximately penetrates the sample 1.66 μm in depth; distance measured at 1000 cm⁻¹ for a ZnSe crystal. This depth of penetration varies with the crystal used (ZnSe, Ge,
diamond, etc), angle of incidence, and refractive index of the sample. In this work all the experiments were performed using a ZnSe crystal as the ATR element.

Enhancement of the signal with an ATR crystal. The evanescent field penetrates the sample 1.66 μm approximately for a ZnSe Crystal

\[ d = \frac{\lambda}{2\pi n_{1} \sin^2 \theta - \left( \frac{n_{2}}{n_{1}} \right)^2} \]

\( \lambda = \) wavelength of the energy
\( \theta = \) angle of incidence
\( n_{2} = \) refractive index of the sample
\( n_{1} = \) refractive index of the crystal

(a) (b)

**Figure 14.** ATR-FTIR Spectroscopy Principles. (a) Two main and general vibrational energies. (b) Schematic representation of an ATR accessory, where the main element is a ZnSe crystal used to enhance the sensitivity of detection. The crystal is beveled in the two edges where the beam enters and exists in the crystal, allowing 12 points of contact with the sample. The equation explains the depth of penetration that the beam has inside the media under study, which depends on the wavelength of the energy, the angle of incidence on the crystal, and the refractive index of both the sample and crystal

The enhancement of the detection is of particular importance for this study in which the concentration used is relatively small (100 μM).
It is important to mention that ATR-FTIR spectroscopy works in the Mid-Infrared region of the electromagnetic spectrum (Figure 15), going from 400 through 4000 wavenumbers.

**Figure 15.** Electromagnetic Spectrum. The Mid-IR region is located between 400 and 4000 wavenumbers

The range can be slightly modified by the ATR crystal used. For instance, in the case of ZnSe crystal the range goes from 650 to 4000 wavenumbers.

### 3.3.1.2. Peptides and Amide Vibration Modes

As explained in Chapter 2 section 2.2, amino acid residues formed the peptides. The functional group of amino acids is the amide group, -C(=O)NH-. Therefore, central vibrational energy characteristic of the peptides is the amide type [6, 62, 63]. The Mid-IR region of the electromagnetic spectrum shows 5 regions correspond to the amide groups (Figure 16(a)).
Figure 16. Amide Bands. Showing the mid-IR range going from higher vibrational energy to lower: (a) All the amide bands encountered in the mid-IR range, (b) Amide I, the most straightforward band holding the secondary structure information of the peptide, (c) NH stretching and bending energies are shown (NH stretching also is present in the Amides A and B). Amide II can be related to protein absorption to the crystal, (d) Amide III region. The example spectrum shown here corresponds to 5 days of incubation Aβ40/42 Peptide.
From the amide bands or regions shown in Figure 16 the more relevant are the amide I and amide II regions; ranging from 1600 to 1700 cm\(^{-1}\) and 1500 to 1600 cm\(^{-1}\) respectively. The amide I region, reveals the changes suffered by the backbone of the peptide without neglecting the side group’s effects [64]. Because, interactions of the type NH and NH\(_2\) allow the peptide to anchor to the crystal’s surface and this interactions are detected in the amide II region, this interactions serve to investigate and understand the process of peptide’s surface adsorption and desorption; simply by calculating the area under the amide II region.

The amide I region is analyzed by its deconvolution in different peaks, each corresponding to an existing secondary structures of the peptide. Table 1 summarizes approximately the ranges in which the secondary structures of amyloid peptides are localized.

The amide I band is sensitive to the secondary structure change in peptide and polypeptides [62]. However, explaining the large splitting into two bands of antiparallel \(\beta\)-strands structures, has become a challenge for researchers. In this sense, some light on this issue has been shown by the use of the transition dipole coupling mechanism theory [65]. This mechanism serves to explain how the \(\alpha\) structures split in two bands, one positioned with center at 1630 cm\(^{-1}\) and the other center at 1690 cm\(^{-1}\). Other effect such as hydrogen bonding also modifies the amide I frequency of the polypeptide.
Table 1. Peak Assignment of Aβ Secondary Structures. The assigned peaks have been selected by gathering data from related literature [66, 67] and adapted from the current study observations during secondary structure study.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Range [cm⁻¹]</th>
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</thead>
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</tr>
<tr>
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<tr>
<td>High frequency, Intramolecular</td>
<td>1680-1700</td>
</tr>
</tbody>
</table>

3.3.1.3. Hydrogen Bonding

The nature of hydrogen bonding has an evident effect on shifting the position of peaks for different structures [68] as explained below.

The peak absorbance for structures in the amide I region correlates to the strength of the hydrogen bonding; this is true for most cases except in the case of the main frequency band of β-sheets. In descending order of hydrogen bonding, the secondary structure bands are the following: intramolecular antiparallel β-strands extended chains (1610-1628 cm⁻¹), intermolecular β-sheets (1630-1640 cm⁻¹), α-helices (1648-1658 cm⁻¹), 3₁₀-helices (1660-1666 cm⁻¹) and non hydrogen bonded amide groups in DMSO (1660-1665 cm⁻¹) [69]. This range is slightly varied from those shown in Table 1. However, at the moment of
the analysis both are considered. The peak frequency of the amide I group is lowered by 20 to 25 cm\(^{-1}\) and is influenced by the two possible hydrogen bonds connecting to the C=O group.

In the case of polypeptides, experiments indicate that amide I main absorption band can vary by 30 cm\(^{-1}\) because of different hydrogen bond strengths among peptide residues.

Alpha-helices hydrogen bonding to solvent influences absorption frequency by approximately 20 cm\(^{-1}\); solvated α-helices induce a 20 cm\(^{-1}\) lower absorbance maximum for amide I.

### 3.3.1.4. Transition Dipole Coupling Mechanism (TDC)

It is a resonant mechanism that causes secondary structures to influence amide I absorbance frequencies. Transition Dipole Coupling (TDC) is dependent on distance between amide groups in a peptide chain, and it is caused by resonance interaction due to oscillation of dipoles of such neighboring amide groups. TDC manifests itself in two ways on the FTIR spectra [70, 71] and is explained as follows.

#### 3.3.1.4.1. Exciton Transfer

In an analogous way to fluorescence (Förster) resonance energy transfer (FRET), energy is absorbed and transmitted to nearby structures delocalizing excitation states over a length of 8 Å; typically exciton transfer energy for α-helix has a constant of 0.5 ps (pico seconds).
3.3.1.4.2. Exciton Splitting

Additionally, TDC results in the splitting of bands since coupled oscillators result in different excitation energy levels in phase and out of phase. This, results in the splitting of the absorbent contribution on amide I for the case of antiparallel β-sheet structures, with an approximately separation of 70 cm$^{-1}$.

3.3.1.5. General ATR-FTIR Data Analysis Protocol

Each spectrum collected with ATR-FTIR spectroscopy was smoothed and baseline corrected in Omnic (version 7.2a, Thermo Fisher Scientific). The spectroscopical regions of interest were the amide I (1600-1700 cm$^{-1}$) and amide II region (1500-1600 cm$^{-1}$). To the amide II region the area under the curve was simply calculated and this value represented the absorption or desorption of the peptide to or from the crystal respectively.

The amide I region (1597-1710 cm$^{-1}$) was selected and saved in Comma Separated Values (CSV) format. The file was then analyzed with OriginPro (version 8 SR5, OriginLab). In OriginPro the analysis was performed by peak fitting using the parameters shown in Table 2. This operation is known as spectrum’s deconvolution by interactive least-squares curve-fitting technique, using a Gaussian distribution model.
Table 2. Fitting Parameters and Boundary Conditions

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<td>1680</td>
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</tr>
<tr>
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<td>0</td>
<td>A_7</td>
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<td></td>
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</tbody>
</table>

The resulting peaks after the curve fitting represent the contribution of each secondary structure, this quantity is calculated by determine the area under each fitted peak, and consequently the addition of these peaks represent the amide I region. Each peak is assigned as a particular structure depending on the position. In this way it is possible to monitor the secondary structure percentage (as a normalized value) and the raw value. The latest value can be related to the kinetic of each particular secondary structure followed with time. An example of this fitting is shown in Figure 17.
Figure 17. Sample’s ATR-FTIR Data Analysis. It Depicts Aβ₁₋₄₂ after 48 hours of incubation. The colored areas correspond to the estimated contribution of each specific structure to the total absorbance calculated by deconvolution of the amide I region. Also the amide II region is shown and it represents the surface protein adsorption.

It is important to mention, that in previous studies (Jimenez, 2005 [72]) the investigation of the amide I has been performed considering the band assignment in a more general fashion. Assuming only one frequency of antiparallel β-sheets (called intermolecular interactions, 1610-1625) and considering the high frequency range (1670-1700 cm⁻¹) as β-turns. In this new study to give to the antiparallel β-sheets structure the importance they have and to understand the role they play during aggregation/dissolution of the peptide the bands assignment in the amide I region has been judiciously modified assigning
β-turn to a range of 1670-1690cm\(^{-1}\) and a introducing a new band between 1680-1710cm\(^{-1}\) corresponding to high frequency antiparallel β-sheets [73, 74].

3.3.1.6. Some Relevant Consideration for the ATR-FTIR Analysis

In order to understand the possible molecular interactions taking place in a solution and related to the absorption energies present at determined points of the spectra, it is important to consider the following important phenomena.

3.3.1.6.1. Protonation or Deprotonation

For instance, at pH 7.4 the Aβ\(_{1-40}\) and Aβ\(_{1-42}\) are deprotonated (see Appendix A), having a net charge (theoretically) of -2 and -3 respectively. This unbalance of net charge contributes to the conformational change of the peptide molecules as well as their absorption energies.

3.3.1.6.2. Chemical Properties of Neighboring Groups

For instance amide group, allows for localizing a particular absorption band and differentiating it from a C=O originated from an aldehyde or ketone group. In this work this is not relevant because it is known that only peptides would be showing this C=O bonding.

3.3.1.6.3. Bond Angles and Conformations

Under the effect of Coulomb interactions, proteins modify their bonding angles; this affects the three-dimensional structure of their backbone originating
changes (conformational changes). This combination is responsible for the amide I band sensitivity to secondary structure.

3.3.1.6.4. Hydrogen Bonding

Another important interaction encountered in peptides is the hydrogen bonding; this type of interaction stabilizes the peptide molecules in their lower energy state depending on the media conditions. The strength of hydrogen bonding can be directly observed as mentioned earlier in section 3.3.1.3.

3.3.1.6.5. Conformational Freedom

A very important aspect of FTIR spectroscopy is the fact that as structures are more flexible, the band that they represent in the vibrational spectrum is wider. Heterogeneity of structures caused by conformational freedom results in broader spectrum bands defining structures. On the other hand, more rigid structures have less conformational freedom and result in tighter spectrum bands. When structures interact through binding, conformational freedom is usually reduced; this is also the case for molecules binding peptides and proteins and for peptide-peptide binding, resulting in the reduction of bandwidth by a factor of two [75]. The incremented rigidity and reduced conformational freedom sometimes increases rigidity in distant parts of large molecules such as proteins as well.
3.3.1.6.6. Overlap of Conformational Changes in the Spectrum

The fact that different structures overlap in the spectrum makes analysis of signals more complex. However, spectra can be subtracted, with the limitation that the infrared difference spectrum reveals only the net change of secondary structure. In this work, by amide I deconvolution it is possible to distinguish the structures during overlapping of conformational changes.

3.3.1.6.7. Rigid Domain Movement

Movements of relative large structures are not detected in near infrared spectra. Hinge regions of proteins are an interesting example in which the larger rigid domains are not detected while the hinge flexible region is prevalent in the spectra. An example of two rigid domains moving about a flexible domain is shown in Figure 18 (black line). In this case, the detection of the flexible domain would be prevalent, established by comparing it to the larger and more rigid domains attached to it. The detection would be limited to the conformation of the relative orientation of neighboring amide groups in the flexible domain. However, there is increased sensitivity in the detection of conformational changes where the backbone portion of a molecule is intimately linked to flexible domains; for example, if an α-helix shortens, there are changes not only in the amide modes of the unwinding backbone portion, but also in others amides related to the remaining portion of the helix.
3.3.1.6.8. Subtle Changes within Secondary Structures

Finally, subtle changes within secondary structures, such as twists in β-sheet, bending of an α-helix, or hydrogen bonding to backbone carbons, are detectable in the amide I of the mid-infrared spectrum.

![Diagram of protein structures](image)

**Figure 18.** IR Spectrum Analysis and Rigid Domain Movement. Adapted from Barth 2007 [76]

3.3.1.7. General Experimental Methodology

The experiments were performed with a 6700 Nicolet spectrometer (Thermo Fisher Scientific, US). Two different accessories were used: an ATR trough plate for the study of monomeric solutions (with more flexibility to evaporate and redissolve the peptide’s in different solvents), and a closed ATR flow cell accessory for the peptide solution in HFIP, and the oligomeric and fibrillar solutions (hermetically closed to avoid evaporation during long incubation times, and with temperature controller).
The IR beam was focused at normal incidence onto one of the 45° beveled faces of the ATR zinc selenide (ZnSe, \(n_s = 2.4\)) trapezoidal crystal, where the IR beam undergoes multiple internal reflections resulting in signal enhancement. A crystal made of ZnSe was the preferable used because of its low reflection losses in the infrared [77], wide infrared range (between 20,000 and 650 cm\(^{-1}\)), compatibility to aqueous solutions, and chemical inertness (Appendix B).

After each measurement, the flow cell was dismantled and thoroughly cleaned with isopropyl and Millipore water; in addition the crystal was exposed 5 minutes to UV to eliminate organics, and rinsed again with Millipore water. All the flow cell parts were finally dried with an ultrapure nitrogen stream and re-assembled.

3.3.1.8. General Data Analysis

For the cases of long incubation times (i.e., 24h, 48h, or longer) and with several spectra collected by hour (i.e., 6 spectra per hour), high variability is recorded from spectrum to spectrum. For these cases, set of spectra with time ranges of 4 hours were averaged and the variance among them was calculated.

To calculate the average absorbance (Eq.1) for a time frame (for example 4 to 8 hours), all curves under that timeframe are added and averaged by the number of curves recorded during that time; this yields an average spectrum:
Average Absorbance(w) = \frac{\sum_{t=1}^{t=n} \text{Absorbance}(t)}{n} \quad \text{(Eq. 1)}

The software calculates then the variance for the curves during that time frame as Eq.2 shows:

\[ \text{Variance}(w) = \frac{\sum_{t=1}^{t=n} (\text{Absorbance}(t) - \text{Average Absorbance})^2}{n} \quad \text{(Eq. 2)} \]

Finally, the number to calculate the Relative Spectral Variance (Eq.3), the variances (Eq.2) for each wavelength in the region are integrated to calculate the area under the variance curve, divided by the average absorbance (Eq.1), and multiplied by 100.

\[ \text{Relative Spectral Variance} = \frac{\int_{w_1}^{w_n} \text{Variance}(w).dw}{\int_{w_1}^{w_n} \text{Average Absorbance}(w).dw} \times 100 \quad \text{(Eq. 3)} \]

This relative spectral variance or %Variance is a good indicator of stability of the processes undergone by Aβ peptides in their stabilization at different conformations; typical values for stable Amide I and Amide II regions are around 10%; as shown in Figure 30.
3.4. Reagents

Table 3 lists all reagents used in this work.

**Table 3.** Reagents Used in this Work

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Manufacturer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,1,1,3,3,3-Hexafluoro-2-Propanol (p³: &gt;99%), HFIP</td>
<td>Fisher Sci.</td>
<td>Very volatile transparent liquid</td>
</tr>
<tr>
<td>Methyl Sulfoxide Anhydrous (p:≥99%), DMSO or Me₂SO</td>
<td>Acros</td>
<td>Viscous transparent liquid</td>
</tr>
<tr>
<td>Tris (Crystallized) p:≥99%)</td>
<td>Fisher Sci. BP152-500</td>
<td>White powder</td>
</tr>
<tr>
<td>Phosphate-buffered saline, PBS</td>
<td>Sigma-Aldrich P3813</td>
<td>White powder</td>
</tr>
<tr>
<td>Hydrochloric Acid, HCl</td>
<td>Acros 124630010</td>
<td></td>
</tr>
<tr>
<td>Nitrogen</td>
<td>Airgas NI UHP300,</td>
<td>Ultrapure gas</td>
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CHAPTER 4
AGGREGATION PATHWAYS OF AMYLOID BETA PEPTIDE

Amyloid beta (Aβ) peptides and proteins in general modify their molecular configuration (i.e., secondary structures) influenced by the media conditions in order to minimize their overall energetic state [78, 79]. This chapter will analyze and discuss how media conditions affect the secondary structure of Aβ peptides. The conditions evaluated here have been used to identify features corresponding to each Aβ species during aggregation. The species studied are: monomers, which are the single molecules of Aβ peptide characterized by their hydrophobic character (Aβ1-42 in particular); oligomers, which are found during the nucleation or seeding state and that are also known as the intermediate species during fibrillization [80]; and finally the fibrils, the main components of neuritic plaques in Alzheimer’s disease brain tissue. In later chapters the findings from this chapter will be used as guidelines to elucidate changes in aggregation and effects of antibodies on the Aβ peptide species.

4.1. Protocols and Methods

Each peptide in this study, Aβ1-40, Aβ1-42, and their equimolar mixture Aβ40/42, were prepared following a common protocol explained below.
4.1.1. Peptide’s Homogenization

Amyloid Beta (Aß) peptides were purchased from American Peptide INC. (Sunnyvale, CA). Table 4 below shows the general specifications of Aß peptides used in this study.

The peptides were homogenized and aliquoted in HFIP, as described by Stine et al. 2003 [37]. Briefly, HFIP was injected into the peptide’s flask using a gas-tight Hamilton glass syringe with a Teflon plunger, at a peptide’s concentration of 1 mM.

**Table 4. General Specifications of Amyloid Beta Peptides Used in this Study**

<table>
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<tr>
<th>Characteristic</th>
<th>Aß₁₋₄₀ (Amyloid Beta 1-40)</th>
<th>Aß₁₋₄² (Amyloid Beta 1-42)</th>
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<td>4,514.1</td>
</tr>
<tr>
<td>Formula</td>
<td>C₁₉₄H₂₉₅N₅₃O₅₈S₁</td>
<td>C₁₉₉H₃₀₇N₅₃O₅₉S₁</td>
</tr>
<tr>
<td>Purity (HPLC Analysis)</td>
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<td>87.2%</td>
</tr>
<tr>
<td>Solubility</td>
<td>1 mg/ml in water</td>
<td>1 mg/ml in 0.05M Tris buffer</td>
</tr>
<tr>
<td>Counter ion</td>
<td>Trifluoroacetate</td>
<td>Trifluoroacetate</td>
</tr>
<tr>
<td>American Peptide Cat. No.</td>
<td>62-0-78</td>
<td>62-0-80</td>
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</tbody>
</table>
The peptide immediately dissolves because of the high polarity of the HFIP, that helps to break down the peptide’s pre-existing tertiary structures [37]. After 10 minutes in HFIP at 25°C, the peptide was aliquoted in sterile microcentrifuge tubes and left overnight in a laminar-flow cabinet (Class 100) allowing the HFIP to evaporate. This protocol created a clear peptide film in the bottom of each vial. Each film was also exposed to 30 in Hg of vacuum for 8 hours to eliminate traces of HFIP and humidity. Each vial was closed with nitrogen (gas), and sealed with Parafilm-M. Finally, all the vials were stored at -20°C in a jar containing molecular sieves and closed with nitrogen to maintain a low humidity environment.

4.1.2. Aβ Monomers under Different Media Conditions

Figure 19 illustrates the protocol followed to understand and identify the initial molecular conformation and morphology of Aβ peptides under media conditions that at different incubation times promote defined monomers, oligomers, and fibrils. Aβ1-40, Aβ1-42, and Aβ40/42 peptides were exposed to the conditions explained below and monitored until stability was reached. That is, the peptides were monitored 30 minutes at conditions (a)-(c); and for 1 hour at conditions (d) and (e).
Figure 19 explains the details of this study as follows:

Figure 19(a): each peptide was dissolved in HFIP at 1mM concentration, as described in the pre-treatment step (section 4.1.1), and allowed to equilibrate for 30 minutes in a gas tight ATR flow cell. Continuous spectra were collected during this period.

Figure 19(b): Once the HFIP evaporates a film is formed, it was determined if monomeric species were maintained. Thus, in a second experiment each peptide dissolved in HFIP as described in Figure 19 (a) was deposited on an open ATR trough plate. Then, the HFIP was removed by evaporation assisted by a dry nitrogen (gas) stream. Continuous spectra were collected for each peptide film during 30 minutes. In addition, a 5 µl drop of this solution was deposited on freshly cleaved mica and the film was later imaged with atomic force microscopy (AFM) to relate peptide’s structure to its morphology.

Figure 19(c): When oligomers or fibrils are prepared, the peptide’s film is always re-dissolved in DMSO to 5 mM concentration.

Figure 19(d): The above solution (c) is taken to 100 µM (0.1 mM) using 20 mM Tris buffer solution (2.42g Tris Base pH 7.4 w/HCl, prepared in 1L of solution) to prepared oligomers.

Figure 19(e): To prepare fibrils, solution (c) is diluted to 100 µM with 0.01 M phosphate-buffered saline solution.

AFM was used to analyze the morphology of Aβ1-40, Aβ1-42, and Aβ40/42, as prepared in Figures 19(d) and 19(e).
Figure 19. Systematic Study of Aβ Monomers under Different Media Conditions. 
(a) Aβ peptide is dissolved in HFIP and monitored with ATR-FTIR spectroscopy; 
(b) the HFIP is evaporated from the peptides’ solution leaving a film; 
(c) the peptide’s film is redissolved with DMSO; 
(d) The peptide dissolved in DMSO is diluted to 100μM with TRIS solution to obtain stable oligomers; 
(e) The peptide dissolved in DMSO is diluted to 100μM with 0.01M PBS solution to obtain fibrils

4.1.3. Oligomers Stability and their Characteristic Secondary Structure

The morphology and secondary structure dynamics of the oligomeric intermediate species of the Aβ peptides was characterized. The oligomers were prepared following a method established in a preliminary study [81]. Briefly, the peptide film was dissolved in DMSO (5 mM) and diluted to 100 μM (0.1 mM) with a 20 mM Tris buffer solution (2.42 g/L Tris Base at pH 7.4 adjusted with HCl).
The solution prepared to form oligomers was split in two portions, one portion was injected in an ATR flow cell to follow in situ the evolution in secondary structures at 25°C; the second portion of the oligomeric solution was aliquoted and incubated at the same temperature (25°C); each aliquot was retrieved at different times during 48 hours for later AFM analysis.

4.1.4. Aβ Species and Kinetics of Fibril Formation

The first step to promote fibril formation was to re-dissolve the peptide film with DMSO until it reached concentration of 5 mM, the dissolution process was assisted by 5 minutes of sonication. This was followed by the addition of PBS (0.01 M, pH 7.4), diluting the peptide solution to 100 µM. After 1 minute of sonication the solution was split in two portions, the first one was injected in the ATR flow cell for spectroscopy analysis (incubated at 25°C) and the second portion was aliquoted for AFM analysis. Aliquots retrieved at different time points.

4.1.5. Peptide Secondary Structure Study: Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Spectroscopy

Once the accessory was assembled and prior to injecting the peptide solution, a portion of fresh peptide-free incubation media solution was flushed through the cell in each new experiment. Spectra and background of this media in contact with the crystal were obtained. The peptide-free solution background was used to eliminate the solvent contribution in the peptides solutions. The experiments were run for 30 minutes for monomer characterization, and for 48
hours for oligomeric and fibril formation. Oligomers and fibrils solutions were set up to collect a new spectrum every 10 minutes.

4.2. Results, Analysis and Discussion

This section shows and discuses the particular characteristics of the Aβ species, beginning with the monomeric species of the two isoforms Aβ peptides (Aβ1-40 and Aβ1-42) and their equimolar mixture; moving on to the oligomers and fibrils characterization for each peptide.

4.2.1. Species of Aβ Peptides

During peptide aggregation different species can be found and defined. However, referred here as monomer, oligomers, and fibrils as species of the Aβ peptide.

4.2.1.1. Aβ Peptide Monomers

This first study was designed to determine the characteristic content of secondary structure present in the monomeric specie of Aβ peptides.
Figure 20. Amide I and II Regions of Aβ_{1-40} Monomers in HFIP. The spectra displayed here were collected for Aβ_{1-40} during 30 minutes of incubation in HFIP at 1mM. The amide I region shows that the antiparallel β-sheet are the dominant structures within the monomeric species. The lack of absorbance in the amide II region is evidence of the absence of protein adsorption, hence all the monomeric species are in solution.

Aβ_{1-40} peptide dissolved in HFIP was injected in a hermetic ATR flow cell accessory and monitor over 30 minutes with ATR-FTIR spectrometry. Figure 21 shows that monomers are unequivocally detected in HFIP, confirmed by the lack of protein adsorption revealed by the amide II region.

After deconvolution of the amide I region for each time shown in Figure 20 with clear center in the antiparallel β-sheet band (1624 cm\(^{-1}\)), it is possible to obtain the information plotted in Figures 21(a) and 21(b), showing the evolution of secondary structures during 30 minutes of incubation.
Figure 21. Secondary Structures of Aβ1-40 Monomers in HFIP. (a) Secondary structures evolution of the monomers in solution. (b) Secondary structure content change for the monomeric species. Each point in this plot was calculated by integrating the area under the curve corresponding to the different specific IR bands defining each secondary structure (Shown in Table 1) at the times shown in (a). Observe in that after 10 minutes the structures start reaching equilibrium and between 27 and 30 minutes stabilize.

Figure 21(a) shows the increase of antiparallel and parallel β-sheets secondary structures during 30 minutes of incubation in HFIP, this figure suggests that the monomers are constantly evolving but they do not aggregate. However the dominant configuration of the monomeric species is the antiparallel β-sheet type, Figure 21(b).
Figure 22. Secondary Structure Content of Aβ1-40 Peptide Monomeric Film. (a) The shift of amide I and the appearance of amide II show a significant difference when protein adsorption occurs. (b) Comparison of secondary structure content change before (light color bars) and after (solid bars) HFIP has evaporated. Main differences are observed, as the switch from antiparallel β-sheet structures to parallel β-sheets with a larger influence of α-helix structures.

Continuing with the study of Aβ1-40 peptide, once the HFIP solvent evaporates, it leaves a film with characteristic secondary structures, shown in Figure 22. The shift of the amide I region in this figure confirms that when the peptides absorb to the crystal, parallel β-sheet structures are highly involved in this process. Protein adsorption (film formation) is verified by analyzing the amide II region (Figure 22(a)).

Figure 22(b) compares the content of secondary structures of the peptide before and after the HFIP is evaporated (film formation). The monomeric film is characterized by the dominant content of parallel β-sheets and α-helix structures, with decrease of antiparallel β-sheet structures, this confirms that the peptide is
not in solution anymore. It is important to note that the antiparallel β-sheet structure was significantly reduced as the film was formed.

The results for Aβ₁₋₄₀ shows that 30 minutes (Figure 21(b)) is a reasonable incubation time in HFIP for the peptide (also found for the cases of Aβ₁₋₄₂ and Aβ₄₀₋₄₂) to have its secondary structure in equilibrium. The two peptides and their 1:1 combination under study (Aβ₁₋₄₀, Aβ₁₋₄₂ and Aβ₄₀₋₄₂) were incubated for 30 minutes in HFIP as an initial practice for all experiments involving these peptides. Comparison of the monomeric species in HFIP for Aβ₁₋₄₀, Aβ₁₋₄₂ and their equimolar combination is shown in Figure 23.

It is important to note that Aβ₁₋₄₂ peptide shows an antiparallel β-sheet to α-helix ratio close to 1 (0.85) in its monomeric state. In previous work (Jimenez, 2005 [72]) α-helix structures were found to play an important role during fibril formation of Aβ₁₋₄₂ peptide. Hence, it is not surprising to find antiparallel β-sheet structures competing with α-helix structures in its monomeric state.

Prion proteins capable of amyloid formation exhibit similar characteristics when forming fibrils; with similar roles played by antiparallel and α-helix structures. This is evidenced in a NMR study performed to the prion protein domain PrP(121-231). This domain contains most of the point-mutation sites known to infectious prion proteins that form two-stranded antiparallel β-sheet and three α-helices [82].
Figure 23. Secondary Structure of Amyloid Peptides Monomers in HFIP. (a) Amide I region, spectra corresponding to 30 minutes of incubation in HFIP for $\alpha$B$_{1-40}$, $\alpha$B$_{1-42}$, and $\alpha$B$_{40/42}$. (b) Secondary structure content corresponding to the monomers of $\alpha$B$_{1-40}$, $\alpha$B$_{1-42}$, and $\alpha$B$_{40/42}$ in HFIP. The values shown in this plot were obtained by averaging the spectra shown in (a) and the error bars are the standard deviations. Notice that, antiparallel $\beta$-sheet (blue) is the dominant structure in $\alpha$B$_{1-40}$ and $\alpha$B$_{40/42}$ monomeric species. When in the case of $\alpha$B$_{1-42}$ both antiparallel and $\alpha$-helix (green) structures dominate the monomeric species (c) Schematic approximation of two $\alpha$B monomers mainly showing antiparallel $\beta$-sheet and small regions of $\alpha$-helix structures (image representing the dimer of transthyretin, shown with permission of Landry, 1996)

The results found in this study, revealed the importance of antiparallel structures to stabilize folded monomers in solution. Note that one would find parallel $\beta$-sheet structures as well as part of the overall structure. However, the dominant secondary structure in all 3 cases is the antiparallel $\beta$-sheet. This observation, is in agreement with the name given to the Alzheimer's disease amyloid peptide, that is amyloid “Beta” peptide, due to the high content in $\beta$-sheet structures in its monomeric folded state [83, 84]. Figure 23(c) is an schematic
approximation of two amyloid forming monomers, with permission of Landry, 1996, this picture represents the antiparallel beta sheet in equilibrium with small domain of α-helix structures. This model is in agreement with our results.

The study performed to Aβ1-40 of HFIP evaporation (Figure 22) and film formation, was repeated for Aβ1-42 and the peptide equimolar mixture Aβ40/42, the results comparing the secondary structure content for each case are shown in Figure 24.

Figure 24. Change in Content of Secondary Structure for Amyloid Peptides during Monomeric Film Formation. (a) Amide I and II regions for Aβ1-40, Aβ1-42, and Aβ40/42. (b) Comparison of secondary structure content before and after the HFIP evaporates, Aβ peptides dissolved in HFIP (light color bars) versus their films (solid bars)
In general, Figure 24(a) shows that when a film is formed, there is a significant shift in the secondary structures (amide I), and a prominent absorption in the amide II region. Figure 24(b) shows that the deposition is mainly dominated by parallel β-sheet structures, while the monomeric state in solution is dominated by antiparallel β-sheet structures.

Figure 25(a) shows the characteristic morphology of Aβ_{1-40} and Aβ_{1-42} monomeric films; which were obtained by depositing the peptide dissolved in HFIP on freshly cleaved mica as explained in 3.1.2.1. Figure 25(b) shows the height distribution analysis for the films from Figure 25(a) corresponding to Aβ_{1-40} and Aβ_{1-42} peptides. Aβ_{1-42} peptide formed more dense films than Aβ_{1-40}, this can be explained by the hydrophobic nature of Aβ_{1-42} peptide, which reduces steric interaction promoting a denser film. Two other studies (Harper in 1997, and Shao in 1999) are in agreement with the hydrophobic nature and ability to pack conferred to Aβ_{1-42} peptide [85, 86].

In addition to these studies with HFIP, Figure 26 shows the results after the peptide films are redissolved with DMSO, since this is the protocol followed prior to oligomeric or fibrillar preparations.

As Figure 26 shows the action of DMSO is to redissolve the peptide and stabilize the secondary structure content. However, when oligomeric and fibrillar solutions are prepared, sonication is an important step to ensure the redissolution of the film. Notice that the film after DMSO addition shown in Figure 26 was not sonicated.
**Figure 25.** Morphology of Monomeric Peptides Film. (a) AFM image of Aβ_{1-40} (top) and Aβ_{1-42} (bottom) peptide monomers on mica (scan size: 0.5x0.5-µm x-y); (b) Gaussian distribution of the heights found in each case (topography), please observe that the Aβ_{1-42} film has a less dispersed distribution (red line, centered at 1nm)

In general, this study has proven that when peptides are pre-treated with HFIP, their conformations in solution are reduced to their monomeric form. This results in a homogeneous (monomeric) film when the HFIP is allowed to evaporate. This is the rationale for pre-treating the peptides with HFIP as a homogenizing point prior to prepare any peptide solution.
Figure 26. Spectroscopy Analysis for Aβ Peptide Monomers in DMSO. (a) Amide I and II regions for Aβ1-40, Aβ1-42, and Aβ40/42, the amide I region shows that the peptides dissolved in DMSO are structurally stable (no shift), the absorption observed in the amide II region shows that there is peptide remaining as a film. (b) Secondary structure content by peptides, comparison between the dry peptide film (light color bars) and the peptide redissolved in DMSO solid bars. Note that in all three cases antiparallel β-sheet structures increased in DMSO.

4.2.1.2. Oligomeric Species of the Aβ Peptides

Oligomers are the intermediate species in the formation of fibrils [87]. Understanding the stability and secondary structure characteristics of these species is key to explain fibril formation and disruption (which will be discussed in chapters 5 and 6 in detail). This study also characterized the kinetics of secondary structures responsible for the formation and stability of oligomeric species of Aβ peptides. The study is presented by peptide type.
4.2.1.2.1. Oligomers of Aβ₁₋₄₀ Peptide

Two stages of the oligomerization process were monitored in this study. First, the peptide dissolution and hydration taking place in the initial 60 minutes of incubation (Figure 27); and second, the formation and stabilization of the oligomeric species during 48 hour of incubation (Figure 28).

Molecular rearrangement is observed in the first 60 minutes of incubation (Figure 27). During the first 10 minutes antiparallel β-sheets and α-helix are the dominant structures; at this point monomers are mainly in solution. After 10 minutes of incubation, the structures shift with increase in parallel β-sheets structures, this is the moment that marks the beginning of the oligomerization process (Figure 27(c)).

Figure 28 (a) shows the spectra corresponding to 48 hour of total incubation, represented by the multicolored lines. Figure 28(b) shows the same 48 hour of total incubation in grey but representing time ranges of 4 hours in red. This visualization technique provides a quick way to observe progressive spectral changes, and assess variance visually; for the whole amide spectra, or for the amides I and II independently.
Figure 27. Spectroscopy Study of Aβ₁-₄₀ Peptide during the First 60 Minutes of Incubation in DMSO+TRIS Solution at 25°C. (a) Amide I and II regions, spectra from 0 to 60 minutes, amide II shows protein adsorption, on amide I region shifting can be observed showing secondary structure instability. (b) Comparison of the total integrated absorbance of amide I vs. amide II from 0 to 60 minutes. (c) Reorganization of the secondary structure that assists the process of aggregation into stable oligomers. (d) Evolution of secondary structure content of Aβ₁-₄₀ the first 60 minutes of incubation.
Figure 28. Kinetics of Aβ_{1-40} Oligomers in DMSO+TRIS Solution at 25°C. (a) Spectra of the Amide I and II regions for Aβ_{1-40} peptide during 48 hours of incubation at 25°C. (b) Sets of spectra selected each 4 hours (red spectra), during 0 to 48 hour of incubation

Figure 29(a) shows the average spectra calculated from the time frames shown in Figure 28(b). The average spectra were calculated using (Eq.1) from Section 3.3.1.4. Figure 29(b) shows the %Variance calculated with (Eq.3) also from Section 3.3.1.4.
Figure 29. Aβ₁₋₄₀ Average Spectra and Variance Percentage. (a) Spectra obtained from averaging the 4 hours range as shown in (Eq.1); (b) %Variance for each time point corresponding to Amide I and II separately and individually, observe that when values are under 30% of variance (Eq.3) the calculation is considered to be statistically acceptable, more variability than this values reflects instability of the sample in that range of time.

The analysis of the amide I and II regions for Aβ₁₋₄₀ peptide incubated in DMSO+TRIS solution is shown in Figure 30(a), it represents the behavior of Aβ₁₋₄₀ during the formation of stable oligomeric species during 48 hours. (b) Shows the changes exhibited by the amide I and II regions each 4 hour time frame (averaged spectra) with overall activity in secondary structure (amide I) and adsorption (amide II). As the deconvolution of amide I spectra reveals in (c), all secondary structures increase for every structure in absolute absorbance values. However, when these values are normalized Figure 30(d) reveals that the...
overall content of parallel structures increases while antiparallel structures decrease during the formation and stabilization of Aβ1-40 oligomers.

**Figure 30.** Analysis of the Amide I and II for Aβ1-40 Oligomers in DMSO+TRIS Solution. (a) Averaged spectra using (Eq.1) from Section 3.3.1.4. (b) Change of integrated absorbance under the amide I and II during the incubation period. (c) Secondary structure kinetics of Aβ1-40 in DMSO+TRIS during 48 hour of incubation. (d) Change in secondary structure content for Aβ1-40 in DMSO+TRIS during 48 hour of incubation
Figure 31. Morphology of Aβ$_{1-40}$ Oligomers over 48 Hours of Incubation. (a) 1x1-μm x-y scans of Aβ$_{1-40}$ from 0.5h to 48h of incubation. (b) Average diameter of the oligomers encountered in solution at different incubation times.

Figure 31(a) shows the morphology of Aβ$_{1-40}$ oligomers through time, these images have a scan size of 1x1-μm x-y. Results in Figure 31(b) show the average diameter of the oligomeric species shown in Figure 31(a), and it can be observed that these species maintained their structure and shape over 48 hours incubation in DMSO+Tris (2:98 volume to volume) at 25°C.

4.2.1.2.2. Oligomers of Aβ$_{1-42}$ Peptide

As in the previous study (4.2.1.2.1), this section investigates the stability of Aβ$_{1-42}$ oligomeric species using two timeframes: during the first hour (Figure 26) of incubation (in DMSO and TRIS solution at 25°C, Figure 32) and during 48 hour to monitor stability.
Figure 32. Change of Secondary Structures of Aβ₁₋₄₂ Dissolved in DMSO after Diluting with TRIS Buffer Solution to 100 μM. (a) Spectra of the Amide I and II regions from 0 to 60 minutes, Amide II shows protein adsorption, on Amide I region shifting can be observed showing instabilities; (b) Comparison between Amide I and Amide II integrated absorbance from 0 to 60 minutes; (c) Reorganization of the secondary structure that trigger a process of aggregation into oligomers is shown; (d) Change of secondary structure content of Aβ₁₋₄₂ in the first 60 minutes of incubation

Figure 32 shows progression of spectra collected during the first hour of Aβ₁₋₄₂ oligomeric species production in DMSO+TRIS buffer at 25°C. It is important to indicate that the predominant species are antiparallel β-sheet and α-
helix as it can be seen in 32(d); this behavior was also observed in the case studied in section 4.2.1.1 for Aβ_{1-42} monomers in HFIP (Figure 23(b)).

For the case of 48 hours study, the spectra of the Aβ_{1-42} oligomers (Figure 33(a)) showed some instability as evidenced by the Figure 33(b) and Figure 34(b), not reaching and stable spectra trajectory and reflected on the variability from time to time. However, this was not an impediment for the oligomeric structure to form and be stable (Figure 35). The explanation for this movement among the spectra might be due to the fact that these oligomers are in a reactive state, therefore the structures and even the adsorption varied from spectrum to spectrum.

Even as the stability of Aβ_{1-42} oligomers lags behind that of Aβ_{1-40}, it is evident that at all times α-helix and antiparallel β sheet are the dominant structures contained and stabilizing the oligomeric species (Figures 34(c) and (d)) of Aβ_{1-42} peptide. Figures 34(c) and (d) are the result of deconvoluting the average spectra shown in Figure 34(a).

**Figure 33.** Amide I and II of Aβ_{1-42} Oligomers during Oligomerization. (a) Spectra of the amide I and II regions for Aβ_{1-42} in DMSO+PBS during 48 hours of incubation at 25°C. (b) Spectra selected in 4 hours ranges (red spectra)
Figure 34. Kinetics of Aβ_{1-42} Oligomers in DMSO+TRIS Solution. (a) Average spectra obtained from the 4 hours range shown in Figure 33(b). (d) Variances calculated from 33(b) for the average spectra. (c) Secondary evolution of Aβ_{1-42} in DMSO+TRIS during 48 hour of incubation. (d) Change of secondary structure content for Aβ_{1-42} in DMSO+TRIS during 48 hour of incubation.
Figure 35. Morphology of Aβ1-42 Oligomers. (a) 1x1-µm x-y scans of Aβ1-42 from 0.5h- 48h of incubation. (b) Average diameter of the oligomers encountered in solution at different incubation times, even when at 12 hour and 24 fibrils are shown, they are rarely found, this fibrils have diameters in the range of the oligomeric species (35±10nm)

The progression of AFM scans of Aβ1-42 oligomeric species in Figure 35 (a), and the analysis of particle sizes in (b) show changes in size of oligomers that stabilizes after 24 hours. Even though fibrils were observed in AFM experiments, these were scarce and did not play an important role in the morphology of aggregates, which were dominated by Aβ1-42 oligomeric species.
4.2.1.2.3. Oligomers of Aβ_{40/42} Peptide

The study in this section mirrors the two preceding studies but tests a combination of both peptide Aβ_{1-40} and Aβ_{1-42} at a molar ratio of 1:1. Similarly to Aβ_{1-40} and Aβ_{1-42} oligomeric study, ATR-FTIR spectroscopy and AFM analysis were performed for 1 hour and 48 hour time scopes.

Figure 36. Change of Secondary Structures of Aβ_{40/42} Incubated in DMSO and TRIS at 25°C. (a) Spectra of the Amide I and II regions from 0 to 60 minutes, Amide II shows protein adsorption, on Amide I region shifting can be observed noting instabilities; (b) Reorganization of the secondary structure that trigger the process of oligomers formation; (c) Change in secondary structure present in the Aβ_{40/42} peptides during the first 60 minutes of incubation in DMSO+TRIS solution
Figure 36(a) shows the spectra (amide I and II regions) corresponding to the initial 60 minutes of incubation, (b) shows the decreased and stabilization of the evolution for antiparallel structures and the rest, respectively. Figure 36(c) shows that antiparallel β-sheet structures are dominant in this first hour of incubation.

Figure 37. Kinetics of Aβ_{40/42} Oligomers in DMSO+TRIS Solution. (a) Spectra of the Amide I and II regions for Aβ_{40/42} peptide spectra during 48 hours of incubation at 25°C; (b) Sets of spectra selected each 4 hours (red spectra), during 0 to 48 hour of incubation

Figure 37(a) shows the spectra collected from 0 to 48 hours of incubation, representing only the amide I and II regions of the spectrum, (b) explains schematically the process of time sets selection. The stability spectral analysis for Aβ_{40/42} oligomers shown in Figure 38 (b) and corresponding to the average spectra in (a), shows that after 12 hours of incubation the oligomers reach stability. This can also be visually observed in figure 37(b).
Figure 38. Kinetics and Change of Secondary Structure Content of Aβ_{40/42} Oligomers Prepared in DMSO+TRIS Solution. (a) Spectra obtained from averaging the 4 hours range as shown in Figure 37(b); (b) Percentage of variance calculated for each average spectra in (a) from the data shown in Figure 37(b). (c) Secondary structure evolution of Aβ_{40/42} in DMSO+TRIS during 48 hour of incubation; (d) Change in secondary structure content for Aβ_{40/42} in DMSO+TRIS during 48 hour of incubation.

During the first 60 minutes of the stability study, the oligomeric equimolar mixture of Aβ_{40/42} seems to reach relative stable conformations (Figure 36(c)), after this short initial time, the peptide mixture shows an increase in antiparallel β-sheet dominating over the second structure increasing, the parallel β-sheets.
AFM analysis was performed to this peptide combination, finding no evidence of adsorption to the mica at any time, suggesting that the oligomers in the ATR crystal are in a dynamic equilibrium of adsorption and desorption, explaining the adsorption shown in Figure 39, and at the same time the absence in the mica surface (not shown).

**Figure 39.** Kinetics of Aβ Oligomeric Species Adsorption. The values shown in this plot are the integrated areas under each amide II region corresponding to each different time, and as explained in section 3.3.1.2 it corresponds to the peptide adsorption on the ATR crystal.

A comparison of the first 48 hours of oligomeric adsorption values from the Amide II region in Figure 39 reveals that Aβ_{1-40} has the most adsorption tendency to the FTIR crystal while Aβ_{1-42} has a comparable adsorption to the peptides mixture; still Aβ_{40-42} has a value in between the individual peptides.
4.2.1.3. Fibrillization of Aβ Peptides at 25°C

The main focus of this work is to understand and obtain the necessary knowledge to interpret the mechanisms of peptide fibrillization, to then be able to target a dissolution process (this will be discuss in Chapters 5 and 6). In the amyloid hypothesis, it is stated that when an imbalance in the amyloid peptide production and clearance equilibrium is altered, this drives to Aβ plaque formation [88, 89], initiating a cascade of effects that ultimately lead to neuronal death, hence understanding the process of peptide aggregation at the molecular lever is important to treat the disease.

This study parallels the previous sections for monomers and oligomers of Aβ40, Aβ42 and the equimolar mixture of Aβ40/42. In this set of experiments, the peptide film dissolved in DMSO was diluted to 100μM with PBS and incubated at 25°C, protocol that yields to fibril formation [72].

4.2.1.3.1 Aβ1-40 Fibrils

Aβ1-40 fibrils were studied for both time scopes of 60 minutes and up to 96 hours, the samples were analyzed with ATR-FTIR spectroscopy and AFM.

The first 60 minutes of Aβ1-40 fibril formation of process have very little stability as it is evidenced by the changes in spectra shown in Figure 40(a), the integrated absorbance of the Amide I and Amide II in (b), and absolute and relative structure content values from (c), and (d).
Figure 40. Change of Secondary Structures of Aβ1-40 Prepared in DMSO+PBS Solution and Incubated at 25°C. (a) Spectra of the Amide I and II regions from 0 to 60 minutes, Amide II shows protein adsorption, on Amide I region shifting can be observed showing the molecular rearranging of the molecules; (b) Comparison between Amide I and Amide II integrated absorbance from 0 to 60 minutes; (c) Reorganization of the secondary structure that trigger the process of peptide aggregation; (d) Change of secondary structure content of Aβ1-40 in the first 60 minutes of incubation.
Figure 41. Fibrillization of Aβ₁₋₄₀ in DMSO+PBS. The fibril are formed at 25°C and monitored during 96 hours. (a) Amide I (1700-1600 cm⁻¹) and amide II (1600-1500 cm⁻¹) spectra. (b) Amide I region, spectra selection shown up to 48 hours; this study is performed to evaluate the stability and structural change of the peptides. (c) Average spectra calculated from the selected times in (b).

The stability analysis presented in Figure 41 allows observing the limited stability of the fibrils formed by Aβ₁₋₄₀ in PBS until more than 80 hours as average spectra show in (c); time frame not shown in (b).
Figure 42. Fibrillization of Aβ_{1-40} and Secondary Structures Change. (a) Morphological change monitored with AFM. (b) Most relevant secondary structures kinetics. (c) Most relevant secondary structure content

Figure 42(a) shows the progression of fibril formation for up to 240 hours for the AFM scans, and 96 hours for FTIR amide I secondary structure content. The analysis shows that fibrils are stable at more than 80 hours and the predominant structures are antiparallel β-sheet and α-helix for formed fibrils.

4.2.1.3.2. Aβ_{1-42} Fibrils

Aβ_{42} fibrils were studied under FTIR-ATR and AFM analysis for both time scopes of 60 minutes and 240 hours, the analyzed data is presented in this section.
Figure 43. Change of Secondary Structures of Aβ₁₋₄₂ Prepared in DMSO+PBS Buffer Solution to 100 μM and Incubated at 25°C. (a) Spectra of the Amide I and II regions from 0 to 60 minutes, Amide II shows protein adsorption, on Amide I region shifting can be observed showing instabilities; (b) Comparison between Amide I and Amide II integrated absorbance from 0 to 60 minutes; (c) Reorganization of the secondary structure that trigger a process of aggregation into oligomers is shown; (d) Change of secondary structure content of Aβ₁₋₄₂ in the first 60 minutes of incubation

Aβ₁₋₄₂ process of fibril formation reaches relative stability at 30 minutes as it is evidenced by the changes in spectra in Figure 43(a), this is further confirmed
by the changes in absorbance of the Amide I and Amide II in (b), and absolute and relative structure content values from (c), and (d).

**Figure 44.** Fibrillization of Aβ<sub>1-42</sub> at 25°C. (a) 0 to 72 hours spectra during the Aβ<sub>1-42</sub> peptide aggregation. (b) Aggregation evolution by time ranges. (c) Averages spectra calculated from (b)

The stability analysis presented in Figure 44 shows the relatively high stability of the fibrils formed by Aβ<sub>1-42</sub> in PBS which increases in the 72 hours of the study, as stability visualization in (b), and as average spectra show in (c).
Figure 45. Secondary Structure Change during Fibrillization of Aβ_{1-42} at 25°C. (a) Rate of aggregation per structure. (b) Percentage change of secondary structure content. (c) AFM morphological characterization of the Aβ_{1-42} fibril formation.

Figure 45 shows secondary structure analysis of FTIR-ATR data for up to 96 hours shows that the remaining dominant secondary structure over the long term evolution of fibrils is parallel β-sheets structure.

The AFM scans on Figure 45(c) show that the morphology of the fibrils change over the 240h scan which is in agreement with the secondary structure analysis in Figure 45(b) in which only parallel β-sheet structure remains dominant among other secondary structures.
4.2.1.3.3. Aß$_{40/42}$ Fibrils

The fibrillation process of Aß$_{1-40}$ and Aß$_{1-42}$ at a ratio of 1:1 and at 25°C was investigated in this section. As for the previous cases, the study was done for the first hour and then for up to 96 hours.

**Figure 46.** Change of Secondary Structures of Aß$_{40/42}$ Dissolved in DMSO after Diluting with PBS Buffer Solution to 100 μM. (a) Spectra of the Amide I and II regions from 0 to 60 minutes, Amide II shows protein adsorption, on Amide I region shifting can be observed showing instabilities; (b) Comparison between Amide I and Amide II integrated absorbance from 0 to 60 minutes; (c) Reorganization of the secondary structure that trigger a process of aggregation into oligomers is shown; (d) Change of secondary structure content of Aß$_{40/42}$ in the first 60 minutes of incubation.
Figure 46 shows the first hour evolution of the fibrillization process the Aβ$_{40/42}$ equimolar mixture, which initially has as dominant secondary structure, antiparallel β-sheets.

Figure 47. Fibrillization of Aβ$_{40/42}$ at 25°C. (a) 0 to 72 hours spectra during the Aβ$_{1-42}$ peptide aggregation. (b) Aggregation evolution by time ranges

The stability analysis of the Aβ$_{40/42}$ depicted in Figure 47, shows little stability of the fibrils during the 72 hour timeframe.
Figure 48. Fibrillization of Aβ_{40/42} at 25°C. (a) Spectra average by time range, (b) AFM morphological characterization of the Aβ_{40/42} fibril formation (c) Most relevant structures changing during fibrillation, (b) Percentage change of most relevant secondary structures.

The morphology of the Aβ_{40/42} fibrils depicted in Figure 48, shows parallel β-sheets as the dominant structure for most of the process on the long term analysis.
Figure 49. Comparing the Morphological Evolution of Aβ Peptides during Fibril Formation at 25°C. (a) AFM morphology characterization. (b) TEM images of Aβ peptides at 10 days of incubation.

The AFM scans and TEM images in Figure 49 provide an opportunity to compare the morphology of fiber formed by either Aβ_{1-40} and Aβ_{1-42} peptide, as well as their 1:1 combination. AFM scans at 1x1 μm reveal that fibrils formed by the mix of peptides Aβ_{40/42} are generally thicker and formed of globular aggregates; this is the case for initial fibril structures formed in Aβ_{1-40}.
Figure 50. Morphology of Aβ Peptides Films Prepared in DMSO + TRIS and DMSO + PBS Solution. The Films shown in (a) were obtained 30 minutes after the samples were prepared. (b) Topographical analysis of oligomers in DMSO + TRIS solution; (c) Topographical analysis of oligomers in DMSO + PBS solution.
The observations of morphology about fibrils formed from the peptide equimolar mixture of Aβ_{40/42} is mirrored by the morphology of oligomeric aggregates shown in Figure 50(a). Quantification of such aggregates from AFM scans show that in the case of the TRIS solution, the size of oligomeric aggregates from Aβ_{1-40} are smaller with smaller average height, Aβ_{1-42} are larger in size, and the size of oligomers from the mix Aβ_{40/42} appear in between those of the peptides. This is not the case for oligomeric aggregates in PBS solution shown in 50(b); which are considerably larger for the mix Aβ_{40/42} than those for each peptide on their own. This morphological behavior is similar to that shown for fibrils in Figure 49.

4.2.1.4. Effects of Temperature during the Fibrillization of Aβ Peptides

This section introduces two new studies complementary to the Aβ peptides fibrillization assessment. Now, varying the incubation temperature to 37°C. these studies are only performed for Aβ_{1-42} and Aβ_{40/42} peptides.

4.2.1.4.1. Aβ_{1-42} Fibrils

Figure 51 shows the evolution of both amides (I and II) during the process of fibril formation at 37°C. Amide II shows adsorption of protein. Amide I region shows molecular rearrangement better appreciate it in Figure 51(b). As previously explained, Figure 51(c) represents the average spectra for the 4 hours ranges selected from Figure 51(b), and the analysis of secondary structure is (performed to the amide I region) is shown in Figure 52(a) and (b).
Figure 51. Aβ₁-₄₂ Peptide Fibrillization at 37°C. (a) Spectra from 0 to 96 hours of incubation. (b) Time selection by ranges. (c) Average spectra calculated per time range selected in (b)

Figure 52(b) shows the parallel structures as the dominant conformation along with the unordered structures. To assess the effect of temperature on the evolution of parallel and antiparallel structures, these structures are compared in Figures 52(c) and (d); it can be observed in these two figures that at 25°C the fibrillization process is more stable than at 37°C, however they both follow same path of aggregation. In addition the fibrils encountered at 48 hours of incubation have similar morphology in both cases (Figure 52(e).)
Figure 52. Aβ1-42 Secondary Structure Change Effects of Temperature during Fibrillization. (a) Kinetics of the secondary structures at 37°C. (b) Content of the secondary structures change. (c) Temperature effect on the change of parallel β-sheet structures. (d) Temperature effect on the change of antiparallel β-sheet structures. (e) Morphology of the Aβ40/42 fibrils at 48 hours of incubation.
4.2.4.1.2. Aß40/42 Fibrils

Figure 53(a) shows the evolution of amide I and II for Aß40/42 when the incubation temperature is 37°C. In Figure 53(b) the changes per 4 hour ranges of the two amides can be explored, and the averages of the time ranges are shown in Figure 53(c), the changes in morphology during the aggregation process is shown in Figure 53(d).

Figure 53. Fibrillization of Aß40/42 at 37°C. (a) AFM morphological characterization of the Aß40/42 fibril formation. (b) Secondary structure as function of preferable energetic state during aggregation.
**Figure 5.4.** Temperature Effect during the Fibrillization Process of Aβ_{40/42}. (a) Rate of aggregation per structure AFM morphological characterization of the Aβ_{40/42} fibril formation. (b) Percentage change of secondary structure content. (c) Temperature effect on the change of parallel β-sheet structures. (d) Temperature effect on the change of antiparallel β-sheet structures.

From Figure 5.3(c), the evolution of secondary structures in the amide I region was calculated and represented in Figures 5.4(a) and (b). These graphs correspond to the change in integrated absorbance and its normalized values respectively.

Figures 5.4 (c) and (d) compares the evolution of parallel and antiparallel β-sheet structures at 25°C and 37°C; showing that at 37°C the fibrillization takes
place rapidly (first 12 hours of incubation) when it reaches equilibrium. In comparison, the fibrillization process at 25°C, the stability of this process is reached at 40 hours of incubation. In addition Figure 55 shows the morphology of fibrils, which have similar appearance at 48 hours of incubation.

![48 hours of incubation](image)

**Figure 55.** Comparison between Aβ_{40/42} Fibrils Prepared at 25°C and 37°C. Images taken at 48 hours of incubation with AFM at a scan size of 10x10μm x-y

### 4.3. Chapter Remarks

One important finding of this study is that preferentially, fibril formation is characterized by the increase of parallel β-sheet structures, while if peptide species are in solution the preferential secondary structure is antiparallel β-sheet configuration.

The experiments and analysis in this chapter allowed the combinatorial study of two different Aβ peptides isoforms Aβ_{1-40}, Aβ_{1-42}, and their equimolar mixture, Aβ_{40/42}. The peptides were studied under 5 different set of conditions designed to control in different ways the aggregation of amyloid proteins. The conditions allowed for the formation of monomers in solution, monomers on a film, oligomers, fibrils formed at 25°C, and fibrils formed at 37°C. The stability of
the aggregation process and the dominant secondary structures were assessed
to provide key knowledge in the understanding of the effect of monoclonal
antibodies (mAb) on Aβ peptides and their aggregation in later chapters.

The results from this chapter have been summarized in a Table 4, which
allows comparing the different cases from the entire study.

Table 5. Dominant Structures and Stability Table

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<th>Aβ₁₋₄₀</th>
<th>Aβ₁₋₄₂</th>
<th>Aβ₄₀/₄₂</th>
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<td>(stable)</td>
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<td>α-Helix</td>
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**Table 5.** (Continued)
A simple assessment of Aβ1-40 and Aβ1-42 aggregation in oligomeric and fibril formation conditions shows how the likelihood of stability of the process flips. Oligomers of Aβ1-40 reach stability much faster than oligomers of Aβ1-42 while the opposite is true for the fibrillar species where Aβ1-42 reaches stability considerably faster than fibrils formed by Aβ1-40 peptide.
CHAPTER 5
MONOCLONAL ANTIBODIES MODIFY FIBRILLOGENESIS

The main goal of this study was to elucidate and analyze the deviation from a typical fibrillization process caused by targeting Aβ peptide monomers and oligomers in solution (before fibrillization occurs) with different anti-Aβ monoclonal antibodies (mAb). In addition to the two isoforms, the equimolar mixture of the Aβ1-40 and Aβ1-42 (Aβ40/42) was studied. The rationale of this relates to the idea of having antibodies with different specificities that would better target the peptide mixture. All the experiments were performed at 37°C and at a substoichiometric molar ratio of 1 to 1000 (antibody to peptide).

5.1. Summary

In this chapter, the effects of antibodies were studied at a condition close physiological conditions in the brain. For this, temperature was kept constant at 37°C, and solutions were kept at pH 7.4. A 1:1000 antibody to peptide molar ratio was chosen to approximate therapeutic conditions. The antibodies were applied to monomers and oligomers in solution (with exception of the first study using 6E10 and 2H6 on preformed fibrils) at the initial stage of the Aβ40/42 fibrillization process.
It is worth mentioning, that the oligomeric species of Aβ peptide is recognized by different groups as the toxic specie from the aggregation process, due to its reactivity [15, 17-20, 87, 90-92], suggesting this species as a possible effective target for the antibody application.

Table 6 describes the specificity for the monoclonal antibodies tested in this study. Briefly, 6E10 can bind to the N-terminus of either Aβ_{1-40} or Aβ_{1-42} peptide, 2H6 binds to the C-terminus of Aβ_{1-40}; 4G8 binds to the mid domain (17-24) of either Aβ_{1-40} or Aβ_{1-42}; and finally mlgG2b with not specificity to neither Aβ_{1-40} or Aβ_{1-42} peptides, that is used as a control antibody.

<table>
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<th>Antibody</th>
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<th>Binding</th>
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<td>Aβ 1-x</td>
</tr>
<tr>
<td>2H6*</td>
<td>c-term 35-40</td>
<td>40 only</td>
</tr>
<tr>
<td>4G8 (Signet Dedham, MA)</td>
<td>mid-domain 17-24</td>
<td>Aβ 17-x</td>
</tr>
<tr>
<td>mlgG2b*³</td>
<td>Control antibody</td>
<td>does not bind Aβ species</td>
</tr>
</tbody>
</table>

* Antibodies provided by Rinat-Pfizer

All the antibodies tested were provided either by David Morgan’s group (USF Health Byrd Alzheimer’s Institute) or Rinat-Pfizer (San Francisco, CA) directly.

³ Antibody raised against a pseudomonas aeruginosa antigen.
5.2. Protocols and Methods

ATR-FTIR, AFM, and TEM analytical techniques were used in the same capacity as described in previous chapters. Moreover, some of the experiments with antibodies were also characterized using Western Blot.

5.2.1. Antibody-Peptide Solution

An equimolar mixture of Aβ₁₋₄₀ and Aβ₁₋₄₂ (Aβ₄₀/₄₂) was first prepared as explained in section 4.1.4. In brief, Aβ₁₋₄₀ and Aβ₁₋₄₂ peptides were prepared separately. Each peptide’s film was redissolved with DMSO and diluted to 100 μM with 0.01 M PBS. These two solutions were then combined in a 1 to 1 molar ratio stock solution.

Amyloid peptides were incubated with each antibody from the beginning of the fibril formation process. However, 6E10 and 2H6 antibodies were also tested against 5 days preformed fibrils.

5.2.1.1. Effects of Low Antibody Concentration on Aβ₄₀/₄₂ Fibrils

Amyloid-β peptide fibrils were prepared following the protocol described in 5.2.1. The Aβ₄₀/₄₂ solution at 100 μM was aliquoted in three portions; one aliquot was injected in the ATR-FTIR flow cell, where the aggregation was monitored over time; the second portion was subdivided in different vials, each corresponding to different incubation times, later analyzed by AFM and TEM. The third portion was incubated for later addition of antibody (at day 5) and analyzed by AFM and TEM at different time over 5 days after antibody addition. Hence, the
processes of fibril formation and antibody addition were verified with ATR-FTIR spectroscopy, AFM and TEM. The antibody was added in a molar ratio of 1 to 1000, antibody to peptide, all the samples were incubated at 37°C.

The control solution for this study was the equimolar mixture of Aβ_{40/42} peptides incubated with no addition of antibody for a period of 10 days at 37°C.

5.2.1.2. Targeting Aβ_{40/42} Monomers and Oligomers with Low Concentration of Different Monoclonal Antibodies

As in section 5.2.1.1, each solution was prepared using a 1:1000 substoichiometric molar ratio, antibody to amyloid peptide respectively. As described previously, the peptide’s film was first redissolved with DMSO (0.2 mM) and diluted to 100 μM in 0.01 M PBS (pH 7.4), Aβ_{1-40} and Aβ_{1-42} peptides were prepared separately and then the solutions were combined in a 1 to 1 molar ratio. Immediately after combining the two peptide solutions the antibody was added to the peptide’s mixture in a molar ratio 1:1000 of antibody to peptide.

The solution antibody-peptide was divided in two portions each incubated at 37°C. The first portion of this solution was injected in the ATR-FTIR flow cell accessory where it was monitored over 10 days of incubation (by spectroscopy). The other portion of the antibody-peptide solution was subdivided for AFM, TEM, and Western Blot analysis. Samples were collected over a period of 10 days.

The control for this study was a solution of Aβ_{40/42} peptide prepared with an antibody (mlgG2b) with not specificity to either peptide.
In addition to the control above, and to prove that our results correspond to the antibody-peptide interaction rather than the single antibody and peptide contribution, a predicted model of secondary structures was calculated, this by mathematical adding the secondary structures calculated for Aβ40/42 peptide incubated without antibody and the secondary structures of antibodies 2H6 and 6E10 when incubated alone over the same period of time. This is Aβ40/42 + 2H6 and Aβ40/42 + 6E10. The Aβ40/42, 2H6, and 6E10 controls were solutions prepared and incubated as if they were combined in a 1:1000 antibody to peptide system.

The antibodies used for these experiments (see Table 6) were received from the provider dissolved in PBS solution at variable concentrations (1-2 mg/ml), hence calculations of the appropriate volume of antibody were performed to assure the same molar ratio for all experiments. In order to avoid antibody contamination and unnecessary freeze-unfreeze cycles, all the antibody stock solutions were aliquoted (in 5 portions) and kept at -20°C once received from the provider. Prior to use, 1 of the 5 vials containing the antibody was retrieved from the freezer and allowed to equilibrate to 25°C.

5.2.2. AFM and TEM Characterization

AFM and TEM assisted to determine the state of aggregation of the peptides in contact with antibodies. In the initial state of aggregation, the peptide tends to spread fairly uniformly across the substrate where it is deposited.

However, as the incubation time increases, and peptides aggregate into fibrils, it becomes challenging for the nanoscale-size tip of the AFM to depict
where fibrils are located on the substrate. At this point, it is better to combine TEM with AFM to determine the aggregation state and to examine the sample’s surface distribution. Therefore, some results illustrating the morphology and/or topology of the samples will be shown with both techniques (TEM adapted to the AFM scale).

5.2.3. ATR-FTIR Analysis

The background solution used for these studies was the solvent in which the peptides/antibody systems were prepared, that was 2% DMSO to 98% 0.01M PBS (v/v). Therefore, all the collected spectra represent the changes of secondary structures of the antibody-peptide mixture.

5.2.4. SDS-PAGE and Western Blot Analysis

SDS-PAGE and immunoblotting was performed as described by Dahlgren et al., 2002 [19]. Briefly, individual aliquots of the stock solution collected at the testing times were centrifuged at 14,000g for 10 min at 4°C in order to remove large aggregates such as potential fibrils in the supernatant. Iced cold Millipore water was used to dilute the monomer- and oligomer-containing Aβ_{40/42} (or mAb-Aβ mixture) supernatant at 1:5 ratio and mixed with a buffer. Note that there is no heating nor use of additional reducing agents. These sample were then combined with a 12 % Bis-Tris NuPage gel using MES as the running buffer (Invitrogen, US). See Blue pre-stained molecular mass markers were run on each gel (Invitrogen, US). Peptides separated at 160 V for 1 h and subsequently
transferred to 0.22 mm PVDF membranes at 35 V for 1 hr. The membranes were
blocked for 1 hour at room temperature (RT) using 5 % non-fat dry milk
(International, Diagnostics Group, UK) in 20 mmol/L Tris-buffer, pH 7.6, with 137
mmol/L NaCl and 0.05 % (w/v) Tween 20. Subsequently, the membranes were
incubated with a primary antibody in blocking buffer overnight at 4°C. The
primary antibody 6E10 (a mouse monoclonal antibody directed against the
human Aβ amino acid residues 1-17) was used at a dilution of 1:1000. The
membrane was then rinsed in Tris-buffer, pH 7.6, 137 mmol/L NaCl and 0.05 %
(w/v) Tween 20 three times and incubated with secondary antibody solution of
HRP-labeled rabbit antibodies against mouse IgG (diluted 1:2000) for 1 h at RT.
Rinsing with 20 mmol/L Tris-buffer, pH 7.6, with 137 mmol/L NaCl and 0.05 %
(w/v) Tween 20 was applied again.

Immunostained peptides were visualized using chemiluminescence, in
accordance with the manufacturer’s recommendations (ECL, Amersham,
Denmark). Western blots were scanned using a Fujifilm Lass 300 Intelligent dark
box scanner, and band density of each blot was quantified within linear range of
detection using AlphaEase software.

This immunnochemical analysis was used to monitor the presence of
monomers and oligomers during the aggregation process.
5.3. **Results, Analysis and Discussions**

The outline for subsequent sections is as follows: 5.3.1. the effects of 6E10 and 2H6 antibodies on Aβ_{40/42} pre-formed fibrils (at 5 days of incubation) are compared; 5.3.2. the effects 6E10 and 2H6 antibodies added at the beginning of the aggregation process (when only monomers and oligomers are present) are compared; and 5.3.3. the effects of 2H6, 6E10, and 4G8 antibodies are compared against mIgG2b, a non-specific antibody.

5.3.1. **Effects of Antibodies on Species of Aβ_{40/42} Targeting the Peptides C-Terminus or N-Terminus**

In this section, it is first shown the effects of 6E10 and 2H6 antibodies on fibrils pre-incubated for 5 days, testing a molar ratio of 1:1000 (antibody to peptide). Then, these same antibodies were tested against an initial aggregation stage solution, were mainly oligomers and monomer were present.

The rationale for comparing the effect of antibodies against fibrils or monomers+oligomers is to determine which species are more likely to be influenced by the addition of antibodies. It is desired that if an antibody is going to be used in passive immunization, it should be able to break preformed fibrils or to stop or prevent their aggregation in the first place. The effect of the antibodies in this study has been measured depending on how effective they are to induce antiparallel beta sheet structures or to decrease parallel beta sheets structures (those structures were associated with monomeric and fibril species, respectively, in Chapter 4).
5.3.1.1. Effects of 6E10 and 2H6 on A\textsubscript{ß}\textsubscript{40/42} Pre-Formed Fibrils

As explained in previous sections, an equimolar mixture of A\textsubscript{ß}\textsubscript{40/42} peptides was used to form fibrils, and a substoichiometric molar ratio of 1:1000, antibody (either 6E10 or 2H6) to peptide respectively was used. Figures 56 (a)-(c) show the spectral evolution for amide I and II bands with respect to incubation time for the control system (A\textsubscript{ß}\textsubscript{40/42} peptides mixture), the A\textsubscript{ß}\textsubscript{40/42} + 6E10 antibody and the A\textsubscript{ß}\textsubscript{40/42} + 2H6 antibody, respectively. These average spectra were obtained as described in the Chapter 3 (Section 3.3.1.3). Briefly, continuous spectra corresponding to a selected time range were averaged into a single spectrum.

As described above, at day 5, the antibody was added to a preformed fibril solution, indicated with an arrow (→ mAb) in the spectrum for the control (A\textsubscript{ß}\textsubscript{40/42}) peptide solution (Figure 56(a)). It is worth mentioning that the background spectrum for Figures 56(b) and (c) was obtained from the control system at day five of incubation (e.g., prior to adding the antibodies). Moreover, the Amide II band progression in Figure 55 shows a significant increase for the case when 6E10 antibody was added (Figure 56(b)). This means that 6E10 antibody promoted surface adsorption of the peptides to the surface. This same band did not increase, and if anything, it shows a slight decrease when 2H6 antibody was added (Figure 56(c)). This implies that the preformed fibrils did not necessarily detach from the surface completely. Rather, it indicates that the fibrils attached to the surface were attacked by 2H6 antibody.
Figure 56. Influence of Low Concentration Antibodies Targeting Aβ_{40/42} Fibrils. Average spectra obtained at 37°C, amide I and II regions are shown for (a) the control mixture of Aβ_{40/42} peptides, (b) 6E10 antibody added to Aβ_{40/42} peptide mixture (1:1000). This antibody targets the N-terminus of both peptides, (c) 2H6 antibody added to Aβ_{40/42} peptide mixture (1:1000), antibody with affinity to the C-terminus of Aβ_{1-40} peptide only. The arrow shown in the spectrum for the control system at day 5 represents the point where antibodies were added to the peptide solution.

Figure 57 shows the most dominant secondary structures after the amide I region was deconvoluted for the control and the two cases of the antibody to peptide systems. Only parallel and antiparallel beta sheet structures are shown because these two structures provide the most relevant information regarding
any dissolution effect on the preformed peptides fibrils. Figure 57(a) shows that in the control system, parallel beta sheet structures increase continuously until they reach a plateau, which is when saturation has been reached after 48 h of incubation. Conversely, the progression for the peptide mixture +2H6 shows a peak with a maximum value at approximately 4 days of incubation in the parallel $\beta$-sheet structures. Then, there is an abrupt decay of such structures, which implies that the antibody was able to render conformational changes tied to dissolution effects (Figure 57(a)). In the case of peptide mixture +6E10, the effect is better depicted in Figure 57(b), where the parallel beta sheet structures decreased to less than 5% of total structure content after day 3.

Figure 57(d) shows the appearance of the fibrils found in the three samples after 10 days of incubation. It is observed that fibrils are still found in the case of 2H6 addition, but the morphology differs from the control system. That is, the fibril density is lower and fibril bundles are thinner than fibrils in the control picture (Top scan compare to bottom scan). In the case of 6E10, fibrils appear to be spread on the surface, but more abundant in comparison to the control system (+6E10 TEM Scan in Figure 57(d)).
Figure 57. Secondary Structure and Morphological Changes of Aβ_{40/42} Fibrils after Combined with 6E10 or 2H6 Antibodies. Analysis of the secondary structures performed from the spectra shown in Figure 56. The values of the control (labeled as Aβ_{40/42}) peptides represent the change after the 5 days of incubation. The peptide mixture and the peptide mixture + antibody were further incubated for 10 days. (a) Comparison of the kinetics of parallel β-sheet structures between the control and the peptide mixtures affected by 2H6 and 6E10 antibodies in a molar ratio 1:1000. (b) Comparison of the parallel β-sheets content for all three systems described above. (c) Comparison of the antiparallel β-sheet structures content with respect to incubation time for the systems described above. (d) TEM scans of the control after a total of 15 days of incubation at 37°C (Top), peptide mixture + 6E10 (Middle), and peptide mixture + 2H6 (Bottom). Both systems with the antibodies added were incubated for 10 days at 37°C.
5.3.1.2. Effects of 6E10 and 2H6 on Aβ_{40/42} Monomers and Oligomers

The study on preformed fibrils on the previous section allows the statement that the effect of antibodies is limited on preformed fibrils. Continuing with this study, the effects taking place when antibodies are added at the initial stage of aggregation are presented.

Figure 58 show the average spectra comparing the amide I and II regions of the control (Aβ_{40/42}), and Aβ_{40/42} peptide treated with either 6E10 or 2H6 antibodies. The main differences are in the amide I region for the peptide mixture+2H6 antibody, where most of the secondary structures undergo sudden transformations at day three of incubation (Figure 59a and (b)). This implies that the fibril formation is dramatically disrupted. On the other hand, the amide II region shows significant growth. This increase in the peptide adsorption is expected since peptide structures undergoing conformational changes may not be able to go back into solution because they have been corrupted (Figure 58c). This finding is verified by analyzing the deconvolution of the spectra and the anatomic morphology of these spectra shown in Figures 59(a), (c) and (d), where the parallel beta sheet structures decrease, antiparallel beta sheet structures increase, and surface globular aggregates appear instead of fibrils. Note that even though antiparallel beta sheets content increased, peptide aggregation on the surface was relatively high. As for the case of having peptide mixture+6E10 antibodies, the Amide I and II regions follow the control system behavior (Figure 58(b) and Figure 59(a)-(c)). Therefore, this antibody was unable to prevent the
fibrillization process under these conditions and did not prevent peptide adsorption to the surface since Figure 59d shows that fibrils were still formed.

Figure 58. Monitoring Aβ_{40/42} Fibrillation Process from the Beginning of the Incubation Process with N-Terminus and C-Terminus Antibodies. Spectra were collected at 37°C. Amides I and II changes for (a) Aβ_{40/42} control system (b) 6E10 added to the beginning of the incubation process of Aβ_{40/42} peptides (1:1000 molar ratio). This antibody targets the N-term of both Aβ_{40} and Aβ_{42} peptides. (c) 2H6 antibody incubated with Aβ_{40/42} peptides. 2H6 is affined to the C-term of the Aβ_{1-40} peptide
Figure 59. Effects of 2H6 and 6E10 Antibodies on Aβ_{40/42} Monomers + Oligomers. (a) Compares the parallel β-sheet structures evolution for the control system and for when antibodies were added to the peptide mixture, (b) Compares the parallel β-sheets content, (c) Compares the antiparallel β-sheet content, (d) AFM images representing the morphology of the fibrils immediately after antibody addition and after 10 days of incubation for the control system (Top), and for the aggregates formed with 6E10 (mid) and +2H6 (Bottom) addition.

In order to investigate the changes in the initial species of the fibrillization process, that is, monomeric and oligomeric species, Western Blot analysis was also done to these samples. Results are shown in Figure 60. Figure 60(a) shows the results from Western Blot analysis performed on samples at the same conditions as in Figure 58. That is, Aβ_{40/42} (control), Aβ_{40/42} + 6E10, and Aβ_{40/42} + 2H6. Figure 59(b) shows the AlphaEase analysis of the soluble species.
(monomers, low molecular weight (LMW) oligomers, and high molecular weight (HMW) oligomers). Note that monomers and oligomers disappear from the solution after day 2 for all three cases. This indicates that during the incubation process, fibrillization is forced and monomeric as well as oligomeric species undergo configurational changes into much higher molecular weight structures, which are transparent to/undetected by this technique (fibril aggregates).

However, the HMW oligomeric species abruptly increases after day 3 for the case of Aβ40/42 peptide mixture treated with 2H6 antibody. This means that the antibody was able to reverse the production of fibril aggregates and induce the production of oligomers. Conversely, the control system and the samples treated with 6E10 antibody show that monomers and oligomers disappear and this antibody is not able to reverse this action. These results obtained with Western Blot are in line with the data from ATR-FTIR spectroscopy and AFM/TEM topological studies as depicted in Figures 58 and 59.
Figure 60. Effect of 2H6 and 6E10 on Soluble Species of Aβ_{40/42} Peptide Equimolar Mixture during Fibrillogenesis. (a) Western Blot of the control system (Aβ_{40/42}), Aβ_{40/42} + 6E10 antibody, and Aβ_{40/42} + 2H6 antibody. (b) AlphaEase analysis of soluble species for Monomers (Right), low molecular weight (LMW) oligomers (Middle), and high molecular weight (HMW) oligomers (Left)

5.3.2. Proving Suitability of Results

Before continuing with this study, it is necessary to prove that the results obtained correspond to the antibody-peptide interaction rather than to a pure contribution of antibody of peptide separately.

For this, different control solutions were ran over 10 days and analyzed. The control solutions were, Aβ_{40/42} with no antibody addition, antibody 6E10, and antibody 2H6. In order to prove the results presented here, the secondary structures corresponding to peptide + 2H6, peptide + 6E10, and peptide +
average of 6E10 and 2H6 were mathematically added. The results show (Figure 61) that when comparing to the experimental data, the secondary structure kinetics is completely different from the predicted values. Thus depicting completely different behaviors.

**Figure 61.** Comparison of Predicted Secondary Structures vs. Experimental Structures. (a) Predicted behavior for the average values of antibodies control 6E10 and 2H6 when added to Aβ40/42 control. (b) Predicted behavior for the average values of antibodies control 6E10 when added to Aβ40/42 Control. (c) Predicted behavior for the average values of antibodies control 2H6 when added to Aβ40/42 control. (d) Experimental values for 6E10: Aβ40/42 at 1:1000. Control. (e) Experimental values for 2H6: Aβ40/42 at 1:1000.
This study proves that when any antibody (that binds the peptide) is added to the peptide mixture, the resulting spectra correspond to the interaction antibody-peptide in solution, and not to the independent spectra. Hence, our results show a fair representation of the effect of antibodies on amyloid beta peptides during fibril formation or disruption.

5.3.3. Effects of Antibodies on Aβ_{40/42} Monomers and Oligomers

This study compares the effects of antibodies on an initial system mainly composed by monomers and oligomers in solution. The study compares three antibodies that bind either or both peptide against a non specific antibody.

The results in Figure 62 show the existence and increase of a peak centered in the parallel β-sheet frequency region (1630cm\(^{-1}\)) for the cases of the control, peptide treated with 6E10 and peptide treated with 4G8, when in comparison to the peptide treated with 2H6 this peak seems to decrease and with the shifting of peaks in other regions. Once Figures 62(a)-(d) are deconvoluted and integrated, the comparison can have greater detail as shown in Figure 63(a)-(c), proving that from all the antibodies only when the peptide is treated with antibody 2H6, there is a noticeable decrease in parallel β-sheets and an increase in antiparallel β-sheets structures after day 3 of incubation.

In addition, Figure 63(d) compares the morphological appearance of the four systems under study, showing the formation of fibrils in the system of peptide treated with IgG2b, 6E10, and 4G8, when in the case of peptide treated with 2H6 amorphous aggregates are found rather than fibrils.
Figure 62. Monitoring Aβ_{40/42} Fibrillization Process from the Beginning of the Incubation Process with N-Terminus and C-Terminus Antibodies. Spectra were collected at 37°C. Amides I and II changes for (a) Aβ_{40/42} control system (b) 6E10 added to the beginning of the incubation process of Aβ_{40/42} peptides (1:1000 molar ratio). This antibody targets the N-term of both Aβ_{40} and Aβ_{42} peptides. (c) 2H6 antibody incubated with Aβ_{40/42} peptides. 2H6 is specific to the C-term of the Aβ_{1-40} peptide. (d) 4G8 antibody incubated with Aβ_{40/42} peptides. 4G8 has specificity to the mid domain of both Aβ_{1-40} and Aβ_{1-42} peptide.
Figure 63. Effects of 2H6, 6E10, and 4G8 Antibodies on Aβ40/42 Monomers+ Oligomers. (a) Compares the parallel β-Sheet structures evolution for the control system and for when antibodies were added to the peptide mixture, (b) Compares the parallel β-sheets content, (c) Compares the antiparallel β-sheet content, (d) AFM images representing the morphology of the fibrils immediately after antibody addition and after 10 days of incubation for the control system (Top), and for the aggregates formed with 6E10, 4G8, and 2H6 (Bottom) addition.

This study proves that it is possible to discern and evaluate the effect of antibodies by analyzing the content and evolution of secondary structures of the system, and that this results can be corroborated by the physical characterization of the sample using AFM and TEM.
5.4. Chapter Remarks

The experiments in this chapter tested various monoclonal antibodies with different specificity against Aβ peptides. Aβ\textsubscript{1-40} and Aβ\textsubscript{1-42} peptides were ratio in a molar mixture of 1 to 1, and for every case, the antibody to peptide molar ratio was 1 to 1000.

2H6 antibody targeting the C-terminus of Aβ\textsubscript{1-40} decreased the content of parallel β-sheets and increased the content of antiparallel β-sheets, these results are summarized in Table 6. The rise in the content of antiparallel β-sheets might indicate the presence of unstable Aβ\textsubscript{40/42} oligomeric species (Chapter 4, Table 5).

The effectiveness of the evaluation methods was tested as a platform to evaluate the effects of antibodies used against amyloid peptides.

In general, the use of antibodies to target Amyloid Beta during the fibrillization process seems to be more effective that targeting fully formed fibrils.
Table 7. Summary of Observations for the Antibodies Tested in this Chapter

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Observable Effect</th>
</tr>
</thead>
</table>
| 6E10     | Aβ<sub>1-40</sub> and Aβ<sub>1-42</sub> n-terminus (1-16) | • No effect to preformed fibrils  
          |             | • No effect on secondary structure content  
          |             | • No effect on fibrillization process |
| 2H6      | Aβ<sub>1/40</sub> c-terminus (35-40) | • Decrease Parallel β-sheet content (both on preformed and in fibrillization)  
          |             | • Increase antiparallel β-sheet content (only in fibrillization)  
          |             | • Limited Effect on preformed fibers  
          |             | • Stopped fibrillization only allowing oligomers |
| 4G8      | Aβ<sub>1/40</sub> and Aβ<sub>1/42</sub> Mid-domain (17-24) | • No effect on secondary structure content  
          |             | • Stopped fibrillization only allowing oligomers |
| mlG2b    | Non-specific | • No effect on secondary structure content  
          |             | • No effect on fibrillization process |
CHAPTER 6

MIMICKING THE NEURON MEMBRANE SURFACE

6.1. Lipid Bilayer Membranes and their Relevance to Aβ Plaque Formation

Lipid bilayer membranes are an important element of living cells, supported by a network of structural proteins called the cytoskeleton. An important characteristic of lipid bilayer membranes is their natural fluidity, which allows free movement of lipid molecules on their own monolayer. This fluidity shows differences throughout the cell membrane, and varies depending on the composition of the principal molecules forming the lipid bilayer: phospholipids, cholesterol, and glycolipids. The biophysical characteristics of the lipid membrane also allow certain proteins to embed and anchor or penetrate through them.

The Alzheimer's disease Aβ-hallmark supposes the formation of extracellular Aβ plaques; these plaques are formed on the cell membrane by binding to membrane molecules and by partial insertion into the cell membrane. It is evident that altering the homeostasis of membrane components such as cholesterol, leads to accelerated plaque formation that might be related to the anchoring or the release of Aβ [93]. Besides the biological implications, the
biophysical role of higher cell membrane contents of cholesterol and other intramembrane components is yet not well understood. However, recent studies suggest that cholesterol and membrane rigidity might hinder Aβ penetration resulting in amyloid accumulation on cell membranes [94].

This chapter presents two methods for building *in vitro* lipid bilayers with the potential to be used as a platform for studying biophysical phenomena related to Aβ plaque formation on lipid bilayer membranes. Two techniques for creating lipid bilayers were explored: Langmuir-Blodgett deposition technique and self-assembled bilayer membranes from lipid vesicles. In both cases, the lipid bilayer membranes were deposited on a thin film of polyethylene glycol (PEG), which acts as a cushion layer to the lipid bilayers and serves to mimic membrane fluidity [95].

The composition of model *in vitro* membranes can be made to mimic different conditions important to the understanding of Aβ plaque formation by reproducing in a monolithic way local characteristics of membrane domains. One of the challenges encountered in reproducing cell membrane characteristics *in vitro* lipid bilayer is reproducing membrane fluidity; this can be overcome by using a cushion layer below the *in vitro* lipid membrane.

6.2. Soft-Support Layer Using Polyethylene Glycol

Polyethylene glycol (PEG) is an amphiphilic oligomer used to build the soft-supported layer. PEG is a linear or branched neutral polyether that is soluble in water and most organic solvents. The following is the chemical formula for
PEG: HO-(CH2CH2O)n-CH2CHOH. PEG is utilized in this study because its physicochemical properties allow its use as a cushion substrate or coating polymer, and it has been a part of previous studies by our group [96]. When in aqueous solution, PEG rejects other polymers and proteins, and can form two-phase systems with other polymers. This property is based on its molecular conformation in aqueous solution, whereby PEG exposes uncharged hydrophilic groups and shows very high surface mobility (steric exclusion).

PEG can also be used to coat surfaces with very little chemistry modification to these surfaces. When surfaces are coated with PEG, they become hydrophilic and protein rejecting. This protein-rejecting characteristic of PEG makes it of value as a cushion layer under a lipid bilayer, as it would reduce the probability of proteins adhering to the substrate underneath the lipid bilayer membrane. Additionally, when chemically attached to a substrate, PEG maintains its biological and biocompatible properties. For all these reasons, PEG is an attractive substrate for using it as a support or cushion layer to increase in vitro fluidity of lipid bilayer membranes.

6.2.1. Construction of the Soft-Support Layer Using PEG

Following a protocol developed by Alcantar et al. in 2000 [97], a polished glass surface (rectangular hemacytometer cover glass, 12-519-10, Fisher Sci) was activated by submerging it in a 10 % w/v sodium hydroxide (NaOH, 1310-73-2, Acros Organics) solution and sonicating (Model FS30, Fisher Sci.) it for 5
minutes. During the surface activation of polished glass, silanol groups (Si-OH) are produced at the solid-air interface (Figure 64).

The glass was retrieved from the NaOH solution and cleaned by rinsing it multiple times with Millipore water. Ultrapure nitrogen was then applied to remove any water traces until the surface was completely dry. The activated glass surface was then submerged in the polyethylene glycol (PEG, 400 Da, P3265-500G, Sigma) for one hour at 100°C. This resulted in the PEG being grafted to the surface of the activated polished glass (Figure 64.b). This process occurs through a reaction between the end alcohol groups of the PEG molecules with the Si-OH molecules on the activated glass (Figures 64.a and 64.b).

(a)                                                     (b)

**Figure 64.** PEG Cushion Layer Construction. (a) During the surface activation of polished glass, silanol groups (Si-OH) are produced at the solid-air interface. (b) Schematic of the PEG surface grafting reaction, where silanol groups on the surface of the activated polished glass lose the hydrogen and forms water with the end hydroxyl group of the PEG molecules. This result in the PEG being grafted to the surface of the activated polished glass.
The PEG grafted glass was retrieved from the PEG solution while still hot and immediately rinsed avoiding direct contact with the water flow. Finally, the surface was dried by gently exposing it to a flow of ultrapure nitrogen.

6.3. Lipid Bilayer Construction Techniques

6.3.1. Langmuir-Blodgett Deposition Technique

This technique is used to deposit multiple organized and compact layers, one monolayer at a time. Any organic or inorganic molecule of amphiphilic nature can be deposited [98, 99]. Typically, a bed of water is used as the initial surface for deposition (subphase) in which the molecules to be deposited are gently placed. For this, a suitable solvent with lower density than the subphase (and with high vapor pressure) is used to dissolve the molecules. This solvent is easily evaporable leaving a monolayer of the molecule of interest on the water’s surface.

Once a monolayer of the molecule is formed on the subphase, the deposition takes place by slowly pulling out a previously submerged substrate, where the monolayers will be deposited in an upward followed by a downward direction. These movements are perpendicular to the subphase surface (Figure 65.a).
This technique is extremely simple and allows deposition of a single layer, bilayers, or multiple layers one at a time. Depending on the orientation of the amphiphilic molecule layers with respect to the substrate and to each other, the deposition can take one of three forms: X-type, Y-type, or Z-type (Figure 65.b).

![Diagram](image)

**Figure 65.** Langmuir-Blodgett Deposition Technique. (a) Schematic of the deposition methodology. (b) Types of deposition based on the order of layers.

The Langmuir-Blodgett (LB) trough (Figure 66) requires an environment completely free of contamination to avoid particles clinging to the surface. These particles can contaminate and perturb the deposition quality. The trough is located inside a laminar flow cabinet to avoid potential contamination. However, the airflow is turned off during lipid deposition in order to protect the trough from the environment. When necessary, Millipore water and sometimes organic
solvents (for instance: chloroform) are used to clean the trough. A common indicator of cleanliness is the absence of air bubble formation. The presence of bubbles with the addition of water indicates potential contamination. Hence, bubbles are eliminated by suction.

![Image of Langmuir-Blodgett Trough](image)

**Figure 66.** Langmuir-Blodgett Trough. The principal components of the trough are highlighted in this photo.

The LB trough is connected to a computer system, and is run by software that allows the control of parameters such as surface area, superficial tension, and substrate deepness necessary for the actual deposition. The area, deposition pressure, and lipid concentration used for the depositions are chosen based on calibration experiments that are run beforehand and are a function of the lipid’s nature.
6.3.1.1. Layer-by-Layer Lipid Membrane Construction

Lipid bilayers composed of 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine (DSPE, 850715X, Avanti Polar Lipids Inc.) were deposited onto the grafted PEG layer using the Langmuir-Blodgett deposition technique (LB trough, Model 611D, Nima Technology) as explained earlier in this chapter. The polished glass surfaces containing the soft-supported PEG layer were kept inside of a laminar flow cabinet (Class 100) to minimize contamination.

The initial uncompressed surface area was fixed at 300 cm². The lipids were compressed using a LB barrier speed of 25 cm²/min. The parameters used for this experiment, such as area, deposition pressure, and lipid concentration, were developed in a previous study [100]. A volume of 100 µL of DSPE was dissolved in chloroform (HPLC grade, 67-66-3, Acros Chemicals) at a concentration of 15 µg/µL. This solution was gently poured on the air/water interface using a gas tight Hamilton syringe (allowing 5 minutes for the solvent to evaporate). During the process of deposition, the surface pressure was kept constant at 30 mN/m. The process of deposition of the first lipid monolayer took place by dipping the activated polished glass with the PEG layer at a velocity of 2 cm/min; the second lipid layer is deposited when the dipping motion is reversed, removing the glass from the trough and placed in a fluid reservoir for transportation (Figure 67).
**Figure 67.** Schematic of Lipid Bilayer Construction. (a) Schematic of first lipid monolayer deposited on the cushioned layer (PEG). (b) Representation of the soft-supported lipid bilayer. Hydrophilic and hydrophobic domains are shown. A double layer of lipid is deposited on top of the PEG layer using Langmuir-Blodgett deposition technique.

### 6.3.1.2. Soft-Supported Lipid Bilayer Characterization: Surface Topography

Atomic Force Microscopy (AFM, MFP-3D, Asylum Research, Santa Barbara, CA) was used to physically characterize the surface topography/topology of the soft-supported lipid bilayer membranes. Tapping mode was used to scan the surfaces, which were at all times submerged in deionized water to preserve the integrity of the lipid bilayer. The cantilevers used had typical spring constant < 1 N/m. Scan sizes were 10x10 μm. (Figure 68).
Figure 68. Characterization of Soft-Supported Lipid Bilayer Membrane. (a) RMSR change at different steps of construction. (b) AFM images (0.5x0.5-μm x-y scan size) at the different steps of the lipid bilayer assembly.

The lipid bilayer membranes were kept in contact with saturated lipid solution at all times to avoid air exposition, which could consequently cause damage to the samples.

While LB is a simple and very reliable technique that allows deposition of homogenous monolayers, there are some limitations to this technique. First, the substrate geometry has to be open and flat, second, the size of the substrate cannot be greater than 4 cm x 4 cm, and third, the transfer from the LB-trough (where the deposition takes place) to a container is difficult because any exposure of the bilayer to the air would destroy it. This makes it difficult to store and unsuitable for certain characterization techniques (like closed ATR-FTIR flow cell). Therefore, other methods to construct lipid bilayers must be considered, as is the case of vesicle based self-assembled lipid bilayers.
6.3.2. Vesicle Based Self-Assembled Lipid Bilayers

Vesicle self-assembly technique provides an attractive alternative to overcome the limitations of the LB lipid deposition technique, such as size and geometry of the starting substrate, and difficult handling.

The process consists of the spontaneous fusion of small unilamellar vesicles [101] onto a surface substrate. Vesicles are made of lipid aggregations when they are forced to associate in cores as micelles for instance (unilamellar) at controlled media conditions.

A general methodology is to dissolve the lipids in a solvent, i.e. chloroform, and then, the lipid in suspension is placed in a rounded flask. The solvent is left to evaporate overnight and vacuum for 4 hours before further preparation. Once the film is completely dry, water is used to hydrate the film by heating and gently agitating it; once the transition temperature of the lipid is overcome the lipid will completely be dissolved. The last step in the preparation of vesicles is to slowly cool down the solution allowing the lipids to organize, resulting in vesicles formation. The deposition by self assembly technique, is accomplished by the interaction between the prepared vesicles and the treated surface substrate. This interaction burst the vesicles and drives a self-assembled lipid bilayer formation.

6.3.2.1. Self-Assembled Lipid Bilayers from Vesicles

As a second stage of this study, the formation of lipid bilayer membranes by self-assembly technique was performed.
Lipid bilayers were prepared with the same lipid used in the Section 6.3.1.1.: 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine (DSPE). Following the principles explained in the previous section, for this study the vesicles were prepared by using 2 mg of lipids dissolved in CHCl3. The solvent was evaporated from the lipid solution using an ultrapure stream of nitrogen. The lipids were then hydrated for 20 minutes (in a concentration of 2 mg/ml in Millipore water) at a temperature above their highest transition temperature (100°C).

In the next step, the solution was sonicated 5 times for periods of 2 minutes (to form small unilamellar vesicles evenly distributed in size) [102]. Finally, the vesicle solution was used to submerge the PEG grafted glass slides. The temperature was kept in the 90°C range, which was gradually lowered to allow vesicles to diffuse onto the PEG cushion layer.

6.3.2.2. Self-Assembled Soft-Supported Lipid Bilayer Characterization

In the preparation of self-assembled membranes using vesicles, the first step into characterization is the analysis of the vesicles size. Using the dynamic light scattering (DLS) technique and instrument (Zetasizer Nano-S, Malvern, PA).

The second characterization step is the study of the surface topography of the bilayer, and as explained for membranes deposited with LB, this surface characterization is to be done with atomic force microscopy. However, because the technique to provide the lipid bilayers using vesicles is still under investigation, only the characterization of the vesicles size was performed.
The results showed high instability in the formation of self-assembled lipid bilayers. The sizes of the vesicles obtained, were highly variably in dimensions (as shown in Table 8), and they were between 485 nm and 2348 nm.

**Table 8.** Dynamic Light Scattering. Results giving the sizes of the multilamellar vesicles formed

<table>
<thead>
<tr>
<th>Peak</th>
<th>Particle’s Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2347.5 ± 401.9</td>
</tr>
<tr>
<td>2</td>
<td>484.8 ± 189.3</td>
</tr>
</tbody>
</table>

The presence of large vesicles is an inconvenience in the formation of the lipid bilayer with this method (the optimum size should be between 40 nm to 100 nm). The lipid used in this study (DSPE), has a long hydrophobic tail, which determines first, the creation of rather stables and large vesicles. Second, causes a high transition temperature (100°C). This high transition temperature is similar to the one required for PEG surface grafting, which could be altering this last process. These reasons might explain the adverse results obtained for this particular lipid.

In order to overcome the inconvenience in this technique, further testing should be accomplished using short hydrophobic tail lipids with low molecular weights, such as the following: 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC), Sphingomyelin (SM. Ceramide-1-Phosphocholine. Brain, Porcine), and 1,2-Distearoyl-sn-Glycero-3-Phosphocholine (DSPC); all with MW between 730-
Additionally, cholesterol (Ovine wool, MW 387) can also be used in combination with these lipids to attain the same effect.

6.4. Chapter Remarks

In this study, a fluid cell membrane in vitro model was constructed to present a brain-like environment approach [103] with the purpose of providing a platform for future Aβ aggregation studies.

In the first stage of this proposed project, lipid (DSPE) bilayers were formed on polyethylene glycol cushion support (PEG) using the Langmuir-Blodgett (LB) deposition technique [104, 105]. The PEG layer was successfully used as a stabilizer for the bilayers. It was observed that the PEG cushion layer offered high mobility to the lipid bilayer membrane (Figure 68). Also, the PEG layer was used to support self-assembled lipid bilayers formed from small unilamellar vesicles, an alternative technique to prepared lipid bilayers.

Two important characteristics make this model a potential good platform for in vitro Aβ aggregation studies: First, the high fluidity provided by the cushion PEG layer underneath the lipid bilayer [105] gives the system fluidity that better represents that of cell membranes in mammalian cells. Second, protein exclusion characteristic of PEG also prevents proteins such as Aβ from breaching the lipid bilayer and adsorbing directly on the substrate; this property makes this platform also ideal for studies involving antibody-peptide interactions by confining these to the surface of the lipid bilayer.
CHAPTER 7
CONCLUSIONS

Alzheimer disease (AD) represents the main leading cause of dementia typically among individuals over the age of 65. As life expectancy increases (76 year in 2006), also does the number of AD cases increases. As of today the number of AD cases worldwide is approximately 26 million of individual affected by the disease; and this number is estimated to increase to 106 million of cases by the year 2050.

AD is known to affect the cognitive function, alter the social behavior and body motor skills, among other affections related to the nervous system. Nowadays, the causes of Alzheimer disease and onset mechanisms are yet unclear. There are still pieces missing in the puzzle to fully understand the disease and discern effective therapies to avoid and eradicate AD.

From the pathology of the disease, are known three hallmarks commonly found in patients with AD, the extracellular formation of amyloid beta (Aβ) plaques, the presence of intracellular neurofibrillary tangles, and finally the neuronal death. The hypothesis that gathers these hallmarks (amyloid hypothesis) to explain the possible mechanism states that imbalance on the catabolic process of Aβ peptide leads to its accumulation forming neuronal
plaques that trigger the formation of intracellular neurofibrillillary tangles and finally terminate the neurons [51, 88, 89].

This work was studies and characterizes the mechanisms involved in the process of Aβ plaque formation and its possible clearance by using an immunotherapeutic approach.

Two isoforms of the amyloid beta peptide were investigated separately and combined in an equimolar mixture. These were, Aβ1-40, and Aβ1-42 peptides. These two Aβ isoforms are the main component of the neuritic Aβ deposits (Aβ plaques) found in the brain of patients with AD.

In order to understand the process of aggregation, initial attention was rendered to the study and characterization of the different species involved in the aggregation process. When many species might be found during the process of aggregation, for the effects of this study the species were classified as monomeric, oligomeric, and fibrillar.

It is relevant to mention that amyloid beta owes its name to the characteristic of its native secondary structure composition, which is mainly the β-structure type. β-structures or more commonly known as β-sheet structures are structures composed by lateral connecting β-strand structures, through 5 or more hydrogen bonds. β-strands are extended parts of the peptide formed by 5 to 10 amino acids.

The β-sheet structures can be either parallel or antiparallel, depending on the orientation of the strands forming the β-sheet structures.
Understanding these molecular configurations was important to interpret the results obtained with ATR-FTIR spectroscopy; since, this technique allows monitoring the secondary structures change by detecting the interactions occurring through hydrogen bonding within and between the peptide molecules.

There are different points of view and disagreements on which is the most energetically stable β-strands configuration (parallel or antiparallel). However, the results in this work showed that the stability of these structural configurations highly depends on the peptide isoform under study. For instance, it was observed that when Aβ₁₋₄₀ fibril were forming the dominant structural configuration (in percentage) present along the process was the antiparallel type, in addition to α-helix structures while parallel structures were reduced considerably. In contrast, in the case of Aβ₁₋₄₂ peptide fibrillization, the structural configuration of the parallel type seems to dominate the aggregation.

When oligomeric species are formed the configuration of the strand structures also varied in accordance with the peptide, as for Aβ₁₋₄₀ oligomeric species, the dominating arrangement was the parallel type, flipping to antiparallel when the peptides are set to form fibrils, and as for the Aβ₁₋₄₂ oligomeric species the antiparallel arrangement seems to dominate (and α-helix) flipping to parallel configuration or rearrangement type.

Results in this study also suggested that the combination of the two peptide isoforms shows competition between the secondary structures making the fibrils morphologically different (thicker) from the peptides alone.
This work included the addition of monoclonal antibodies (mAb) with specificity to Aβ with the purpose to modify or reverse the fibrillization process. All experiments were carried out at close to physiological conditions (37°C and pH 7.4). The antibodies used were chosen for their particular specificity to Aβ peptides and some for their potential use in therapeutic settings. These antibodies were 6E10 (binds the N-terminus of both peptide isoforms), 2H6 (preferentially binds to the C-terminus of Aβ1-40), 4G8 (binds the mid domain either isoform), and a control antibody mIgG2b that has no specificity for either Aβ peptide.

Preformed fibrils targeted with antibodies 6E10 and 2H6 suffer secondary structural change but not in a degree that allow fibril dissolution. The initial state of aggregation resulted a convenient phase for the addition of antibodies. This is evidenced by experiments in which the target point for antibody addition where monomeric and oligomeric species. This resulted in antibody binding and altering the fibrillization process (Chapter 6). This phenomenon was observed when in two cases under equal incubation conditions but varying the species targeted by the antibody. Preformed fibrils in solution were not easily dissolved, but targeting monomer + oligomers in solution (at the initial fibrillization phase) resulted in an evident effect of the antibody causing the disruption of the fibrillization process.

This study points to the structural configuration of the β-strands forming the β-sheet structures as the key to understand and explain at the molecular
level the effects caused by antibodies to the different species of the peptides isoforms studied in this work.

The combination of the analytical techniques (ATR-FTIR, AFM, TEM) allowed the testing and evaluation of the peptide characteristics during aggregation and allowed to explain the effects of antibodies observed when targeting the different species of the Aβ peptides.

Finally, it was possible to prepare a soft substrate mimicking the cell membrane's surface (Chapter 7), laying the groundwork for future experiments. A polymeric film (PEG) grafted to SiO2 served as a cushion layer for the deposition of a double layer of lipids. Langmuir-Blodgett resulted as the preferential deposition technique due to the control on monolayer uniformity before and during the lipids deposition. In the future, this membrane could be optimized by inserting other cell membrane molecules; such as cholesterol, other lipids, sugar groups, or proteins. This interface could potentially serve to test the surface role on the process of plaque formation as function of rigidity, for instance varying the amount of cholesterol inserted in the bilayer membrane.
CHAPTER 8
FUTURE DIRECTIONS

The studies presented in this work leave a door open for new possible paths to investigate, as described below.

• Measurement of surface energy of Aβ peptide species, in particular oligomers and protofibrils, which are the intermediate species in the process of fibrillization, this can be performed with surface force apparatus (SFA).

• The effects observed in the ATR-FTIR spectroscopic analysis can be used to investigate synthetic analogs to antibodies that act on Aβ peptides. ATR-FTIR information can be utilized to detect secondary structure fingerprints in molecule screening processes.

• Other amylogenic processes such as the hyperphosphorylation of Tau protein (other hallmark of AD) can be studied using the methods presented in this dissertation.

• Complementary techniques could be used to study binding and rate of aggregation and dissolution to complement the ATR-FTIR such as quartz crystal microbalance (QCM).
• It is suitable to introduce polarized light to the ATR-FTIR spectrometer to produce more complete information in regards to the structural orientation of the molecules.

• Lipid bilayer membrane construction by self-assembled vesicles can be improved by introducing a mixture of lipids with different tail lengths, which might allow a controlled system at lower temperatures more suitable for the preparation of these membranes.
LIST OF REFERENCES


[99] K.B. Blodgett, Monomolecular Films of Fatty Acids on Glass Journal of the American Chemical Society 56 (1934) 495.


APPENDICES
Appendix A. Isoelectric Points of Aβ Peptides

Figure 69. Titration Curve of Aβ₁₋₄₀ Indicating its Isoelectric Point. Obtained from http://biophysics.cs.vt.edu/H++/ (Virginia Tech) using the Protein Data Bank for the peptide sequence.

Figure 70. Titration Curve of Aβ₁₋₄₂ Indicating its Isoelectric Point. Obtained from http://biophysics.cs.vt.edu/H++/ (Virginia Tech) using the Protein Data Bank for the peptide sequence.
### Appendix B. ATR Crystals

#### Table 9. Limitations and General Specifications of ATR Crystals

<table>
<thead>
<tr>
<th>Material</th>
<th>$n_1$</th>
<th>LWL</th>
<th>dp</th>
<th>$S_{\text{water}}$</th>
<th>pH Range</th>
<th>Hardness [Kg/mm²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMTIR</td>
<td>2.5</td>
<td>625</td>
<td>1.46</td>
<td>Insoluble</td>
<td>1-9</td>
<td>170</td>
</tr>
<tr>
<td>Diamond/ZnSe</td>
<td>2.4</td>
<td>525</td>
<td>1.66</td>
<td>Insoluble</td>
<td>1-14</td>
<td>5,700</td>
</tr>
<tr>
<td>Diamond/KRS-5</td>
<td>5</td>
<td>250</td>
<td>1.66</td>
<td>Insoluble</td>
<td>1-14</td>
<td>5,700</td>
</tr>
<tr>
<td>Germanium</td>
<td>4.0</td>
<td>780</td>
<td>0.65</td>
<td>Insoluble</td>
<td>1-14</td>
<td>550</td>
</tr>
<tr>
<td>KRS-5</td>
<td>2.4</td>
<td>250</td>
<td>1.73</td>
<td>0.05</td>
<td>5-8</td>
<td>40</td>
</tr>
<tr>
<td>Silicon</td>
<td>3.4</td>
<td>1500</td>
<td>0.84</td>
<td>Insoluble</td>
<td>1-12</td>
<td>1150</td>
</tr>
<tr>
<td>Silicon/ZnSe</td>
<td>3.4</td>
<td>525</td>
<td>0.84</td>
<td>Insoluble</td>
<td>1-12</td>
<td>1150</td>
</tr>
<tr>
<td>ZnS</td>
<td>2.2</td>
<td>850</td>
<td>2.35</td>
<td>Insoluble</td>
<td>5-9</td>
<td>240</td>
</tr>
<tr>
<td>ZnSe</td>
<td>2.4</td>
<td>525</td>
<td>1.66</td>
<td>Insoluble</td>
<td>5-9</td>
<td>120</td>
</tr>
</tbody>
</table>

$n_1 =$ refractive index of ATR crystal, LWL = long wave length cut-off, dp = depth of penetration at 1000 cm⁻¹, $S_{\text{water}} =$ Solubility in Water
ABOUT THE AUTHOR

Jeffy Pilar Jiménez received her Bachelor’s degree in Chemical Engineering from Los Andes University in Mérida-Venezuela in 2001 and her Master’s degree in Chemical Engineering from the University of South Florida (USF) in 2005. In 2005 she started her Ph.D. in Chemical Engineering program at USF, in 2006 she had a summer internship experience at the R&D department for Dow Chemical. In every step of her career she has been moved by the passion for research. Jeffy has been exchanging and leaving scientific, mentoring, and cultural experiences in the group of Norma Alcantar (Ph.D.) since 2003, also guided by David Morgan (Ph.D.). Jeffy’s research focused on elucidating mechanisms responsible for Aß aggregation and dissolution by the mean of monoclonal antibodies, studies that have been conducted in-vitro at the Nanosurface-Chemistry and Green Materials Chemistry Laboratory. She successfully defended her doctoral dissertation in April 2010 at the University of South Florida.