Characterization of Beta-amyloid Peptide Aggregation and Acceleration with Non-fibrillar Forming Peptide-based Mediators

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CHARACTERIZATION OF BETA-AMYLOID PEPTIDE AGGREGATION AND ACCELERATION WITH NON-FIBRILLAR FORMING PEPTIDE-BASED MEDIATORS

A Dissertation

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

in

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by

Nadia J. Edwin
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For Nadlee,
thanks for making this part of my journey unforgettable …
ACKNOWLEDGEMENTS

Acknowledgements can sound cliché, devoid of the heart of true thanks. This is not the case here. As an island girl, once, I had felt as did Simon and Garfunkel in their recording of “I am a rock’, with [only] my books and poetry to protect me” until I came to depend upon a few special persons who helped prove to me that indeed, no girl is an island.

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LIST OF SYMBOLS

\[ c \] concentration

\[ C(t) \] contrast

\[ D \] diffusion coefficient

\[ g^{(l)}(t) \] normalized electric field correlation function

\[ h_r \] hour

\[ I \] Intensity

\[ k \] Boltzmann constant

\[ K \] spatial frequency

\[ L \] period of repeat pattern

\[ \text{min} \] minute

\[ q \] scattering vector magnitude

\[ R_h \] hydrodynamic radius

\[ T \] absolute temperature

\[ T_m \] melting temperature

\[ \Gamma \] decay rate

\[ \Theta \] scattering angle

\[ \eta \] solvent viscosity
LIST OF ABBREVIATIONS

Aβ  β-amyloid peptide
CMC  critical micelle concentration
DLS  dynamic light scattering
FPR  fluorescence photobleaching recovery
KOH  potassium hydroxide
MES  2-[(n-morpholino) ethane-sulphonic acid
NaCl  sodium chloride
mg  milligram
mM  millimolar
µM  micromolar
NBD-PC  1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine
PBS  phosphate buffered saline
POPC  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
TEM  transmission electron microscopy
TRIS  tris(hydroxymethyl)aminomethane
ABSTRACT

Protein aggregation occurs under many circumstances, from the dynamic assembly of tubulin to form microtubules, the aggregation of actin into filaments, as well as plaque formation by amyloid precipitation. One important requirement in studying the mechanism of amyloid aggregation is the ability to monitor the growth kinetics over a wide range in size scales (10 nm to microns) with time that spans microseconds to seconds. Understanding the mechanisms of the aggregation may then lead to improved design of drugs to help control or suppress the aggregation process.

In this dissertation, the physical characterization of the β-amyloid peptide and its interaction with αα-amino acid peptide-based mediators was investigated from the early, rapidly evolving stage to the later, slowly diffusing peptide stage by the application of fluorescence photobleaching recovery (FPR). The diffusion rates of β-amyloid peptide and β-amyloid assemblies under the effects of variables including concentration of β-amyloid, blocker peptide, ionic strength, pH, time and temperature were accessible by this method. In some instances, dynamic light scattering (DLS), which acquire signals greater than 10 decades of lag times, without requiring a dye label was used for comparison and to account for the limitations of any given approach. Attachment of 5-carboxyfluorescein did not affect the integrity of the protein and the measured diffusion coefficients were similar to those measured by diffusion ordered spectroscopy (DOSY) and from theoretical expectations. FPR proved more sensitive than DLS for detection of low oligomer aggregates of the β-amyloid peptide coexisting with much larger fibrils. We were able to reverse the conformation of the peptide from the low oligomeric state to the
aggregated state under neutral and acidic pH conditions and confirmed that the peptide
growth increased with increasing ionic strength.

The interaction of β-amyloid peptide with membranes results in several
membrane-perturbing effects which may play a pivotal role in the pathogenic cascade
leading to Alzheimer’s disease. Model phospholipid bilayer membranes consisting of 1-
Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC) and 1-Oleoyl-2-[12-[(7-nitro-
2-1,3-benzoxadiazol-4-yl)amino] dodecanoyl]-sn-Glycero-3-Phosphocholine (18:1-12:0
NBD PC) were prepared on mica. FPR proved to be a useful technique for obtaining
information of the nature of membrane fluidity upon interaction with the β-amyloid
peptide.
CHAPTER 1
INTRODUCTION

1.1 Proteins and Peptides

Proteins are macromolecules that are essential to the structure and function of all living organisms. The primary structure of a protein consists of a linear chain of amino acids linked by amide bonds. There are twenty standard amino acids, and it is possible to create many unique sequences of very long chains of amino acids, resulting in proteins with high molecular masses. Shorter chains with typically less than fifty amino acids are called peptides. The function of proteins and peptides are dependent on their shape.

In order for proteins to perform their biological function, they must fold into the correct higher order three-dimensional structures. The folding and unfolding kinetics of proteins ranges from being fast (microseconds) and simple, with one single exponential function of time\(^1\) to being slow (years). Regardless of the wide range of time frames, proteins fold into complex shapes whereby minimum energy is used to maintain stability in its native conformation. The ability of proteins to spontaneously fold into their lowest energy native conformation depends on the interaction of their amino acid sequence with environmental conditions like (solvent, pH, ionic strength, presence of other components such as metal ions or prosthetic groups, temperature, and other).\(^2\) Pioneering studies by Christian Anfinsen demonstrated that an unfolded protein (ribonuclease) could spontaneously refold (by removal of certain chemicals or lowering the temperature) into its native conformation in a test tube, thus concluding that proper protein folding is primarily dependent upon the interactions of the amino acid sequence in a given environment.
While normal physiological conditions predisposes the manner in which a protein’s amino acid sequence folds into its native structure, there exists conditions (extremes of pH, temperature) under which a protein will misfold or become unfolded. Disruption in the normal protein folding pathway can result in potentially detrimental consequences in the cellular environment. Incorrectly folded proteins that are essential to normal cellular function may encounter difficulty in performing their biological function, thus leading to malfunctioning of living systems and ultimately disease.3

Several human diseases are associated with the improper folding of proteins that result in cellular malfunction.4 These include Alzheimer’s disease, Parkinson’, cystic fibrosis, cancer and Tay-Sachs. While the exact mechanisms by which loss of function occurs vary, researchers were able to categorize some diseases into three main groups – defective proteins due to mutagenesis, amyloidosis and mislocalization. Mutations often alter a protein sequence causing changes in the overall protein structure, which may result in partial or complete loss of protein function. In contrast, amyloidosis results from the accumulation of misfolded protein aggregations outside the cell forming insoluble amyloid fibrils which have been associated with having a toxic function. A list of diseases grouped according to mutant proteins and the associated molecular defect appears in Table 1.1.5

Alzheimer’s disease (AD) is a neurodegenerative disorder and the leading cause of dementia affecting majority of the elderly population. While neuropathologically, the brains of individuals with the disease are characterized by insoluble extracellular amyloid plaques and fibrils6, clinically, the disease progresses with gradual memory loss, changes in behavior and disorientation. As the population ages, it is estimated that the number of
Table 1.1  Some putative protein folding diseases  (adapted from Ref. 5)

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<th>Disease</th>
<th>Mutant protein/protein involved</th>
<th>Molecular phenotype</th>
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<td></td>
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<td>Cystic fibrosis</td>
<td>CFTR</td>
<td>Misfolding/altered Hsp70 and calnexin interactions</td>
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<tr>
<td>Scrapie/Creutzfeldt-Jacob/Familial insomnia</td>
<td>Prion protein</td>
<td>Aggregation</td>
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<td>Alzheimer’s disease</td>
<td>β-Amyloid</td>
<td>Aggregation</td>
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<td>Familial amyloidosis</td>
<td>Transthyretin/lysozyme</td>
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<td>Cataracts</td>
<td>Crystallins</td>
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<td><strong>Mislocalization owing to misfolding</strong></td>
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<td>Familial hypercholesterolemia</td>
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<td>α₁-Antitrypsin Deficiency</td>
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<td>Tay-Sachs disease</td>
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<td>Retinitis pigmentosa</td>
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<td>Leprechaunism</td>
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people with the disease will continue to increase. Thus, development of therapeutic targets for inhibition of the disease is of utmost importance. Despite rapid advances in the research findings in the past decade, at the moment, there is no cure for the disease. Nevertheless, researchers are continually striving to design new strategies for curbing the progression of the disease.
1.2 Aims of This Study

The main emphasis in this dissertation research is the use of fluorescence photobleaching recovery (FPR) and dynamic light scattering (DLS) in the detection of the early intermediates in protofibril formation and subsequently β-amyloid peptide self-assembly. This study will use β-amyloid1-40 (Aβ) and fluorescently (5-carboxy-fluorescein) labeled β-amyloid1-40 mixtures at a variety of concentrations and physiological conditions to determine the diffusion rates of β-amyloid peptide and β-amyloid assemblies. The effects of variables including concentration β-amyloid, ionic strength, pH, time and temperature are accessible by these methods. Following reliable studies of the β-amyloid monomer association and aggregation, the physical properties of ‘inhibitor’ molecules, including, de novo peptide-based molecules designed by Robert P. Hammer and their effectiveness at mediating amyloidoses was assessed.

Dynamic light scattering (DLS) was initially used to assess the earliest stages of β-amyloid self-assembly. Monomeric Aβ, which is a 4KD peptide, scarcely scatters above solvent level at the concentrations of interest. Thus, DLS in the early stage of Aβ self-assembly proved challenging, but with a choice of powerful frequency doubled diode and Argon ion lasers, a variety of detectors, and high-coherence single-mode fiber optic detection as an option, signals could be obtained. Even so, and despite discriminating custom software to analyze multiple runs in an efficient graphical fashion, distinguishing the expected aggregates from dust remained a major issue. Moreover, aggregates were sometimes present at low numbers in the detected volume, which leads to unwanted number fluctuations. Taken together, these problems made it difficult to establish “clean start” conditions to assess ‘inhibitor’ efficiency. It was decided that the best use of DLS
was to screen inhibitor molecules for efficiency by testing their effect on larger assemblies. To further establish “clean starting” conditions to study the effect of inhibitor molecules on Aβ assembly, the dissociation of the peptide was monitored under high pressure.

Fluorescence photobleaching recovery technique was a suitable alternative to remediate the deficiencies of DLS from the standpoint of aggregation mechanism, despite the requirement of having a fluorophore attached to the molecule of interest. FPR would improve the economy of experimenting with the expensive Aβ peptide and inhibitor molecules, in addition to addressing questions such as the reversibility of the Aβ peptide. An in situ dialysis cell was designed and used to determine diffusion coefficients by FPR. The diffusion coefficients were easily converted to size through Stoke’s law because the system is very dilute. FPR was also used to assess the fluidity of lipid membranes upon interaction with the Aβ peptide.
2.1 Cleavage of β-Amyloid Peptide from APP

It has been suggested that the accumulation of amyloid-β (Aβ) is the primary influence driving AD pathogenesis.\(^8\,^9\) In 1984, Glenner and Wong\(^10\,^11\) reported the first sequence of Aβ isolated from the principal protein component in AD brain. The Aβ peptide was found to vary in length from 39-43 amino acid residues due to proteolytic cleavage of the transmembrane glycoprotein, amyloid-β precursor protein (APP) by α, β, and γ-secretases, as shown in Figure 2.1. The α-secretase cleaves APP within the Aβ sequence producing soluble nonamyloidogenic fragments, which is a normal constituent of human biological fluids,\(^12\) whereas cleavage by the β-secretase (extracellular, produces the NH\(_2\) terminus), and γ-secretase (intracellular, produces the COOH terminus) releases the toxic Aβ fragment from APP. The Aβ1-40 and Aβ1-42 are the predominant forms and the peptide. The normal physiological function of APP remains uncertain, but it has been shown that some possible functions may include cell proliferation\(^13\), cell adhesion\(^14\), neurite outgrowth\(^15\), and neuronal migration.\(^16\)

2.2 Properties of β-Amyloid Peptide

The term amyloid was originally used by German physician and scientist, Rudolf Virchow, to describe proteinaceous aggregates associated with diseases as shown in Table 1.1 because some of their properties resembled those of (amylase, cellulose) starch.\(^4\,^17\) Virchow was able to recognize amyloid by tinctorial properties elicited when amyloid-laden tissues were treated with iodine at the autopsy table.\(^18\) Later, amyloid deposits were characterized by diagnostic staining methods, x-ray diffraction and electron
microscopy to investigate the molecular structure of the fibrils. The early investigations indicated that all amyloids, regardless of the disease involved or the source of the fibrils, share the following morphological, tinctorial and structural characteristics\(^{19}\).

Figure 2.1 Schematic of β-amyloid (1-40) peptide location within the amyloid precursor protein and its representative amino acid sequence.

(i) the molecular structure of all amyloids is such that the fibrils bind Congo Red and interact with the dye in such a way that it appears red microscopically in normal light and has a characteristic apple green birefringence\(^{20,21}\) under polarized light;

(ii) in the electron microscope amyloid deposits can be seen to be composed of uniform, straight unbranched fibers, approx. 10 nm in diameter and of indefinite length – longer than 200 nm \(^{22}\); the fibers are usually straight or slightly curved which suggests they have a particularly rigid molecular structure;
(iii) amyloid x-ray diffraction patterns show that the ordered, repeating molecular structure of the fibrils consists of polypeptide chains in an extended \( \beta \)-conformation, hydrogen-bonded together into sheets which run parallel to the axis of the fibril and which have their constituent \( \beta \)-strands arranged perpendicular to this axis, the so-called ‘cross-\( \beta \)’ conformation\(^\text{23;24}\). An x-ray diffraction pattern\(^\text{25}\) from paired helical filaments (PHF) purified from Alzheimer’s patients cerebral cortex is shown in Figure 2.2.

(iv) amyloid protein aggregates were also found to be insoluble in common solvents and detergents, and protease resistance.\(^\text{26}\)

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**Figure 2.2**  
(a) X-ray diffraction pattern from a partially oriented, dried PHF pellet from Alzheimer cerebral cortex.\(^\text{25}\) The beam was directed normal to the fiber axis, which is vertical. The meridionally accentuated arcs at 0.476- nm spacing are indicated by the arrowheads, and the equatorially accentuated arcs centered at about 1.06- nm spacing are indicated by the arrows.  
(b) Schematic diagram summarizing the analysis of the x-ray diffraction pattern from (a) indicating \( \beta \) conformation.\(^\text{23;24}\)
These early studies have now been extended by more detailed examinations of ex vivo amyloid and synthetic amyloid with a range of techniques (mentioned below), and it is now evident that while amyloids composed of different proteins belong to the same class of substance, with similar or identical core structures, there are some significant differences between them.

2.3 Literature Review

Many researchers are involved in understanding the role of β-amyloid peptide in the pathogenesis of Alzheimer’s disease and designing strategies to prevent or slow the disease progression. A wide variety of instrumental techniques, such as analytical ultracentrifugation (AUC), dynamic light scattering (DLS), static light scattering (SLS), diffusion-ordered spectroscopy (DOSY), circular dichroism spectroscopy (CD), electron microscopy (EM), atomic force microscopy (AFM) and scanning force microscopy (SFM), have been used to study the formation and aggregation properties of β-amyloid peptide. The results from all these methods gave interesting insights into the mechanism of amyloid formation and growth, but with the availability of new techniques, additional progress will be made to enrich our understanding of the protein aggregation process.

This brief review of previous studies will highlight the strengths and limitations of individual instrumental techniques used in the characterization of β-amyloid peptide and cases when it was necessary to use a combination of techniques for comparison and to augment the deficiencies of a given method.

Plaques that develop in the brains of Alzheimer’s patients are built up from fibrils composed of hundreds or more β-amyloid peptide (Aβ) molecules. Thus, a central question in the etiology of AD is the mechanism(s) by which these Aβ molecules are
converted into plaque-associated fibers.\textsuperscript{32,33} Two instrumental techniques that have been used to provide information about fiber formation\textsuperscript{34-37} and inhibition\textsuperscript{38,39} are dynamic and static light scattering. Analysis of scattered light from particles in a solution illuminated by a coherent light source provides a means for monitoring macromolecular association in solution in real time.\textsuperscript{40} The data are heavily weighted toward larger particles; thus, the method is most sensitive to the species of most interest-aggregated Aβ.

Pallitto \textit{et al.} combined static and dynamic light scattering as a means to characterize the interactions of several hybrid peptides with Aβ in solution. One advantage to using light scattering was the fact that no probe molecules or labels that could potentially interfere with Aβ aggregation was needed. Furthermore, quantitative information on changes in the average size and morphology of particles is readily extracted from the data. The DLS results of this study found that the best inhibitors as measured by protection from toxicity, appeared to alter Aβ aggregation by increasing the rate of aggregation. The inconsistency of this result was removed when SLS measurements showed that the altering aggregate morphology was due to branching of the fibrils. That is, the growth proceeded by consecutive association of Aβ onto the ends of existing fibrils.\textsuperscript{41-43}

Another instrumental technique that has been useful for providing information about aggregate morphology is electron microscopy. Several studies have utilized EM to obtain structural information about the fibril formation process.\textsuperscript{24,44-49} In a “seeded” polymerization mechanism, a small amount of preformed amyloid fibrils is added to a solution of Aβ peptide.\textsuperscript{20,50} It is believed that prior to fibril formation, oligomeric intermediates must form. Early kinetic studies of Aβ fibrillization failed to detect any
intermediate species.\textsuperscript{51} Thus, while EM is useful for visualizing changes in fibril formation, it is difficult to quantify and provides limited information about the aggregation process. Other methods that have failed to distinguish between fibrillar morphologies of Aβ are turbidimetry and sedimentation. Although small oligomeric Aβ species have been separated by analytical ultracentrifugation, (it was observed that at 450 µM, Aβ1-40 produces a slowly sedimenting species, which in addition to the rapidly sedimenting, probably fibrillar material\textsuperscript{52} detailed structural information is still lacking.

This shortcoming was corrected by the use of AFM analysis, a technique providing three-dimensional information about species adsorbed to surfaces without the need for extensive sample preparation.\textsuperscript{45,53} Because AFM provides a three-dimensional characterization of the fibril, it offers distinct advantage over traditional electron microscopy.\textsuperscript{54} In a study by Harper \textit{et al.}, AFM was used to follow amyloid fibril formation \textit{in vitro} by the Aβ variants Aβ1-40 and Aβ1-42. Small elongated Aβ oligomers, which was termed ‘protofibrils’, (possible intermediates in the assembly of amyloid fibrils) was observed.\textsuperscript{55} It is difficult to decide whether the protofibrils are assembly precursors of the fibrils or whether the protofibrils are in fast equilibrium with monomeric Aβ. This demonstrates a limitation of AFM to provide quantifiable kinetic effects. An alternate technique would be fluorescence photobleaching recovery (FPR), although the structural detail would be lacking.

Although FPR has never been used to study amyloid systems, a comparative method, fluorescence correlation spectroscopy (FCS), has been used to overcome the lack of selectivity in light scattering.\textsuperscript{56,57} FCS detects spontaneous fluctuations in the fluorescence emission of small molecular ensembles.\textsuperscript{56,58-60} Theoretical advantages of
FCS are balanced by practical experimental advantages of FPR.\textsuperscript{61} Another self-diffusion technique, pulsed field gradient NMR, has been used to study the extent of Aβ aggregation; however, the methodology was deemed of limited utility for very large aggregates due to unfavorable nuclear relaxation properties.\textsuperscript{62,63} Thus, FPR should be a valuable complement to both FCS and pulsed field gradient NMR.

One important requirement in studying the mechanism of protein aggregation is the ability to monitor continuously the kinetics of fibril growth and to identify the intermediate stages of this process. Experimental data relating to the early stages of amyloid aggregation are difficult to obtain.\textsuperscript{64} In an attempt to elucidate information about the kinetics and different sizes spanning the range expected during peptide aggregation, the experimental technique must be able to measure both rapidly and slowly diffusing peptide aggregates. Fluorescence photobleaching recovery (FPR) is a well-established technique for the measurement of diffusion coefficients. This study will investigate the formation of β-amyloid aggregation at various stages by FPR, thus marking the first application of the highly selective FPR method to amyloid research. It should prove a valuable complement to additional characterization techniques that has been used to determine the stability of the Aβ peptide aggregates.
CHAPTER 3

EFFECT OF HIGH PRESSURE ON β-AMYLOID AGGREGATES

3.1 Introduction

High pressure has been known to cause conformational changes in proteins for over a hundred years. In 1899, B. H. Hite reported the use of high pressure for milk (β-lactoglobulin) preservation.\textsuperscript{65} Fifteen years later, P.W. Bridgman discovered that raw egg (albumen, lysozyme) appeared cooked after high hydrostatic pressure treatment.\textsuperscript{66} Although few papers exist in the literature since the earliest studies, in the past decade there has been a resurgence in the use of high pressure technology ranging from understanding the fundamentals of protein disaggregation and refolding\textsuperscript{67-70}, to application in the food industry.\textsuperscript{71-73} High pressure is becoming an increasingly popular biophysical technique for studying protein folding and structural dynamics; this is important to gain insight into diseases associated with protein misfolding. The immense interest in the food industry is due to adaptation of existing high pressure technologies for utilization in both food preservation and preparation (processing and packaging of materials). High pressure treatment may potentially preserve several properties of food ingredients (color, flavor, nutritional value, texture, taste)\textsuperscript{72} with minimal effect on final product quality. For example, high pressure treatment of milk inactivates the enzymatic activity of microbes to prevent spoilage, in addition to enhancing the coagulation properties of milk.\textsuperscript{71} In order to further exploit the use of high pressure technologies for applications in biotechnology (protein therapeutics, design of novel vaccines and antiviral drugs\textsuperscript{74}), and the food industry (dairy, meats, and vegetables), it is paramount to
understand the impact of high pressure on the structure and conformational changes of proteins.

Several researchers have investigated the effect of high pressure on protein structure and self-assembly properties. High pressure has been used successfully to dissociate both oligomeric and complex-aggregated proteins. Paladini et al. reported that hydrostatic pressure in the range of 1 bar – 3 kbar was able to dissociate proteins (enolase, yeast hexokinase) from the oligomeric form into subunits.\textsuperscript{75,76} Much more complicated protein systems (brome mosaic virus\textsuperscript{77} and mitochondrial ATP synthase\textsuperscript{78}) have also been dissociated with high pressure.\textsuperscript{79} High pressure treatment was shown to reduce the aggregation rates of denatured proteins during refolding.\textsuperscript{67,80} St. John et al. used high pressures (1-2 kbar) combined with low, nondenaturing concentrations of guanidine hydrochloride (GdnHCl) as an alternative to strong chaotrophic salts to disaggregate and refold proteins.\textsuperscript{70} Thus high pressure can eliminate the need for dilution of reagents, buffer changes, or modification of reaction conditions.\textsuperscript{70,80}

The mode of action of high pressure on protein dissociation has been experimentally confirmed to depend on the penetration of water into the protein matrix.\textsuperscript{68,81-86} Proteins have been found to contain small “free volumes” within their folded conformation due to restrictions in amino acids proximity to each other. This results in internal cavities and packing defects that make them sensitive to pressure.\textsuperscript{68} Application of high pressure disrupts the hydrophobic and electrostatic interactions within the protein structure which results in a decrease in the volume of the system.\textsuperscript{67,72,84} Consequently, packing of solvent molecules is more efficient following protein dissociation.
Several amyloidogenic proteins aggregate into stable, predominantly β-sheet fibrillar structures which are insoluble in common solvents.\textsuperscript{50,51} These protein aggregates are implicated in several diseases. In Alzheimer’s disease, the β-amyloid (Aβ) peptide is the major component of these aggregates. There is an urgent need for efficient methods to hinder aggregate formation as this may lead to design of potential therapeutics.

Although significant progress has been made in understanding the mechanisms of protein aggregate formation, several challenges remain. Among these, obtaining reproducible starting materials (monomeric Aβ peptide) for biophysical characterization has been of utmost priority. The existing chemical protocols to disaggregate the Aβ peptide are time-consuming, and/or expensive; worse, the likelihood that some pre-seeded Aβ may still exist in the starting material cannot be ignored. As high pressure treatment has proven to be a successful tool in dissociating proteins, it may help circumvent some of these problems.

The objective of this study is to investigate the application of high pressure to dissociate Aβ peptide into monomeric subunits. High pressure is a powerful, physical method that has been shown to dissociate the amyloidogenic protein transthyretin, with the possibility of achieving stable intermediates when pressure and temperature are carefully “tuned”.\textsuperscript{68} The aggregation state of the Aβ peptide prior to experiment is critical for obtaining reproducible results. This is particularly important when screening and characterizing new peptides. Use of an inadequate array of biophysical techniques would be costly overall. Thus, we used a high pressure system in order to obtain ‘stable’ Aβ starting material for testing the efficiency of blocker peptides upon interaction with the Aβ peptide.
3.2 Materials and Methods

- Reagents and Chemicals

All reagents were of analytical grade. Hydrostatic liquid was spectroscopic-grade ethanol with a low-fluorescence background signal. Distilled water was filtered and deionized through a Millipore water purification system (18 MΩ resistance). β-amyloid(1-40) (Catalog No. 24236) was purchased from Anaspec, Inc. (San Jose, CA). Phosphoric acid (99.999%, Catalog No. 34,524-5) and semiconductor-grade potassium hydroxide (99.99%, Catalog No. 30,656-8) were obtained from Aldrich Chemical Co. Sodium chloride (99.999%, Catalog No. 10862) was obtained from Alfa Aesar. (2-[N-Morpholino] ethane-sulfonic acid, MES) was purchased from Sigma (Catalog No. M-8250).

- Sample Preparation

The high pressure experiments were performed at two locations, identified as Location A (UNICAMP, Sao Paulo, Brazil) and Location B (Louisiana State University, LA). Both locations were equipped with similar high pressure system with the difference being the information obtained. In Location A, the information obtained was the change in light scattering intensity of the sample with response to increasing high pressure (recorded with an Edingburg FL 900 spectrofluorometer). In Location B, the information obtained was the change in size (radius) of the sample with response to increasing and decreasing high pressure (recorded with a dynamic light scattering (DLS) instrument). The β-amyloid(1-40) (Aβ) samples (500 µM stock solution) were solubilized according to the method of Aucoin. Briefly, stock solutions were prepared by dissolving the peptide in filtered 10 mM KOH (pH 11) and vortexing with a Daigger Vortex Genie 2 until
completely dissolved. The Aβ peptide is known to exist in a monomeric state under sufficiently basic conditions.\textsuperscript{45} Otherwise, the stock was adjusted to the appropriate concentration and pH values. In location A, the buffers used were 100 mM MES buffer at pH 5.5 and 50 mM Tris chloride buffer, 150 mM NaCl, at pH 7.4. In Location B, several buffers were used but the presence of large aggregates in solution exceeded the capabilities of DLS. Results were obtained in the 50 mM phosphate buffered saline, 150 mM NaCl, pH 7.4 and 50 mM Tris chloride buffer, 150 mM NaCl, at pH 7.4.

- Light scattering and fluorescence under pressure (Location A, UNICAMP, Sao Paulo, Brazil)

The high-pressure system has been described elsewhere.\textsuperscript{88} An ISS model (HPCell\textsuperscript{TM}, Champaign, IL USA) high-pressure cell with sapphire windows connected to a pressure generator (HIP) was used. The intensity of light scattered at 340 nm was recorded in an Edinburg FL 900 spectrofluorometer and was measured at an angle of 90° relative to the incident light using the same wavelength for the excitation and emission monochromators. The fluorescence data were obtained at an excitation wavelength of 280 nm and an emission of 290-400 nm.

The pressure system was automated and controlled by software denominated “Automa” written in Delphi 5.0 language and compatible with Windows. A computer controlled a series of devices, including: (1) a servomotor connected to an induction motor that was coupled to the pressure generator, (2) the fluorometer monochromators for excitation and emission, and (3) the step motors for the excitation and emission slits. The software received information from a pressure gauge via an analog interface connected to a 14-bit analog-digital board that allowed the control of pressure in real
time, while data from the detectors were obtained via an RS232 serial port. All data were processed in ASCII format and displayed on x-y plots.

- Dynamic light scattering under pressure (Location B, Louisiana State University, LA)

A similar setup as described previously was used for DLS with the exception that the pump was manually operated. The light scattering instrument is described in Chapter 4. Details and photograph (courtesy of Rafael Cueto) of the set-up is provided in Appendix B.

3.3 Results

- Light scattering intensity results (Location A)

To examine the effect of pressure on Aβ aggregates, an initial sample of peptide was prepared in MES buffer under conditions known to produce immediate aggregates. Figure 3.1, (top) shows the effect of pressure on a 50 µM Aβ solution in 100 mM MES buffer at pH 5.5. Light scattering showed a decrease of intensity with increasing pressure, implying that the Aβ aggregates were being dissociated. Upon the return to atmospheric pressure, the samples were inverted to check for precipitation of the sample at the bottom on the sample cell, but no aggregates were observed visually. After about two hours at atmospheric pressure, the same sample (Aβ run #1) was subjected to high pressure a second time (Aβ run #2). The light scattering results followed an identical trend to the first sample. The only intrinsic fluorophore present in the Aβ peptide sequence is the aromatic side-chain of the tyrosine (Tyr 10) molecule. Although tyrosine has a low quantum yield, its sensitivity was measurable by fluorescence spectroscopy. Thus, the effect of pressure on the fluorescence of the sample was probed to ascertain whether changes due to the tyrosine residues occurred. Figure 3.1, (middle) shows the
fluorescence intensity response of the Aβ peptide to increasing pressure. After an initial decrease in the fluorescence intensity, at pressures above 10,000 psi the fluorescence intensity stabilized.

Figure 3.1 Effect of pressure on the light scattering intensity and fluorescence properties of β-amyloid peptide (Aβ). Top: light scattering intensity of an Aβ solution with a concentration of 50 µM in 100 mM MES buffer, pH 5.5 subjected to high pressure. Middle: Fluorescence properties of β-amyloid peptide solution in the top figure.

(figure continued)
Figure 3.1 (continued) Bottom: Fluorescence emission spectra of Aβ solution in the middle figure and corresponding plot of maximum fluorescence as a function of pressure. The excitation wavelength was 280 nm. Each spectrum data was collected 5 minutes after the pressure value had stabilized.

The increasing pressure had negligible effect on the tyrosine groups in the sample. This suggests that perhaps there are no major structural changes in the microenvironment of the fluorophore. The emission spectra characteristic of tyrosine groups as a function of increasing pressure is illustrated in Figure 3.1, (bottom).
To further test whether the Aβ peptide aggregates were being dissociated by high pressure, the supernatant of the starting 50 µM Aβ solution in 100 mM MES buffer, pH 5.5 (collected after centrifuging the sample at 14,000 x g for 30 minutes) was subjected to high pressure. Figure 3.2 (top) shows that the light scattering intensity is decreasing as a function of pressure and leveled off at about 30,000 psi. The light scattering change is not as dramatic as in Figure 3.1 (top), suggesting that further dissociation of the sample may be due to the presence of minor amounts of aggregates in the solution. The light scattering data of the Aβ supernatant solution is plotted with the aggregated sample for comparison (Figure 3.2, bottom).

Figure 3.2 Changes in light scattering of Aβ peptide as a function of pressure. Top: light scattering intensity of an Aβ ‘supernatant’ solution with a concentration of 50 µM in 100 mM MES buffer, pH 5.5 subjected to high pressure. (figure continued)
After observing that it was possible to dissociate aggregated Aβ solution prepared in MES buffer, a more ‘common’ buffer system was used. A fresh 50 µM Aβ solution in 50 mM Tris-Cl buffer at pH 7.4 was subjected to high pressure. Figure 3.3, top and bottom shows the light scattering and the corresponding fluorescence intensity results obtained. Both plots show similar trend in the light scattering and fluorescence intensity. At low pressure up to 7,000 psi a sharp increase in the scattering and fluorescence intensity was observed, followed by the opposite effect, namely a decrease in intensity at higher pressures up to a value of about 17,000 psi. The experiment was terminated at this pressure value due to technical problems (malfunctioning pressure gauge) with the instrument.
Although the instrumental problems mentioned above was solved, it turned out to be ‘temporarily fixed’ as the problem recurred during acquisition of the data in Figure 3.4. The effect of high pressure on the light scattering intensity of Aβ in the presence of Amyl peptide was investigated. First, the light scattering of a 50 µM Aβ peptide prepared in 50 mM Tris-Cl buffer, pH 7.4, under high pressure was monitored. The increase and subsequent decrease in the light scattering intensity at low and high pressure respectively, suggest that at low pressure the peptide’s structure is changing (increasing size) perhaps due to interaction with the solvent molecules, while at higher pressure values the opposite effect (decreasing size) may be due to dissociation of the peptide.

![Figure 3.3 Effect of pressure on the scattering and fluorescence intensity of β-Amyloid peptide. Top: Light scattering intensity of 50 µM Aβ in 50 mM Tris-Cl buffer, pH 7.4.](figure continued)
Figure 3.3 (continued) Bottom: Fluorescence intensity of 50 µM Aβ in 50 mM Tris-Cl buffer, pH 7.4.

The high pressure effect on the light scattering of the 50 µM Amy1 peptide prepared in water was then monitored. The inset in Figure 3.4 shows an enhanced scale of the trend in the scattering intensity of the Amy1 peptide under high pressure. The light scattering signal remained uniform at both low and high pressure, implying that the Amy1 peptide was stable under high pressure treatment. The sample containing a 1:1 molar ratio of Aβ and Amy1 petide was then subjected to high pressure treatment. The general trend in the data suggests that the combinded Aβ and Amy1 peptides were being dissociated, as the light scattering intensity signal steadily decreased with increasing pressure. Additional experiments could not be performed due to continued problems with the instrument’s pressure gauge.
Figure 3.4 Effect of high pressure on the light scattering of β-amyloid peptide and Amy1 peptide-based mediator. Inset shows adjustment of y-axis to show trend in the effect of high pressure on the light scattering of 50 µM Amy1 peptide dissolved in water.

- Dynamic light scattering results (Location B)

Although it would have been advantageous to measure the light scattering intensity and corresponding size of the Aβ peptide, the instrument in Location A lacked that capability. An opportunity to measure the effect of high pressure on the size of Aβ peptide at a different location (Location B) was presented. The initial experiment to
investigate the dissociation of Aβ aggregates prepared in MES buffer, as done at Location A proved very challenging for dynamic light scattering as the aggregates were too large. The alternate buffer choice used was 50 mM Tris-Cl, pH 7.4. Figure 3.5 shows the effect of increasing and decreasing pressure on the hydrodynamic radius of Aβ peptide in Tris-Cl buffer. The initial hydrodynamic radius calculated was about 116 ± 4.0 nm. This value very slightly increased to 148 ± 18 nm at the highest pressure of 35,000 psi. Upon decreasing the pressure, differences between the initial and final radius of the sample was negligible, suggesting stabilization of the sample. There was no substantial effect (within experimental error) of high pressure on the size of the Aβ peptide in Tris-Cl buffer.

As most of the initial investigations of Aβ peptide were performed in phosphate buffer, this buffer system was chosen (neglecting the effect of pressure on the buffer itself) to study the effect of high pressure on Aβ peptide in this solvent environment. A 50 µM Aβ sample was subjected to high pressure in 50 mM PBS buffer, pH 7.4. Figure 3.6 shows the changes in hydrodynamic radius of Aβ as a function of increasing and decreasing pressure. There is a steady increase in the radius of the peptide with increasing pressure. At the highest pressure, the radius increased to 480 ± 4.3 nm, a difference of about 100 nm from the initial radius (376 ± 1.8 nm) at atmospheric pressure. The radius decreased to a value of 434 ± 3.2 nm at 10,000 psi and appeared to level off at 5,000 psi, suggesting stabilization of the sample. One of the most likely explanations to the increasing radius is that in the phosphate buffer environment, any dissociation of the Aβ peptide induced by pressure is exposing the hydrophobic sites on the peptide to the solvent, promoting interactions which leads to the observed increased in hydrodynamic radius.
Figure 3.5 Effect of high pressure on the hydrodynamic radius of Aβ. A 50 µM Aβ sample subjected to high pressure in 50 mM PBS buffer, pH 7.4. Error bars are standard deviation of triplicate runs.

The sample was kept at atmospheric pressure for one day and subsequently subjected to high pressure. Figure 3.6, middle, shows the effect of high pressure on the radius of the Aβ peptide after one day at atmospheric pressure following the first pressure cycle. The peptide radii were identical with negligible error (standard deviation of triplicate runs) during both the increase and decrease of pressure. A final radius of 325 nm was obtained which is slightly smaller than the initial radius of 376 nm at the start of the experiment. The results obtained for both pressure cycles of the effect of pressure on the radius of Aβ are plotted for comparison (Figure 3.6, bottom).
Figure 3.6 Dynamic light scattering results of increasing and decreasing pressure on β-amyloid Aggregation. Top: Hydrodynamic radius plotted against increasing and decreasing pressure. Middle: Hydrodynamic radius plotted against increasing and decreasing pressure. (figure continued)
3.4 Conclusions

The main goal of this study was to use hydrostatic pressure to dissociate Aβ peptide to obtain reproducible starting Aβ solution. The results obtained with light scattering and fluorescence intensity measurements demonstrated that high pressure is an efficient technique to dissociate Aβ aggregates and obtain stable material. For the effect of high pressure on the size of Aβ peptide in both 50 mM Tris-Cl and 50 mM PBS, 150 mM NaCl, pH 7.4, the results did not show the expected trend corresponding to Aβ dissociation. Rather, in the 50 mM Tris-Cl solvent environment, there was negligible change in the size of the Aβ peptide for both increasing and decreasing pressure. In the PBS environment, there was an initial increase in radius with increasing pressure and the

Figure 3.6 (continued) Bottom: Superposition of hydrodynamic radius for both cycles.
opposite effect upon decreasing the pressure, with stabilization of the radius at low pressures. Subjecting the Aβ peptide in the PBS environment to a second cycle of increasing and decreasing pressures demonstrated that the sample had stabilized with no changes in the peptide radius during both increasing and decreasing pressure.

The data in Figures 3.5 and 3.6 were measured at an angle of 90° at various pressures, using a conventional dynamic light scattering instrument (modified for accommodating the high pressure cell). As only a single angle was used, the hydrodynamic radii represent apparent values. In both figures, the presence of large aggregates at low and high pressure, were easily detected by DLS. Small aggregates were not detected, but their presence could not be confirmed or denied; indicating one limitation of DLS for preferentially detecting larger species. Nonetheless, the results obtained using single angle measurements were adequate for observing any effect of high pressure on the aggregation properties of Aβ. Overall, these results suggest that it is possible to obtain stable samples (within the experimental time frame) of Aβ peptide following high pressure treatment in PBS.
CHAPTER 4

TECHNIQUES FOR MEASURING β-AMYLOID AGGREGATION

4.1 Introduction

Protein transport plays a critical role in how the mammalian central nervous system communicates with the rest of the body. Important to the function of this sophisticated transport system is the blood-brain barrier (BBB). If the structural complexity of the system is ignored, the BBB can simply be described as a “gatekeeper” of the central nervous system which regulates the passage of substances. Protein and peptide ligand gain entry into or out of the brain and spinal cord by either simple diffusion or saturable transport. An example of this concept is the transport of the β-amyloid peptide (Aβ) out of the brain. It is believed that the normal function of the transport system is to facilitate the removal of Aβ from the brain. Failure of the transport system to clear the Aβ results in formation of amyloid plaque. Alzheimer’s disease is a detrimental consequence of amyloid plaque accumulation in the brain. A major goal is to understand the mechanisms of Aβ transport and plaque formation in an effort to design therapeutic approaches to solve this problem.

While it is often a challenge to exactly emulate nature, scientists continue to find ways to explore and answer many fundamental questions. For instance, fluorescence photobleaching recovery (FPR) was originally developed to examine the dynamics of living cells in vitro. The first application of FPR was geared towards understanding the effect of membrane transport on the physiological states of cells. Recent reviews of this technique describe the proliferation of this method as well as a host of applications. Like FPR, dynamic light scattering (DLS) is also a powerful tool for studying
molecular diffusion. The theory of DLS relevant to this study is described below. Complete theoretical background of DLS can be found elsewhere.\textsuperscript{106-108}

### 4.2 Theoretical Background

Mutual diffusion is a transport process in which there is a net flow of molecules, normally from a region of high concentration to one of low concentration. Self diffusion is also “natural” and does not involve the high-to-low change. Fick proposed that the flow of matter along a concentration gradient should be analogous to the law of heat flow along a temperature gradient.\textsuperscript{109} The product of diffusion coefficient and concentration gradient is a measure of the flux and can be seen in the equation below, known as Fick’s first law of diffusion:

\[
J = -D_m \left( \frac{dc}{dx} \right)
\]

where \( J \) represents the flux with units of square centimeter per second, \( c \) has concentration units, and \( D_m \) is the mutual diffusion coefficient with units of centimeter squared per second. The subscript represents mutual diffusion coefficient and it is the diffusion coefficient obtained from measuring relaxation of the concentration gradient. Diffusion depends on molecular size according to the Stokes-Einstein equation:

\[
D = \frac{kT}{f}
\]

where \( k \) is the Boltzmann constant, \( T \) is the Kelvin temperature and \( f \) is the size-, shape-, and concentration-dependent friction coefficient. For a spherical particle with a hydrodynamic radius \( R_h \) when dispersed at low concentrations in a solvent of viscosity \( \eta_o \), the friction coefficient is given by \( f = 6\pi\eta_oR_h \). Translational diffusion can be used to
determine the size and shape of individual molecules as well as molecular aggregates. Dynamic light scattering is a useful technique for the measurement of motion of molecules by the fluctuations in intensity. One measures the autocorrelation function of the scattered light, $g^{(1)}(t)$, which contains information from its distribution of the relaxation rate that can be used to determine molecular size. The decay follows a single exponential profile:

$$g^{(1)}(t) = e^{-\Gamma t}$$  \hspace{1cm} (3)

where $\Gamma$ is the decay rate, proportional to the diffusion coefficient by:

$$\Gamma = q^2 D_m$$  \hspace{1cm} (4)

in which $q$ is the scattering vector magnitude, $q = (4\pi n/\lambda_o) \sin(\theta/2)$, $n$ is the refractive index of the solution, $\lambda_o$ is the in vacuo incident light wavelength and the scattering angle is $\theta$. A plot of $\Gamma$ vs. $q^2$ should be linear with a zero intercept and slope $D$. The apparent mutual diffusion coefficient, $D_{app} = \Gamma/q^2$ is computed to test whether this is the case. A plot of $D_{app}$ vs. $q^2$ should be flat. Failure of $\Gamma$ vs. $q^2$ to rise linearly, or a slanted $D_{app}$ vs. $q^2$ plot, indicates particle heterogeneity and/or a shape other than spherical. This behavior can then be used to evaluate the apparent hydrodynamic radius, $R_{h,app}$ of molecules via the Stokes-Einstein equation:

$$R_{h,app} = \lim_{c \to 0, t \to 0} \frac{kT}{6\pi \eta D_{app}}$$  \hspace{1cm} (5)

where $k$ is the Boltzmann’s constant, and $\eta$ is the solvent viscosity at temperature $T$.

In addition to the mutual diffusion, there exists a self-diffusion that also depends on concentration, but self-diffusion happens even without a concentration gradient. Self-
diffusion is a measure of the translational motion of a molecule. The appropriate relationship is due to Einstein and it appears in the equations below:

\[ <x^2> = 2D_s t \] \hspace{1cm} (6)

\[ D_s = \frac{<x^2>}{2t} \] \hspace{1cm} (7)

The self-diffusion coefficient is referred to as optical tracer self-diffusion coefficient when optical methods such as fluorescence photobleaching recovery experiments are used to obtain it. The experimental setup for FPR is explained below. A distinguishing factor observed between mutual and self-diffusion is that for mutual diffusion, spontaneous or artificially created fluctuations help drive the diffusion from a region of high concentration to one of low concentration, while for self-diffusion, the concentration remains almost uniform. This behavior is observed because in FPR, the concentration of the sample is proportional to the concentration of dye before bleaching a stripe pattern into the sample. After bleaching, the concentration of the sample does not change, while that of the dye changes due to diffusion. If the period of the pattern in the sample is \( L \), and the spatial frequency of the pattern is \( K \), then:

\[ K = \frac{2\pi}{L} \] \hspace{1cm} (8)

In a FPR system equipped with modulation detector, a Ronchi ruling is translated perpendicular to its stripes (bleached in the sample). Immediately after bleaching, the maximum current at the detector represents a triangle wave, but the high harmonics fade so quickly that the sharply defined pattern softens and approaches a sinusoidal wave form. The contrast, \( C(t) \), of the stripe pattern, which is proportional to a voltage output from the modulation detector system, decays exponentially:
\[ C(t) = C_0 e^{\Gamma t} + B \]  
\( \text{(9)} \)

The baseline, \( B \), represents noise attributed to electronic jitter, imperfections in the cell, or artifacts in the sample. The decay rate, \( \Gamma = DK^2 \) where \( D \) is the tracer self diffusion coefficient, \( K \) is the grating constant, and \( t \) is the time since photobleach. The diffusion coefficient is obtained as the slope of \( \Gamma \) versus \( K^2 \) plots of the data. Like DLS, the Stokes-Einstein relation, \( D_s = kT/(6\pi\eta R_h) \) is used to obtain size information and is only valid at \( c = 0, K = 0 \). Normally, it is safe to assume \( K = 0 \) in FPR, while taking the \( q = 0 \) limit in DLS is much less secure.

4.3 Characterization Background of FPR

Fluorescence photobleaching recovery is a powerful diffusion tool used to determine the physical properties of fluorescently tagged macromolecules. The theory of FPR appears in detail in other sources.\(^{110-113} \) A brief summary of FPR is described.

The FPR apparatus is set up such that either a spot or fringed pattern is bleached on the sample. In this work, only the modulated fringed pattern was used. The fringe pattern is obtained from a Ronchi ruling (a coarse grating in which black stripes are etched onto glass at uniform intervals) placed at the back focal plane of the microscope objective lens. A short, intense bleach pulse (writing beam) with duration of less then one-tenth of the recovery time irreversibly destroys about 5 to 10% of the fluorescently labeled sample. After bleaching, a striped image resulting from the fringed pattern is written in the sample. A weak continuous ‘reading’ beam subsequently excites the unbleached fluorophores from the ground state to the first excited state. The recovery of fluorescence due to redistribution of unbleached molecules into the bleached region of
the sample is measured. The rate of recovery provides information about the diffusion of the fluorescent molecules.

4.4 Instrumental Setup of FPR

The FPR apparatus is shown in Figure 4.1. The central component of the FPR is an Olympus BH2 epifluorescence microscope whose illuminator assembly is modified to allow illumination by a light source. The laser, either a Coherent Innova 90 argon ion laser capable of producing 0.9 Watts at 488 nm or a Lexel EXCEL 3000 argon ion laser capable of producing 0.9 Watts at 488 nm was used. The laser beam is passed through an acousto-optic modulator (AOM, Newport Research N35085-3) driven by a modified radio-frequency source (Newport Research 31085-6DS) which splits the beam into two diffraction beams. The first-order diffracted beam, which is about 85% of the laser output, is used for photobleaching. In this study, a fringed pattern is obtained by placing a 1” Ronchi ruling (50, 100, 150, and 300 lines/inch, Edmund Scientific), cradled between two loudspeakers (4” woofers – Radio Shack catalog number 40-1022B) that face each other at the rear image plane of the microscope objective. The speakers are driven in push-pull fashion by a low-frequency (typically 16 Hz) triangle wave whose amplitude is adjusted to produce an oscillation one half the period of the ruling (or multiples thereof). The input is shorted to stop the speakers during the photobleaching step.

The patterned beam is deflected by a dichroic mirror and focused by the microscope objective (4X, 7X, 10X, and 18X) onto the sample, which sits on a temperature-controlled stage. An RCA 7265 photomultiplier tube (PMT) receives the fluorescence from the sample. The PMT is protected by a shutter (Newport 846HP) that
remains close during the photobleaching pulse. The intensity readout from the photodetector is fed to a Stanford Research Systems model SR560 low noise preamplifier to filter out unwanted frequencies. The signal is then transmitted to both a Tektronic 221A 100-MHz digital oscilloscope (to monitor the quality of the sinusoidal wave generated in the experiment) and an analog-digital card from National Instruments (# AT-M10-16D, Part #320489-01). The output signal of the A/D card is read by a Labview generated program which analyzes the sine wave amplitude. The Labview program also controls the switching of the AOM, the shutter, and the duration of signal collection. The exponentially decaying contrast signal is then analyzed by all the usual analysis methods—cumulants\textsuperscript{114}, single or multiple exponentials with floating baseline, and even Laplace inversion\textsuperscript{115}.

Figure 4.1 Schematic of fluorescence photobleaching recovery experimental setup (variant of the Lanni and Ware instrument) used in this lab. AOM=acousto-optic modulator; M = folding mirrors (an accommodation to space); D=diaphragm (stray light reduction); RR=Ronchi Ruling; L= Lens (depends on one’s microscope); DM=dichroic mirror; OBJ=Objective (4X – 40X; low N.A.);
4.5 FPR Experimental Protocol

One requirement of samples for FPR study is the attachment, preferably covalent, of a fluorophore to the molecule of interest. It is important to choose a fluorophore that is not easily photobleachable; on the other hand, the fluorophore should not be so stable that it is barely bleachable. In the case of proteins, it is critical to establish that attachment of a fluorophore does not affect the functioning of the protein. Once the sample has been prepared, a very minute amount is required for doing FPR. The sample is typically loaded in 0.1- or 0.2-mm-path-length rectangular microslides (Vitrocom) by capillarity, and the microslide is flame-sealed. A fluorescently labeled dextran is prepared to ensure that the instrument is in working condition, particularly after focusing and alignment.

A sample data trace appears in Figure 4.2, along with the DC signal (equivalent to a spot photobleach, but the depth is only about 5%). The quality of the decaying contrast signal approaches that of a typical DLS correlogram after conversion to a first-order correlation function. The inset of Figure 4.2 shows a plot of decay rate $\Gamma$ vs. $K^2$; the lack of an intercept confirms the absence of nondiffusive (spontaneous or chemical) recovery of the photobleached dye.

4.6 Characterization Background of DLS

When laser light is used to illuminate macromolecules, the electrons in the molecules are induced to oscillate, due to the oscillating electric field of the light. As the molecules will in turn radiate that light, the intensity of the scattered light will depend on the magnitude of the dipole induced in the molecules.
Figure 4.2 Modulation detector trace for aqueous FITC-Dextran (Sigma FD150). Top: clean contrast recovery (AC) signal after only 5% photobleach of the original intensity, DC. Middle: after baseline subtraction, semilogarithmic representation of a single-exponential behavior. (figure continued)
Thus, the larger the dipole induced, means the greater the intensity of the scattered light. In solution, the random motion of the molecules will impart randomness to the light scattered by these molecules. The relative position of two molecules will determine whether the scattered light arriving at a particular position will interfere constructively (higher intensity) or destructively (lower intensity). The combined intensity of light scattered from several scattering molecules will vary in time, fluctuating around some average intensity as the molecules move relative to each other. The rate of the fluctuations is related to the rate the molecules are moving through the solvent. On a short time scale, these fluctuations will be similar and correlated, thus; analyzing these fluctuations can provide information about the molecule size.
4.7 Instrumental Setup of DLS

A schematic of the DLS experimental set-up appears in Figure 4.3. The lasers used were either a HeNe laser with wavelength of 632.8 nm or a Coherent Innova 90, with wavelength of 488 nm. The device consists of a sample holder with an index matching bath containing toluene. The sample is normally placed in 13 mm culture tube or borosilicate glass cells (6 x 13 mm, Kimble) which is nearly isorefractive with the bath solvent. The electronics consisted of a pulse amplifier/discriminator (PAD, Pacific Precision model 126), a Hamamatsu R928p photomultiplier, and an ALV-5000 digital multi-tau autocorrelator card installed in a personal computer. Measurements were made at multiple scattering angles ranging from 30° to 110° by manually moving a detector arm to each new angle needed.

Figure 4.3 Schematic of dynamic light scattering experimental setup.
4.8 DLS Experimental Protocol

Light scattering is very sensitive to large particles and does not distinguish them from less interesting particles, such as dust or undissolved material. Measures are taken to verify that the sample cell (13 mm culture tube) is clean by rinsing repeatedly with dust-free deionized water from a Barnstead Nanopure water purification system. The cleaned tubes are then filled with the same ultrafiltered water and checked for any dust particulate by viewing at 30 degrees in the light scattering system. Before the actual samples are measured, an unimportant sample is measured to verify that the instrument is correctly aligned, and in proper working condition.

In this case, a latex sample with an advertised diameter of 0.087 µm was used as a reference. Figure 4.4 shows the dynamic light scattering results for the latex sample. The calculated diameter of the latex particle was 0.089 µm which is in close agreement with the advertised diameter. This shows that the instrument is almost perfectly aligned.

Figure 4.4 Light scattering data for Latex particles. Top: $I'$ versus $q^2$ plot.

(figure continued)
Figure 4.4 (continued) Bottom: Apparent diffusion coefficient plotted as a function of the squared scattering vector magnitude.
CHAPTER 5

USE OF FLUORESCENCE PHOTOBLEACHING RECOVERY AND DYNAMIC LIGHT SCATTERING TO STUDY β-AMYLOID AGGREGATION

5.1 Introduction

The β-amyloid (Aβ) peptide is believed to play a causative role in the pathogenesis of Alzheimer’s disease (AD). The exact mechanism through which the Aβ induces its toxicity is still unclear. The conformational change from a random coil/α-helix, soluble Aβ to a β-sheet fibrillic intermediate may be a crucial link in this pathogenic cascade. Small aggregates (oligomers, protofibrils) were reported to be more neurotoxic than the larger aggregates (fibrils). Several approaches to the reduction of Aβ and its associated aggregates are under development. Challenges of quantifying heterogeneous protein mixtures are key limitations of existing techniques. Regardless of whether protofibrils or fibrils are responsible, it is certain that there is a need for additional biophysical techniques to help solve this problem.

The main aim of this research was to apply new biophysical tools to characterize the Aβ peptide aggregates and to test the behavior of the peptide in the presence of mediators. Novel peptide-based mediators containing α,α-disubstituted amino acids (ααAA), dipropyl glycine (Dpg), diisobutylglycine (Dibg) and dibenzyl glycine (Dbg) were incorporated at alternating positions in peptide sequences forming the hydrophobic core (KLVFF) of Aβ. These mediators, referred to as Amy1, Amy2 and modified-Amy were designed with the idea that they would block amyloid fibril formation.

The observation that Aβ can bind a peptide fragment corresponding to the central hydrophobic region of Aβ (16-20) and disrupt fibril formation underlies several competing research efforts, including those mentioned above. Due to its ability to bind
and prevent amyloid fibril formation without forming fibrils itself, the KLVFF fragment of Aβ has been termed the ‘recognition element’. Several groups subsequently developed inhibitor peptides with this recognition element and an additional ‘disrupter’ group to either block hydrogen bonding or solubilize any Aβ-inhibitor byproduct. Soto et al.\textsuperscript{124;125} included proline residues (known β-sheet breakers) within the recognition element and reported that the compound was able to prevent fibril formation, as well as partially disaggregate preformed fibrils. Murphy et al.\textsuperscript{38;126} designed peptides with the sequence (15-25) as the recognition element with the addition of hydrophilic poly(Lys) units as the disrupting groups. They reported that their compounds were able to alter Aβ aggregation by increasing the rate of protofibril assembly as well as blocked toxicity \textit{in vitro}.

Meredith et al.\textsuperscript{127} designed inhibitors that contained N-methyl amino acids, and those that replaced amide bonds with ester bonds in alternating positions within the recognition element. They found that their compounds both blocked fibril formation and disassembled preformed fibrils. It can be concluded that compounds containing both the recognition element and disrupting groups are capable of altering Aβ aggregation kinetics and morphology.

The ααAA-containing mediators were designed using Murphy’s inhibitors model as a template. Previous work\textsuperscript{128-130} showed that ααAA had the ability to stabilize extended peptide structural conformations. Fu \textit{et al}.\textsuperscript{131} showed that peptides containing the Dbzg residues at alternating positions were capable of adopting an extended conformation, thereby preventing β-sheet formation. The peptide sequences of the Amy mediators are shown in Scheme 1 below. Complete synthetic protocol of the Amy mediators has been described.\textsuperscript{132;133}
Scheme 1: Code name and sequence of ααAA-containing peptide blockers of Aβ assembly

AMY-1: H-Lys-Dibg-Val-Dbzg-Phe-Dpg-(Lys)₆-NH₂
AMY-2: H-(Lys)₇-Dibg-Val-Dbg-Phe-Dpg-NH₂
AMYB₃: H-Lys-Aib-Val-Aib-Phe-Aib-(Lys)₆-NH₂

Scheme 2: Code name and sequence of modified ‘recognition element’ peptide blockers of Aβ assembly

MURPHY: H-Lys-Leu-Val-Phe-Phe-Ala-(Lys)₆-NH₂
MEREDITH: H-Lys-Leu(Me)-Val-Phe(Me)-Phe-Ala(Me)-Glu-NH₂

A combination of techniques was used to determine the stability of Aβ peptide and kinetics of fibril growth in an attempt to identify the early and intermediate stages of assembly. Physiological factors that induce the aggregation of soluble Aβ are of interest in determining the cause of Aβ fibril formation. It is known that the peptide tends to self-assemble under conditions of low and neutral pH (as a function of time). The peptide remains in a monomeric or low oligomeric state at high pH values. The effect of variables including concentration, ionic strength, pH and temperature were monitored. Subsequently, the interaction of ααAA-containing peptide blockers with Aβ was tested.

5.2 Materials and Methods

• Reagents and Chemicals

For this study, synthetic β-amyloid(1-40) and fluorescently labeled β-amyloid(1-40) were obtained from Anaspec, Inc. San Jose, CA (catalog #’s 20698 and 23513). Phosphoric acid (99.999%, Catalog No. 34,524-5) and semiconductor-grade potassium
hydroxide (99.99%, Catalog No. 30,656-8) were obtained from Aldrich Chemical Co. Sodium chloride (99.999%, Catalog No. 10862) was obtained from Alfa Aesar. The filters used were from Whatman, 0.02 µm (Anotop 10, Catalog No. 6809-1102).

- Sample Preparation

The biggest challenge in preparing β-amyloid samples is controlling the initial, unaggregated state of the peptide. This is particularly important in order to understand the early stages of amyloid aggregate formation. The presence of ‘seeds’ was reported to greatly accelerate the aggregation of Aβ. To circumvent this problem, stock solutions were prepared by dissolving the Aβ peptide in 10 mM aqueous potassium hydroxide. This initial step was important as environments of high pH was found to produce ‘stable’ solutions of Aβ. The word ‘stable’ is taken to mean, not immediately forming protofibrils when subjected to physiological phosphate buffer conditions; in other words, the sample should be ‘non-aggregating’ for weeks.

Stock solution (500 µM) was prepared by dissolving the peptide in 10 mM potassium hydroxide and vortexing until completely dissolved. In those instances where the sample was difficult to solubilize, it was sonicated in a Branson Model No. 2510 sonicator. The sonication was done in five seconds cycles to prevent heat from affecting the sample. An aliquot (20 µl) was removed for AAA and the remaining solution was filtered through a 0.02 µm Whatman Anotop filter. Following filtration, an aliquot was removed for AAA. A 10X phosphate-buffered saline (PBS), pH 7.4 (0.5 M phosphoric acid, 1.5 M sodium chloride, 5 M potassium hydroxide) was filtered through 0.2 µm Whatman Anotop filter, after which it was mixed with the desired volume of stock to make final solutions of desired peptide concentration in 1X PBS, pH 7.4. Note that water
was first added to the peptide filtrate prior to adding the desired amount of PBS needed for 50 mM, in order to prevent rapid disruption of sample in such high ionic strength. Dilute solutions were made by adding buffer to the stock solution. The samples were loaded in 0.2-mm-path-length rectangular microslides (VitroCom) by capillarity, and the microslides were flame-sealed.

5.3 Results

5.3.1 Solution Properties of β-amyloid Peptide

- Effect of variables on Aβ peptide aggregation

The application of fluorescence photobleaching recovery (FPR) and dynamic light scattering techniques to the Aβ protein aggregation process are presented. In the case of Aβ, it has been shown that labeling exclusively at the N-terminus of the peptide minimizes modifications to the peptide conformation caused by the fluorophore’s presence and preserves the original biological activity.135;136 The first experiment was to determine the effect of varying bleaching time on the diffusion values of a solution composed of 5-carboxyfluorescein labeled Aβ and mixtures of 5-carboxyfluorescein labeled Aβ with unlabeled Aβ peptide. Figure 5.1 shows the diffusion coefficient as a function of increasing bleach time at a laser power of 0.5 Watts. Both a plot for a 100% labeled peptide and a 25/75 ratio labeled to unlabeled peptide at a concentration of 50 µM dissolved in 50 mM PBS, 150 mM NaCl, pH 7.4 are displayed. There is a slight difference in the diffusion coefficient values obtained for the two samples; however, both display decreasing diffusion coefficient values as a function of increasing bleach time. At bleach times up to two seconds, the 100% labeled sample has slightly bigger diffusion values than the mixed (25/75 ratio labeled to unlabeled peptide) sample, except at the
highest bleach time of three seconds, where it is slightly smaller. It is evident (25% difference in diffusion coefficient values from the initial bleach at 0.5 s to final bleach time of 3.0 s) in the results that the increasing bleach times is causing changes to the sample, however, it is difficult to attribute the decreasing trend in the diffusion values to degradation of the samples due to heating by the laser. If cleavage of the fluorophore occurred, then faster diffusion values representative of free dye would have been expected. The smaller diffusion values suggest that some sort of binding or Aβ peptide interaction may be more likely. These results indicate that care should be taken to avoid high bleaching times as this affects the diffusion values of the peptide.

Figure 5.2, top and bottom shows a screen capture of the raw FPR data for the 100% labeled peptide (at the conditions above) bleached for 3.0 s (top) and 0.5 s (bottom). Both conditions produced reliable diffusion coefficients. The arrow in each figure demonstrates the DC signal before and immediately after the bleaching pulse. In the top figure, about 50% of the sample is bleached and less than 10% in the bottom figure. In the orginal and most common form of doing FPR experiments, very deep bleaching (exceeding 50%) of a circular or Gaussian spot are required. In most of those studies the samples measured are typically live cells or proteins. Apparently sample damage due to prolong laser beam exposure has not hindered the use of this method. The lack of defined boundary conditions for diffusion in Spot photobleaching may give rise to partial photobleaching recovery. In heterogeneous samples where multiple diffusers are present, the possibility that slow and fast diffusers may exchange on the same time scale is highly likely and problematic. Shallow bleaches, typical 5-10% are sufficient in the
modulation detector FPR system and this method is well-suited for analyzing polydisperse systems.

Figure 5.1 Diffusion as a function of bleach time of labeled and mixture of labeled and unlabeled Aβ. The filled square symbol represents 100% 5-carboxyfluorescein Aβ_{1-40} and the open circle symbol represents a mixture of 25% 5-carboxyfluorescein Aβ_{1-40} with 75% Aβ_{1-40} expressed as percentage of 50 μM peptide. Conditions: 50 mM PBS, 150 mM NaCl, pH 7.4; laser power, 0.5 Watts. Error bars are standard deviations from triplicate runs.

The effect of bleach time on the diffusion of Aβ peptide established that overheating during the photobleaching pulse may have potentially caused changes to the overall sample integrity and caution should be taken to avoid over-bleaching. Next, the effect of a fluorophore’s presence on the Aβ peptides’ normal property was examined. Samples were prepared having different ratios of 5-carboxyfluorescein labeled to unlabeled Aβ_{1-40}. 

50
Figure 5.2 Representative raw FPR data for 100% 5-carboxyfluorescein labeled Aβ bleached at 3.0 s and 0.5 s respectively. The x-axis is time and the y-axis is the contrast (signal amplitude) for all plots. The arrows indicate the pre and post-bleach DC signal. (A) Linear-Linear scale representation. (B) Information about the sample file. (C) Logarithm-Linear scale representation and (D) Linear-Logarithm scale representation.
These samples were measured and compared to 100% labeled Aβ1-40 as a test that attachment of a probe had little effect on the sample properties. Figure 5.3 shows the diffusion coefficient of the 100% labeled sample was within error of that measured in the mixed samples, indicating that the dye moiety does not itself preferentially interact with unlabeled Aβ1-40 molecules. In the studies done with a mixture of labeled and unlabeled Aβ1-40 peptide, a ratio of one to three or twenty-five to seventy-five percent labeled to unlabeled peptide was used, mostly to compensate for any given strength of the laser power. If a protein is purchased fully labeled, cutting in some unlabeled material may prove useful and practical if the labeled peptide is three times as costly as the unlabeled peptide.

![Graph](image)

Figure 5.3 Diffusion as a function of dyed to undyed Aβ, expressed as percentage of 50 µM 5-carboxyfluorescein Aβ1-40 mixed with 50 µM Aβ1-40 Conditions: 50 mM PBS, 150 mM NaCl, pH 7.4. Error bars are standard deviations from triplicate runs.
• Effect of Concentration on Aβ Assembly

The kinetics of Aβ aggregation are known to depend on the peptide concentration, pH and ionic strength. In addition, negligence in sample preparation such as impurities in the sample containers, surface roughness of sample containers, introduction of salt, metal ions, and contact with metals can all induce aggregation. The effect of concentration on the diffusion coefficient of Aβ samples was examined. Figure 5.4 shows the effect of increasing concentration on the diffusion coefficient of 75/25 % labeled to unlabeled Aβ peptide in 10 mM KOH. The samples were measured on three consecutive days, stored in a dark chamber, and measured again on the twenty-sixth day following sample preparation. As Aβ peptide was observed to be non-aggregating at high pH, no concentration dependence on the diffusion coefficient of the peptide was expected and none was observed.

The diffusion coefficients at each concentration and at the given times measured are almost identical within experimental error. The slight decrease in the diffusion coefficient measured on day 2 may be attributed to variation in measuring conditions, such as increased laser intensity or bleached time. The measurement on day 26 was done at a different objective setting and consequently spatial frequency value in an attempt to improve the signal quality. Improvement in the signal quality does not however explain the faster diffusion values obtained on day 26. At instances where the Aβ was bleached too deeply, slower diffusion values resulted, thus the most likely explanation for the faster diffusion values may be attributed to stabilization of the sample due to the presence of the 5-carboxy-fluorescein fluorophore. The dye may be hindering the Aβ peptide’s ability to self associate with a likely consequence being contraction of the peptide.
Figure 5.4 Effect of concentration on the diffusion coefficient of 75/25 % labeled and unlabeled Aβ peptide. The samples were measured on three consecutive days and stored for a period of time and measured again on the twenty-sixth day following sample preparation. The filled circles represent data from day 1. The open circles represent data from day 2. The open upside-down triangles represent data from day 3. The filled triangles represent data from day 26. Conditions: 10 mM KOH, (Ronchi Ruling = 50 lines per inch; objective = 4X, K = 157 cm\(^{-1}\) and objective = 7X, K= 253 cm\(^{-1}\)).

- **Effect of pH and Ionic Strength on Aβ Assembly**

FPR measurements were made on samples containing 25% labeled and 75% unlabeled peptide at three pH values, spanning the range where the peptide is believed to exist in the high-to-low oligomeric states. Figure 5.5 shows diffusion coefficient results for a mixture of labeled and unlabeled Aβ (100 µM in 50 mM phosphate buffer at pH 2.7, 6.9 and 11) over a one month period. The diffusion values are consistent with theoretical predictions.\(^{63}\) They also match experimental values for monomeric β-amyloid\(_{1-40}\).
measured elsewhere using diffusion ordered NMR spectroscopy. These results demonstrate that attachment of a dye does not radically alter the hydrodynamic size of the peptide; however, these findings are perplexing as the \( \beta \)-amyloid\(_{1-40} \) peptide is widely thought to aggregate at neutral and lower pH values. Smaller values of diffusion coefficient might have been expected as the A\( \beta \)\(_{1-40} \) peptide was thought to aggregate within the experimental time frame at neutral pH. One possibility is that the 5-carboxy fluorescein dye attached to the peptide is contributing to its stability. Figure 5.6 shows the corresponding semilogarithmic plot for representative data demonstrating almost single exponential characteristic of a monodisperse sample.

![Graph illustrating pH effects on the diffusion coefficient of A\( \beta \).](image)

Figure 5.5 Trend of pH effects on the diffusion coefficient of A\( \beta \). Conditions: sample was composed of a 100 µM 25/75 % mixture of labeled and unlabeled A\( \beta \) in 50 mM PBS, 150 mM NaCl at pH ▶ 2.7, ○ 6.9, and ■ 11. The error bars are standard deviation of triplicate runs.
In order to further explore possible explanations for the results in Figure 5.5, it was necessary to perform additional experiments. While repeating the experiment with a fresh stock of peptide would have been best (that was done and those results are discussed below), it was simpler to verify that the peptide was capable of assembling by varying a condition known to induce aggregation. One such condition is the presence of high concentrations of salts in the aqueous solution. FPR measurements were made on 100 µM Aβ samples containing 25% labeled and 75% unlabeled peptide in different concentrations of sodium chloride environments. The effect of added sodium chloride on the diffusion coefficient of the Aβ sample was monitored. At low salt concentrations, only one decay mode was observed.

Figure 5.6 Semilogarithmic FPR traces for three Aβ samples at different pH values. After baseline subtraction, each semilogarithmic representation highlights the almost single exponential characteristic of a monodisperse sample. Conditions: 100 µM 25/75 % mixture of 5-carboxyfluorescein Aβ and unlabeled Aβ in 50 mM PBS, 150 mM NaCl at pH values ▶ 2.7, ○ 6.9, and ■ 11.
At ionic strengths of 100 µM up to a sodium chloride concentration of 200 µM, double exponential behavior was observed. Subsequently, the effect of pH on Aβ aggregation experiment was repeated using a fresh batch of only labeled Aβ.

Figure 5.7 FPR measurements on samples containing 100% 5-carboxyfluorescein-labeled peptide in 50 mM PB, at pH 7.4 with increasing sodium chloride concentrations. The monomeric diffusion coefficient of Aβ(1-40) was found to be $1.58 \times 10^{-6}$ cm$^2$s$^{-1}$.

In Figure 5.8 are shown results for labeled Aβ1-40 peptide at three different pH values (▶ 2.7, ○ 6.9, and ■ 11). The diffusion coefficient values at pH 6.7 and 11 were almost identical, (as observed in Figure 5.5 with the mixed Aβ sample), and remained constant over a period of two weeks. At pH 2.7, the diffusion coefficient did decrease significantly with time. When this result is converted to diameter via the Stokes-Einstein equation ($D_h = kT/6\pi\eta R$, where $\eta$ represents the viscosity), one finds an enormous
increase in hydrodynamic diameter from about 2.6 nm to about 860 nm. This observation correctly indicates the ability of FPR to follow a very wide range of diffusers. The decreases in diffusion in Figure 5.8 do not fully reflect the presence of macroscopically large aggregates that were visible immediately after lowering the pH.

Figure 5.8 Diffusion of 100 µM 5-carboxyfluorescein Aβ1-40 (100% labeled) in 50 mM PBS, 150 mM NaCl, pH values, ▶ 2.7, ○ 6.9, and ■ 11. Error bars are standard deviation of triplicate runs.

A fluorescence microscopy image of the aggregated peptide (100 µM 5-carboxyfluorescein Aβ1-40, 50 mM PBS, 150 mM NaCl, pH 2.7), is shown in Figure 5.9a, accompanied by an image of the stripe pattern in Figure 5.9b. Visible aggregates are present throughout, but they are not uniformly distributed; thus, the measured diffusion can vary within the sample cell, depending a little on the position chosen for measurement. One advantage of FPR is that one may choose visually to measure regions that do not possess extraordinarily large aggregates. If the striped pattern were to illuminate regions with many of these very large aggregates, the recovery of the signal
would be incomplete on the time scale of observations in this study. Very long observations might then reveal the rate of exchange of molecules into and out of the very large aggregates and or very slow diffusion along the fibrils.

Figure 5.9 (a) Fluorescence microscopy image of 100 µM 5-carboxyfluorescein Aβ1-40 in 50 mM PBS, 150 mM NaCl, pH 2.7. (b) microscopy image of Ronchi ruling stripe pattern in labeled gelatin.

The log-log curves of Figure 5.10a, demonstrate the effectiveness of FPR as a tool to detect simultaneously both the large, slow diffusers and the small, fast ones within a given sample. Over 99% of the contrast was relaxed in these measurements, indicating that immobile fragments were avoided. Even in cases where a large fragment is illuminated, such that the recovery levels out after some time, it is possible to determine the size of the mobile fraction by treating the immobile fraction as a baseline term during analysis.

Further analysis of the data curves in Figure 5.10a, was done by estimating the inverse Laplace transform, ILT, using an adaptation of Provencher’s\textsuperscript{143;144} CONTIN program. The corresponding CONTIN distributions of the data are shown in Figure 5.10b.
Figure 5.10 (a) Log-log FPR traces for Aβ samples at three pH values. After baseline subtraction, each semilogarithmic representation highlights double exponentiality characteristic of a polydisperse sample. Conditions: 100 µM 25/75 % mixture of 5-carboxyfluorescein Aβ and unlabeled Aβ in 50 mM PBS, 150 mM NaCl at pH values ▲ 2.7, ○ 6.9, and ■ 11. (b) CONTIN distributions of data in (a). (figure continued)
Figure 5.10 (continued) (c), (d), screen capture of the overlay of data, multiple exponentials and CONTIN. The roman numerals indicate: (I) simulated diffusion data, (II) ILT from CONTIN, (III) exponential analysis, (IV) error in CONTIN amplitude, (V) residuals of ILT calculated in CONTIN. (figure continued)
Figure 5.10 (continued) (e), screen capture of the overlay of data, multiple exponentials and CONTIN.

The major peak at pH 11 indicates that the majority of the diffusing species are narrowly distributed. The major peaks corresponding to pH 2.7 and 6.9, demonstrates slightly broader peaks than that of pH 11, with one (pH 6.9) and two (pH 2.7) additional smaller peaks. The broader peak represents the diffusion of the fast molecules, while the smaller peaks are assigned to the slowly diffusing species. The well-defined CONTIN peaks were an indication of the size range of the various species in solution. These results indicate that each pH environment induced different aggregation properties of the Aβ peptide.

Figure 5.10 (c), (d), and (e) displays CONTIN fits with compressed data and overlaid exponential fits corresponding to the decay behavior of single (c, pH 11) and multiple diffusers (d, pH 6.9, and e, 2.7). In Figure 5.10c, for the single diffusing species at pH 11, the average decay rate obtained by the ILT agreed with the single exponential
result (indicated by magenta vertical line dissecting the major peak). A fast diffuser with narrow distribution and large error bars is evident in both Figure 5.10c and d. This can be attributed to failure of the beginning data points to follow the trend in the exponential analysis. In Figure 5.10d, the fast decay mode of the ILT distribution failed to overlay with the results from triple exponential analysis. This suggests that a double exponential analysis would fit the data best. In Figure 5.10e, almost perfect agreement between the overlay of the ILT and triple exponential analysis is demonstrated.

![Graph showing effect of temperature on diffusion coefficient](image)

**Figure 5.11 Effect of temperature on the aggregation properties of Aβ peptide.**

Conditions: 100 µM Aβ in a 100 mM PBS, 150 mM NaCl, pH 7.4.

- **Effect of Temperature on Aβ Assembly**

  The effect of temperature on Aβ assembly was also investigated. Figure 5.11 shows the response in diffusion coefficient values as a function of increasing temperature. The high temperature was expected to induce the self-assembly of the Aβ
peptide. The results indicate that only a fast and slowly diffusing species were present from the initial physiological temperature, and the duration of the experiment. The trend in the diffusion coefficient implies that perhaps the Aβ sample had stabilized and the increasing temperature did not affect the structural integrity of the peptide.

5.3.2 Development of a Convenient Tool to Study β-amyloid Assembly

- Dialysis fluorescence photobleaching recovery

It was demonstrated that FPR was able to successfully characterize Aβ peptide. The sensitivity of this technique made it possible to distinguish between the sizes of several species in a heterogeneous solution. To further improve the economy of experimenting with the expensive ($640/mg) 5-carboxyfluorescein (labeled) Aβ and to test the efficacy of mediator peptides, an in situ FPR dialysis cell was developed. The advantages of this set-up include simplified studies on the effect of salt and pH on Aβ aggregation, as well as addressing the reversibility of the peptide in solution.

A schematic of the constructed dialysis FPR cell is shown in Figure 5.12. The housing (a top cover and a bottom chamber) of the cell was fabricated from PTFE. A mini peristaltic pump (Crouzet, France) with a motor speed of 60 rpm was used to circulate the exchange fluid through the subchamber from a 250 mL filter flask at a rate of about 20 mL/min. The microscope viewing piece was constructed with round microscope cover glass that was bonded for about 24 h at room temperature, under modest pressure, to an etched Teflon sheet with a thickness of 0.3 mm (Small Parts, Inc.) using Super Glue (a fast-curing cyanoacrylate formulation, Walmart). The dialysis membrane (Spectrum® Laboratories Inc. Spectra/Por #7) that forms the semi-permeable partition between the sample and exchange fluid had a 2000 Da MWCO. To prepare the
set-up, typically 250 µL of sample is sandwiched between the microscope viewing piece and the dialysis membrane. The inside of the bottom chamber (area underneath the dialysis membrane) is filled with 15 mL of the exchange fluid. When the dialysis housing is closed, the dialysis membrane is pressed against the etched Teflon space and in turn against the cover glass and the bottom chamber, using O-rings. The large dialysis area (~0.65 in.²), together with the thinness of the sample, about 0.3 mm, facilitates rapid dialysis.

Figure 5.12 Schematic and photograph of the dialysis FPR cell.
The kinetics of Aβ aggregation was monitored by dialyzing 50 µM of labeled Aβ sample against 10 mM citrate buffer at pH 5.0. The sample was initially solubilized in 10 mM KOH. Figure 5.13 shows the slow decline in diffusivity of Aβ under exposure to the weak citrate buffer. After about 1 hr, a slow diffuser appears, representing assemblies with a hydrodynamic radius of about 23 nm. This larger species, which accounted for about 18% of the Aβ present, would have been expected to grow to many times larger in time. This growth was not observed due to a problem with the dialysis cell. The problem commonly encountered is that the microscope cover glass tends to crack with prolonged dialysis. This may be attributed to the fragility of the glass or perhaps as a result of changes in the pressure of the system.

Figure 5.13 Evolution of protofibrils from labeled monomer after dialysis against a weak citrate buffer at pH 5.0. After ~ 1 hr, large aggregates appear and represent about 18% of the signal.
The dialysis of Aβ against citrate buffer demonstrated that it was possible to change the size of the peptide in a relatively short period of time. Thus, an alternate buffer in which the aggregation of the peptide was ‘delayed’ or rather, representative of typical growth kinetics of the peptide (where rapid aggregation was not induced) was desired. To achieve this, a new sample, composed of 50 µM labeled Aβ in 10 mM KOH was dialyzed against 100 µM Acetate buffer at pH 4.0. Figure 5.14 shows the time response of the diffusion coefficient of the peptide after first dialyzing against acetate buffer, followed by the addition of increasing concentrations of calcium chloride, 0.1 N HCl, and finally 50 mM phosphate buffer, pH 7.2. Both fast and slow diffusers were present after about 30 mins of sample dialysis. This was indicated by the biexponential analysis of the data. There was a slight decrease in the diffusion coefficient values, but, after 19 hrs of dialysis, the diffusion coefficient was almost identical within experimental error, to the initial diffusion coefficient value. The trend in the diffusion coefficient suggested that the aggregates had stabilized; otherwise the diffusion values would have been expected to continue to decrease after the first 5 hrs of dialyzing. At this point, it was best to change the course of the experiment.

Several choices were possible, but the effect of calcium chloride on the Aβ species present in solution was investigated. The required amount of CaCl₂ corresponding to 5 mM was added to the dialysis fluid and the sample was dialyzed for about 1 hr. The diffusion coefficient response remained biexponential and decreased with increasing calcium chloride concentration, up to 15 mM. After addition of 25 mM calcium chloride to the dialysis fluid, the diffusion coefficient remained almost identical to that obtained for 15 mM. The sample was dialyzed for an additional 2 hrs and measured again. The
The diffusion coefficient value increased slightly higher than that obtained with 15 mM \( \text{CaCl}_2 \), but overall, still smaller than when the sample was dialyzed against acetate buffer. The addition of \( \text{CaCl}_2 \) (up to 15 mM) facilitated the aggregation of A\( \beta \). Additional \( \text{CaCl}_2 \) (up to 25 mM), suppressed this effect and appeared to instead dissociate the A\( \beta \) aggregates. The reason for this is not obvious but can be explained as a change in the pH of the dialyzing solution due to the addition of calcium chloride. It can be proposed that calcium chloride binds certain amino acid residues in the A\( \beta \) peptide. Increasing the calcium chloride concentration may have created slightly acidic pH conditions which perhaps destabilized the aggregates. This can be compared to the effect of the divalent metal Zn(II) on A\( \beta \) aggregation. Zn(II) was found to promote A\( \beta \) aggregation at normal pH while suppressing A\( \beta \) aggregation at slightly acidic pH conditions. At this point in the experiment it appeared that the formation of aggregates was sequestering the fluorophore. This was evident by a significant loss of fluorescence in the sample and the appearance of black (non-fluorescent) aggregates. One explanation why the sample had become difficult to bleach may simply be that with extensive dialysis, any source of oxygen present in the sample had become depleted. The pH of the sample was adjusted with 0.1 N HCl, to a final pH of about 1. The sample was dialyzed for almost 17 hrs and subsequently measured. The sample had regained full fluorescence intensity and apparently the biexponential nature had disappeared and the diffusion coefficient was now a single exponential value. The dialysis fluid was then exchanged with 50 mM phosphate buffer, pH 7.2. The diffusion coefficient remained single exponential and increased to a value higher than that of the 0.1 N HCl.
Figure 5.14 One Pot dialysis of 50 µM 5-carboxyfluorescein labeled Aβ starting at pH 11, then dialyzing against 100 µM acetate buffer, pH 4, increasing concentrations of calcium chloride, 0.1 N HCl and 50 mM phosphate buffer, pH 7.2.

The percent amplitude corresponding to each biexponential point plotted in Figure 5.14 is displayed in Figure 5.15. Since each point represent an average of triplicate runs, the signal amplitude of each individual run was plotted. It appeared that the larger species were dominant in solution and represented about 75% of the sample. This value fluctuated slightly between 60% and 75% when the sample was dialyzed for a period of time in acetate buffer. Since different regions within the sample were chosen for measurement, the variation in the signal amplitude suggests that different species were present. This variation may also indicate that perhaps some regions within the cell experienced more perturbation from the pulsating action of the dialysis fluid circulating through the subchamber. This experiment showed the potential of the in situ dialysis cell to vary pH in small steps and also follow the changes from the addition of salt. If one
considers the variation of pH environments that proteins encounter within the cell, then
the in situ dialysis cell may be able to simulate such cellular environments to provide
information about the structural changes of a proteins’ passage through such changing
environments.

![Graph showing percent amplitude of fast and slow components of FPR diffusion coefficient values in Figure 5.14.](image)

Figure 5.15 Percent amplitude of fast and slow components of FPR diffusion coefficient values in Figure 5.14.

The reversibility of Aβ aggregation through pH changes was studied by
alternating the pH within the dialysis cell. First, the effect of pH on the diffusion
coefficient of labeled Aβ, without addition of unlabeled material was monitored. Figure
5.16 shows the diffusion coefficient of 100 µM labeled Aβ initially dissolved in 10 mM
KOH alternately dialyzed against 50 mM PB, pH 2.7, and 50 mM PB, pH 7.2. Since the
system is dilute, the diffusion coefficient can be converted to a hydrodynamic radius
through Stokes’ law. The hydrodynamic radius of the peptide at pH 7.2 is about 1.5 nm,
typical of monomeric Aβ peptide. At pH 2.7, the formation of protofibrils resulted in decreased diffusion coefficient, corresponding to a hydrodynamic radius of about 90 nm. The sample was then dialyzed back against the phosphate buffer at pH 7.2. The diffusion coefficient rose to the starting value. After periodically measuring the diffusion coefficient while the sample was being dialyzed, for about 15 hrs, there was no significant change in the diffusion coefficient value. The sample was not dialyzed back against low pH as the microscope cover glass broke. The same experiment was repeated with an alternate sample that contained unlabeled Aβ peptide.

Figure 5.16 Diffusion from in situ FPR of 50 µM 5-carboxyfluorescein-Aβ starting at pH 11, then alternately dialyzed between pH 2.7 and pH 7.4.

Figure 5.17 shows the time response of the diffusion coefficient of 100 µM 5-carboxyfluorescein-Aβ (25% mixed with unlabeled 75% Aβ), starting at pH 11 and alternately dialyzed against 50 mM phosphate buffer, pH 2.7 and 50 mM phosphate buffer, pH 7.4. The trend in the diffusion coefficient was similar to that obtained for the
labeled Aβ peptide in Figure 5.16. The diffusion coefficient was converted to hydrodynamic radius and at pH 7.4 the Aβ had a hydrodynamic radius of about 1.5 nm. At the lower pH, 2.7, the hydrodynamic radius was 85 nm at the first cycle and decreased to 46 nm on the second. Additional studies were done to determine the aggregation limit of the Aβ peptide through dialysis. When the Aβ peptide was extensively dialyzed for a week at pH 7.4, the diffusion coefficient started and remained stabilized at the same value \((1.75 \times 10^{-6} \text{ cm}^2 \text{s}^{-1})\) representative of a monomeric Aβ peptide.

![Figure 5.17 Diffusion from in situ FPR of 100 µM 5-carboxyfluorescein-Aβ (25% mixed with unlabeled 75% Aβ) starting at pH 11, then alternately dialyzed between pH 2.7 and pH 7.4.](image)

**5.3.3 Interaction of Aβ Peptide with Peptide Mediators**

- Non-fibril forming Accelerators

  The hydrophobic core, KLVFF, of the Aβ peptide has been used by several researchers to design inhibitors of Aβ aggregation and dissolution of pre-formed fibrils.
Peptides containing alpha-alpha disubstituted amino acids (ααAA) were designed and prepared at LSU as potential inhibitors of Aβ fibril formation. The Murphy and Meredith peptides (designed by Regina M. Murphy at University of Wisconsin and Stephen C. Meredith at the University of Chicago, respectively) were also prepared at LSU and used as control. FPR was used to identify mechanistic differences in how the different mediator peptides interact with Aβ. Samples were prepared at a 1:1 ratio with 100 μM 5-carboxy-fluorescein Aβ and mediator peptides in 50 mM PBS, 150 mM NaCl, pH 7.4. A sample composed of 100 μM 5-carboxy-fluorescein Aβ and a mixture of 5-carboxy-fluorescein Aβ with unlabeled Aβ peptide was also measured for comparison. Figure 5.18 shows the diffusion coefficient as a function of time for 5-carboxy-fluorescein Aβ and mediator peptides: Amy1, Amy2, Modified Amy, Murphy, and Meredith. The diffusion coefficient of all the Aβ and mediator peptide mixtures tended to decrease after fifteen hours of sample preparation, with the exception of fluorescein-Aβ: Amy1 which seemed to maintain a constant diffusion coefficient value for the duration of the experiment. The sample mixture of 5-carboxy-fluorescein Aβ and unlabeled peptide had the fastest diffusion coefficient values while the fluorescein Aβ: Modified Amy mixture had the lowest diffusion coefficient value overall. These results are an early indication that the mediator peptides all have different modes of interaction with the Aβ peptide. The results mostly seem to indicate that the mode of inhibition may not involve shrinkage of the Aβ peptide, as the decreasing trend in the diffusion coefficient values implies increasing particle size. The diffusion coefficient results of the mixture of 5-carboxy-fluorescein Aβ and unlabeled peptide is a bit perplexing as the 5-carboxy-fluorescein Aβ would have been expected to have the fastest diffusion coefficients. Perhaps, the unlabeled peptide is
causing the 5-carboxy-fluorescein Aβ to collapse on itself, becoming smaller as a result. Another explanation might be that the unlabeled Aβ peptide repels the 5-carboxy-fluorescein Aβ molecules, making them freer to move in solution. Whatever the scenario, the diffusion coefficient results are indicative of small molecules with increased mobility.

The results from FPR studies on the interaction of Aβ with mediator peptides indicated that the 5-carboxy-fluorescein labeled Aβ peptide could track changes in the Aβ peptide aggregation kinetics in the presence of peptide-based mediators. Thus, the question of whether the labeled Aβ peptide could be substituted for Thioflavin T (ThT) as an alternate means of monitoring fibril growth or disassembly was addressed.

Figure 5.18 Diffusion coefficients of Aβ and peptide-based mediators as a function of time. Conditions: 100 µM 5-carboxy-fluorescein labeled Aβ and 100 µM 5-carboxy-fluorescein labeled Aβ with 100 µM: Amy1, Amy2, Modified Amy, Murphy, and Meredith in 50 mM PBS, 150 mM NaCl, pH 7.4.
Thioflavin T is a fluorescence probe that exhibits enhanced fluorescent properties upon binding to amyloid fibrils. Several studies have utilized ThT fluorescence for monitoring Aβ kinetics. While ThT fluorescence is a highly sensitive probe for detecting fibril formation, its earliest binding activity has been reported to commence with the oligomeric form of the Aβ peptide. Perhaps, the 5-carboxy-fluorescein labeled Aβ peptide would be able to detect even earlier stages of Aβ aggregation and therefore eliminate the need for ThT. Ultimately, it could be used for screening the efficacy of Aβ mediators. Fluorescence spectroscopy, using 5-carboxy-fluorescein labeled Aβ as the probe, was used to monitor the kinetics of amyloid fibril formation and the effect of peptide mediators on Aβ aggregation. FPR measurements were done on the same samples for quantitative assessment of the data.

Initial fluorescence spectroscopy experiments were done with different concentrations of the 5-carboxy-fluorescein labeled Aβ and mixtures with unlabeled peptide to determine the optimal concentration whereby the sensitivity limit of the detector would not be exceeded. Subsequently, a series of samples were prepared by diluting appropriate concentrations of Aβ peptide and peptide-based mediator stock solutions directly (to take advantage of its high-throughput capabilities) into a 96-well plate for fluorescence spectroscopy measurements. Aliquots of the same samples were loaded in 0.1-mm-path-length rectangular microslides (Vitrocom) by capillary action, and the microslides were flame-sealed for FPR measurements.

The fluorescence spectroscopy results had no meaningful interpretation. A screen capture of the results plotted in real time in an Excel spreadsheet by the FLUOstar Galaxy software is shown in Appendix A. A table showing a 96-well plate layout with
the corresponding sample identification is also shown in Appendix A. While the 96-well plate is highly efficient for fluorescence spectroscopy, it proved a rather daunting task to perform the FPR experiment with all the samples on the same day. Table 5.1 summarizes the diffusion coefficient results for Aβ and Aβ in the presence of various peptide mediators obtained by FPR. The LAβ samples are composed of 0.1 µM 5-carboxy-fluorescein labeled Aβ and LAβAβ represent mixtures of 0.1 µM 5-carboxy-fluorescein labeled Aβ and 25 µM unlabeled Aβ. The FPR results show that under physiological conditions (50 mM PBS, 150 mM NaCl, pH 7.4, incubated at 37 °C for one day), all the samples (Aβ and Aβ mixed with peptide-based mediators) had a diffusion coefficient representative of a predominantly low oligomeric state (monomer-dimer in equilibrium) value, with the exception of LAβAβ-Amy1 and LAβAβ-Amy2. Both the labeled Aβ and the mixture of labeled Aβ and unlabeled Aβ had diffusion coefficient values that were similar within experimental error. In subsequent experiments a mixture of labeled Aβ and unlabeled Aβ was chosen in an effort to limit the presence of the fluorophore and have a more native-peptide-like sample. The additional diffusion coefficient value for the LAβAβ-Amy1 peptide was only 3.5% of the single amplitude. This suggests that a very small amount of higher order aggregates were present in the solution. The FPR measurements of the LAβAβ-Amy2 sample gave diffusion coefficient values representative of predominantly slowly diffusing molecules, corresponding to large aggregates (observed visually at 18× magnification in the light microscope). The slowly diffusing species represented about 80% of the signal amplitude. The data for two of the three runs of the LAβAβ-Amy2 sample were biexponential and the third run fitted best with a triple exponential analysis. The significant difference in the percentage of
aggregates present in the LAβAβ-Amy1 and LAβAβ-Amy2 samples indicate that the
peptides interact with Aβ via different mechanisms. It was necessary to perform
additional measurements on fresh samples of LAβAβ-Amy1 and LAβAβ-Amy2 at
shorter time scales to determine how quickly those aggregates formed.

Several samples composed of different ratios of 5-carboxyfluorescein labeled Aβ,
unlabeled Aβ and Amy2 peptide were prepared to monitor the kinetics of Aβ and Amy2
mediator peptide assembly. Table 5.2 shows the summary of diffusion coefficient results
for Aβ and Aβ: Amy2 peptide mediator obtained by FPR.

Table 5.1 Summary of FPR diffusion coefficient results for 5-carboxy-fluorescein labeled
Aβ (LAβ) and peptide-based mediators. LAβAβ represents the code name for mixtures of
5-carboxy-fluorescein labeled Aβ and unlabeled Aβ. The error represents standard
deviation determined by triplicate measurements of the sample. a Only one of the
replicate runs fitted with a triple-exponential analysis. b The percent amplitude of the
single run with triple-exponential analysis.

<table>
<thead>
<tr>
<th>Sample (50 mM PBS, 150 mM NaCl, pH 7.4 – incubated at 37 °C for 1 day)</th>
<th>Diffusion Coefficient (cm²s⁻¹) (% - Average Percent Amplitude of Signal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 µM LAβ</td>
<td>(1.92 ± 0.193) × 10⁻⁶</td>
</tr>
<tr>
<td>0.1:25 µM LAβAβ</td>
<td>(2.15 ± 0.031) × 10⁻⁶</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Murphy</td>
<td>(1.76 ± 0.0669) × 10⁻⁶</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Meredith</td>
<td>(2.24 ± 0.0305) × 10⁻⁶</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Modified</td>
<td>(2.15 ± 0.170) × 10⁻⁶</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Amy1</td>
<td>(1.46 ± 0.198) × 10⁻⁶</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Amy2</td>
<td>3.8 × 10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>(2.45 ± 1.62) × 10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>(1.94 ± 0.017) × 10⁻⁹</td>
</tr>
</tbody>
</table>

a Only one of the replicate runs fitted with a triple-exponential analysis.
b The percent amplitude of the single run with triple-exponential analysis.
There was no significant difference in the diffusion coefficient values for different ratios of Amy2 in the presence of 5-carboxy-fluorescein labeled Aβ. In the presence of almost equal molar ratios of Aβ and Amy2 mediator, the data fitted best with triple exponential analysis for all three runs.

The largest species represented about 60% of the signal amplitude, while each of the remaining species represented about 20% of the signal amplitude. When the molar ratio of the Amy2 peptide increased to 2:1 of Amy2: LAβAβ, only two exponentials were observed with the slower diffuser dominant at 78%. The molar ratio of Amy2 peptide was increased to 10:1 of Amy2: LAβAβ. At this ratio, only one diffusing species was observed with a diffusion coefficient value representative of monomeric Aβ. The interaction of Amy2 mediator with the Aβ peptide indicated that large aggregates were formed almost immediately upon placing the mediator in the presence of Aβ. At the highest molar ratio (10:1) of Amy2 to Aβ peptide, the characteristic formation of large aggregates was absent, indicating that the excess mediator peptide may be blocking the assembly of the Aβ peptide. A three dimensional, 3-D, representation of the distributions of the various species present is a mixture of LAβAβ: Amy2 at a ratio of 0.1:25:25 µM is shown in Figure 5.19. Immediately after preparing the samples, there were three species in solution, the largest represented the broad peak and the smallest represented the small peak. After one day of incubation at room temperature, the broad peak has became bigger, while the small and middle peaks are now represented by one small peak. Three days after sample preparation, only one broad peak is observed. The distribution trends suggest that the initial amounts of monomeric and low oligomeric peptide got depleted to compensate for the growth of the larger aggregates.
Table 5.2 Summary of FPR diffusion coefficient results for 5-carboxy-fluorescein labeled Aβ (LAβ) and Amy2. LAβAβ represents the code name for mixtures of 5-carboxy-fluorescein labeled Aβ and unlabeled Aβ. The error represents standard deviation determined by triplicate measurements of the sample.

<table>
<thead>
<tr>
<th>Sample (50 mM PBS, 150 mM NaCl, pH 7.4– incubated at 37 °C for 1 day)</th>
<th>Ratio</th>
<th>Diffusion Coefficient (cm²s⁻¹)</th>
<th>(% - Average Percent Amplitude of Signal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1:25 µM LAβ-Amy2</td>
<td>0.1:1</td>
<td>(2.72 ± 1.27) × 10⁻⁸</td>
<td>32%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.65 ± 1.92) × 10⁻⁹</td>
<td>68%</td>
</tr>
<tr>
<td>0.1:50 µM LAβ-Amy2</td>
<td>0.1:2</td>
<td>(3.74 ± 0.938) × 10⁻⁸</td>
<td>27%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.61 ± 0.353) × 10⁻⁹</td>
<td>73%</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Amy2</td>
<td>0.1:1:1</td>
<td>(9.86 ± 1.57) × 10⁻⁸</td>
<td>20.7%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.49 ± 0.380) × 10⁻⁸</td>
<td>17.83%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.4 ± 0.033) × 10⁻⁹</td>
<td>61.5%</td>
</tr>
<tr>
<td>0.1:25:50 µM LAβAβ-Amy2</td>
<td>0.1:1:2</td>
<td>(1.72 ± 2.06) × 10⁻⁷</td>
<td>22%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.31 ± 0.437) × 10⁻⁹</td>
<td>78%</td>
</tr>
<tr>
<td>0.1:25:250 µM LAβAβ-Amy2</td>
<td>0.1:1:1</td>
<td>(2.53 ± 0.153) × 10⁻⁶</td>
<td>100%</td>
</tr>
</tbody>
</table>

This clearly implies that Amy2 alters the rate of Aβ assembly by forming large aggregates. Etienne et al.¹⁵⁰ suggested that the Amy2 mediator peptide, (oligolysine on the N-terminus) disrupts Aβ assembly only at the hydrophilic N-terminus of the Aβ peptide. When the Amy2 mediator peptide was interacted with only labeled Aβ, which has a 5-carboxy-fluorescein molecule attached to the N-terminus of the Aβ peptide, the formation of large aggregates was still observed, similar to studies done with Amy2 and
Aβ peptide without an attached fluorophore. This may be further validation to the claim that the attachment of a fluorophore does not disrupt the normal structural activity of the Aβ peptide or it may be an indication that the Amy2 peptide has alternate binding sites other than the N-terminus of the Aβ peptide.

Similar FPR studies were done with the Amy1 mediator peptide in the presence of a mixture of 5-carboxy-fluorescein labeled Aβ and unlabeled Aβ (LAβAβ). Table 5.3 summarizes the diffusion coefficient of the Amy1 mediator peptide in the presence of a mixture of 5-carboxy-fluorescein labeled Aβ and unlabeled Aβ at different time periods. The sample composition was 0.1 µM 5-carboxy-fluorescein labeled Aβ and a one to one molar ratio of unlabeled peptide and Amy2 mediator peptide at 25 µM, in 50 mM PBS, 150 mM NaCl, pH 7.4.

Figure 5.19 3-D representation of the diffusion coefficient data from LAβAβ: Amy2 at a ratio of 0.1:25:25 µM.
The diffusion coefficient result obtained at zero hours and eight hours were almost identical within experimental error. After one day of incubation at 37 °C, the presence of a small percentage (4%) of a larger species was observed. After three days, the percentage of the larger species remained almost constant with a negligible 1% increase in the signal amplitude on day 3.

Table 5.3 Summary of FPR diffusion coefficient results for Amy1 in the presence of 5-carboxy-fluorescein labeled Aβ and unlabeled Aβ (LAβAβ). LAβAβ represents the code name for mixtures of 5-carboxy-fluorescein labeled Aβ with unlabeled Aβ. The error represents standard deviation determined by triplicate measurements of the sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ratio</th>
<th>Diffusion Coefficient (cm²s⁻¹)</th>
<th>(% - Average Percent Amplitude of Signal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(50 mM PBS, 150 mM NaCl, pH 7.4– incubated at 37 °C for 1 day)</td>
<td>0.1:1:1</td>
<td>(2.05 ± 0.185) × 10⁻⁶</td>
<td>100%</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Amy1</td>
<td>0hrs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Amy1</td>
<td>8hrs</td>
<td>(2.24 ± 0.046) × 10⁻⁶</td>
<td>100%</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Amy1</td>
<td>1 day</td>
<td>(1.46 ± 0.198) × 10⁻⁶</td>
<td>96%</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Amy1</td>
<td>2 days</td>
<td>(1.61 ± 0.101) × 10⁻⁶</td>
<td>96%</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Amy1</td>
<td>3 days</td>
<td>(1.66 ± 0.014) × 10⁻⁶</td>
<td>95%</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Amy1</td>
<td></td>
<td>(3.82 ± 1.22) × 10⁻⁹</td>
<td>4%</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Amy1</td>
<td></td>
<td>(1.01 ± 0.342) × 10⁻⁸</td>
<td>4%</td>
</tr>
<tr>
<td>0.1:25:25 µM LAβAβ-Amy1</td>
<td></td>
<td>(1.82 ± 0.225) × 10⁻⁸</td>
<td>5%</td>
</tr>
</tbody>
</table>

Figure 5.20 shows a graphical representation of the diffusion coefficient values found in Table 5.3. The fast diffusers are clearly dominant throughout the experimental time.
frame. Although the percentages of the larger diffusers are consistently low, the general trend in the diffusion coefficient values indicates that the sizes of these species are getting smaller with time. This suggests that the Amy1 mediator is disrupting the assembly of the Aβ to create smaller particles.

Figure 5.20 Diffusion coefficients as a function of time for Amy1 in the presence of 5-carboxy-fluorescein labeled Aβ and unlabeled Aβ (LAβAβ). Conditions: 0.1 µM labeled Aβ and a 1:1 molar ratio of unlabeled Aβ and Amy1 at 25 µM in 50 mM PBS, 150 mM NaCl, pH 7.4.

FPR studies were performed with all the mediator peptides (Amy1, Amy2, Modified Amy, Murphy, Meredith) including the addition of another potential mediator molecule, one-directional [9]-6 arborol. One-directional arborols ([9]-n) are amphiphilic, tree shaped dendrimers with nine hydroxyl groups ([9]-) forming the hydrophilic head and an alkyl chain as the hydrophobic moiety (n stands for the number of carbons in the alkyl chain; in this case, n = 6). Sun discovered that the arborol molecules
exhibited almost similar fibrillar self-assembly properties to the Aβ peptide. Could the shorter, one-directional [9]-6 arborol molecule interact with the Aβ peptide to inhibit the amyloid fibril assembly? The amphiphilic nature of the one-directional [9]-6 arborol, plus the fact that it has a much smaller molecular weight than most of the designed and costly peptide-based amyloid inhibitors are some desirable properties that makes this potential amyloid fibril mediator worth investigating. The structure of the one-directional 9-6 arborol is shown in Figure 5.21.

![Figure 5.21 Structure of one-directional [9]-6 arborol.](image)

The diffusion coefficient values as a function of time for each mediator and Aβ peptide combinations are shown. For the purpose of clarity, the data is presented as individual plots for each sample. Figure 5.22 shows the diffusion behavior of 10 µM 5-carboxy-fluorescein Aβ as a function of time. For a period of one week the diffusion coefficient remained at an almost constant value with a slight decrease at days 5 and 7. The diffusion coefficient was representative of values for monomeric Aβ (refer also to
Figure 5.23 shows the diffusion behavior of 10 µM 5-carboxy-fluorescein Aβ mixed with 25 µM unlabeled Aβ as a function of time. For the first three days the diffusion coefficient remained at an almost constant value, characteristic of the ‘lag phase’ that precedes fibril growth. At days 5 and 7, the presence of larger aggregates was observed.

![Graph showing diffusion coefficients as a function of time for 5-carboxy-fluorescein Aβ.](image)

Figure 5.22 Diffusion coefficients as a function of time for 5-carboxy-fluorescein Aβ (5-CF-Aβ). Conditions: 10 µM 5-carboxy-fluorescein Aβ was prepared in 50 mM PBS, 150 mM NaCl, pH 7.4.

Initially, the larger species represented only 2% of the signal amplitude, but by the seventh day, that value had increased dramatically to 32%. This demonstrates the ‘seeding’ effect of the Aβ. The presence of small amounts of seeds was sufficient to initiate Aβ peptide assembly.

Figure 5.24 shows the diffusion behavior of 10 µM 5-carboxy-fluorescein Aβ and a one-to-one molar ratio of unlabeled peptide and Amy1 mediator peptide at 25 µM, in 50
mM PBS, 150 mM NaCl, pH 7.4. Initially, only a single diffusion coefficient is observed. Measurements on subsequent days indicated that larger species were present in solution. The large difference in the diffusion coefficient from the first day of sample measurement suggests that the Amy1 peptide is most likely inducing the aggregation state of the peptide as results for the control peptide indicated that only small species were present in solution up to about the fifth day of measurement. The large differences (randomness) in the percent signal amplitudes simply reflect the heterogenous nature of the sample. As different regions of the sample are selected for photobleaching during FPR measurement, this implies that the aggregates are not uniformly distributed within the sample cell.

Figure 5.23 Diffusion coefficients as a function of time for 5-carboxy-fluorescein Aβ (5-CF-Aβ) mixed with unlabeled Aβ. Conditions: 10 µM 5-carboxy-fluorescein Aβ mixed with 25 µM unlabeled Aβ prepared in 50 mM PBS, 150 mM NaCl, pH 7.4.
Figure 5.25 shows the diffusion behavior of 10 µM 5-carboxy-fluorescein Aβ and a one-to-one molar ratio of unlabeled peptide and Amy2 mediator peptide at 25 µM, in 50 mM PBS, 150 mM NaCl, pH 7.4. In contrast to Amy1 and the control samples, large aggregates were observed immediately after sample preparation. This is reflected in the range of diffusion values shown in Figure 5.25. There was no time dependence on the aggregation behavior of the Amy2 peptide with Aβ as the lowest diffusion values seem to be stabilized around \((3.51 \pm 1.8) \times 10^{-10} \text{ cm}^2\text{s}^{-1}\).

Figure 5.24 Diffusion coefficients as a function of time for 5-carboxy-fluorescein Aβ (5-CF-Aβ) mixed with unlabeled Aβ and Amy1. Conditions: 10 µM 5-carboxy-fluorescein Aβ mixed with 1:1 molar ratio of unlabeled Aβ and Amy1 at 25 µM, prepared in 50 mM PBS, 150 mM NaCl, pH 7.4.

Figure 5.26 shows the diffusion behavior of 10 µM 5-carboxy-fluorescein Aβ and a one-to-one molar ratio of unlabeled peptide and Modified Amy mediator peptide at 25 µM, in 50 mM PBS, 150 mM NaCl, pH 7.4. The trend in the diffusion coefficient results appears identical to that of the control peptide in Figure 5.23. The only difference is in
the percent signal amplitude on day 7, where the Modified Amy peptide has a higher percentage of large diffusers than the control Aβ peptide. This suggests that the Modified Amy mediator peptide has a *cat and mouse* approach to mediating Aβ peptide aggregation.

![Graph](image)

**Figure 5.25** Diffusion coefficients as a function of time for 5-carboxy-fluorescein Aβ (5-CF-Aβ) mixed with unlabeled Aβ and Amy2. Conditions: 10 µM 5-carboxy-fluorescein Aβ mixed with 1:1 molar ratio of unlabeled Aβ and Amy2 at 25 µM, prepared in 50 mM PBS, 150 mM NaCl, pH 7.4.

Both peptides co-exist in solution with the Modified Amy being the *watch(cat)* of the Aβ peptide activity. It appears that as soon as any Aβ aggregation begins, the Modified Amy works to destroy those aggregates most likely by binding to Aβ. The Murphy and Meredith peptides, Figure 5.27 and 5.28 respectively, both seem to utilize similar mechanisms for mediating Aβ peptide assembly.
Figure 5.26 Diffusion coefficients as a function of time for 5-carboxy-fluorescein Aβ (5-CF-Aβ) mixed with unlabeled Aβ and Modified Amy. Conditions: 10 µM 5-carboxy-fluorescein Aβ mixed with 1:1 molar ratio of unlabeled Aβ and Modified Amy at 25 µM, prepared in 50 mM PBS, 150 mM NaCl, pH 7.4.

Figure 5.29 top, shows the diffusion behavior of 10 µM 5-carboxy-fluorescein Aβ and a one to one molar ratio of unlabeled peptide and the one-directional [9]-6 arborol molecule at 25 µM, in 50 mM PBS, 150 mM NaCl, pH 7.4. The trend in the diffusion results seems to mimic that of Amy2 with the exception that no large diffusers were observed on day 1. The one-directional [9]-6 arborol has been found to be very surface active molecules. Transmission electron microscopy (TEM) of Aβ in the presence of one-directional [9]-6 arborol revealed the presence of large micellar-type structures similar to results obtained for one-directional [9]-6 arborol alone at concentrations exceeding its critical micelle concentration (CMC). It appears that the one-directional [9]-6 arborol is behaving like a cosurfactant to stabilize the Aβ aggregation.
Figure 5.27 Diffusion coefficients as a function of time for 5-carboxy-fluorescein Aβ (5-CF-Aβ) mixed with unlabeled Aβ and Murphy. Conditions: 10 µM 5-carboxy-fluorescein Aβ mixed with 1:1 molar ratio of unlabeled Aβ and Murphy at 25 µM, prepared in 50 mM PBS, 150 mM NaCl, pH 7.4.

Figure 5.28 Diffusion coefficients as a function of time for 5-carboxy-fluorescein Aβ (5-CF-Aβ) mixed with unlabeled Aβ and Meredith. Conditions: 10 µM 5-carboxy-fluorescein Aβ mixed with 1:1 molar ratio of unlabeled Aβ and Meredith at 25 µM, prepared in 50 mM PBS, 150 mM NaCl, pH 7.4.
To get a better idea of the nature of the species that formed in solution, an aliquot of the sample was examined by TEM. Figure 5.29, middle and bottom, shows TEM images of the 10 µM 5-carboxy-fluorescein Aβ and a one to one molar ratio of unlabeled peptide and the one-directional [9]-6 arborol molecule at 25 µM, in 50 mM PBS, 150 mM NaCl, pH 7.4 negatively stained with 2% uranyl acetate. The top image was taken at a magnification of 50KX and the bottom image was taken at a magnification of 100 KX. The small, spherical-type lighter areas in the images are characteristic of the aggregation behavior of the arborol molecule. Although the top image appeared to have some ‘barely’ visible fibrillar-mesh in the background, closer examination of the sample at higher magnification did not reveal the obvious presence of fibrils.

Figure 5.29 Top: Diffusion coefficients as a function of time for 5-carboxy-fluorescein Aβ (5-CF-Aβ) mixed with unlabeled Aβ and one-directional 9-6 arborol. Conditions: 10 µM 5-carboxy-fluorescein Aβ mixed with 1:1 molar ratio of unlabeled Aβ and Amy1 at 25 µM, prepared in 50 mM PBS, 150 mM NaCl pH 7.4. (figure continued)
Figure 5.29 (continued) Middle and Bottom: TEM images of the 5-carboxy-fluorescein Aβ (5-CF-Aβ) mixed with unlabeled Aβ and one-directional 9-6 arborol negatively stained with 2% Uranyl acetate. Middle image was taken at a magnification of 50KX and the Bottom image was taken at a magnification of 100 KX.

A dynamic light scattering experiment was used to follow the behavior of the mediator peptides in the presence of Aβ. Figure 5.30 shows a simple DLS experiment designed to assess the behavior of the Murphy peptide against large Aβ fibrils. After disrupting the large and slowly growing fibrils by sonication, the system remains stable.
for hours. This suggests that aged, broken fibrils are not easily healed. Addition of the Murphy peptide rapidly accelerates fibril growth. This experiment is effective for screening mediator peptides and can help guide the synthesis of new materials.

Figure 5.30 Growth kinetics of β-amyloid at physiological pH. β-amyloid was dissolved in neat DMSO at a concentration of 1 mg/mL followed by dilution in 50 mM PBS, 150 mM NaCl, pH 7.4, yielding a concentration of 100 µM. The apparent hydrodynamic radius was determined from cumulant analysis of dynamic light scattering data taken at 90° scattering angle.

Figure 5.31 shows the dynamic light scattering results for Aβ in the presence of Amy1 and Amy2 mediator peptides at two different ratios (1:1 and 1:5). The hydrodynamic radius of the Aβ is greatly accelerated in the presence of Amy2. In contrast, in the presence of the Amy1 peptide, the Aβ is only slightly larger than the control.
Figure 5.31 Dynamic light scattering results of Aβ and its interaction with peptide-based inhibitors. The hydrodynamic radius if plotted as a function of time.

5.4 Conclusions

Fluorescence photobleaching recovery (FPR) studies on 5-carboxyfluorescein labeled β-amyloid peptide solutions readily confirmed the coexistence of large and small species. This routine experiment can effectively screen inhibitors and can guide the synthetic enterprise. To address the question of reversibility, and to improve the economy of experimenting with expensive Aβ peptide and inhibitors, *in situ* dialysis cells for DLS and FPR were developed. The reversibility of the peptide conformation from the low oligomeric state to the aggregated state under neutral and acidic pH conditions and confirmed that the peptide growth increased with increasing ionic strength.
6.1 Introduction

The earliest experimental studies to predict that lipid molecules in membranes were organized bilayers were done by E. Gorter and F. Grendel in 1925. Later, studies performed with instrumental techniques such as X-ray diffraction, freeze-fracture electron microscopy and X-ray crystallography helped confirm that the lipid bilayer provides the basic structure of biological membranes. These experiments provided vital evidence to support the fluid mosaic model of membrane structure proposed by S. J. Singer and G. L. Nicolson in 1972. Although the Singer model appears simple in view of the considerable progress that has been made in unraveling the complexity of biological membranes, it remains a useful starting point for comprehending cellular membrane structure.

The major constituent of all biological membranes is lipid molecules (30 – 80%), proteins (20 – 60%) and carbohydrates (0 – 10%). The lipid bilayer has a typical membrane thickness of 4-5 nm and has been described as a fluid matrix, within which protein molecules are attached, adsorbed, inserted or embedded. Among the major types of lipids present in the cell membrane, the phospholipids are the most common, followed by cholesterol and glycolipids. The structure, composition and interaction of a lipid bilayer with membrane proteins determine its function. An important property of a lipid layer which is crucial to many of its function is its fluidity. The degree of fluidity may vary from being highly fluid to a partially fluid state depending on the arrangement and composition of membrane components. For example, cholesterol molecules are important
in maintaining the flexibility and stability of the cell membrane, but affect the lipid bilayer by decreasing its fluidity. Cell membranes must be in a relatively fluid state to maintain their normal biological function. Thus, it is necessary to understand the structure and dynamic properties of lipid molecules within the membrane environment, combined with their interaction with proteins as this may provide insight into the orientation and structural transformation that occurs upon insertion of proteins into lipid membranes.\textsuperscript{158}

The discovery of liposomes (small artificial lipid vesicles) by A. Bangham in 1962\textsuperscript{159} provided a suitable model system that closely mimicked natural cell membranes to study such fundamental processes.\textsuperscript{160,161} Several unique properties make vesicles ideal artificial membrane mimics.\textsuperscript{162,163} One advantage of lipid vesicles is their ability to form spontaneously in aqueous environments and self-assemble into structures such as monolayers or bilayers on solid supports (glass, quartz, mica, silicon chips).\textsuperscript{158,164-168} Additional properties such as permeability to ions, and versatility (it is possible to vary and control composition, pH, and ionic strength) have made it possible to study a variety of physical parameters of these systems. For instance, tremendous effort is being applied to the study of the interactions and binding properties of various proteins and other molecules to lipid membrane surfaces. Extensive documentation exists on the potential uses of liposomes in a range of biological applications.\textsuperscript{169-171}

Although scientists continue to gain deeper understanding of lipid vesicles and have, in some cases, been able to correlate these fundamental properties with those of natural membranes, major areas of research remain to be explored.\textsuperscript{172} Tremendous effort is being directed towards development of biophysical techniques, and progress is being made in the areas of pathogen attack,\textsuperscript{173} inflammatory response, design of more realistic
and sophisticated model membrane systems (tethered polymer-supported lipid bilayer artificial membranes),\textsuperscript{174-176} and membrane pathologies that result in cellular dysfunction.\textsuperscript{177}

The objective of this work is to develop model membrane systems to study the interaction between \(\beta\)-amyloid peptide (\(\text{A}\beta\)) and lipid bilayers and understand how the lipid membrane affects the properties of \(\text{A}\beta\) (and vice versa). Specifically, the effect the \(\text{A}\beta\) peptide attribute to the fluidity of the lipid membrane will be addressed. The results from several studies are misleading as some indicate that \(\text{A}\beta\) alters the membrane by increasing its fluidity while others report the opposite effect. The reader is directed to a review article by Wood \textit{et al.}\textsuperscript{178} for a summary of the studies dealing with the effects of \(\text{A}\beta\) on membrane fluidity both in model and biological membranes. The experimental results are clearly controversial and further investigations with suitable model systems and biophysical techniques are needed.

\textbf{6.2 Interaction of \(\beta\)-amyloid with Lipid Bilayers}

Many researchers have investigated the interaction of \(\text{A}\beta\) with natural\textsuperscript{179-182} and artificial (brief review follows) lipid membranes. Based on its amphiphilic nature, \(\text{A}\beta\) can interact with membranes resulting in several membrane perturbing effects, which may play a pivotal role in the pathogenic cascade leading to Alzheimer’s disease. It remains a difficult task to pinpoint the exact mechanism via which \(\text{A}\beta\) becomes toxic to cells. Nevertheless, it is evident that the concentration of \(\text{A}\beta\) found in cerebrovasular fluids \textit{in vivo} is insufficient to initiate fibril growth \textit{in vitro}. Thus, it is possible that the toxicity of \(\text{A}\beta\) to cells may result from a direct consequence of specific interactions upon binding to the membrane surface. In fact, several researchers have found evidence that the \(\text{A}\beta\)
changes conformation from a random coil to β-sheet upon binding the membrane. The β-sheet structure adopted by the Aβ peptide is crucial for formation of fibrils; thus, if indeed interaction with the membrane surface initiates fibril formation, then this may account for the low concentrations of the peptide found in vivo.

Terzi et al.\textsuperscript{183} demonstrated that the binding of monomeric A\textsubscript{β25-35} peptide to negative lipid vesicles is possible primarily through electrostatic interactions. Subsequent studies with the A\textsubscript{β1-40} peptide by Terzi et al.\textsuperscript{184,185} and later, Bokvist et al.\textsuperscript{186} revealed that a conformational change from random coil to β-sheet structure occurs when the peptide binds negatively charged, small unilamellar lipid vesicles (SUV) (under conditions of low ionic strength; about 5 -10 mM MOPS or Tris buffer, without NaCl); neutral membranes had no effect on the monomeric form of the peptide. Terzi et al.\textsuperscript{185} failed to observe a conformational change at physiological ionic strength. Studies by McLaurin et al.\textsuperscript{187} also confirmed that negatively charged membranes can induce a conformational change from random coil to β-sheet in the A\textsubscript{β1-40} peptide in the presence of SUVs but only under conditions of lowered pH values.

In alternate studies, Choo-Smith et al.\textsuperscript{188} varied the membrane system to include ganglioside as it was believed that the ‘lipid rafts’ are the preferential site for the formation of the Aβ fibrils. Several other studies have confirmed the accelerated formation of Aβ fibrils in the presence of membranes composed of sphingomyelin and cholesterol.\textsuperscript{189-192} Other effects that have been reported to result from the interaction of the Aβ peptide with membranes include: formation of Ca\textsuperscript{2+} channels and ion pores\textsuperscript{193-195}. Despite the limitations of these studies, it is plausible that interaction of the Aβ peptide
induces changes (subtle or otherwise) in the membrane environment which may be deleterious to the cells.

6.3 Measuring Diffusion with FPR

To study the effect of Aβ on lipid membranes a combination of fluorescence photobleaching recovery (FPR) and fluorescence microscopy was used. The detailed technical information of FPR is presented in Chapter 4. Hence, a brief summary of the suitability of this technique will be given. Many of the physiological functions of the cell membrane depend on its mobility: transport of nutrients and waste into and out of the cell, membrane fusion and cell interaction, responding to signals in the cellular environment. The classic experiment by L. Frye and M. Edidin in the 1970s demonstrated that membrane proteins diffuse freely in the lipid bilayer, implying that they were fluid. Frye et al. labeled antigens at the surface of mouse and human cells with green and red fluorescent dyes respectively. The cells were fused with Sendai virus to form heterokaryons with both mouse and human surface antigens localized within specific regions of the ‘newly-formed’ cells. The cells were incubated and later observed with a microscope. While initially the red and green dyes were separated, through diffusion, the molecules were found randomly distributed over the entire cell surface.

One great advantage of FPR is the fact that it was developed (and has been successfully used) for studying the diffusion of molecules in biological systems, including proteins and lipid within membranes. Fundamentally, the FPR technique was designed with a similar concept to the experiment of Frye et al. Briefly, a small spot in a fluorescently labeled sample is photobleached and the region is monitored to detect the return of fluorescence as bleached and unbleached molecules randomize
their positions through translational diffusion. Thus, FPR has been established as an ideal technique for measuring mobility of molecules within membranes and should prove effective for obtaining information on the nature of membrane fluidity upon interaction with the Aβ peptide.

6.4 Interaction of Melittin with Lipid Bilayers

Melittin, the major toxic component of the venom of the honey bee, Apis mellifera, is among the best-studied amphipathic α-helical polypeptides. Its 26-amino-acid-sequence is arranged such that the amino-terminal part (residues 1-20) is predominantly hydrophobic, whereas the carboxy terminal region (residues 21-26) is hydrophilic and strongly basic, due to four positively charged (Lys-Arg-Lys-Arg) amino acid residues. As a consequence of its structure, melittin binds natural, as well as negatively charged and zwitterionic, phospholipid artificial membranes resulting in different but profound effects in each case. Studies have reported that melittin forms transmembrane pores via a “barrel-stave” mechanism in zwitterionic lipid bilayers, while it acts like a detergent, (solubilizing membranes) via a “carpet-like” mechanism in negatively charged membranes. Melittin has also been found to induce the fusion of both small and large zwitterionic and acidic vesicles resulting in large structures. Although characterization of the various effects of melittin on membranes is not complete, it has been a convenient model in providing insight into complex membrane protein interactions.

Several of the characteristics of melittin were found to be similar to the properties of the Aβ peptide; thus, melittin was chosen as a useful reference for comparison of the lipid-peptide interactions in lipid bilayers. A few striking parallels between the two
peptides are: their ability to change conformation upon binding to membrane, resulting in changes in the membrane morphology; their ability to exert their toxic effects upon interaction with membranes; and, their ability to form defects in membrane structure, such as pores and channels. In this study, the interaction of melittin with vesicles and lipid bilayers on mica was examined with fluorescence photobleaching recovery and fluorescence microscopy.

6.5 Materials

![Chemical structures of POPC and NBD-PC](image)

1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC)

1-Oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phosphocholine (18:1-12:0 NBD PC)

Figure 6.1. Chemical structures of the phospholipids POPC and the fluorescent probe NBD-PC used in this study.

For this study all lipids used, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) in chloroform, and 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC) in chloroform, were purchased from Avanti Polar Lipids, Inc. Chloroform (99.9% HPLC grade) was purchased from Aldrich Chemicals. Melittin was purchased from Sigma.
peptide was purchased from Anaspec, Inc. Imaging chamber (20 mm diameter, 1 mm deep was purchased from Sigma-Aldrich. All fluorescent-probe-containing solutions were wrapped in aluminum foil and stored in the dark to prevent photobleaching from room light. Nanopure water for all experiments was obtained by a Barnstead Nanopure Water System (18 MΩ cm) resistivity.

6.6 Preparation of Lipid Vesicles

The preparation of lipid vesicles of the right size and structure involves three basic steps: first, prepare the lipid for hydration by first drying off organic solvents from the lipid, resulting in a film; second, hydrate the lipid film in aqueous media; third, downsize the formed hydrated lipid vesicles by sonication or extrusion. In this study, the lipids POPC (1.9 mg) and NBD-PC (0.1 mg) were both dissolved in chloroform at the concentration of 2 mg lipid/mL of chloroform in a Pyrex test tube (13 × 100 mm) to ensure a homogeneous mixture of lipids. Additional chloroform (1-2 mL) was added and thoroughly mixed to ensure optimal film formation. The chloroform was subsequently removed by rotatory evaporation (Buchler Instruments) for about 20 minutes, yielding a thin lipid film in a round bottom flask. Residual chloroform was removed by placing the flask in a vacuum oven overnight. The dried lipid film was hydrated with a 2-mL aliquot of a 50 mM phosphate-buffered saline buffer, 150 mM NaCl at pH 7.4 and placed on a rotatory evaporator without a vacuum – spinning the round bottom flask in a water bath to maintain a temperature above the $T_m$ of the lipid suspension for about one hour.

The resulting hydrated large, multilamellar vesicles (MLVs) were disrupted by either sonication or extrusion to form small, unilamellar vesicles. Initially, the lipid solution was sonicated with a micro tip Branson Sonifier 450 on an on-off cycle for five
minutes total. The sonication tip was found to release titanium particles into the lipid suspension, which need to be removed by centrifugation. In other cases, the sample was often heterogeneous between batches. It was difficult to control and reproduce the conditions of sonication, thus an alternate method was used.

The disruption of the MLV suspension was achieved by using the lipid extrusion technique. Prior to disruption, the MLVs were alternately frozen in liquid nitrogen and thawed in hot water (60 °C) for five freeze-thaw cycles to improve homogeneity of the final lipid suspension. The MLVs were then extruded for 20 complete cycles through an Avestin polycarbonate filter (100 nm pore size) on a liposofast-basic manual extrusion apparatus. The diameter of the vesicles was measured by dynamic light scattering and a value of 100-120 nm was found (near the pore size of the filter used). All vesicles in this study were prepared by the extrusion technique because this method proved more reproducible than sonified lipid vesicles.

6.7 Formation of Lipid Bilayers

Lipid bilayers have a natural tendency to form spontaneously215 (i.e. $\Delta G < 0$), by fusion or adsorption into a continuous bilayer membrane on several materials.216 The formation of a lipid bilayer by vesicle adsorption on a hydrophilic surface involves three steps: adsorption, rupture and spreading.217 The adsorption of lipids onto hydrophilic surfaces is spontaneous due to the presence of the hydrophilic phosphate headgroups at the outer surface of the vesicles. The electrostatic environment with fixed charges due to the phosphate and choline groups218 causes the vesicles to flatten out onto the surface, after which the vesicles are ruptured. Spontaneous spreading is facilitated by the presence of a thin (1-2 nm) film of water216;219-222 (‘precusor film’) located between the vesicles
and the hydrophilic surface. Strong self-assembly forces causes phospholipid membranes of almost constant thickness to form as the membrane is very thin and close to the hydrophilic surface. A schematic representation for vesicle interactions with hydrophilic surfaces commonly presented in the literature is shown in Figure 6.2.

![Schematic representation of vesicle fusion on a hydrophilic surface.](image)

**Figure 6.2.** Schematic representation of vesicle fusion on a hydrophilic surface. Adapted from reference 221.

The supported lipid bilayers were formed by pipetting a drop of the vesicle solution (lipid concentration of 2 mg/mL) onto a freshly cleaved mica disk (10 mm) which had been glued onto a mica puck and placed on a microscope slide. The sample was allowed to incubate at room temperature for one hour in a covered Petri dish that had been layered with a moist filter paper. After the incubation period, the sample was rinsed with phosphate buffer to remove any unfused vesicles and a small volume of the buffer was kept on the membrane at all times to prevent interference with the liquid-air interface. The sample was then sealed with an imaging chamber to prevent air flow that
could cause the liquid interface to move and also to prevent evaporation of the buffer which would result in drying of the sample. For FPR studies involving the lipid vesicles in solution, the samples were loaded in 0.2-mm-path-length rectangular microslides (Vitrocom) by capillary action, and the microslides were flame-sealed.

6.8 Results

6.8.1 Fluorescence Imaging and FPR Analysis of Supported Lipid Bilayers

To obtain information on the structural and dynamic properties of supported phospholipid bilayer on mica, fluorescence microscopy and fluorescence photobleaching recovery (FPR) techniques were used. Representative fluorescence microscopy images for mixed POPC/NBD-PC bilayers formed by direct vesicle fusion on mica observed at different time points during experimentation are shown in Figure 6.3. It should be noted that images such as those appearing in Figure 6.3 a-c were rarely observed, but the patterns evidenced therein are recognized as important steps in the formation of defect-free supported membranes. The appearance of mobile unruptured spherical vesicles can be seen in Figure 6.3a. This may be attributed to residual unfused vesicles that were not completely rinsed away during sample preparation. Some defects can be observed in Figure 6.3b due to incompletely formed membrane. Due to the mobility of the lipid membrane, complete coverage of the surface is possible with additional time. The image in Figure 6.3c was taken near the edge of the mica supported bilayer. A portion of the lipid bilayer has holes and is separated by a portion that is defect free. It is not known what caused the holes, but one can speculate that some tension, perhaps at the air-water interface may have contributed to its formation. These holes can be likened to similar ‘circular defects’ observed in cholesterol containing planar ‘raft’ model membranes.
Figure 6.3. Fluorescence microscopy images of supported phospholipid bilayers on mica. (a) spherical unfused vesicles floating on the membrane, (b) incompletely formed membrane with defects, (c) holes within the lipid bilayer (d) uniform supported lipid bilayer on mica. (Images were false colored with Adobe Photoshop).

Crane et al.\textsuperscript{219} reasoned that the circular defects in their fluorescence microscopy images were possibly due to surface defects in the solid support or stretching during deposition of the first lipid layer by the direction of the Langmuir-Blodgett. The uniformly (within optical resolution) continuous supported membrane shown in Figure 6.3d was typically used for experimentation.
There are several methods to test the integrity of supported membranes formed by vesicle fusion. While fluorescence microscopy can be used to obtain structural information, it would not be an appropriate choice to determine the fluidity of a defect-free bilayer that is uniformly fluorescent. If the membrane is scratched, however, then it should be possible to observe the fluidity of the membrane by its ability to spread into the scratched region. In Figure 6.4a, the mica was mechanically scratched with a razor blade prior to deposition of the vesicles onto its surface. The observed scratch was not ‘healed’ following incubation for one day, suggesting that spreading was unfavorable.\textsuperscript{164} Groves \textit{et al.}\textsuperscript{216} demonstrated that under neutral or basic conditions, scratch barriers are very stable, whereas they heal in minutes under mildly acidic conditions. The scratch-free regions within the bilayer were found to be ‘fluid’ by measurement of diffusion coefficient with FPR. The stability of the scratch in the bilayer can be viewed as an example of a barrier to lateral diffusion, in keeping with the natural ability of cell membranes to restrict access to unwanted components. A stripe pattern photobleached into a lipid bilayer is shown in Figure 6.4b.

FPR is an established technique for measuring lateral mobility and hence determining the fluidity of lipid membranes.\textsuperscript{175} Supported membranes have been demonstrated to exhibit many features of natural membranes including lateral fluidity. To determine whether the prepared lipid membrane maintained its dynamic properties on the mica support, its lateral diffusion was measured by FPR. The diffusion coefficient of both NBD-PC/POPC vesicles and lipid bilayer on mica support was assessed by this method.
Figure 6.4. Fluorescence microscopy images of supported phospholipid bilayers on mica. (a) lipid bilayer which has been scratched (b) image of stripe pattern bleached into the supported lipid bilayer, acquired with a 10× objective. The black spot is due to photobleaching during a previous exposure at 63×, final objective 10× magnification. (Image 6.4a was false colored with Adobe Photoshop).

The diffusion coefficient of the NBD-PC/POPC vesicles was determined to be \((4.93 \pm 0.27) \times 10^{-8} \text{ cm}^2\text{s}^{-1}\) (Figure 6.5a). A typical FPR recovery profile appears in Figure 6.5a. The inset shows a plot of \(\Gamma\) vs \(K^2\) whose slope provides the diffusion coefficient. The zero intercept, within experimental error, indicates true Brownian diffusion rather than relaxation due to convection or reversible photobleaching. A semilogarithmic FPR trace of the data points in Figure 6.5a displays almost single exponential behavior, indicating that the lipid vesicles were uniformly composed (Figure 6.5b).

After fusion of the NBD-PC/POPC vesicles with the mica surface to form a lipid bilayer as in Figure 6.3d, the NBD-PC was observed to have a diffusion coefficient of \((5.99 \pm 0.55) \times 10^{-8} \text{ cm}^2\text{s}^{-1}\) (Figure 6.6a). The semilogarithmic plot also displays almost single exponential behavior similar to the lipid vesicles (Figure 6.6b).
Figure 6.5. Diffusion measurement of phospholipid vesicles in suspension. (a) Recovery profile for NPD-PC/POPC (5:95 molar ratio) phospholipid vesicles. Inset: $I'$ vs $K^2$ plot of the same sample. (b) Semilogarithmic FPR trace of the data points in Figure 6.5a.
Figure 6.6. Diffusion measurement of NBD-PC in a phospholipid bilayer. (a) Recovery profile for NPD-PC/POPC (5:95 molar ratio) phospholipid bilayer on mica support. Inset: $\Gamma$ vs $K^2$ plot of the same sample. (b) Semilogarithmic FPR trace of the data points in Figure 6.6a.
The diffusion coefficient values obtained for NBD-PC probe lipid in both mica supported bilayers and vesicles composed of NBD-PC/POPC (5/95 mol/mol) suggests that membranes diffuse. The measured diffusion coefficients were also in the range \((1-8 \times 10^{-8} \text{ cm}^2 \text{s}^{-1})\) reported for diffusion of phospholipid molecules in fluid bilayers. After obtaining the diffusion coefficient of the lipid vesicles and bilayers, it was determined that the membrane was sufficiently fluid and within the expected values in literature. Thus, similar experiments were performed to determine the interaction of the \(\beta\)-amyloid peptide and, later, melittin with lipid vesicles and lipid bilayers.

### 6.8.2 Fluorescence Imaging and FPR analysis of the Interaction of \(\beta\)-amyloid with Lipid Bilayers

Most studies have used fluorescence anisotropy to determine how A\(\beta\) affects the fluidity of lipid membrane. As discussed earlier, discrepancies exist as to whether the A\(\beta\) peptide increases or decreases the fluidity of the membrane. To address how A\(\beta\) alters membrane fluidity, FPR was used to determine the diffusion of a fluorescent lipid probe, 1-Oleoyl-2-[12-[[7-nitro-2,1,3-benzoxadiazol-4-yl]amino]dodecanoyl]-sn-Glycero-3-Phosphocholine (NBD-PC) on a mica-supported model membrane system made of 5% NBD-PC and 95% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) as the major component. Figure 6.7 shows the trend of the surface diffusion of NBD-PC in a bilayer with POPC as a function of time. The bilayer was incubated with pre-formed A\(\beta\) protofibrils, followed by rinsing with buffer to remove any unbound material - the buffer was used to keep the bilayer moist throughout the experiment. The data implies that the fluidity of the lipid is compromised by the presence of fibrils, as indicated by the decreasing diffusion results with time.
Figure 6.7. Diffusion of 5% NBD-Labeled PC fluorescent tracer molecule in 95% unlabeled POPC on mica with preformed β-amyloid1-40 fibrils.

To further explore the interaction of Aβ with lipid bilayer model membranes, lipid bilayers were prepared as described previously and an aliquot of a 50 µM unlabeled Aβ sample prepared in 50 mM PBS, 150 mM NaCl, pH 7.4 was applied to the wet lipid bilayer. Fluorescence microscopy images taken at different areas in the sample revealed that the presence of the bilayer induced the aggregation of the peptide (Figure 6.8). To further explain the appearance, it is hypothesized that the Aβ picks up some NBD-PC as it forms aggregates. To determine whether the prepared lipid bilayers maintained its dynamic properties on the mica support, its lateral diffusion was measured by FPR. Figure 6.9 shows the trend in the diffusion coefficient of NBD-PC in a bilayer with POPC as a function of time. Two diffusion modes which differed by orders of magnitude were calculated. The slow mode was dominant with about 80% of the signal amplitude.
There was almost no change in the diffusion coefficient for the duration of the experiment.

![Figure 6.8](image)

Figure 6.8. Fluorescence microscopy images showing the interaction of unlabeled β-amyloid peptide with supported phospholipid bilayers on mica. (a-d) changes in the lipid membrane at different regions in the sample.

The interaction of Aβ was further investigated with lipid vesicles in suspension. Figure 6.10 shows the diffusion values as a function of time. In contrast to the results obtained for the interaction of Aβ with lipid bilayers only one diffusion mode was obtained. The diffusion coefficient was only slightly lower than that of the vesicles alone.
Figure 6.9. FPR diffusion coefficient values of NBD-PC in the presence of β-amyloid on mica-supported lipid bilayer as a function of time. The percent of fast and slow signal amplitude are displayed.

Figure 6.10. Diffusion of NBD-PC in vesicles composed of NBD-PC/POPC (5/95 mol percent) in the presence of preformed β-amyloid$_{1-40}$ fibrils (1:10 molar ratio).
Like the results obtained for the interaction of Aβ with lipid bilayers, there was almost no change in the diffusion value as a function of time. These later results are consistent with findings in the literature that the Aβ does not alter the properties of zwitterionic lipid vesicles.

6.8.3 Fluorescence Imaging and FPR Analysis of Interaction of Melittin with Lipid Bilayers

Melittin was chosen as a control molecule due to its profound ability to affect the properties of natural, as well as negatively charged and zwitterionic, phospholipid artificial membranes. FPR was used to determine whether melittin induced any changes in the dynamic properties of the lipid bilayer on a mica support. Figure 6.11 shows the trend in the diffusion value as a function of time.

Figure 6.11. FPR diffusion coefficient of NBD-PC in the presence of melittin on mica-supported lipid bilayer as a function of time. The percent of fast and slow signal amplitude are displayed.
The results are almost identical to those obtained for the interaction of Aβ with lipid bilayers, with the exception being that the percent signal amplitude was lower by almost 20% for the slow diffusers. Additional experiments were performed to investigate the interaction of melittin with lipid vesicles.

Figure 6.12. Fluorescence microscopy image showing the interaction of melittin with vesicles composed of 5% NBD-Labeled PC/95% unlabeled POPC (1:1 molar ratio). (a) after 3hrs, (b) after 7.5 hrs.

Figure 6.12 shows fluorescence microscopy images of a one to one molar ratio of melittin with lipid vesicles at 3 and 7.5 hours after sample preparation. The initial presence of small spherical vesicles shown in white against a green background is observed in Figure 6.12a. The growth of the vesicles became more apparent after 7.5 hours. The trend in the diffusion values as a function of time is shown in Figure 6.13. The fact that the sample was a polydisperse population of several species is clearly demonstrated by the multiple exponential analysis results and better yet by the 3-D representation of the CONTIN distribution of the data in Figure 6.13 bottom.
Figure 6.13. Top: Diffusion of NBD-PC in vesicles composed of NBD-PC/POPC (5/95 mol percent) in the presence of melittin. Bottom: 3-D representation of CONTIN distribution of data.
Figure 6.14. Contrast of melittin at zero and 7.5 hours in lipid vesicles.

The deviation from single exponentiality of the signal contrast at time 3 and 7.5 hours in Figure 6.14 also suggests accurate analysis of the sample composition. Unlike the Aβ peptide, the results clearly show that melittin interacts with zwitterionic lipid vesicles and induces swelling of the vesicles.

6.9 Conclusions

FPR was used to investigate the effect of Aβ on membrane fluidity. For comparison, melittin was chosen as a control molecule as it has been found to induce changes in the dynamic properties of lipid bilayers. The results from initial FPR experiments for the diffusion of tracer lipid molecules composed of the fluorescent lipid probe, 1-Oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phosphocholine (NBD-PC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) at a ratio of 5% NBD-PC to 95% POPC (supported on mica) was consistent with diffusion coefficient values reported for phospholipids molecules in
fluid bilayers. Upon incubating the Aβ on the bilayers, there was a marked decreased in the diffusion coefficient values, indicating that the presence of the fibrils hindered the diffusion of the lipid probe, suggesting that the Aβ acts to decrease the fluidity of the lipid membrane. Fluorescence microscopy images revealed that the Aβ peptide perturbs the composition of the bilayers and increased aggregation of the peptide was observed. In studies examining the interaction of Aβ with zwitterionic lipid vesicles, there was no change in the diffusion values. The melittin control on the other hand, vastly induced the aggregation of the lipid vesicles. This suggests that bilayer surface may play a pivotal role in the Aβ aggregation mechanism. Perhaps, the lipid membrane is acting as a nucleation site for initiating the aggregation of the Aβ peptide.
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214) Preparation of Liposomes. 


# APPENDIX A: FLUORESCENCE SPECTROSCOPY

Table with sample identification using a 96-well plate layout.

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<td>25 µM unlabeled Aβ</td>
<td>0.1 µM Labeled Aβ + 25 µM unlabeled Aβ</td>
<td>0.1 µM Labeled Aβ + 25 µM unlabeled Amy1</td>
<td>0.1 µM Labeled Aβ + 25 µM unlabeled Amy2</td>
<td>0.1 µM Labeled Aβ + 25 µM modified Amy1</td>
<td>0.1 µM Labeled Aβ + 25 µM modified Amy2</td>
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<td>B</td>
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<td>0.1 µM Labeled Aβ + 25 µM unlabeled Aβ</td>
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<td>0.1 µM Labeled Aβ + 25 µM unlabeled Amy2</td>
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A screen capture of fluorescence spectroscopy results plotted in an Excel spreadsheet using FLUOstar Galaxy software.
APPENDIX B: HIGH PRESSURE INSTRUMENTS

High Pressure Cell Set-up

Both vial and cap are filled with the sample and covered – care should be taken to avoid air bubbles. The vial is subsequently inserted into a sample holder within the ethanol filled high pressure cell chamber.

Photograph of high pressure cell set-up (Location A, UNICAMP, Campinas, Sao Paulo, Brazil)
The high pressure cell is then placed within a modified Edinburg FL 900 spectrofluorometer chamber and the pressure generator connected to the top of the cell.

Photograph of high pressure cell set-up (Location A, UNICAMP, Campinas, Sao Paulo, Brazil)
Photograph (courtesy of Dr. Rafael Cueto) of high pressure cell set-up (Location B, Louisiana State University, LA)
Photograph (courtesy of Dr. Rafael Cueto) of high pressure cell set-up (Location B, Louisiana State University, LA)
VITA

Nadia J. Edwin was born on the island of St. Croix, U. S. Virgin Islands. She completed her secondary education at the Castries Comprehensive Secondary School on the island of St. Lucia, West Indies, in 1994. She received a Bachelor of Science degree in chemistry in 2000 from the University of the Virgin Islands, St. Thomas campus. In 2000, she became a doctoral candidate in the department of chemistry at the Louisiana State University, where she is currently completing the requirements for the degree of Doctor of Philosophy.