A STUDY ON THE APPLICATION OF BIOPHYSICAL TECHNIQUES TO TERMINAL DISEASES VIA THE METABOLOMIC-BASED ANALYSES OF CANCERS AND THE ANALYSIS OF ALZHEIMER'S DISEASE THROUGH THE ACTION OF AMYLOID-BETA

by

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READ AND APPROVED

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TABLE OF CONTENTS

DEDICATION

To my family and friends who have helped me complete this work:

Without you, none of this would have been possible.

To those chemistry students who follow me at the University of Michigan:

May this work inspire you as previous works have inspired me.

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LIST OF FIGURES AND TABLES

SECTION 1: METABOLOMICS

Chapter 1: Introduction

Chapter 2: On the Resolution of Complex NMR Spectra via Esterification and Phase Separation, with Applications to Urinary Metabolomics

Chapter 3: A Method of Minimizing Ionic Strength to Reduce Chemical Shift Variance in NMR-Based Urinary Metabolomics

SECTION 2: MECHANISMS OF ALZHEIMER'S DISEASE

Chapter 5: Introduction

Chapter 6: Effects of Membrane Compositions on Amyloid-β Aggregation

PREFACE:

A BRIEF STATEMENT ON BIOPHYSICS AND BIOPHYSICAL TECHNIQUES AND THEIR RELATIONSHIP TO PURE CHEMISTRY

Biophysics is, at its roots, the application of physical principles to biological systems at both a gross biological and a chemical level, creating a truly interdisciplinary field at the interface of the three major natural sciences. Biophysics is not limited to pure biology and chemistry, however, as it has influences biochemistry, bioengineering, computational biology, nanotechnology, and even a development of basic physical principles from quantum dynamics and quantum and statistical mechanics. Essentially, biophysics focuses on a quantitative analysis of biological systems, rather than the more qualitative forms seen in direct biological analysis. Examples of biophysical techniques include fluorescent imaging, microscopy, nuclear magnetic resonance, mass spectrometry, circular dichromism, atomic force microscopy, and many others. Theoretical techniques, such as computational analysis via statistical mechanics, are also used. Many of these methods fall under the scope of physical chemistry as well, and it is in that context that the investigations presented herein are discussed. Biophysical analyses were done in this investigation to study two terminal illnesses: cancer and Alzheimer's disease. Though on the surface these two diseases may seem unconnected, they are linked through the physical technique applied to study them. Through the biophysical analysis of urine and lipid membranes, investigations into the early detection of cancer and the mechanism of Alzheimer's disease were carried out. These analyses may be biophysical in nature, but they remain a physical procedure in principle. No science is truly one discipline anymore, and it is in that spirit that we proceed.

SECTION 1:

METABOLOMICS AND CANCER

Chapter 1:

Introduction

1.1. An Introduction to Metabolomics

Metabolomics is a form of scientific analysis that exploits the biochemical processes in the cell to make statements on the health or illness of that cell.[1] In doing so, it looks at the levels and characterization of the metabolic side products from biochemical processes, giving a picture of the state of the global metabolism of the cell or organism.[2-5] Essentially, metabolomics focused on the metabolome, which is the full profile of the small molecules present in cells, tissues, and organisms, and change as a result of processes ranging from gene expression, the Krebs cycle, glycolysis, and cell division, to even system perturbations caused by the consumption of food, pharmaceuticals, and illicit drugs, giving direct measurements of biochemical activity.[6-7] This is in contrast to other "omics" sciences, such as proteomics, which focuses solely on protein expression and levels, and genomics, which concentrates on the DNA and various RNA samples found in a cell. [8-10] In studying these small molecules, metabolomics actually gives a snapshot or chemical fingerprint of the cell and its processes. As they are chemically changed during metabolism, their relative concentrations give insight into the physiological state of a biological system at the moment of sampling, giving a holistic analysis, and improving on genomics and proteomics.[1-5, 8-10] Genes and proteins are limited to epigenetic and posttranslational modification, being less direct in identifying specific biochemistry, affording less specific information as small molecule analyses, nor is their information as good of a representation of the specific, current state of a cell.[11-12] This process is also known as

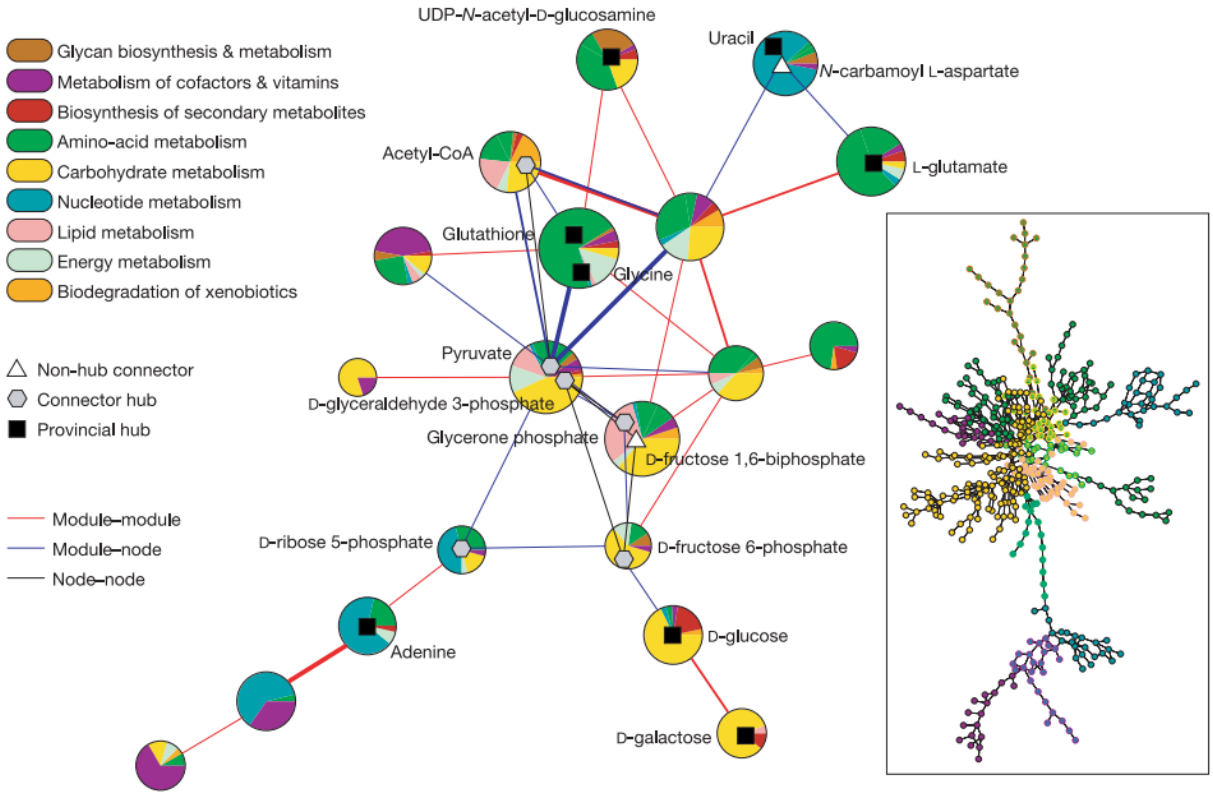

Figure 1.1. A schematic showing the important metabolites and their related processes and interconnections. Figure reprinted with permission from Guimerà, R.; Amaral, L. A. N. *Nature*. 2005, *433*, 895-900. Copyright 2005 by Nature Publishing Group.

metabonomics, metabolic profiling, metanomics, and metabolic fingerprinting, depending on the quantitative level of analysis and the application.[1-5]

In studying the metabolome and metabolomics, the exact range of metabolites to be investigated remains under debate, as do the various components and samples to be analyzed, and the exact mode by which this analysis is done. Though the number of individual metabolites numbers in the tens of thousands, no analytical technique can accurately characterize the full range of metabolites.[13-14] Therefore, many techniques focus on those that are able to be easily characterized. Some important metabolites, with all the processes they are involved in, appear in Figure 1.1.[6-7] In the full analysis of these metabolites, comparisons can be made between a healthy, unperturbed system, and those subject to some kind of stress, whether pathophysiological, pharmaceutical, or otherwise.[15-17] In using metabolomics, the goal is to create a detailed map of these interconnected metabolomic and genetic pathways, allowing for a pinpointing of the change in these processes as determined by the change in the profile itself. An

example map appears in Figure 1.2.[18-20] These changes would ultimately be linked to specific diseases or other environmental stresses, which would allow for a quick and accurate determination of the cause of the stress, taking advantage of the full analytical power of the modern instruments used therein.

Metabolomics has been shown to be especially promising in disease diagnoses.[21-25] Diseases are well-known to disrupt various metabolic pathways, accelerating and retarding their rates as appropriate. These changes are seen in metabolic signatures and profiles, which, when analyzed via metabolomics, can be matched to the disease and thus provide diagnostic information. In fact, diagnostic applications of the metabolome have been made for diseases such as tuberculosis,[26-28] schizophrenia,[29-30] bipolar disorder,[31-32] Alzheimer's disease,[33-35] coronary artery disease,[36-38] hypertension,[39-40] hemorrhages,[41-42] type II diabetes,[43-45] drug overdose,[46-48]

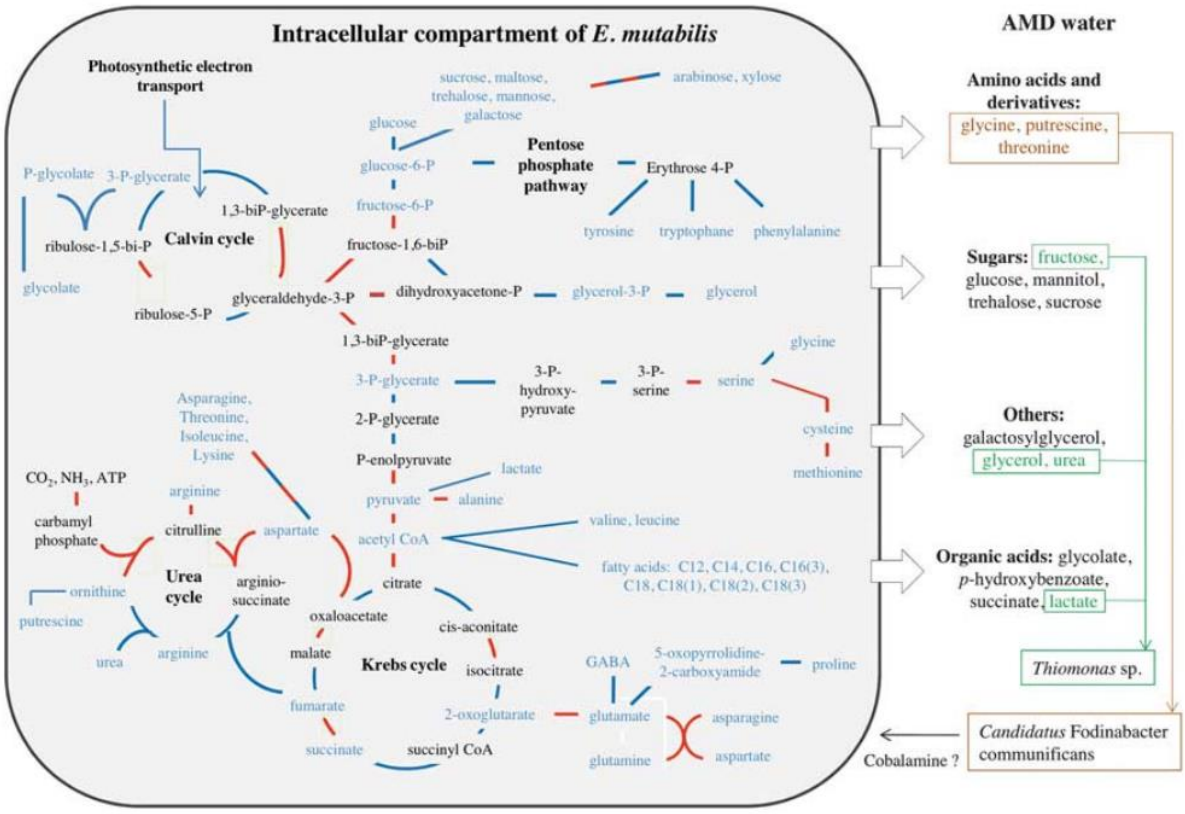

Figure 1.2. A metabolomic map showing important metabolites and pathways in *E. mutabilis*. Figure reprinted with permission from Halter, D.; Goulhen-Chollet, F.; Gallien, S.; Casiot, C.; Hamelin, J.; Gilard, F.; Heintz, D.; Schaeffer, C.; Carapito, C.; Van Dorsselaer, A.; Tcherkez, G.; Arsène-Ploetze, F.; Bertin, P. N. *ISME J.* 2012, *6*,1391-1402. Copyright 2012 by Nature Publishing Group.

and preeclampsia.[49-50] A full list of diseases appears in Table 1.1. However, metabolomics is probably best demonstrated in its applications to the various cancers, as discussed herein.

1.2 Metabolomics and Cancer

As discussed, above, metabolomics has been shown to have a large impact on the ability to diagnose diseases, especially cancers. A full list of cancers analyzed via metabolomics, appears in Table 1.1. Thus, it can be stated that metabolomic approaches are well-established for this form of disease, allowing for a more detailed understanding of disease pathogenesis, and improvements in prognostic and diagnostic approaches for patient care. The ultimate goal is for metabolomics to become the dominant form of clinical tests for the various cancers; however, in

Disease	References
Alzheimer's Disease	33-35
Bipolar Disorder	31-32
Bladder Cancer	51-60
Brain Cancer	61-68
Breast Cancer	69-91
Cervical Cancer	92-94
Colorectal Cancer	95-111
Coronary Artery Disease	36-38
Head and Neck Cancer	112-124
Esophageal Cancer	125-131
Gastric Cancer	132-141
Hemorrhages	41-42
Hepatic Cancer	142-159
Hypertension	39-40
Leukemia	160-169
Lung Cancer	170-183
Ovarian Cancer	184-192
Pancreatic Cancer	193-203
Preeclampsia	49-50
Prostate Cancer	204-218
Renal Cancer	219-228
Schizophrenia	29-30
Tuberculosis	26-28
Type II Diabetes	43-45

Table 1.1. List of disease studied via metabolomics, and pertinent references.

order to achieve that ambitious statement, all aspects of the metabolomic process, including the selection, preparation, analysis, and interpretation of samples must reach a certain level of scientific consensus, having been examined, standardized, and perfected to a degree such that all variation is removed and uniformity is ensured.[1,229] This involves a focus on such minutiae as the type, number, collection time, storage conditions, the various levels, stages, and degrees of metastasis of the cancers in the patients studied.[230-231] The variations in medications ingested and the length of treatment both affect metabolomic patters as well due to the processing of pharmaceuticals in the body. Even with this detailed treatment of data, statistical anomalies may still result from such factors as age, diet, sex, and even the location where the patient resides. Furthermore, patient-researcher interaction must be closely scrutinized to ensure no violations of the safety and rights of the human subjects under study are occurring, and their protection must remain the highest property. Generally, this is done through following the provisions of the Good Clinical Practice, an international quality standard for such studies.[232-235]

Though metabolomics has been shown to be well-suited to cancer analyses, as demonstrated by the sheer number of studies done, many challenges remain to the wide implementation of these techniques. Metabolites, by lieu of them being small molecules that participate in a wide range of metabolic processes, have an intrinsically transient nature, affording a large dynamic range that depends on the time of collection, endemic interindividual variation, and other factors that ultimately cannot be standardized.[236-238] Metabolome variations can indicate diseases, but can also be due to natural physiological fluctuations, and even temporal and seasonal changes in diet and environment. Sample transport, freezing, and storage procedures, if not exactly the same between research groups, can introduce more error into the sample. Even further, such variations may cause usable data to be obscured from analysis as

well, providing more opportunities for false positive or false negative diagnoses.[239-240] Due to this myriad of potential issues, all metabolomic studies have to be under strict and reproducible conditions. As most metabolomic investigations use some form of instrumentation, such as mass spectrometry, nuclear magnetic resonance spectroscopy, or fluorescence spectroscopy, these techniques provide reproducibility, if all acquisitions are done under the same conditions of temperature, ionization procedure, and even on the same instrument.[241-243] It is overwhelmingly crucial to painstakingly establish and follow detailed collection and preparation protocols to standardize all data before making any sort of conclusion. All such standard operating procedures (SOPs) are necessary for the highest level of accuracy in metabolomics.[244]

Despite these drawbacks, metabolomics still provides many advantages over current methods. Currently, most noninvasive cancer diagnostic tests use a similar method to metabolomics in the application of biomarkers, larger molecules that are considered to be representative of a diseased state.[245-247] They tend to be metabolites whose concentrations are correlated to a specific and complex physiological parameter or state, including diseases, though the best-known example of this is the use of the human chorionic gonadotropin hormone as a pregnancy test. However, many cancer biomarker tests have a large amount of variation. The most evident of these is the prostate-specific antigen (PSA) test, used to detect prostate cancer.[248-250] In this test, the levels of PSA are detected in the blood, acting like a biomarker for prostate cancer. Elevated values are purported to correlate to prostate cancer; however, it can also indicate prostatitis or benign prostatic hyperplasia.[251] Only 30% of patients that have elevated levels of PSA detected actually have prostate cancer, leading to overdiagnosis, overtreatment, and a screening process that is generally recommended against by most cancer agencies, including the American Cancer Society and the United States Preventive Services Task.[252-253]

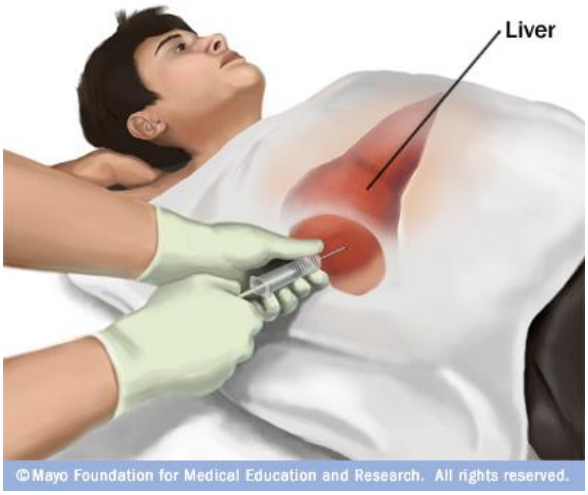

Figure 1.3. Depiction of the invasive nature of a biopsy. Copyright 2015 Mayo Foundation for Medical Education and Research.

Definitive cancer diagnosis truly only occurs after biopsies, however, in which a sample of the potential tumor is taken out of the patient, in a painful, invasive, and potentially expensive manner.[254-255] An image of the biopsy procedure appears in Figure 1.3. For many people who undergo biopsies, no tumor is present, causing them undue harm and stress. Metabolomics improves on all of those. In using a small molecule profile, rather than just individual biomarkers, more accurate statements on the actual state of an individual can be made, as the concentration patterns in the metabolome provide both more holistic forms of analysis and have less variation due to stresses that may falsely elevate the concentration of a single biomarker, introducing error into the procedure currently in use.[1]

As metabolomics searches for metabolite variations that differentiate between healthy and diseased samples, the most accurate form of diagnosis would be from comparing the metabolome of a healthy and diseased sample from the same person. However, as this is unrealistic, a standard picture of a diseased metabolome could be used in comparison to the one obtained from the patient, which would allow for better screening processes, preventing unneeded biopsies and stress on the patient.[1, 51-228] This type of screening test would thus encompass the benefits of the current noninvasive diagnostic modality, while also improving on

its sensitivity, cost-effectiveness, level of patient discomfort. Furthermore, metabolomic techniques, due to the increase in sensitivity, catch diseases earlier in their pathophysiological progression, sometimes before even symptoms manifest.[256-258] Thus, this type of diagnostic test would improve on all current techniques, allowing for a higher rate of survival of these cancers, and a better quality of life during treatment, whether chemotherapy or radiation therapy.[259-260]

Metabolomics does not limit itself to diagnostic applications, however, but also affords a better understanding of the underlying mechanisms of a disease through determining changes in metabolites, and discovering the reasons for these changes, monitoring the progress of the disease and giving insight into pathology while providing biomarkers that can diagnose the disease.[261-265] In doing so, metabolomics is able to connect the basic science to the clinical, and allow for a better characterization of these deadly diseases. In understanding how these ailments specifically affect the human body, targeted drugs can be made to combat cancer, allowing for a higher rate of success in treatment and a better quality of life. In summary, metabolomics will have implications both in biomedical research and clinical practices, as it captures information with regard to the mechanism of the disease and its symptomatic results, allowing for the tracing of the effects on pathways, and applications in the diagnostic field.[1]

Before medical applications can be fully realized, however, the basic principles of metabolomics must be established and standardized, allowing for a comprehensive method that would eliminate the drawbacks in metabolomics discussed above. The development of such a comprehensive metabolomic method, steps to which are taken in these studies, would allow for the definitive application of this analysis to disease diagnosis. This investigation focused on determining elements of a standard protocol for the metabolomic investigation of cancers at a general level. In determining the techniques, aspects such as reproducibility, sensitivity, and ease

of the procedure were considered, as well as how early in the progression of cancer this technique could be used, especially if before metastasis. If successful, the studies herein would be instrumental to revolutionizing the field of oncology and the health care system as a whole, as they would indicate feasibility in other cancers, and potential applications to other techniques.

1.4 Metabolomics Methodology

1.4.1 Introduction to Metabolomic Methodologies

As metabolomic mixtures have an intrinsically large chemico-physical variation, analysis is extremely complicated. To deal with this variation, a plethora of techniques have been applied to metabolomics, including fluorescence probing and spectroscopy.[266-268] The dominant techniques in metabolomics, however, remain nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), as they maintain the necessary analytical power to overcome the issues inherent in metabolic mixtures, especially when combined with chromatographic purification techniques to reduce complexity.[269-270] Overcoming this complexity and improving the overall analysis methodology is the ultimate goal of the study discussed herein, as the need for specialized and customized methods of statistical analysis is paramount.

Generally, metabolomic analysis falls into two categories: targeted and untargeted. Targeted approaches quantify known biomarkers for certain diseases or physiological states to make conclusions on the patient from whom the data originates.[271-273] Essentially, it looks for something expected in the data. Untargeted approaches search for correlations between diseased states and experimental quantities in previously uncharacterized diseases to develop targeting profiles, therapeutic targets, and biochemical mechanisms of fundamental processes.[274-276] Current untargeted approaches have even revealed that metabolites used in biological systems

expand beyond canonical biochemical pathways, which allows for further pathways for biomarker identification to exist beyond the currently known range.[277-278] As both methods acquire data similarly, they have similar limitations, which are shared with metabolomics as a whole. Furthermore, these techniques feed into vast, uncharacterized metabolomic databases, at a chemical to biochemical to even anatomical level. This is a growing research area.

One way in which untargeted metabolomics is improved is via meta-analysis,[279] where cascades of metabolic perturbations are prioritized if unknown, even if unrelated to the disease of interest, revealing hundreds of alterations to pathways that may be useful in other diseases. By comparing the profiles of various diseases, disease pathologies can be identified, allowing for the further understanding of pathophysiology of both diseases.[279-280] This data reduction allows for more aggressive analysis of unknown features that may be medically relevant.

1.4.2 Sample Collection and Preparation

Samples analyzed include tissues, urine, blood, and amniotic fluid.[281-283] However, these data sources contain fewer metabolites as compared to genetic materials and proteins, even fewer of which are detectable, depending on the sample, the analytical platform, and the goals of the study. Furthermore, due to natural variations in biological samples, no single method can identify and quantify all metabolites in a sample, needing instead a combination of applicable procedures to maximize the number of detected and quantified metabolites.[1] Also, homogenization prevents location-based analysis, destroying information that may be able to pinpoint specific anatomical regions and their effects on disease. Though this procedure is not universally accepted currently, a typical metabolomics workflow is presented in Figure 1.4.[1]

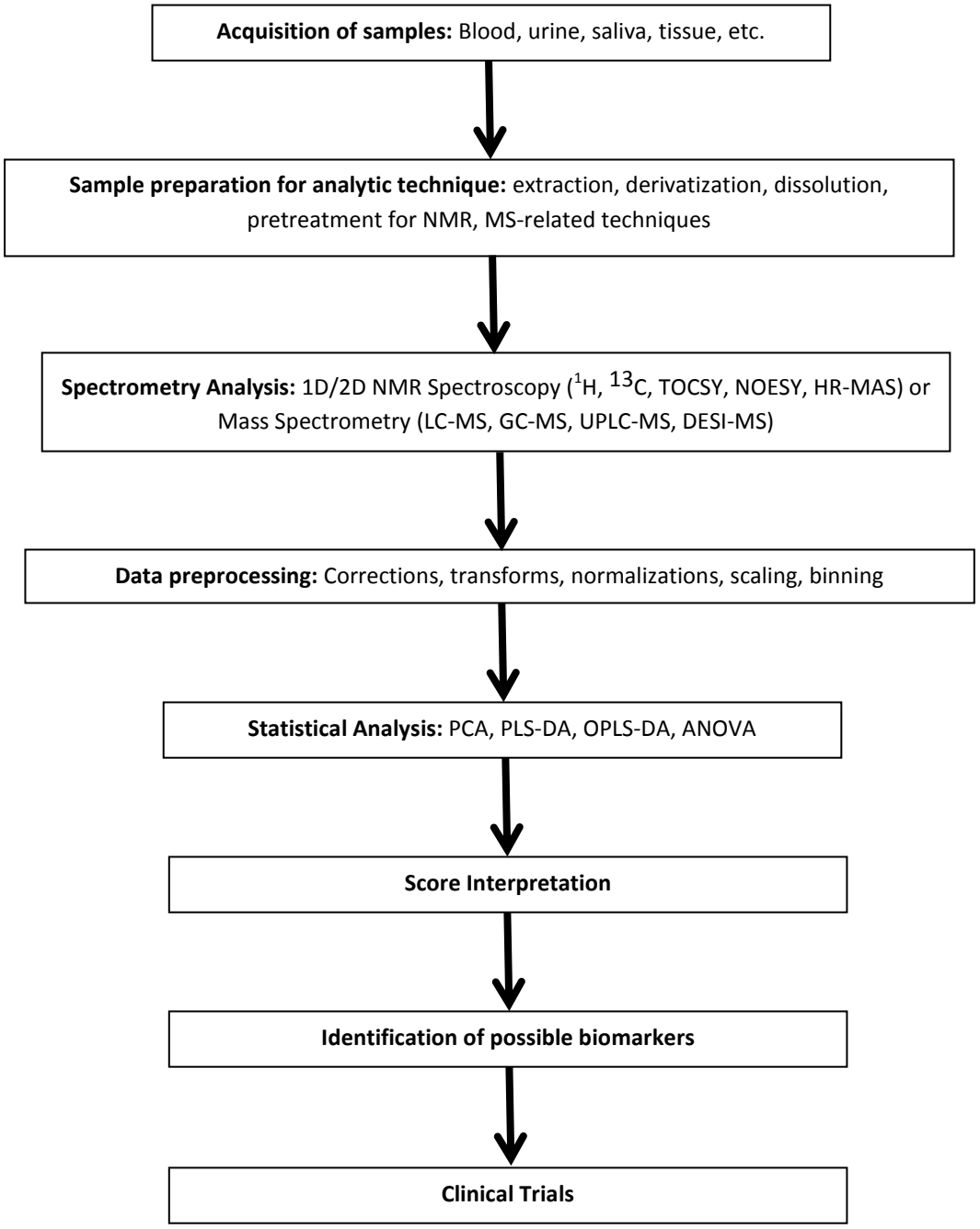

Figure 1.4: Overview of metabolomics methodology for NMR and MS based analysis. Figure reprinted with permission from Jain, N. S.; Dürr, U. H. N.; Ramamoorthy, A. *Chinese Chem. Lett.* 2015. In press. Copyright 2015 Ayyalusamy Ramamoorthy. Published by Elsevier.

Preparation of samples for analysis is very important in metabolomics, as the natural physicochemical variation in biological samples requires specific steps to standardize samples. For example, NMR samples require homogenization and extraction, with an appropriate solvent, if done in liquid phase. Tissue samples can be analyzed using solid-state NMR spectroscopy through magic angle spinning (MAS) or high-resolution magic angle spinning (HR-MAS)

techniques, which study tissues nondestructively.[284-285] Neat, extracted, and incubated samples are used in MS analyses. Specific conditions depend on the analysis being done.

In order to best determine the course of sample collection and preparation, the type of cancer and the goals of analysis must be understood in conjunction with factors such as diet, gender, age, ethnicity, pathophysiology, and location. Furthermore, a large number of samples, controls, replicates, and blanks are always preferable to ensure the broadest and most accurate data.[1] To perform analysis, samples must be preserved through sample-specific techniques. Blood plasma and serum, samples are collected with anti-coagulants or coagulants, respectively, centrifuged and stored frozen. Urine storage uses the same principles, but done so at -80 °C. Saliva samples are preferable if not stimulated, and stored at normally -20 °C for short term use and – 70 °C for long term usage.[286-287] Tissue samples are generally frozen at -80 °C. Sometimes, more drastic storage techniques, such as freezing under liquid N_2, acid treatment, quenching in salt and methanol, or chemical protection is needed to prevent oxidation and degradation.

Sample preparation also depends on the sample, the metabolites analyzed, and the analytical platform. Volatile metabolites, especially organic compounds, are difficult to use, and require special care to prevent sample loss. Extraction techniques can destroy metabolites, or alter them, which affects mass spectrometry, as MS depends on solid-phase or liquid-liquid extraction, or liquid and gas chromatography.[1, 288] However, these techniques can be directly coupled to MS, eliminating transfer steps and allowing for analysis, while still introducing error. Proper separation is one of the problems in metabolomics that this research needs to address.

NMR-based metabolomics uses a different sample preparation methodology, and also have variation on the type of NMR used, whether solution or solid-state. In solution-state NMR, the sample is prepared according to standard methods, desolvated, and suspended in a deuterated

solvent for analysis. Adjustments are made for certain conditions, including a buffer to regulate pH or the introduction of ethylenediaminetetraacetic acid (EDTA) to eliminate salt variations.[289-291] This minimal sample preparation eases the use of solution NMR spectroscopy in metabolomic analysis. Solid-state NMR spectroscopy can be directly applied to tissues, even *in vivo*, and MAS techniques directly to bone,[292-294] limiting sample preparation further. High throughput NMR techniques also exist, using a standardized flow cell for automatic sample introduction, with data nearly as accurate as standard methods.[295] Research addressing peak drift in NMR spectra due to changes in pH or salt concentration, which can cause inaccuracies in comparing signals between spectra collected from different samples, as well as spectrum complexity are addressed herein.

1.4.3 Nuclear Magnetic Resonance Spectroscopy and Metabolomics

NMR spectroscopy is a common technique applied to everything from physical chemistry to medicine.[296-299] It is highly amenable to metabolomic analysis, as a wide variety of nuclei are NMR sensitive (Table 1.2).[1] Furthermore, NMR does not damage or denature the sample, allowing for a reproduction of tests and a corroboration of results. The principle of NMR rests in the half-spin of the fermions that make up nuclei, namely protons and neutrons. Unpaired proton and neutron spin states contribute to the nuclear spin quantum number I,[300-303] with each unpaired spin contributing ½ to I. The number I is related to the magnetic moment, a measure of the torque a system will experience in a magnetic field, essentially representative of a transition

Nucleus	Nuclear spin	Natural abundance	Relative NMR sensitivity
^{1}**H**	½	99.98%	100
^{2}**H**	**1**	0.02%	0.96
^{13}**C**	½	1.1%	1.6
^{15}**N**	½	0.366%	0.1
^{19}**F**	½	100%	83.3
^{31}**P**	½	100%	6.6

Table 1.2: List of NMR-active nuclei used in metabolomics. Figure reprinted with permission from Jain, N. S.; Dürr, U. H. N.; Ramamoorthy, A. *Chinese Chem. Lett.* 2015. In press. Copyright 2015 Ayyalusamy Ramamoorthy. Published by Elsevier.

dipole moment operator. Spin states with no external magnetic field are degenerate; however, introducing an external field induces Zeeman splitting, creating 2I+1 split spin states, with the more spin aligned states stabilized, and those more antiparallel to the field destabilized.[300-303] This spin moment can be related to the gyromagnetic moment through the applied magnetic field, which is unified through shimming, locking, and spinning of the sample.

The spin moment vector can be excited by a radiofrequency pulse, and the precession frequency measured, reflecting the spin transitions to the excited state.[300-303] This motion is measured, and gives the Larmor frequency, which is characteristic of the chemical environment and the nucleus analyzed. The frequency is measured as a free induction decay, in which the relaxation of the spin excited states is measured as t_1, affected by spin-lattice interactions, and the dephasing of spins, or spin-spin relaxation, in t_2. Longer t_1 and t_2 values indicate narrower spectra.[304] Performing the Fourier Transform on the free induction decay converts the time-domain data into frequency data, which is then adjusted to the chemical shift based on the instrumental parameters.[305] These shifts represent different chemical environments of the nuclei studying, mostly affected by electron density and matrix effects. However, as nuclei are not isolated, they can experience coupling, such as *J*-coupling, or other effects such as the nuclear Overhauser effect, which can be exploited in multidimensional NMR.[306]

The instrumentation of an NMR instrument is simple, yet effective. The main components are the cryomagnet, a probe where a sample is loaded for data collection, and a console that comprises of amplifiers, receivers and data processing units.[307] A general schematic appears in Figure 1.5. Cooled probes, also known as cryoprobes, use liquid He and N_2 to achieve higher sensitivities by reducing thermal noise in the probe and detector.[300-307] The cryomagnet strength can be changed, and is often denoted in terms of ^{1}H NMR resonance frequency, where

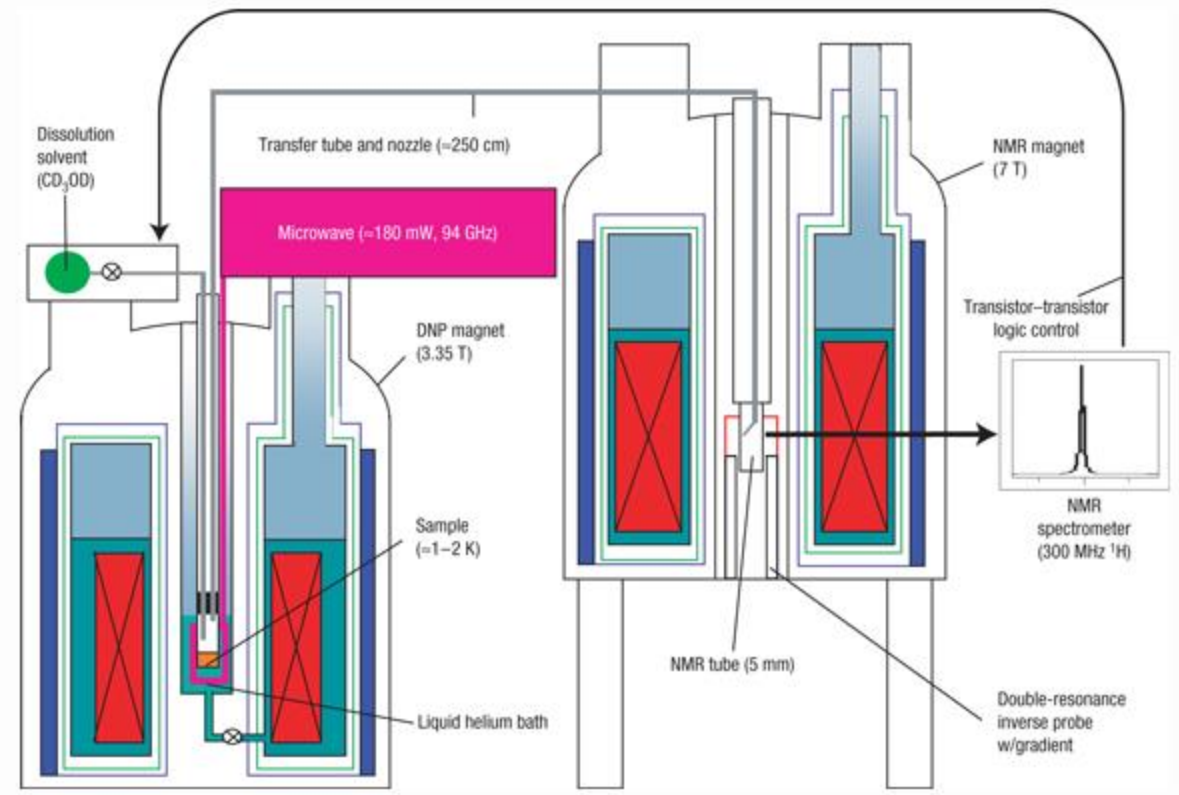

Figure 1.5: Schematic of an NMR instrument. Reprinted with permission from Frydman, L.; Blazina, D. *Nat. Phys.* 2007*, 3*, 415-419. Copyright 2007 Nature Publishing Group.

higher numbers indicate a higher sensitivity and a better resolution, giving more ppm per Hz. Cryomagnets as high as 1000 MHz are presently available for metabolomics studies, though expensive to purchase, maintain, and use.[1] The use of an ultra-high magnetic field does afford a high enough sensitivity to detect low-abundant metabolites, while also giving a high enough resolution to simultaneously detect different types of metabolites present in a biospecimen, giving much better analysis.[1]

As figures of merit increase with magnetic field strength, a 500 MHz spectrometer with a cooled probe is the lower limit for metabolomic studies.[1] Other limitations on the use of NMR in metabolomics, such as the need for an expert operator to ensure data quality, are removed by the automation of operation, making NMR high-throughput as used in commercially available quality control techniques.[308-310] Despite reproducible and quantitative data, standardized procedures, ease of preparation, and automation, the relatively low sensitivity of NMR still is the

major limitation when compared to mass spectrometry.[311] However, the use of latest approaches including fast data collection and cryoprobes can potentially overcome this limitation, as can potentially the advent of higher magnetic fields and dynamic nuclear polarization (DNP) techniques.[312]

NMR experiments also can produce data in one, two, and three dimensions, allowing for more facets of analysis that can identify and fully characterize metabolites without any difficulty in reproducing the data. The Carr-Purcell-Meiboom-Gill (CPMG) sequence helps suppress peaks from large molecular components like proteins, clarifying data, while diffusion edited experiments identify lipids.[313] Other techniques, such as total correlation spectroscopy (TOCSY) can be used in metabolomics to detect low concentration metabolites. Some two-dimensional methods reduce spectral complexity and show connectivities to increase accuracy in identifying metabolites, increase the time of acquisition as well.[314] Recently introduced non-uniform sampling (NUS) approach can dramatically speed up the data collection process.[315] In general, two-dimensional spin-spin correlation techniques such as TOCSY, heteronuclear single-quantum coherence, and heteronuclear multiple bond correlation are used in metabolomics.

As discussed above, a large profile of atoms are available for metabolomic analysis. However, most NMR studies are carried out on ^{1}H nuclei, with ^{13}C and ^{15}N experiments

NMR Methods	Information	Advantages
1D, presaturation, NOESY	Universal detection	Very quantitative, good for screening
CPMG	Selective for small molecules	Easily interpreted, good for blood, HR-MAS
Diffusion-edited sequences	Gives more large molecule peaks	Simple technique, simple spectra
2D *J*-RES	Simplified spectra, elimination of coupling	Simplifies overlapping signals
HSQC	Identifies heteromolecules connections with proton	Simplifies spectra
HMBC	Identifies heteromolecules connections with proton	Simplifies spectra
TOCSY	Identifies heteromolecules connections with proton	Simplifies spectra, detects lower concentrations

Table 1.3: List of NMR-based experiments used in metabolomics. Figure reprinted with permission from Jain, N. S.; Dürr, U. H. N.; Ramamoorthy, A. *Chinese Chem. Lett.* 2015. In press. Copyright 2015 Ayyalusamy Ramamoorthy. Published by Elsevier.

becoming more frequency. The heteronuclear experiments have resisted widespread implementation due to the need for and high cost of isotopically labeled samples. Combining ^{1}H and ^{13}C NMR into 2D experiments, as described above, can cut down on data collection time and give better analyses. The full range of NMR-based metabolomics experiments is in Table 1.3.[1]

NMR is also done mostly on solution samples in deuterated solvents such as D_2O, $CDCl_3$, and CD_3OD, but can be applied to solid samples as well, through solid-state NMR spectroscopy. However, due to the lack of motion of the sample in the solid state, there is an inherent loss of signal intensity, as sharp peaks in the liquid phase become broader in the solid state.[303] Thus, spinning of the sample is done to ensure the uniformity of the magnetic field and reduce signal broadening. Spinning at the magic angle (54.7356°) allows for the acquisition of "liquid-like" spectra, due to the basic physical interactions between a sample and the magnetic field.[316] Samples can be spun up to 110 kHz for bone and rigid tissue, but soft tissue is normally spun at 5 kHz. MAS techniques increase the avenues for metabolomic analysis, including the ability to study non-soluble or large metabolites, and directly analyze bacteria, cells, tissue, and viruses.[317] These measurements depict physiologically relevant metabolomics, giving more access to metabolomic profiles.[318-319] NMR spectroscopy is established in disease and drug metabolomics, as well as other avenues, but expanding NMR applications is an avenue for future research, and discussed herein.

1.4.4 Mass Spectrometry in Metabolomics

Mass spectrometry (MS) is a highly sensitive technique for metabolomic analysis. The theory of MS will not be discussed in great detail here, as the instrumentation was not directly implemented in this investigation.[302] A MS instrument has three main components: an ion source

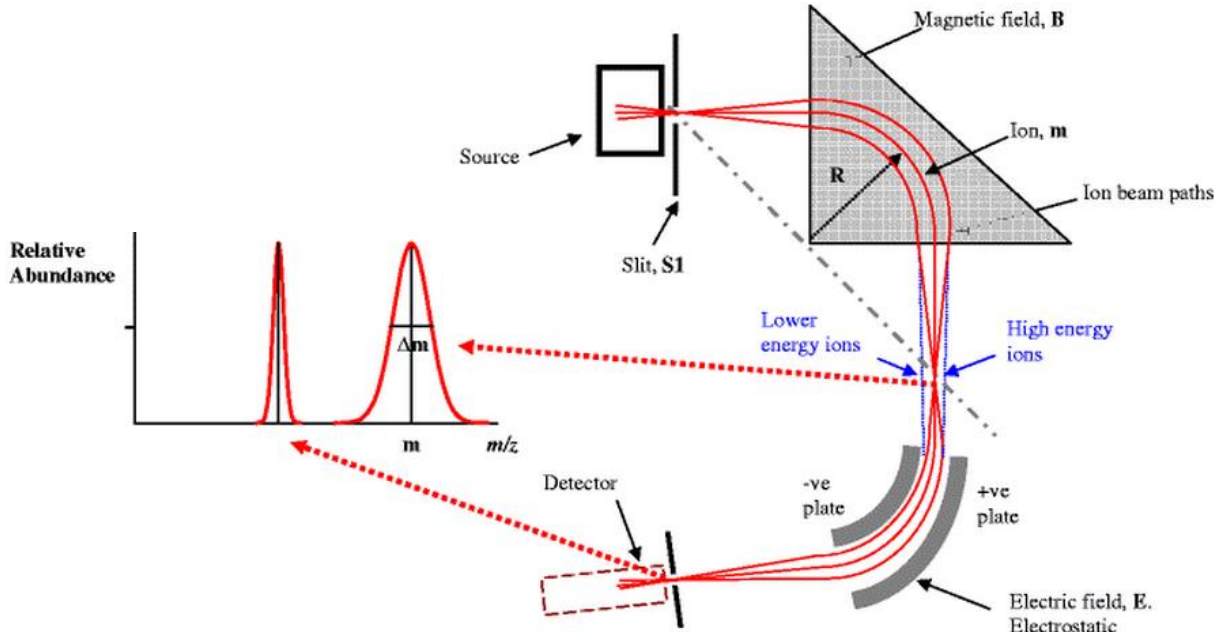

Figure 1.6: Schematic of an MS instrument. Reprinted with permission from Godfrey, A. R.; Brenton, A. G. *Anal. Bioanal. Chem*. 2012, *404*, 1159-1164. Copyright 2012 Springer Link Publishing.

for desolvation and ionization; a mass analyzer, which separates ions based on the mass-to-charge ratio after acceleration and placement in a magnetic field, and a detector, which determines the mass-to-charge ratio. A general schematic appears in Figure 1.6.[320] Multiple ionization methods exist, ranging from the harsh electron impact (EI) to softer electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI).[321-323] Common analyzers include quadrupoles, ion traps, and time-of-flight analyzers, with FT-ICR and orbitraps for a higher resolution, around 1 ppm.[324-326]

Metabolomics generally uses quadrupole and time-of-flight analyzers, which affords the most flexibility and range for analysis. A full list of all MS experiments used in metabolomics is provided in Table 1.5, including in multidimensional and imaging experiments.[1] MS is beneficial in that it is more specific and sensitive toward a complex mixture than NMR. But, MS-based metabolomics has severe limitations, as it can provide irreproducible results, variations across research groups and methodologies, fail to identify established biomarkers, and give too complex

MS Methods	Information	Advantages	Disadvantages
LC-MS/HPLC-MS/UPLC-MS	Molecules separated with liquid chromatography first	Simple preparation	Ion suppression can alter results
GC-MS	Uses gas chromatography first	More sensitive, reproducible, high separation efficiency	Long preparation, not universal technique
2D-GC-MS	Molecules separate in 2D	Applicable to complex samples	Long preparation, not universal technique
EESI-MS	Direct solution analysis method, uses spray sources	No preparation	Less quantitative, damages samples
DESI-MS	Ionization method for direct analysis	Tolerates salts, no preparation	Less quantitative, damages samples
DART-MS	Uses He gas, N_2, real time info, direct analysis	Sensitive, no preparation	Less quantitative, damages samples
TOF-MS	Mass-to-charge ratio determined by time needed	Unlimited mass range, fast	Complex electronics, complicated set up
CE-MS	Samples separated by capillary electrophoresis	High separation efficiency	Needs higher concentration of samples

Table 1.4: List of MS-based experiments used in metabolomics. Figure reprinted with permission from Jain, N. S.; Dürr, U. H. N.; Ramamoorthy, A. *Chinese Chem. Lett.* 2015. In press. Copyright 2015 Ayyalusamy Ramamoorthy. Published by Elsevier.

of a spectrum to identify an individual analyte. In addition, unlike NMR, samples for measurements are not recoverable for further analyses. These are all avenues for future research.

1.4.5 Analytical Techniques in Combination in Metabolomics

As discussed above, these analytical techniques are not used in a vacuum, but can be combined with separation methodologies, such as gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) in so-called hyphenated experiments.[327-329] Due to the combination of separations and analysis, more information is gleaned, as several simpler spectra are obtained as opposed to one complex spectrum, affording more accurate results. LC is favored in metabolomics as it is more sensitive and offers a larger breadth of information, as many samples are fluids that can be directly placed in the instrument for analysis with minimal preparation, such as the removal of proteins from sera. Furthermore, though advances in LC have reduced the detection limits in the systems, the methodology still is time-consuming. GC is also widely seen, but requires more sample preparation. Further methods

can be used for the separation of analytes but may require analysis or alteration of metabolites, affecting the data seen. Separation techniques are investigated herein as well.

1.4.6 Statistical Analysis in Metabolomics

Due to the mass of data obtained via NMR and MS spectral analysis, a large amount of statistical processing is needed before interpretation can even proceed. False signals, matrix effects, and peak drifts that cause misalignment, the removal of complexities and the revealing of low-abundance signals through binning and data scaling and centering are a few of the processing steps needed. Peak drifts are discussed in detail in this study. All these methodologies are at risk of fundamentally changing data, however, by eliminating real biomarkers or giving false positives that are artifacts of the statistical procedure.

Spectra are aligned based on reference compounds from databases such as KnowItAll and Amix.[330-331] After post-acquisition processing, direct statistical methods are used for interpretation. Univariate analyses, such as the students' t-test, are used in metabolomics, but are not the dominate method.[332] Commonly, multivariate methods are used due to the complexity of samples, which may limit the dimensionality of data to ease interpretation.

Multivariate analysis can be performed using supervised and unsupervised methods. Unsupervised methods classify spectra based on given data, not external standards, in untargeted analysis. This is done via principal component analysis (PCA), which ranks components and descriptors to explain variance. Alternative methods include hierarchical cluster analysis (HCA), which defines natural clusters based on comparison between distances of pairs of samples, or variables, and K nearest neighbor analysis (KNN), which shows similarity between classes.[333]

On the other hand, supervised methods compare data against a predicted model in targeted analysis, used mostly as a cross-validation technique, but can force classification of data. Some supervised methods include partial least squares-discriminant analysis (PLS-DA),[334] soft independent modeling of class analogies (SIMCA), orthogonal signal correction (OSC), and OSC and PLS-DA combined to give OPLS-DA. Statistical validation can use permutation tests upward of 200, and validated findings still need secondary samples and biological validation before acceptance of the biomarker, as well as further clinical trials.[335]

Other statistical methods are used, including correlation methods, such as the statistical TOCSY used to compare NMR and MS spectra, and simply the identification of metabolites. PCA, NMR, and MS results are also often combined to aid the interpretation of data when two-dimensional score plots fail to fully capture the information presented.[336-338]

1.4.7 Databases used in Metabolomic Analyses

To ease the interpretation of the vast amount of spectral data generated, databases have been formed to identify metabolites and provide reference values, benefitting metabolomics as a whole. Examples include the Human Metabolome Database (HMDB), backed by the University of Alberta,[339] a free electronic database with 41,154 metabolite entries, including water-soluble, lipid-soluble, abundant, and rare signals. Other commonly used databases are METAGENE, based in the University of Tübingen,[340] which contains genetic errors in metabolic pathways, giving information on the genetic fingerprint and metabolic concentrations found in a disease; MassBank, of Keio University,[341] with vast amounts of MS spectra, and the Biological Magnetic Resonance Data Bank (BMRB) at the University of Wisconsin,[342] which is NMR-specific and metabolomics specific, along with more detailed NMR data such as coupling constants, time-

domain data, and kinetic information. One developed at the University of Michigan is MetaboID, an NMR-specific database used for complex mixture assignment in 1D analysis.[343] AMIX and Chenomx are commercial databases that can verify small molecule structures from NMR and MS, and even quantify compounds accounting for conditions of analysis.[345-346]

1.5 Issues in Metabolomic Analysis

As displayed above, there are certain issues inherent in metabolomic analysis, especially in NMR, the methodology chosen in this analysis. NMR was the focal point due to its reproducibility, ease of analysis, and potential for growth, all of which outstrip MS.[347-350] Furthermore, NMR is faster and requires less sample preparation, as well as is accessible to a wide range of metabolites. However, as discussed above, NMR suffers from low detection limits and can afford overly complex spectra for analysis (Figure 1.7),[351] especially in holistic ^{1}H analysis, the simplest form of NMR done and the method most likely to be implemented on a wider, diagnostic-level scale. This prevents the identification of compounds in the spectrum, and potentially false positives and false negatives in biomarker searches. NMR also suffers from inherent peak shifts, as the chemical environment is highly sensitive to the ionic strength and pH

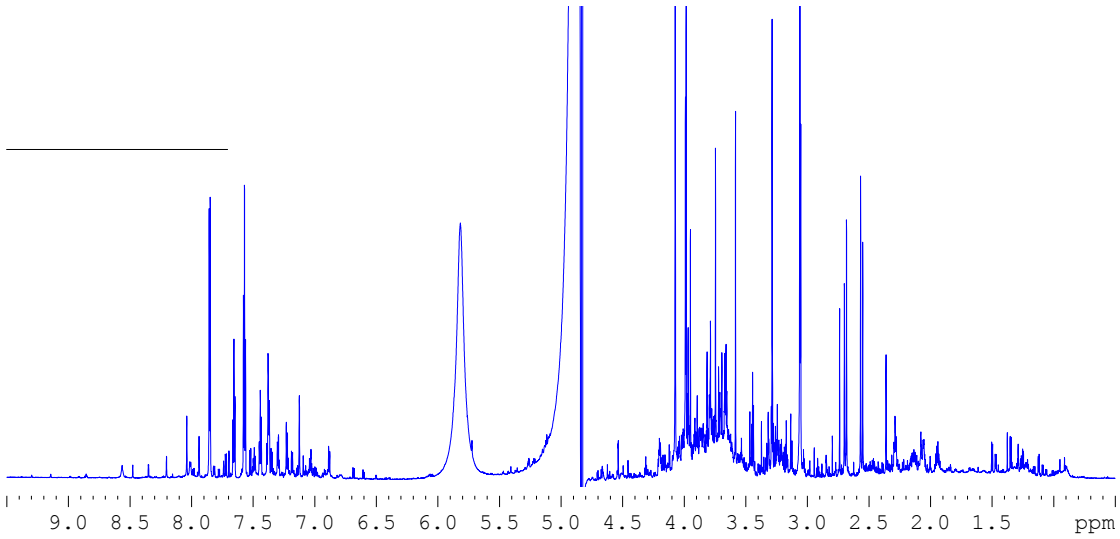

Figure 1.7: 900 MHz ^{1}H NMR scan of neat urine. Notice the complexity across the spectrum.

of the surroundings. Thus, data is no longer reproducible, as a peak cannot reliably appear in the same position, causing a misidentification when compared between two separate patients.[1] Therefore, that biomarker, identified in one patient, cannot be used in the other, as the actual signal of the biomarker may appear as a different analyte.[1] These two issues combined are a major roadblock to the widespread implementation of metabolomic analysis as a cancer diagnosis methodology.

This analysis was done on urine specimen as well, rather than blood, saliva, or tissue, as urine offers a holistic assessment of body conditions, as all systems drain into the kidneys, without the protein and cellular complexities of blood, or the location specificity of tissue and saliva.[281-283] Also, the liquid nature of urine allows for ease of application to NMR, with minimal sample preparation needed. Urine is also obtained in a noninvasive fashion, allowing for easy acquisition and less patient discomfort, affording larger benefits as a diagnostic technique.

It is the addressing of these issues that drove this investigation into metabolomic methodology. First, spectral complexity was tackled by applying a simple two-step phase separation technique, which resolved the complex NMR spectrum into two simpler spectra. Then, spectral drift was assessed through a simple standardization technique by which the urine was treated, again a two-step process which gave uniform spectra across samples from various different patients. By solving these issues, metabolomics would become open to a full-scale implementation in biomarker analysis, allowing for these analytes to be easily detected, and affording universal improvement of cancer diagnosis through a simple urinary biomarker test. If successful, this would allow for further applications to other diseases.

Chapter 2:

On the Resolution of Complex NMR Spectra via Esterification and Phase Separation, with Applications to Urinary Metabolomics

2.1 Abstract

Metabolomics is devoted to determining the metabolic profile of diseased and healthy individuals, in order to discover biomarkers that would be indicative of the selected disease, allowing for earlier and more accurate diagnoses. One technique used widely in metabolomics is nuclear magnetic resonance (NMR) spectroscopy, which exploits the intrinsic spin of nuclei. However, NMR has complications in metabolomics, as the human samples used, such as urine, contain a large variety of compounds that afford extremely complex spectra, and make identifying specific markers impossible. To this end, a simple esterification procedure was proposed, using (trimethylsilyl)diazomethane, known to react with carboxylic acids to form esters, followed by a phase separation to give two distinct, simple spectra from one complex spectrum. Though successful in providing two less complex structures, the procedure still requires optimization before widespread implementation can be carried out in medical diagnoses.

2.2 Introduction

As discussed above, metabolomics is an all-encompassing science, focusing on the study of the metabolic byproducts of biochemical processes in the human body.[1] By profiling these processes, metabolomics has been shown to be able to distinguish between healthy and diseased patients solely by comparing the concentrations of the metabolites profiled.[2-5] As health care costs continue to rise, and diagnostic techniques for diseases such as cancer remain invasive and

expensive, metabolomics fills a need for more accurate, less invasive, and quicker diagnostic techniques, improving on the outcome of patients.[21-25]

Currently, metabolomics focuses on various biological samples, including blood, urine, and saliva,[281-283] and differing analytical platforms, such as mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.[241-243] As discussed above, each has its own benefits and drawbacks. Urinary analysis is especially interesting as it provides a holistic analysis of the body, and simultaneous screening for different diseases. This, combined with NMR, allows for extremely fast and accurate profiling of the patient. However, urine, as the depository fluid for all bodily processes, has a myriad of compounds detectable by NMR spectroscopy, which results in unresolvable spectra.[281-283,351] Though higher order experiments, including two-dimensional spectroscopy and complex derivatization procedures, have been performed to resolve this issue, a simple procedure has not been found.[311-315,352]

Such a procedure would allow for the performance of NMR-based urinary metabolomics by technicians of any skill level. This would allow for the full implementation of this technique as a diagnostic technique, as a major obstacle to metabolomics-based diagnoses is the skill required to operate and interpret the results. Many metabolites are carboxylic acids, such as citric acids, or carboxylic acid-derivatives, such as the full complement of amino acids.[2-5] Thus, they are easy targets for selective esterification with a mild agent, such as (trimethylsilyl)diazomethane (TMSDM), which has been well-established in selective esterifications.[353-355] Furthermore, in removing the carboxylic group, and replacing it with a methyl ester, the solubility profile of these compounds change from aqueous to organic. Thus, a simple phase extraction was proposed herein, allowing for the separation of the newly reacted compounds into a nonpolar solvent, while leaving behind polar, unreacted analytes. Subsequent

analyses of these solutions would then result in two spectra that are easy to understand. It was hypothesized that the procedure discussed above would be simply implemented and optimized, affording two separate, clearly understandable spectra, giving polar, nonacidic metabolites in the water spectrum and the esterified carboxylic acid and nonpolar metabolites in the chloroform spectrum. Herein is discussed the methodology development and subsequent optimization of the phase separation of urinary metabolites via esterification to afford resolved NMR spectra.

2.3 Experimental

2.3.1 General Considerations

All conversions and manipulations were done to the open air. Urine samples were obtained from the University of Michigan Hospital System. Samples were labeled with an ID number, without knowledge of the condition of the patient. All sample procedures followed standard safety and security practices. Urine was kept at -80 °C until use, during which it was kept at -10 °C. All solvents, standard amino acids, urea, and the (trimethylsilyl)diazomethane were purchased from Sigma-Aldrich. Deuterated solvents were purchased from Cambridge Isotope Laboratories. Lyophilization and centrifugation were done on the instruments provided by Biophysics at the University of Michigan. All spectroscopic analysis was done on a Bruker 500 MHz instrument, with one-dimensional and two-dimensional capabilities. All matching, tuning, shimming, and locking of the instrument signal on the resonant proton was done by hand, rather than the automated function. Initial data processing was done using the Bruker interface. Further processing, including spectral annotation was done via ACD/NMR Labs software. All spectra obtained during the course of the experiment appear in the supplementary information.

2.3.2. Esterification Procedure.

Various ratios and solvents were used to optimize the esterification procedure. Each condition used, accounting for each change in solvent, time, and volume, is detailed in the supplementary information, with the general procedure outlined below. Standard carboxylic acid solutions were also made and analyzed, as detailed below.

Standard carboxylic acid preparation: The following L-amino acids and other carboxylic acids were made into 0.1 M solutions, after which 25 μL of each were combined to form the standard solution for esterification as below: citric acid, adipic acid, *trans*-aconitic acid, alanine, phenylalanine, glycine, glutamate, and valine.

General Esterification Procedure: Urine was initially thawed to room temperature. A 2 mL sample was combined with equal parts of methanol and acetone, and then allowed to sit at -10 °C for one hour. After, the sample was centrifuged at 5000 rpm for fifteen minutes, after which the clear yellow supernatant was taken, and the white solid discarded. This supernatant was then divided into four equal aliquots of 1.5 mL each, and lyophilized overnight. After lyophilization, the residual yellow-brown solid was reconstituted in 4 mL of 3:2 (v/v) toluene:methanol to form a clear, colorless solution. Next, 150 μL of (trimethylsilyl)diazomethane was added, and the sample was stirred vigorously until the yellow color disappeared. Gas evolution was observed. This occurred over the course of three hours. After, the sample was dried with N_2 gas. The residual solid was then treated with 1:1 water:chloroform, and then centrifuged at 5000 rpm for five minutes. The water layer was then extracted, and dried via lyophilization overnight, while the chloroform layer was dried over nitrogen. The water layer was then dissolved in 500 μL D_2O

and 50 μL phosphate buffer (10 mM phosphate, 100 mM NaCl, pH 7.4), and the chloroform layer in 5550 μL $CDCl_3$, and both samples were separately analyzed via NMR spectroscopy.

2.4 Results and Discussion

2.4.1 Methodology Development

Initially, standard carboxylic acid solutions were made, containing citric acid, adipic acid, *trans*-aconitic acid, and the following L-amino acids: phenylalanine, alanine, glycine, valine, and glutamate. These compounds were treated with (trimethylsilyl)diazomethane (TMSDM), a known esterifying agent (Figure 2.1). This was done in a 3:2 toluene:MeOH (v/v) solution, with 150 μL of TMSDM and a three hour reaction. After treatment with this agent, the sample was then separated via a nonpolar phase extraction via 1:1 H_2O:$CHCl_3$, and the two phases were analyzed separately via ^{1}H-NMR. The samples were tracked for the peaks relevant to these acids, as displayed in Table 2.1.[339-346] A representative spectrum comparing the initial, untreated urine and both the nonpolar and polar phases of the treated sample appears in Figure 2.2. Full spectral details appear in the supplementary information.

Based on the data presented in Figure 2.2, the esterification was successful. It is evident that the peaks disappear from the polar phase, and appear in the nonpolar phase. Furthermore, there is a large increase in peak intensity around 3.5 ppm, indicative of the addition of a new methyl group to the carboxylic acids. Looking at the polar phase (Figure 2.2b), it is evident that not all peaks went into the nonpolar phase. Most residual peaks were attributed to citric acid; as it has three carboxylic groups and one hydroxyl group, it is possible that the citrate did not fully

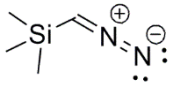

Figure 2.1. (Trimethylsilyl)diazomethane.

Compound	Peaks (ppm)
***Trans*-aconitic acid,**	3.74, 6.93
Adipic Acid	1.51, 2.21, 12.0
Citric Acid	2.883, 3.011
L-Phenylalanine	3.126, 3.286, 3.990, 7.333, 7.383, 7.428
L-Alanine	1.482, 3.787
Glycine	3.567
L-Valine	0.995, 1.045, 2.260, 3.608
L-Glutamic Acid	2.035, 2.390, 2.510, 3.896, 8.590, 13.000

Table 2.1. List of peaks studied in standard sample.

esterify and leave the polar phase for the nonpolar phase. Also, the increase in peak intensity from 0 to 2 ppm in the nonpolar phase is due to degradation of plastic containers used in centrifugation due to chloroform.

2.4.2 Urinary Analysis

After a successful trial with the standard set of carboxylic acids, this procedure was then applied to urinary samples. After thawing and lyophilization, 500 µL of urine was treated with

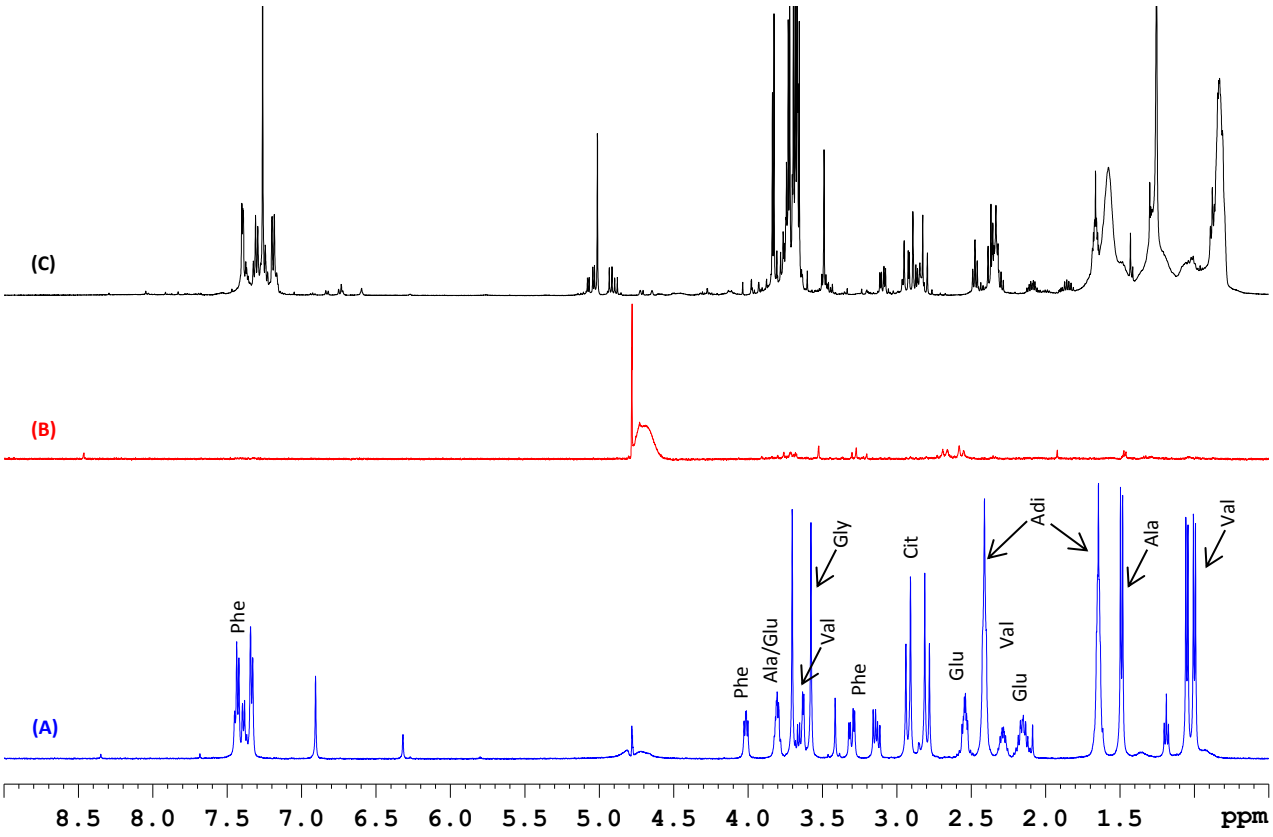

Figure 2.2: 500MHz ^{1}H NMR Spectra of (A) Mixture of amino acids and carboxylic acids in D_2O (B) Water layer after esterification and (C) Chloroform layer after esterification. It is clearly seen that all the carboxylic containing polar mixtures becomes non polar after esterification. The reaction was done with 125 of TMSDM in 3:2 Tol:MeOH (v/v) for three hours. Phase separation was then done with 1:1 water:chloroform.

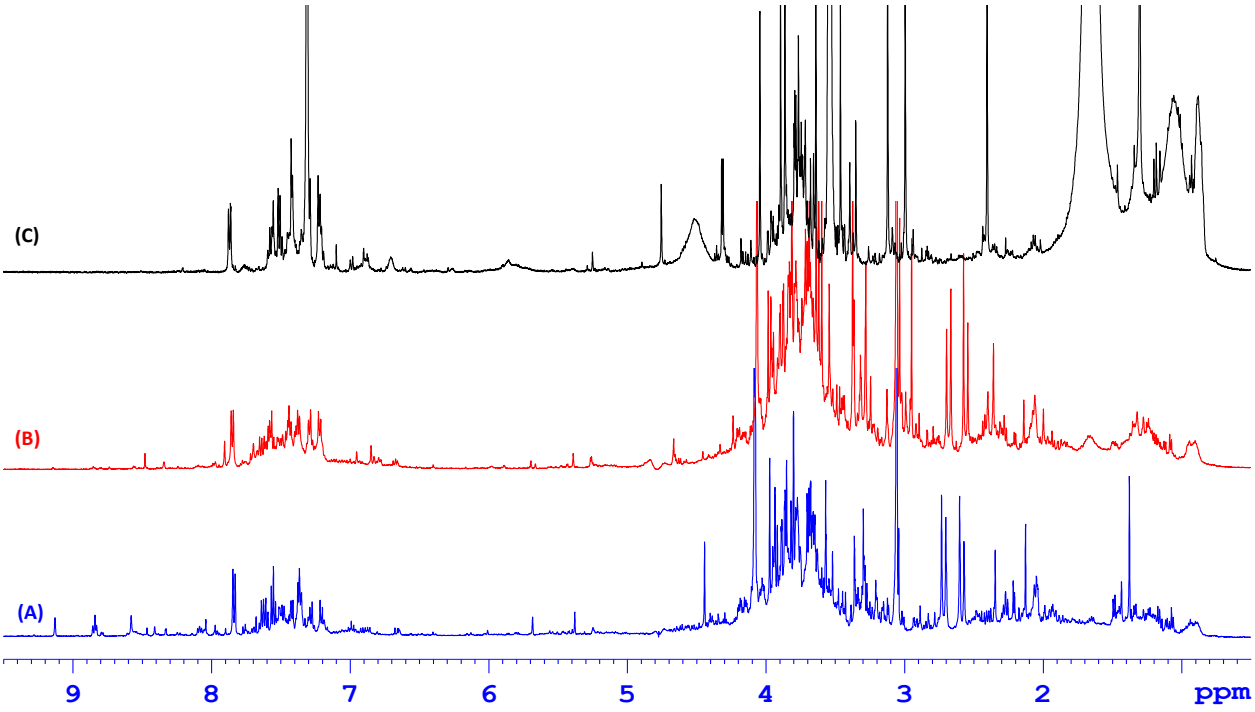

Figure 2.3: 500 MHz ^{1}H NMR spectra of (A) 500 µL urine showing all the metabolites (B) water layer after urine esterification showing only unesterified non-carboxylic containing metabolites (C) Chloroform layer showing only esterified carboxylic containing metabolites. The reaction was done with 125 of TMSDM in 3:2 Tol:MeOH (v/v) for three hours. Phase separation was then done with 1:1 water:chloroform.

150 µL of TMSDM in 3:2 Tol:MeOH (v/v) for three hours. Phase separation was then done with 1:1 water:chloroform, and the two phases separated. Though no peaks were specifically looked for in terms of shifting, the acids studied above were paid special attention to as bellwethers of if this procedure was effective, based on peaks in Table 2.1. An example spectrum of the results of this procedure appears in Figure 2.3. Full spectral details are in the supplementary information.

As seen in Figure 2.3, phase separation was achieved, to a degree. It is evident that the nonpolar layer has a large amount of metabolites in it, as does the polar layer. Therefore, it is hard to definitively state that this procedure was truly effective. It is possible that there are some esterified metabolites in the nonpolar layer, as evidenced by the increase of peak intensity at 3.5 ppm, as well as some natively nonpolar metabolites that were separated into the chloroform layer without interaction with the esterifying agent. Furthermore, it is evident that the water layer contains some nonesterified reagents. Though peaks from the valine and alanine amino acids were quite depressed in the water layer, the amount of aromatic phenylalanine still was high, as

was the various other carboxylic acids present in the 3-4 ppm range, evidenced by the large intensity of those regions. Ideally, if this procedure were to work to its fullest extent, the relative intensities of peaks in the water and chloroform spectra would be roughly the same, or the chloroform would be greater, depending on the patient. It is evident that this is not the case. Therefore, this procedure was further studied to afford an optimized reaction.

2.4.3 Procedure Optimization

After it was determined that this procedure needed to be optimized, a set of screens was set up to see which esterification procedure afforded the best data. TMSDM is known to esterify in not only the toluene and methanol mixture used above, but also in chloroform.[353-355] Various times and separation techniques were used as well. The full set of conditions studied appears in Table 2.2, with the full set of spectra obtained in the supplementary information. Condition quality was graded on a scale from 1 to 10, with 10 being a fully optimized procedure. These grades were based on the completeness of the esterification, as seen in the comparison between the chloroform and water layers of the NMR spectra, as well as the quality of each individual spectrum. Ideally, statistical analysis would have been performed, but no available package was

Condition Number	Solvent (mL)	TMSDM Volume (µL)	Reaction Time (hr)	Separation System	Grade
1	Tol:MeOH 3:2 (4)	150	3	1:1 H_2O:$CHCl_3$	6
2	Tol:MeOH 3:2 (4)	150	4	8:4:3 $CHCl_3$:MeOH:H_2O	0
3	Tol:MeOH 3:2 (4)	300	4	8:4:3 $CHCl_3$:MeOH:H_2O	2
4	Tol:MeOH 3:2 (4)	300	4	1:1 $CHCl_3$:0.9% NaCl	3
5	Tol:MeOH 3:2 (4)	300	4	2:1:0.3 $CHCl_3$:MeOH:0.9% NaCl	3
6	Tol:MeOH 3:2 (2)	150	4	1:1 H_2O:$CHCl_3$	1
7	Tol:MeOH 3:2 (2.5)	50	3	1:1 H_2O:$CHCl_3$	0
8	MeOH:Et_2O 10:1 (4)	150	2	1:1 H_2O:$CHCl_3$	0
9	DCM: Et_2O 1:1 (4)	150	2	1:1 H_2O:$CHCl_3$	0
10	Tol:MeOH 3:2 (2)	150	2	1:1 H_2O:$CHCl_3$	1
11	Tol:MeOH 3:2 (2.5)	60	3.5	1:1 H_2O:$CHCl_3$	0
12	Tol:MeOH 3:2 (2.5)	70	3.5	1:1 H_2O:$CHCl_3$	0
13	Tol:MeOH 3:2 (2.5)	100	18	1:1 H_2O:$CHCl_3$	5
14	Tol:MeOH 3:2 (2.5)	125	18	1:1 H_2O:$CHCl_3$	6
15	Tol:MeOH 3:2 (2.5)	150	18	1:1 H_2O:$CHCl_3$	7
16	$CHCl_3$:CD_3OD 2:1 (0.6)	125	18	1:1 H_2O:$CHCl_3$	1
17	Tol:MeOH 3:2 (2.5)	200	18	1:1 H_2O:$CHCl_3$	3

Table 2.2. List of conditions studied for optimization. Grades were on a 1-10 scale, with 10 being the optimal procedure, based on the completeness of the esterification and the separation. Spectra are available in the Supplementary Information.

able to completely assess the quality of the data at the level required. These grades are in Table 2.

Initially, the extraction procedure was modified, keeping the same reaction conditions. Rather than the 1:1 $CHCl_3$:H_2O condition discussed above, a procedure of 8:4:3 $CHCl_3$:MeOH:H_2O was used to determine if that would induce better spectra. Other separation techniques used were 1:1 $CHCl_3$:0.9% NaCl and 2:1:0.3 $CHCl_3$:MeOH:H_2O. After, urine was acidified before treatment, to determine if that would improve the spectrum quality seen. This procedure was done using the conditions and techniques discussed above. The solvent system was also changed in the search for better data. TMSDM, though generally done in toluene and methanol, can be reacted in various solvent conditions, including 10:1 MeOH:Et_2O and 1:1 DCM:Et_2O.[354-355] Both these conditions were attempted with the TMSDM concentrations as discussed above. None of the above conditions afforded better data, as seen in Table 2.2. The full spectral breakdown of these conditions is in the supplementary information.

Based on Table 2.2, the 3:2 Tol:MeOH solvent system, with the 1:1 H_2O:$CHCl_3$ extraction procedure was determined to be the optimal one, and the concentrations of TMSDM and the time of the reaction were varied instead. The full range of conditions appears in Table 2, and went from 60 μL to 300 μL of esterifying agent, and 2 hours to overnight reaction. A schematic showing the changes in NMR spectra as the TMS concentration was increased appears in Figure 2.4. The corresponding change in spectra due to changes in time is in Figure 2.5. As before, the grades of the esterification procedure quality are in Table 2.2, and a full spectral breakdown of these conditions appears in the supplementary information.

As can be seen from this data, changing the time of reaction and the amount of esterifying agent did not improve the spectral quality to any significant degree. The spectral quality did improve; however, the improvement was not meaningful enough to support the

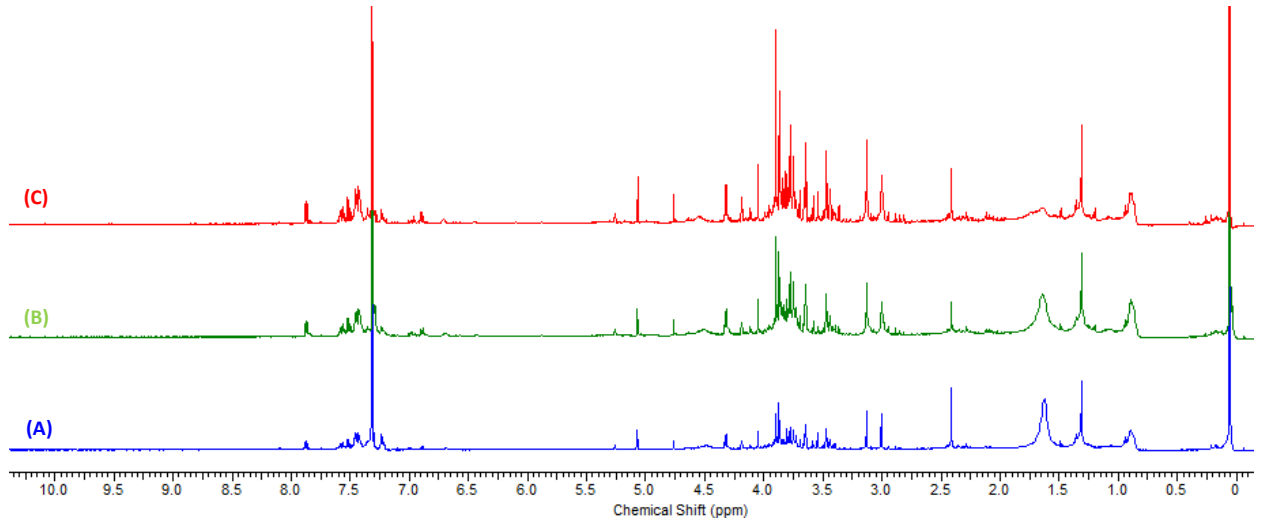

Figure 2.4: 500 MHz ^{1}H NMR spectra of $CDCl_3$ layers of (A) Condition 13 in Table 2.2 (B) Condition 14 in Table 2.2 (C) Condition 15 in Table 2.2. Though the intensity of certain residues increased with the increase in TMSDM, there were still missing peaks corresponding to key amino acids known to esterify. Identified peaks also corresponded to residues that appeared at larger values in the native urine, indicative of incomplete esterification. Chloroform layers only are shown for simplicity.

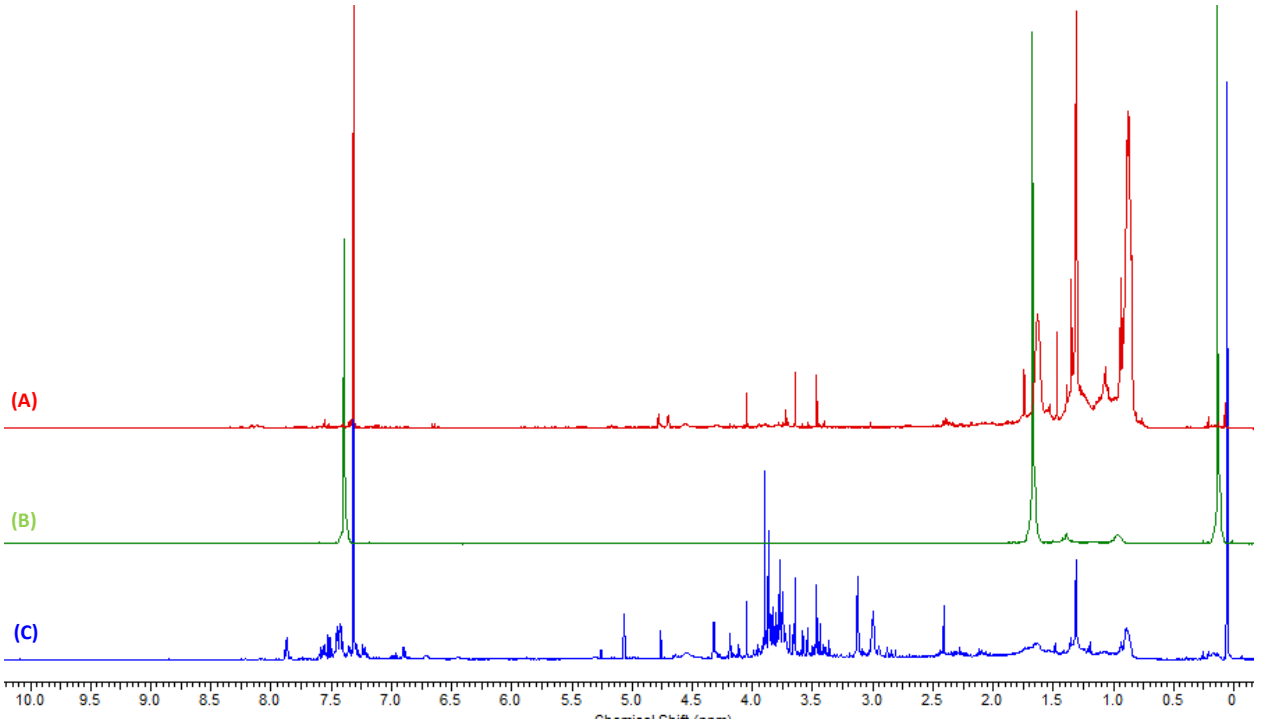

Figure 2.5: 500 MHz ^{1}H NMR spectra of $CDCl_3$ layers of (A) Condition 1 in Table 2.2 (B) Condition 10 in Table 2.2 (C) Condition 15 in Table 2.2. Though the intensity of certain residues increased with the increase in reaction time, there were still missing peaks corresponding to key amino acids known to esterify. Identified peaks also corresponded to residues that appeared at larger values in the native urine, indicative of incomplete esterification. Chloroform layers only are shown for simplicity.

validation of this technique in metabolomic analysis. Peak intensities increased across samples, but no new peaks were formed. Thus, though key metabolites such as adipic acid are present at

higher TMSDM levels at higher intensities in the chloroform layer, key contributors, such as valine and glycine, still are missing from the esterified spectrum. Furthermore, the intensities still pale compare to those found in native urine (Supplementary Information), suggesting that there remains a large amount of these compounds in the water sample. Though the water samples were not analyzed to corroborate this thought, the lack of complete esterification and extraction is eminently clear, and none of these techniques were able to work completely. Increasing TMSDM levels further may help, but impede the cost-effectiveness of this technique.

This negative result inspired an investigation into components of urine that may affect the mechanism of TMSDM action (Figure 2.6).[356] Based on this mechanism, it was thought that components of urine, such as salt, would affect the action of TMSDM. Proteins had already been removed during the pretreatment of urine with methanol under cold temperature. However, a study of the effects of salt on TMSDM action with several standard amino acids revealed that there was no real influence of salt on the mechanism, and thus other components were investigated, such as urea, which showed no change (Supplementary Information). Similarly,

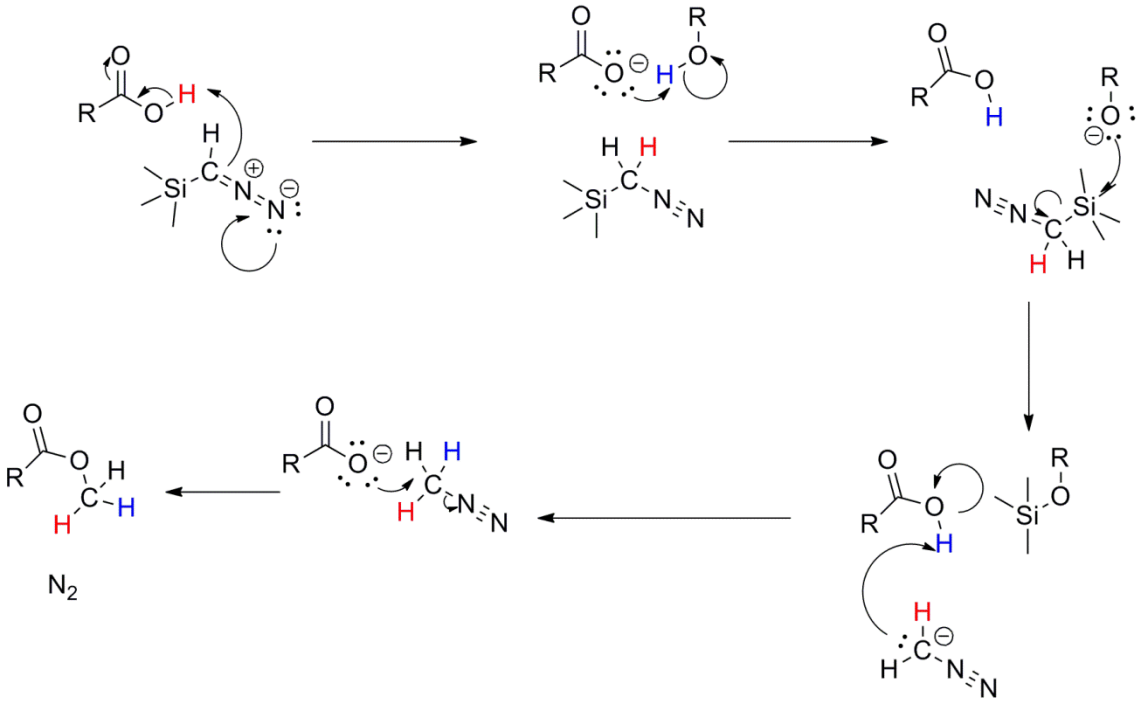

Figure 2.6 Mechanism of TMSDM action on generic carboxylic acids. The transference of hydrogens is highlighted. It is possible that water or salt can contribute to quenching of this reaction.

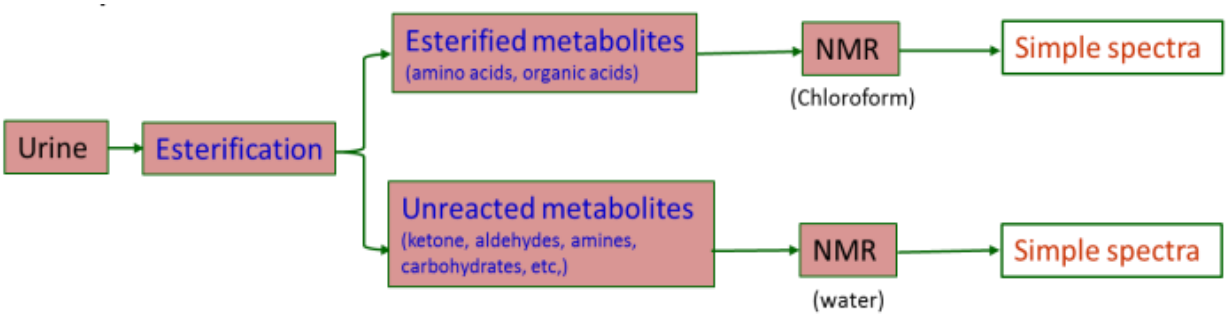

Figure 2.7. The general workflow for this esterification procedure. Each step was carefully done to prevent error. The esterification step includes the reaction and phase separation as detailed above.

acidified urine did not afford any improvement on spectra, as can be seen in the supplementary information as well.

This left many questions as to the reason why there was a lack of optimization. Based on the workflow presented in Figure 2.7, all steps were done with the utmost care to prevent water contamination of esterification, which is known to quench this reaction. It is unlikely that there was not enough of the esterification agent to cause this reaction to occur, as increasing the TMSDM concentration did not change reaction results, nor was there a kinetic barrier, as increasing the reaction time had little to no improvement. Studying the workflow and the mechanism, it is possible that a completely anhydrous environment would be needed to fully perform this reaction with the high concentration of metabolites in urine.[353-355] This would not be necessary for the standard acid solution, as the concentration of sample would have been so low that any ambient water contamination would be inconsequential. As a fully anhydrous reaction was unable to be carried out with the facilities present, the reaction was tabled for further study.

Thus, though optimization of this reaction was unsuccessful, the proof of principle remains. This simple esterification reaction is a viable procedure for the separation of carboxylic acids from other polar metabolites in urinary NMR spectra, allowing for the easing of identification of analytes for metabolomic identification and use.

2.5 Conclusion

In this study, the cumulative effect of a simple esterification procedure performed by (trimethylsilyl)diazomethane and a solvent phase extraction to simplify NMR spectra for urinary metabolomics was investigated. Initial standard carboxylic acid solutions were shown to esterify quickly and effectively, causing a near-complete phase transition. However, applications to urine samples were more complex, mainly due to the complex nature of urine samples, in terms of salt, concentration of metabolites, and other issues. Various solvent conditions were investigated, as were differing concentrations of TMSDM and reaction times, and even different extraction procedures. However, none afforded a fully optimized sample, as both the water layer and the chloroform layer showed signs of carboxylic acid analytes, even after esterification. A study of the mechanism of the reaction and the workflow of the esterification procedure was done to determine where error could be introduced. It was believed that esterification was prevented by water contamination in the samples, which could not be removed by the accessible facilities. Therefore, the procedure could not be fully optimized. Thus, though the principle of the reaction was shown to be successful and effective, the procedure performed above is not ready for widespread use. Many questions and challenges remain before this esterification workflow can be fully implemented. Future directions for this line of thought would be the full optimization of this procedure such that it is effective to its fullest extent. Next, statistical calculations would be performed, to see if this procedure truly does improve metabolite detection. A new method would need development, as current ones do not fully detail the desired information. After optimization, implementation for biomarker development would follow, with hopes of improvements on current disease biomarker identification modalities for full clinical applications.

Chapter 3:

A Method of Minimizing Ionic Strength to Reduce Chemical Shift Variance in NMR-Based Urinary Metabolomics

3.1 Abstract

As NMR-based metabolomics gains popularity as a potential diagnostic technique, the exact mechanisms of the procedure need to be optimized. Though extremely quick and simple, NMR is sensitive to subtle changes in pH and ionic strength, which can cause peaks to shift, creating inconsistencies in peak assignment between patients, and thus unreliable biomarker identification. The standardization of sample conditions was investigated herein, through a simple methodology. It was found that the addition of decanoic acid removed salt and proteins, as shown by the narrowing of the chemical shift of creatinine, citrate, and glycine. Thus, this procedure was shown to be effective, allowing for improvements on technique and the further detection of biomarkers for the treatment of various diseases.

3.2 Introduction

As discussed above, metabolomics is the study of the entirety of a metabolic profile of a given biological structure or compartment, or a representative subgroup therein.[1-5] It identifies and uses the chemical fingerprints of cellular processes to develop real-time profiles of the state of a biological system, representing the total unity of the metabolites and chemical byproducts in a cell, tissue, organ, or organism, depending on the scale under study, allowing for various diagnostic and prognostic applications.[21-25] Metabolomics fills a need for a faster, more accurate, and cheaper diagnostic test for diseases such as the various cancers, due to their previously established utility in metabolomics.

As metabolomic samples generally are body fluids, many choices are present as to the best form. Urine samples provide the advantage of being easily acquired and also a holistic representation of the subject at large.[281-283] Furthermore, multiple analytical platforms exist. NMR is in many ways superior to MS, as it affords more reproducible data, and can be quantified easily. It gives faster data acquisition and more flexible experiments.[347-350] However, challenges remain to the expansion of NMR-based metabolomic experiments. Peaks in NMR spectra can change chemical shift values based on salt concentration and pH, causing metabolites to appear at different position on spectra from different samples.[357] This is especially a problem for metabolomic analysis, as metabolites are intrinsically changing and individuals have various salt and pH levels, which change the spectra seen. This prevents the standardization of data from sample to sample, as samples can appear in one location for one person, and another for the next.[268-238] Thus, the determination of biomarkers for accurate diagnoses would be impossible, preventing approaches that can apply to all patients and alleviate health care costs.

This issue needs to be addressed before metabolomics can be fully implemented as a diagnostic technique. Previous attempts have focused on the use of ethylenediaaminetetraacetic acid (EDTA), known to be strong chelator to the cations that affect peak shifts in solution.[289-291] However, this molecule introduces new peaks into the sample in metabolomically relevant regions, and must persist in the sample during spectral acquisition. Thus, it obscures potentially useful data, complicating interpretations further. Other computational techniques result in artifacts appearing in data, misaligning spectra and giving false positives and negatives.

In order to maximize the applicability of metabolomic analysis, these issues need to be addressed. Toward this goal, it was proposed that peak shift standardization would be achievable by treating urine with decanoic acid to remove the salt from the solution. This procedure is seen

on an industrial scale with regards to producing drinking water from brine, which demonstrated that the mixing of decanoic acid and brine at 80 °C caused a salt layer to settle to the bottom, and afforded a removable decanoic acid, resulting in the recovery of pure water. [358] This procedure was adapted to suit urinary metabolomics. As the decanoic acid is removed from the solution before analysis, it does not have the peak-obscuring issues of EDTA. It was hypothesized that this method will reduce the peak shifts as measured by the standard deviation of the chemical shift, an established measure of sample variation,[348] of three major metabolites, glycine, creatine, and citric acid. Herein we discuss the applications of decanoic acid-based salt precipitation in urinary NMR-based metabolomics.

3.3 Experimental

3.3.1 General Considerations

All conversions and manipulations were done to the open air. Urine samples were obtained from the University of Michigan Hospital System. Samples were labeled with an ID number, without knowledge of the condition of the patient. All sample procedures followed standard safety and security practices. Urine was kept at -80 °C until use, during which it was kept at -10 °C. All solvents, citrate, and decanoic acid were purchased from Sigma-Aldrich. Deuterated solvents were purchased from Cambridge Isotope Laboratories. Lyophilization and centrifugation were done on the instruments provided by Biophysics at the University of Michigan. All spectroscopic analysis was done on a Bruker 500 MHz instrument, with one-dimensional and two-dimensional capabilities. All matching, tuning, shimming, and locking of the instrument signal on the resonant proton was done by hand, rather than the automated function. Initial data processing was done using the Bruker interface. Further processing,

including spectral annotation was done via ACD/Labs software. All spectra obtained during the course of the experiment appear in the supplementary information.

3.3.2. Decanoic Acid Procedure.

Pure decanoic acid (200 μL) was melted at 80 °C and maintained at that temperature in a water bath. To this sample, 600 μL of urine was added. This mixture turned cloudy, and was then mixed at room temperature intermittently over the course of an hour, while the temperature was maintained at 80 °C. After mixing was completed, the mixture stood in the water bath for an additional ten minutes. The top 700 μL formed a clear solution, which was transferred to a separate vial, and allowed to settle into two distinct layers. The top layer was removed and discarded, while the bottom was lyophilized, reconstituted in 500 μL D_2O and 50 μL phosphate buffer (10 mM phosphate, 100 mM NaCl, pH 7.4) and analyzed via ^{1}H-NMR.

3.4. Results and Discussion

3.4.1 Decanoic Acid Studies

Initial decanoic acid procedures followed that seen for industrial-scale removal of salt from brine.[358] Acid was melted and mixed with neat urine samples in a heat bath. After, the supernatant was removed, the decanoic acid separated, and the urine dried and analyzed. This procedure is outlined in Figure 3.1. After, NMR spectra were taken of each urine sample, and overlaid with that of the native and with each other. This is displayed in the supplementary information. The spectra were monitored for three key metabolites: creatinine, citrate, and glycine. These appear in in Figure 3.2, and a table of standard peaks for them is in Table 3.1.[86-92]

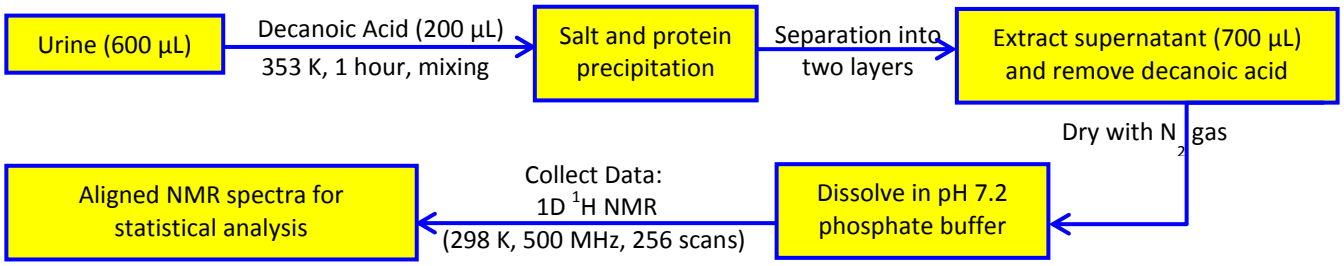

Figure 3.1. The general workflow for the decanoic acid procedure.

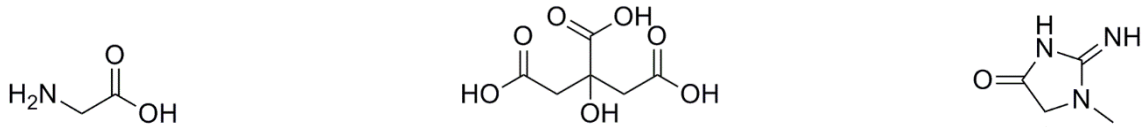

Figure 3.2. Representative structures of glycine (left), citric acid (middle), creatinine (right).

This technique was then applied to fifteen separate urine samples. Each native sample was analyzed, as were two treated samples. The creatine, citrate, and glycine peaks were tracked, and recorded, as seen in Table 3.1. Representative spectra appear in Figure 3.3. Based on obtained spectra, the native urine gave the following data (ppm): 3.02±0.02 and 4.02±0.02 for creatinine; 2.68±0.02, 2.65±0.02, 2.54±0.02, 2.70±0.02, and 2.50±0.02 for citrate; and 3.54±0.03 for glycine. Treated urine, on the other hand, gave the following peaks (ppm): 3.021±0.009 and 4.03±0.01 for creatinine; 2.68±0.01, 2.65±0.01, 2.55±0.02, 2.509±0.008, and 2.70±0.02 for citrate; and 3.538±0.008 for glycine. This data is summarized in Table 3.1, and given in detail in the supplementary information.

Decanoic acid was chosen for this investigation due to the fact that it is removed during the procedure, and that its NMR spectrum does not interfere with the metabolomic-relevant region. An image of decanoic acid appears in Figure 3.4, along with its NMR peaks.[342] As a

Decanoic acid was chosen for this investigation due to the fact that it is removed during the procedure, and that its NMR spectrum does not interfere with the metabolomic-relevant region. An image of decanoic acid appears in Figure 3.4, along with its NMR peaks.[342] As a majority of metabolomics focuses on the aromatic region and the region between 2.5-4.5 ppm, the decanoic acid procedure does not come into that region. Furthermore, decanoic acid is a solid

Metabolite	Standard Peak Value (ppm)	Average Untreated Peak (ppm)	Untreated Std. Dev. (ppm)	Average Treated Peak (ppm)	Treated Std. Dev. (ppm)	Percent Lowered Standard Deviation
Creatinine	3.03	3.020	0.024	3.021	0.009	62.9
	4.05	4.018	0.038	4.033	0.010	72.2
Citrate	2.67	2.684	0.021	2.678	0.014	33.2
	2.64	2.654	0.020	2.647	0.012	41.5
	2.54	2.541	0.024	2.546	0.023	7.5
	2.51	2.503	0.019	2.509	0.009	54.9
Glycine	3.54	3.542	0.023	3.538	0.008	73.2

Table 3.1. Table of analytes studied, with standard values, untreated peak locations and standard deviations, and treated averages and standard deviations. The percent lowered standard deviation represents the narrowing of the variance in peaks after treatment, and are in red for emphasis. All treatments were done with the decanoic acid treatment as detailed above.

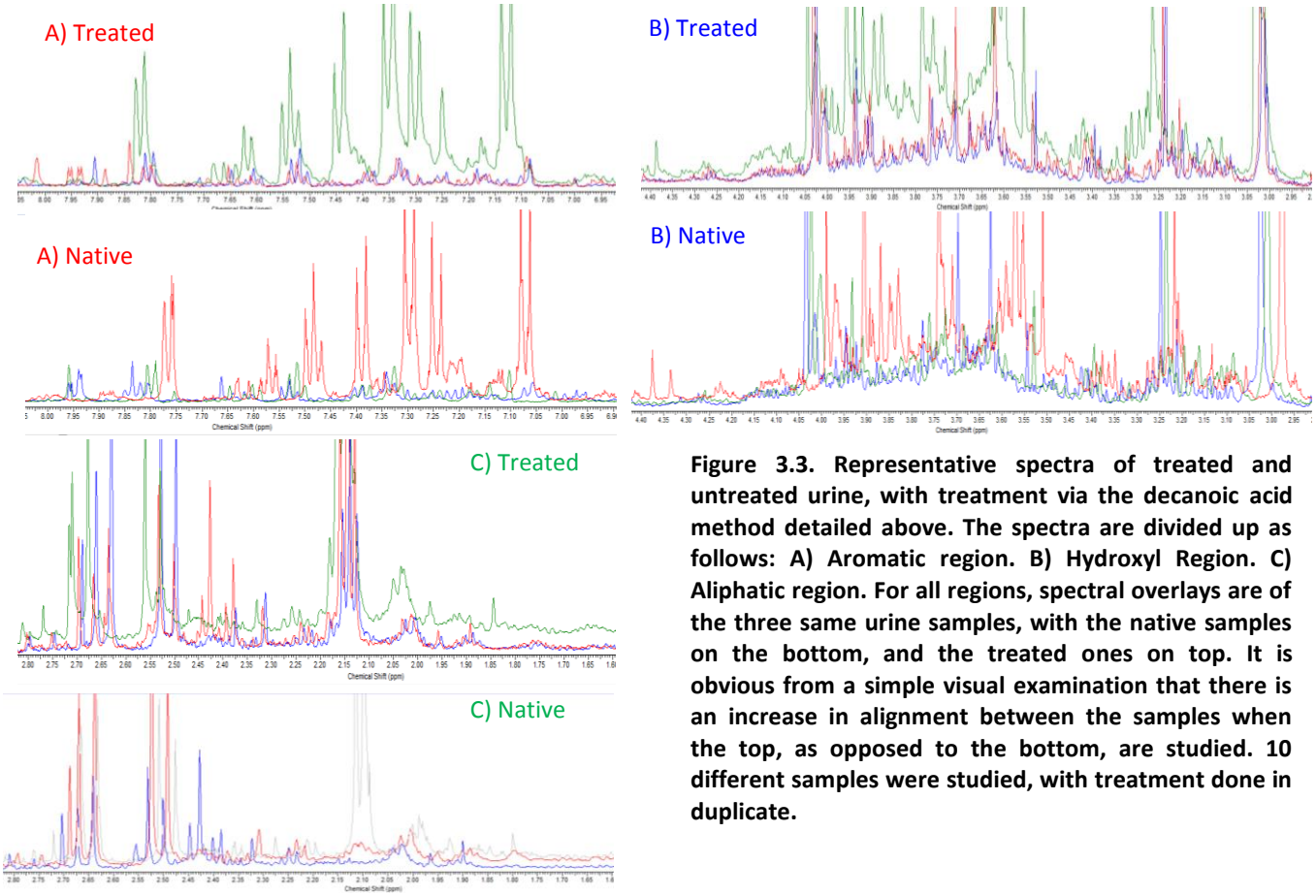

Figure 3.3. Representative spectra of treated and untreated urine, with treatment via the decanoic acid method detailed above. The spectra are divided up as follows: A) Aromatic region. B) Hydroxyl Region. C) Aliphatic region. For all regions, spectral overlays are of the three same urine samples, with the native samples on the bottom, and the treated ones on top. It is obvious from a simple visual examination that there is an increase in alignment between the samples when the top, as opposed to the bottom, are studied. 10 different samples were studied, with treatment done in duplicate.

at room temperature; therefore, it forms an easily removed film on top of the urine sample after cooling, and before drying, giving great separation from the tested water layer.

Based on the peaks presented above, a comparison of the standard deviations of the peaks before and after treatment was done to determine if the variance in the peak location dropped. This data is also presented in Table 3.1. For creatinine, the standard deviation dropped by 62.9% for the upfield peak and 72.2% for the downfield peak. For glycine, the peak variation dropped

by 73.2%. For citrate, on the other hand, the change in standard deviation varied from 7.5% to 54.9%. This data is to be expected. Citrate, due to its carboxylic nature, is highly sensitive to salt variations in solution, being used as a chelating agent and a salt probe previously.[359-360] Therefore, any small amount of salt would cause peak shifts to occur. Residual salt not removed by the decanoic acid procedure would still cause peak shifts to occur. However, for the other two amino acids studied, the drop in standard deviation was remarkable, as they were quite large

Furthermore, the peaks stayed close to the standard value of the metabolites studied. This is important in that there were no artifacts or other false positives inherent in the statistical alignment methods. Also, a comparison of the native to the treated spectra showed that no evident new peaks were formed in the treated sample, indicating that the decanoic acid layer was removed and did not affect the spectra quality or obscure peaks, while still allowing for high

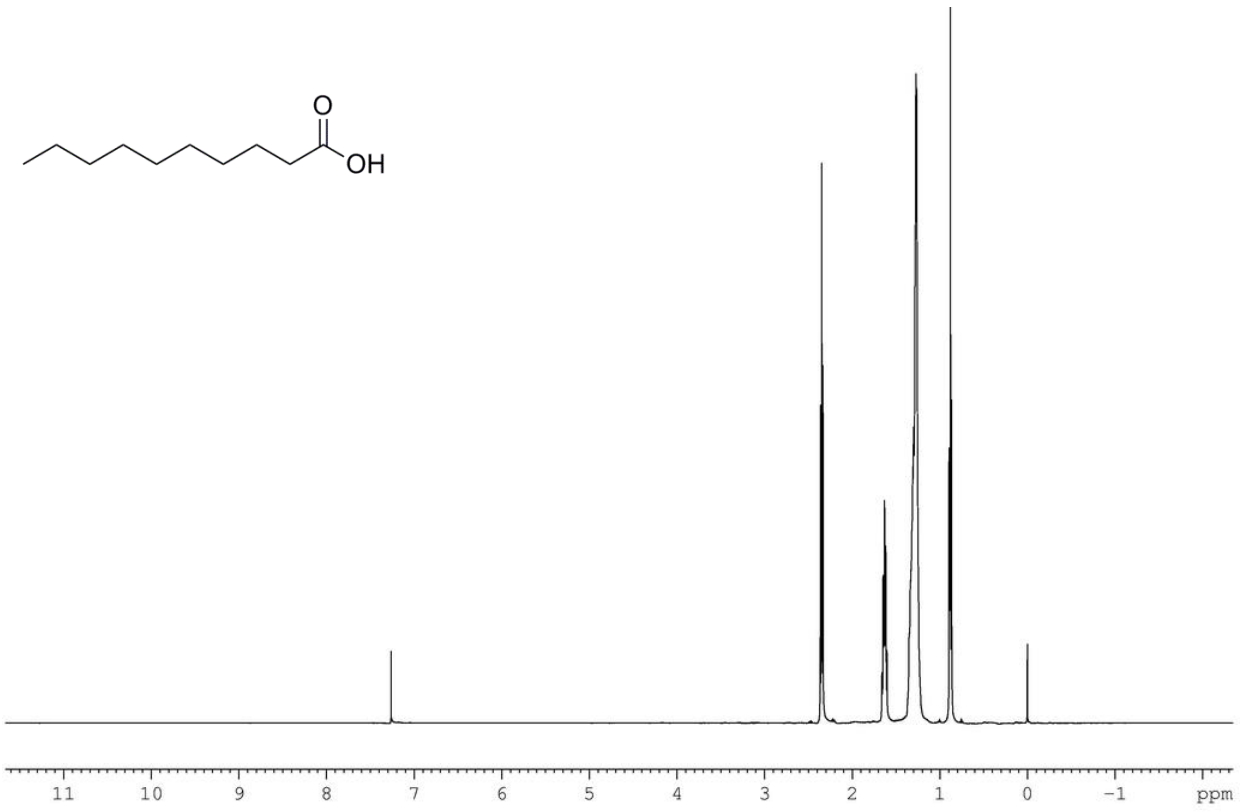

Figure 3.4. Decanoic acid spectrum. Spectrum from the BioMagResBank. Ulrich, E. L.; Akutsu, H.; Doreleijers, J. F.; Harano, Y.; Ioannidis, Y. E.; Lin, J.; Livny, M.; Mading, S.; Maziuk, D.; Miller, Z.; Nakatani, E.; Schulte, C. F.; Tolmie, D. E.; Wenger, R. K.; Yao, H.; Markley, J. L. *Nuc. Acids Res.* 2008, *36*, 402-408. Copyright 2008 Oxford University Press.

quality salt removal. Thus, this improves on the EDTA technique, as no new peaks were added to further complicate data. This salt removal procedure was not perfect, however, as there were still variations in the peak position. Peak variation is not only due to salt concentration but can also be due to the relative concentrations of the various metabolites in solution. Thus, it is not to be expected that all peak variation would disappear. Lowering this variance, however, to a point such that peaks can be consistently identified, as presented above, would be diagnostically useful.

Based on the data presented above, the decanoic acid procedure was successful for these three metabolites. A full statistical analysis needs to be performed before full implementation of this procedure, but its easy process and evident results make it a likely candidate to replace EDTA and statistical methods for peak alignment.

3.5. Conclusion

In this study, the effect of using decanoic acid to lower the ionic strength of solutions to standardize NMR chemical shift values was investigated, with applications to urinary metabolomics of cancers. Creatinine, glycine, and citric acid were chosen for their applicability to metabolomics. It was shown that the decanoic acid procedure worked extremely well for creatinine and glycine, and not quite as well for citric acid, due to the sensitivity of citrate to salt concentrations in solution. Furthermore, decanoic acid did not introduce any novel peaks in regions of the spectrum related to metabolomic analysis, improving on the previously established methodologies based around ethylenediaminetetraacetic acid. Also, peaks remained in their expected regions, removing the false positives and negative seen from statistical alignment procedures. Furthermore, the decanoic acid was a simple procedure, able to be done at all levels

of technical proficiency, affording widespread implementation. This decanoic acid procedure was not perfect, however, as peak variation from other sources still existed, but the improvement seen allows for the improvement of biomarker identification and consistency between patient analyses. Future directions for this procedure involve optimization for a high-throughput method, along with a full statistical analysis to determine the degree to which all peaks decrease in variance. Implementation for biomarker development would follow to determine improvements to disease biomarker identification for full clinical applications.

Chapter 4

Concluding Remarks and Future Directions

As metabolomics has grown as a science, its applications to studying major diseases, such as the various cancers. Metabolomics-related methods have the ability to alter clinical diagnoses for not only these diseases, but a wide profile. As seen above, the potential impact for medical applications is ever-expanding, as the litany of research being done in the various cancers only adds to the general knowledge about how diagnoses can be improved, and raises the likelihood of discovering a definitive biomarker. Thus, metabolomics maintains a positive outlook, improving diagnostic accuracy of current detection techniques upon expanded usage, limiting medical issues and extending lifespans. Furthermore, metabolomics, once established in cancers, can expand to other diseases such as diabetes, cardiovascular disease, pulmonary disease, and even bipolar disorder, though this analysis is still under development.

However, as displayed in this analysis, metabolomic techniques have a large ability to improve on current methodologies, supplying many avenues of future research before even reaching the stage of biomarker development. Metabolomics is not quite as high-throughput as desired. Designing new methodologies and machinery to aid in this would be a large step forward to increasing the power of metabolomics, improving both the speed and accuracy of data collection. This could include synthesizing separation techniques, MS, and NMR analysis, or even finding a methodology to simplify the preparation of each sample, whether by determining optimal samples for each technique or helping techniques such as MS improve on their solid state analysis.

Specific analytical techniques also pose their own problems, which need to be addressed, especially in relation to NMR. Current techniques give overly complex spectra for proton analysis. Though higher order experiments could be performed to improve on the spectral quality, this adds another layer of complexity in the experimental design, preventing the widespread implementation of the technique and impeding its diagnostic capability. These were addressed above by introducing a selective esterification procedure. As displayed above, the esterification procedure proposed showed some promising results. Though optimization is needed, there still exists the potential for the improvement of separations, resulting in a more accurate metabolomic procedure, as biomarkers would be easily identified on simplified spectra.

NMR is also sensitive to peak shifts between different samples mainly due to pH and salt concentrations. These variations are characteristic of metabolomic samples, and are due to variations in age, weight, gender, location, diet, pharmacology, and other subject-specific factors. These changes must be taken into account for accurate metabolomic analysis, whether by statistical or chemical treatment. As shown above, this was dealt with through a simple decanoic acid procedure, which allowed for the removal of salt from the sample, and a narrowing of the variance of chemical shifts across samples from different patients, as determined by the standard deviation of the peaks. A full statistical analysis is needed, but this procedure allows for the standardization of samples without the introduction of new peaks, as with EDTA, or the propagation of false positives and negatives, à la statistical alignment, and affords more consistent detection of analytes for more accurate biomarker development and diagnoses.

NMR-based metabolomics can also benefit from unification with MS data. As discussed above, this can be through the synthesis of NMR and MS instrumentation for high-throughput and simultaneous data acquisition. Improvements to mass spectra would also help support the

conclusions made from NMR data. This can be done by creating standard operating procedures for the type of ionization, the settings of the mass analyzer, and so forth, to remove variation among research groups and afford unified spectra, which would allow for biomarker identification, and remove a major obstacle to metabolomic analysis.

These improvements on metabolomic analysis, however, do not lose sight of the overall goal of this type of analysis; that is, all these methodology improvements are made to better the biomarker detection of this technique. As seen the various cancers, there still is a need for better means to detect diseases. Metabolomics is one of the best candidates to fill this void, but its lack of standardization and difficulty of interpretation prevents it from reaching its clinical potential. The improvements to metabolomics methodology presented in this investigation represent steps toward reaching that potential. Being able to consistently and accurate locate potential biomarkers will allow for the detection of metabolites indicative of diseases. Once these analytes can be detected, they can be compiled and used for diagnoses. This process is the single most important step. Once the procedure is optimized, clinical applications will follow.

With the improvements in methodology above, metabolomics will become the most sensitive and convenient approach for early disease diagnosis. Though these procedures still need optimization, early results have been promising. Thus, the five-year outlook is extremely strong for metabolomics, given rapid recent developments and easily discernible areas of improvement that promise to fix current problems and provide a definitive platform by which diseases can be accurately and specifically detected, diagnosed, and treated.

Appendix A.1:
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Appendix B.1:
Supplementary Information

All spectra are available online at http://www-personal.umich.edu/~nirbs/jain_supplementary_information_section_1.pdf

For the spectra related to the analyses done in Chapter 2, please see pages S1-S136

For the spectra related to the analyses done in Chapter 3, please see pages S137-S206

SECTION 2:
MECHANISMS OF ALZHEIMER'S DISEASE

Chapter 5:

Introduction:

5.1 Alzheimer's Disease

5.1.1 Introduction to Alzheimer's disease

Alzheimer's disease (AD) is a debilitating neurodegenerative disease that is the leading cause of dementia, accounting for up to 70% of all cases seen.[1-3] As with other dementia diseases, AD is characterized by a decrease in the ability of an individual to think and remember to such an extent that the quality of daily life of the patient is negatively impacted. Though the consciousness of the person may not be affected, there can be issues with emotions, language, and motivation.[4-6] These problems may develop as part of the aging process, but only truly become a form of dementia when they outstrip what may be expected to be a natural byproduct of aging.[7]

The rapidity of the onset of AD can vary from patient to patient, but body functions degrade to an extent that death is seen generally three to nine years after diagnosis.[8-10] Alzheimer's disease was found in about 35 million people worldwide in the year 2010, causing 486,000 deaths, making it one of the most widespread diseases seen.[11] Furthermore, this disease tends to affect the elderly, being more common in people above the age of 65, though those younger may suffer from a form known as early-onset AD.[12] As it stands, AD is the single most expensive disease to treat in developed countries.[13-15] This fact, coupled with the large burden not only on the patient but on their caregivers, such as family, friends, and medical staff, greatly expands the impact of this disease beyond the individual to other societal, psychological,

physical, and economic extents. Institutions such as nursing homes and long-term care help support the caregivers, but costs can range up to $77,500 per year in the United States.[13]

Despite the widespread nature of this disease, a solid understanding of its origins is severely lacking.[16] Many factors are thought to be at the root of AD, including genetics,[17-19] head injuries,[20-21] depression,[22] hypertension,[23-25] cerebral plaques,[26-29] obesity,[30] and others. As symptoms are normally mistaken as natural byproducts of aging, this disease can go undiagnosed for years. Furthermore, there are no generally accepted procedures to lower one's risk for AD, nor is there a treatment to stop the progression of this dementia.[31-33] As displayed above, the cost of AD treatment is extremely high. Coupling this cost with the growing age of the baby boomer generation, and the general economic strain accompanying them, indicates that there is a great need for a better understanding of this disease.[34] A mechanistic understanding from studying the molecular nature of AD would allow for more knowledge of how it functions, and thus how to treat it.

5.1.2 Signs and Symptoms

AD is generally split into four phases: pre-dementia, early, middle, and late.[35] The pre-dementia stage is generally conflated with natural processes of aging or stress, such as an increase in forgetfulness, short-term memory loss, a loss of planning ability and abstract thinking, apathy, mild depression, and other slight cognitive impairments.[36] Though these symptoms may affect the most complex of daily life activities, they do not change the everyday enough such that the patient is aware of the potential for AD.

Early onset AD is the stage in which most diagnoses are made.[37] There may be some difficulties with language or perception, or even movement, along with an increase in memory

impairment. Fine motor tasks become more difficult, and sufferers may require assistance with more complex tasks, or at least supervision.

At the moderate stage of AD, independence tends to be eliminated, as sufferers struggle with even the most common daily tasks.[38] Difficulties in speech increase as well, causing word-substitutions and a general drop in vocabulary, accompanied by functional illiteracy. Long-term memory, not affected in the early onset stage, becomes damaged here, along with personality changes and delusion. There are some physical effects as well, including incontinence, and it is at this stage that many patients are moved to long-term facilities.

In the last stage, known as advanced, the patient is completely dependent on their caregiver for all aspects of life, being able to communicate only in short phrases or single words.[39] Aphasia eventually occurs. Though emotional communication is still possible, apathy is the dominant feeling. Muscle mass and motility disappear, and the body is open to more diseases, leading to death. The cause of death is not AD itself, but rather ulcers or pneumonia, which only became deadly due to the effects of Alzheimer's.[40-41]

5.1.3 Diagnostic Techniques

Diagnosis of AD can only be definitively done from a histological brain analysis, but factors such as medical and family history, computed tomography, magnetic resonance imaging, single-photon emission computed tomography, or positron emission tomography are studied in connection with functionality tests.[42-47] These tests all follow rigorous guidelines established by the Alzheimer's Association, which have a strong correlation with AD diagnoses.[48] However, as neurological examinations may provide normal results or even support other forms of dementia, corroboration with blood tests and thyroid function tests are used. Early-onset cases are generally

not diagnosed; however, as the signs of AD may seem like normal stages of aging, and thus are ignored by the patient, before true cognitive impairment leads to the need for a diagnosis.[49-50]

Some metabolite screening procedures have been developed, using cerebrospinal fluid to test for β-amyloid or tau proteins, but must be done in conjunction with neuroimaging techniques before a diagnosis be truly trusted.[51-53]

5.1.4 Pathophysiology

Generally, AD is characterized by neurodegeneration and atrophies of affected cerebral areas.[54-58] This degradation progresses as the disease progresses, and is generally accompanied with amyloid plaques, made up of insoluble collections of β-amyloid peptide, and neurofibrillary tangles, comprised of tubular tau proteins. This protein misfolding may disrupt basic cellular processes, including calcium ion homeostasis, enzyme function, and glucose usage, resulting in apoptosis.[55] This is normally seen in conjunction with inflammatory responses, which help induce neural degeneration. All these processes, however, essentially lead to neuronal cell death, which is the root of AD, though the exact trigger that starts killing cells is unknown.[59]

5.1.5 Prevention, Treatment, and Prognosis

No clear prevention or treatment plan for AD exists, and the prognosis remains poor.[31-33] However, theories abound on how to best combat this disease. One such theory is that cardiovascular risk factors correlate to a higher rate of AD.[23-25] However, statins and anti-inflammatory drugs have had minimal effects in preventing and treating the disease. Intellectual activities and physical fitness, along with diet, have some potential as preventative techniques, but this link remains weak.[60-62] Compounds such as caffeine and flavonoids, along with certain

vitamins, cannabinoids, and curcumin, have shown some potential positive activity, but these correlations remain under further investigation.[63-66]

Various acetylcholinesterase inhibitors have also been developed to combat AD, as well as glutamate and other antipsychotic drugs.[67-69] Further psychological techniques have all been suggested as psychosocial alternatives to pharmaceutical therapy. However, as a whole, none of these potential treatments have been shown to be effective. In fact, their main usage has been as symptom management or palliative care, but has not stopped the onset of the disease, nor prevented death from complications due to Alzheimer's disease.

As discussed above, the symptom progression of AD will eventually lead to death, being the underlying cause of death for 68% of patients, generally due to complications of AD that lead to pneumonia, dehydration.[11] Though the rate of cancer is lower in AD patients, the mean life expectancy is six years, with less than 5% of patients surviving over fourteen years. With this poor prognosis, AD is among the deadlier of diseases.

5.1.6 Causes

The cause of AD is unknown.[59] Though the actual mechanism of cell death is fairly well-characterized, the reason for the process to begin is a mystery. Genetic factors have been identified, which result in mutations in the amyloid polypeptide (APP) and presenilins that cause the production of amyloid-β to increase, resulting in plaque formation (Figure 5.1).[70-73] Other hypotheses include the cholinergic hypothesis, in which a reduction of acetylcholine leads to AD, though this has been generally rejected due to the lack of effectiveness of cholinergic pharmaceuticals.[74-75] The tau hypothesis tends to focus on the tau protein as the initiator of this disease, in which tau protein microtubules degrade the neuronal cytoskeleton and thus the

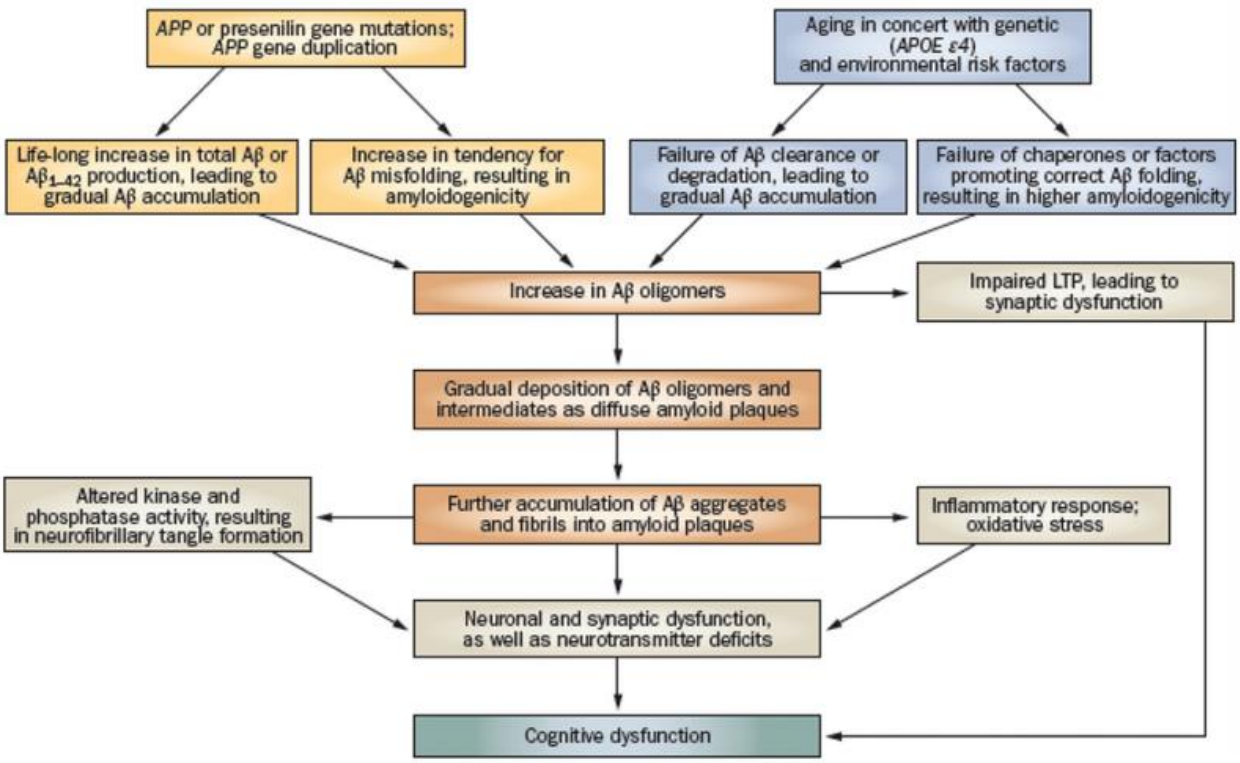

Figure 5.1. A schematic showing the effects of amyloid-β on Alzheimer's disease. Figure reprinted with permission from Blennow, K.; Hampel, H.; Weiner, M.; Zetterberg, H. *Nat. Rev. Neurol.* 2010, *6*, 131-144. Copyright 2010 Macmillan Publishers Ltd.

neuron.[76-77] More esoteric beliefs include metallic causes of AD, mediated by changes in the homeostatic concentrations of Fe, Cu, Zn, and Al.[78-79] Electromagnetic fields,[80] smoking,[81] autoimmune factors,[82] myelin breakdown,[83] oxidative stress,[84] and air pollution[85] have all been suggested as potential causes, but none have been shown to be the true source of AD. A full diagram of all potential AD causes appears in Figure 5.2.[75-88] The most evident cause of this

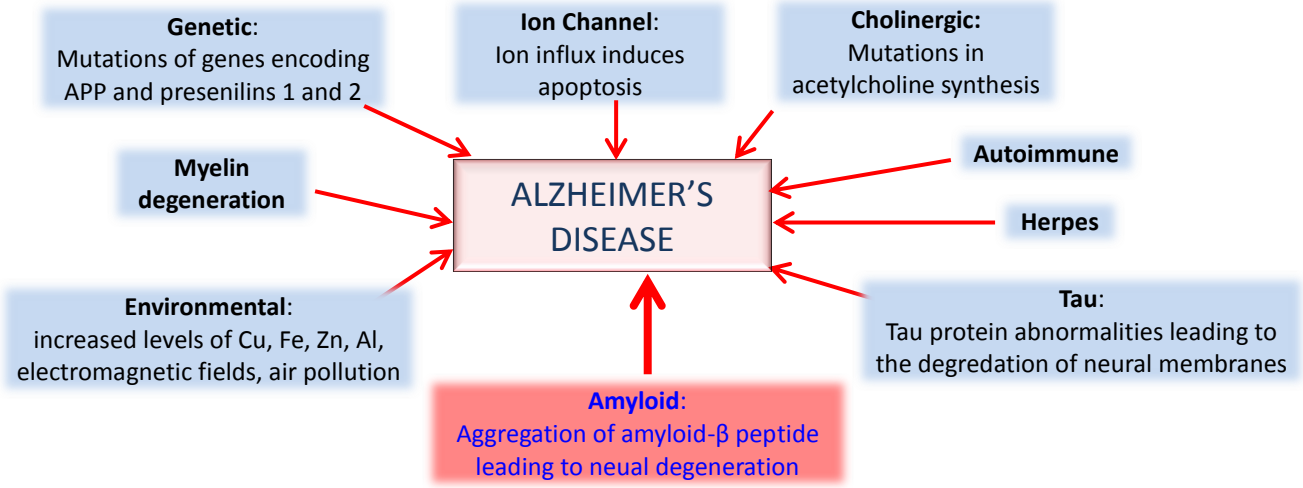

Figure 5.2. A schematic showing the various theories of the cause of Alzheimer's Disease. The prevailing theory, the amyloid cascade hypothesis, is highlighted in red and blue at the bottom.

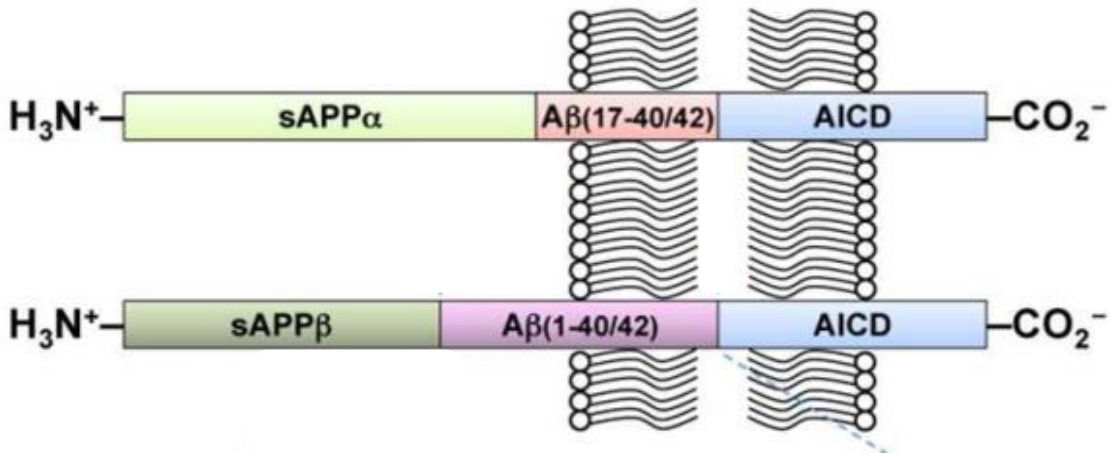

Figure 5.3. A representation of APP in the membrane, showing the location of the amyloid-β peptide within this larger peptide. Reprinted with permission from Savelieff, M. G.; Lee, S.; Liu, Y.; Lim, M. H. *ACS Chem. Biol.* 2013, *8*, 856-865. Copyright 2013: American Chemical Society.

disease remains the amyloid hypothesis,[86-88] discussed further below.

5.2 The Amyloid Cascade Hypothesis and Amyloid-β

5.2.1 Introduction to the Amyloid Cascade Hypothesis and Amyloid-β

The amyloid cascade hypothesis is the most generally accepted cause for AD, stating that extracellular deposits of amyloid-β (Aβ) are the underlying cause of the disease,[86-88] as well as other diseases such as Parkinson's disease,[89-90] Huntington's disease,[91-92] and type II diabetes.[93-94] This hypothesis gained support through the fact that the APP gene is present on chromosome 21, and those with trisomy 21 (Down's syndrome) present AD by the age of 40.[95] Furthermore, identified genetic risk factors correlate to APP and Aβ buildup.[96-98]

Based on the amyloid cascade hypothesis, however, it is not the native form of Aβ that causes AD, but rather an aggregated form, which has been recently developed to exclude the fibrillar, amyloid state of the aggregate, focusing instead on the oligomer as the toxic species.[86-88] Native Aβ find their origin in APP (Figure 5.3), which is proteolytically processed to form Aβ by cleavage via a copper metalloprotein and a secretase.[99-100] This yields fragments ranging from 39-43 residues, with the most common alloforms having 40 and 42 residues (Figure 5.4).[99,101-104]

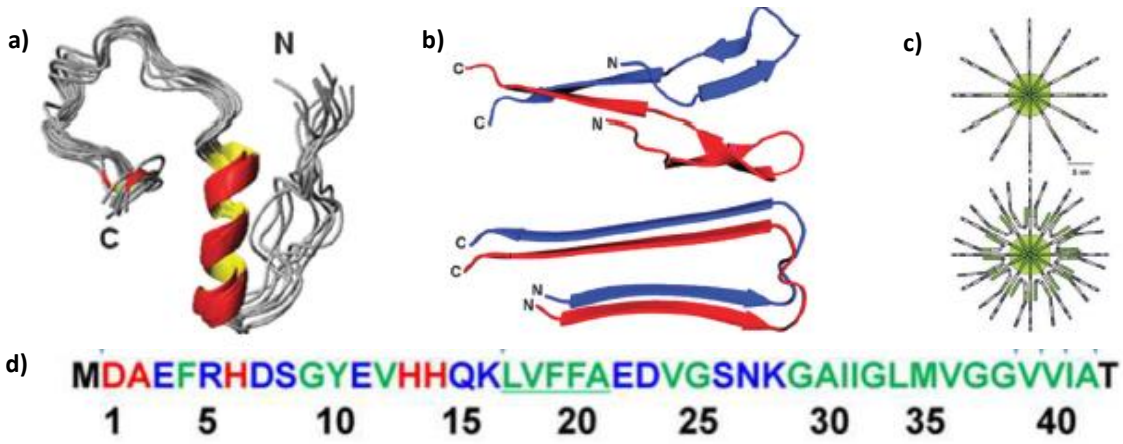

Figure 5.4. Various representations of amyloid-β. a) Amyloid-β as a partially folded structure in the presence of 50 mM NaCl, with residues 13 to 23 forming a 3_{10} helix. Reprinted with permission from Vivekanandan, S.; Brender, J. R.; Lee, S. Y.; Ramamoorthy, A. *Biochem. Biophys. Res. Commun.* 2011, *411*, 312-316. Copyright 2011 Elsevier. b) Solution NMR structure of a 0.05% SDS-stabilized pre-globulomer of amyloid-β(42) (top) compared with the basic fold of the fibrils of amyloid-β(42) (bottom). Adapted with permission from Yu, L.; Edalji, R.; Harlan, J. E.; Holzman, T. F.; Lopez, A. P.; Labkovsky, B.; Hillen, H.; Barghorn, S.; Ebert, U.; Richardson, P. L.; Miesbauer, L.; Solomon, L.; Bartley, D.; Walter, K.; Johnson, R. W.; Hajduk, P. J.; Olejnicazk, E. T. *Biochemistry.* 2009, *48*, 1870-1877. Copyright 2009 American Chemical Society. (c) Structural schematic of amyloid-β balls formed at low pH in the absence (top) and presence (bottom) of DSS. These structures both show a pinwheel or micelle-like arrangement of monomers. Reprinted with permission from Laurents, D. V.; Gorman, P. M.; Guo, M.; Rico, M.; Chakrabartty, A.; Bruix, M. *J. Biol. Chem.* 2005, *280*, 3675-36853. Copyright 2005 Journal of Biological Chemistry. d) The sequence of amyloid-β(42), with labels as follows: black, flanking APP residues that are not in amyloid-β; red, putative Cu2+-binding residues; blue, hydrophilic residues; green, hydrophobic residues; underlined, self-recognition region. The terminal I and A residues are not in amyloid-β(40). Reprinted with permission from Savelieff, M. G.; Lee, S.; Liu, Y.; Lim, M. H. *ACS Chem. Biol.* 2013, *8*, 856-865. Copyright 2013: American Chemical Society.

Both alloforms oligomerize and ultimately fibrilize, however, the Aβ(42) case is clinically correlated, while the 40 case is more commonly formed. A general schematic showing the stages of fibrilization and the effects on the cell to lead to AD appears in Figure 5.5.[99]

The amyloid hypothesis suggests that Aβ aggregates are the pathogenic source of AD, and the signal that commences the chain of physiological effects leading to cell death, especially when in the oligomer form, rather than the insoluble, fully fibrilized form.[105] Though fibers may retain toxic activity, the oligomers are the truly dangerous species. Oligomers are formed through the aggregation of individual monomeric peptides, in which the random coil peptide, with some intrinsic β-sheet and α-helix conformations, interact such that the hydrophilic N-termini and the hydrophobic C-termini are aligned in a parallel β-sheet structure.[106-108] Oligomerization heightened in the 42-residue species, causing it to have more inherent toxic ability.[99]

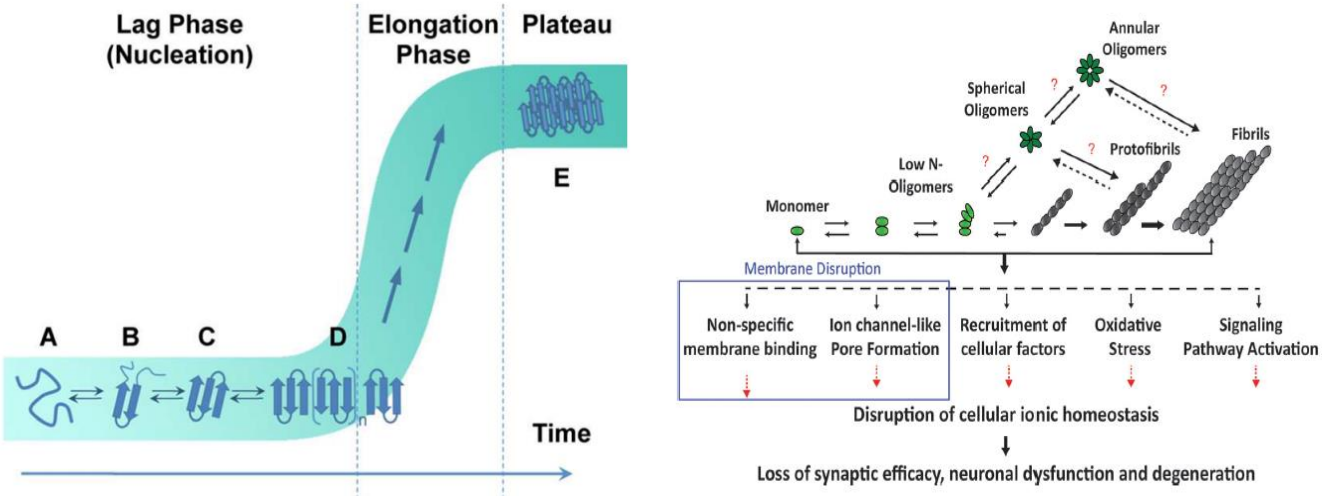

Figure 5.5. Representation of the aggregation of amyloid-β. Left: a diagram representing the three phases of amyloid-β aggregation, including monomer to β-sheet oligomerization and then platueau at a insoluble plaque. Reprinted with permission from Savelieff, M. G.; Lee, S.; Liu, Y.; Lim, M. H. *ACS Chem. Biol.* 2013, *8*, 856-865. Copyright 2013: American Chemical Society. Right: A diagram representing the effects of each stage of amyloid-β aggregation on the size of oligomers and on the cell, with the consequences for Alzheimer's disease. The membrane disruption stage, thought to be the key location of the signal that starts Alzheimer's disease and neural degeneration, is highlighted. Reprinted with permission from Kotler, S. A.; Walsh, P.; Brender, J. R.; Ramamoorthy, A. *Chem. Soc. Rev.* 2014, *43*, 6692-6700.

The mechanism of fiber growth is known as a nucleated growth mechanism (Figure 5.5).[99, 105] In this proposed mechanism a critical nucleating species is formed that triggers the formation of protofibrillar aggregates. The formation of protofibrils is followed by secondary nucleation and elongation to an eventual plateau at which point the fibers are formed and the system reaches its kinetic equilibrium. This is generally modeled as a sigmoidal curve and can be measured via fluorescent techniques. Fibril shape and size is dependent on a wide variety of factors, including monomer conformation, solution pH, salt concentration, peptide concentration, the presence of exogenous species, temperature, agitation, and others.[109-111]

Aggregates take on a β-sheet conformation, which act as seeds to further aggregation, and arise in a variety of morphologies with differing toxic abilities, though these links are not fully understood.[106-108,112] Most studies point to oligomers of Aβ eliciting toxicity through membrane interactions, such as binding and insertion into membranes to various degrees, causing entry of calcium ions into the cell and disrupting ionic homeostasis by forming ion-channel like pores.[113-115] This type of membrane disruption to be correlated with smaller, annular oligomers, before full

fibril formation, and leads to apoptosis of the cell in the ion channel hypothesis.[116-119] Elongation of the fiber to large, spherical aggregates, can change conductance on the cell surface, and lead to the recruitment of cellular factors, oxidative stress, and apoptosis.[105, 120-121] Furthermore, oligomers have shown inherent binding abilities to surface receptors, which could result in a loss of neuronal plasticity and neuronal function.[122] However, *in vitro* studies have failed to display the same level of toxicity as *in vivo* studies.[123-125] Thus, there may be further modifications or interactions *in vivo*, as well as other types of oligomers that underlie the toxicity of this protein. Thus, though a wealth of information that displays the connections between AD and Aβ via the amyloid hypothesis exists, the exact details are unknown.[126]

5.2.2 The Two-Step Mechanism

The ion channel hypothesis of Aβ discussed above is one of the dominant explanations of the amyloid cascade hypothesis, but does not explain the full morphological effects seen in cellular fragmentation and the toxicity of large aggregates.[127] Much of the evidence for the Aβ channel rests largely on findings through studies utilizing atomic force microscopy (AFM), electron microscopy, molecular dynamics simulations, and single channel conductance measurements.[95-127] While the Aβ channel hypothesis is well characterized, it unsatisfactorily describes how large aggregates, such as protofibrils or amyloidospheroids. In the case of large aggregates, it is difficult to fathom how they could form the smaller Aβ aggregates comprising the channel-like structures discussed above. A recent study demonstrated how such large aggregate structures could contribute to membrane disruption through a non-specific mechanism.[128] This study from Sciacca, et al, proposed a two-step mechanism of membrane disruption (Figure 5.6).[128] In this mechanism, initial aggregation of Aβ on the cellular surface

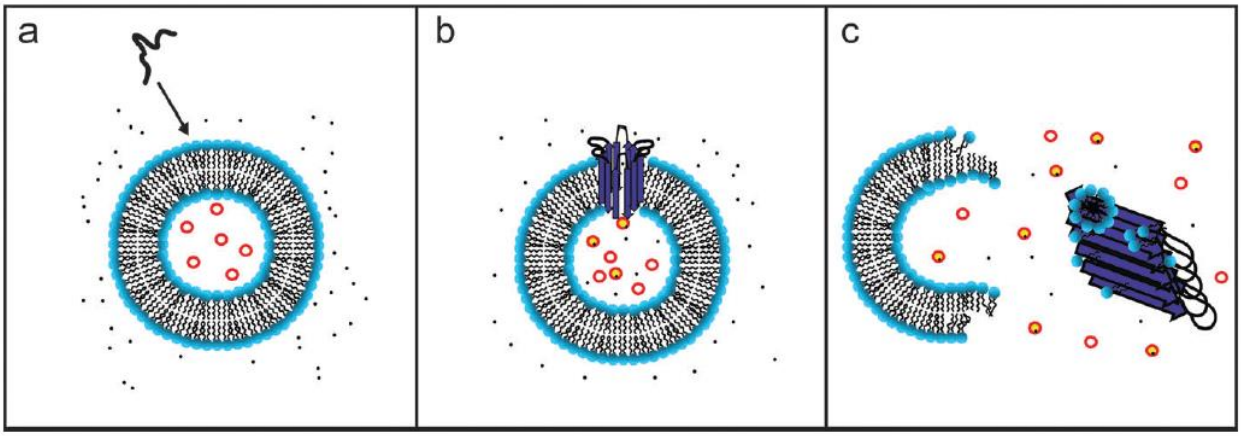

Figure 5.6. A representation of the aggregation of amyloid-β. a) Amyloid-β binding to the membrane and forming ion channel-like pores. b) Appearance of amyloid-β pores increased by the presence of ganglioside content, mediating a fiber-dependent step to induce membrane disruption. c) Membrane fragmentation. Reprinted with permission from Kotler, S. A.; Walsh, P.; Brender, J. R.; Ramamoorthy, A. *Chem. Soc. Rev.* 2014, *43*, 6692-6700.

leads to pore formation, in which ion channels are formed via amyloid insertion into the membrane. This stage of the mechanism is characterized by calcium influx. Channels have been shown to be permeable to calcium, but blocked by zinc. Thus, this step explains the ion channel hypothesis outlined above.[128]

However, this is not the only means by which amyloids affect the cell membrane. In this mechanism, the pore formation step is followed by a fiber-dependent form of membrane disruption step resulting in fragmentation of the lipid bilayer via a detergent-like mechanism. This phase is characterized by the leakage of large dyes from the interior of the cellular membrane, which only appear after fibrillary Aβ structures are formed.[128] Though pores were found to appear almost immediately after introduction of Aβ and initial aggregation, the membrane fragmentation was only found after the oligomers were allowed to form full fibers. This membrane disruption is fundamentally different from the pore formation stage in that it is nonselective, being permissible to positive and negative species, as well as species of various sizes. This lack of permittivity indicates that the integrity of the membrane is lost, further supported by the appearance of micelle-like structures.[128] This stage also required the presence of fiber formation and their continued polymerization. Once fibers were fully formed,

fragmentation stopped. Though the steps in this procedure are not known to be separate phases or connected, this two-step mechanism explains many strange activities of Aβ and membrane interactions, namely the transient nature of pore formation and membrane disruption. This mechanism is promising, but merits further investigation before full acceptance.[128]

5.3 Membrane Modulations and its Effects on Amyloid-β and Alzheimer's Disease

5.3.1 Gangliosides, the Membrane and Amyloid-β

Though many membrane studies have been carried out with Aβ, the role of specific membrane components and their effects on Aβ are still not well-understood. One such component is monosialotetrahexosylganglioside, better known as GM1.[129-131] It is a glycosphingolipid with a sialic acid linked to the sugar chain, known to be a component of cellular membranes in domains known as lipid rafts, especially in neuronal membranes. GM1 is known to have important applications in neuronal plasticity and the release of neurotrophins in the brain, as well as being a binding site for both the Cholera toxin and the *E. coli* toxin.[132-133]

Recently, GM1 has been demonstrated to be a signaling point for Aβ aggregation and AD onset.[134-136] Initially, it was found that GM1 influences the structure of the Aβ fibril during aggregation, mediated by the Aβ:GM1 ratio.[137] At a low ratio, the α-helix is dominant, but increasing the amount of Aβ per GM1 causes more fibrillization. Furthermore, the potential for the formation of amyloid aggregates from Aβ is controlled by GM1, as this effect is increased by the introduction of lipid rafts containing GM1.[138] This binding may be due to the sialic acid moiety on the GM1 molecule, as confirmed by various studies. [139-142]

The process of ganglioside-Aβ binding is known to be a multi-stage event, involving electrostatic interactions with the N-terminus, and further hydrophobic driving forces allowing

for C-terminus insertion within the membrane.[138-142] This is amplified by the fact that GM1 carries a negative charge, helping to facilitate the interaction of the N-terminus with the sugar, allowing for the orienting of the peptide for easy insertion.[143] However, this mechanism still remains in question, and requires further investigation before it can be fully described.

The action of GM1 in the context of the two-step mechanism, however, is less controversial. As discussed above, the cytotoxic nature of Aβ is not fully known, but the two-step mechanism, involving initial pore formation, followed by gross membrane disruption, is one possibility within the framework of the amyloid cascade hypothesis.[128] It was displayed that this mechanism is entirely dependent on the presence of gangliosides in the membrane, as GM1-free membranes displayed only pore formation, but not gross membrane disruption. Given the fact that gangliosides are a generally accepted factor in Aβ aggregation,[144-145] the fact that it has such demonstrable effects on membrane disruption is further support of the two-step mechanism as the source of amyloid toxicity in AD.

Though gangliosides have been universally recognized as a factor in AD progression, its role still remains clouded. The place of ganglioside in the two-step mechanism has not been established in the presence of other important membrane contributors, such as cholesterol and other components of lipid rafts, and the specific nature of its interaction with Aβ is unknown. This remains an area of further study.

5.3.1 Cholesterol, the Membrane and Amyloid-β

Another important membrane component is cholesterol, a major membrane lipid, as well as the main source of lipid rafts.[146-149] The role of cholesterol in membranes remains controversial.[150-152] It is known to contribute to membrane fluidity by modulating the phase

transition temperature of the lipid membrane.[153] Three main types of phases exist: gel phases, in which there is little lateral diffusion of the membrane components;[154-155] liquid ordered, in which lateral diffusion occurs in distinct clusters;[156-157] and liquid disordered, in which lateral diffusion occurs in random structures, maximizing entropy.[158-159] Cholesterol is believed to decrease the fluidity of liquid ordered membranes and increase the fluidity of gel ordered membranes,[160-162] while also creating microdomains of liquid ordered regions in an area of complete disorder, enriched in cholesterol, sphingolipid, ganglioside, and other proteins known as lipid rafts.[146-149] These rafts are considered to be signals for Aβ binding due to the fact that they contain gangliosides known to promote oligomerization, such as GM1.[163-167]

Cholesterol has been shown to modulate lipid-Aβ interaction, with recent studies showing that the gel phase interacts with Aβ at the highest level, indicative of the lowest fluidity.[168-169] This is corroborated by the effects of cholesterol in the membrane itself, where its increased fluidity allow for the sealing of pores and fragments which may be caused by Aβ aggregation, and result in apoptosis.[170-172] This also agrees with other studies in which Aβ affects membrane fluidity.[173-175] Though this effect is not well-characterized, the connection is still evident. Due to this connection, the role of cholesterol in the two-step mechanism, especially with ganglioside, merits further investigation and discussion.

This is especially important due to the fact that connections have been made between cholesterol and AD.[176-179] Though the direct connection between cholesterol and AD remains tenuous at best, it has been shown that increased cardiovascular risks, such as higher levels of cholesterol,[23-25] do correlate to increased risks for Alzheimer's. Furthermore, cholesterol may interact with many important compounds that affect brain activity, such as hydroxymethyl glutaryl-coenzyme A, eventually helping promote AD, resulting in higher risk levels.[180-181] In

fact, lipids such as cholesterol have been shown to modulate the amyloidogenic processing of APP, which leads to the formation of Aβ and AD. There is also an epidemiological connection between higher cholesterol and higher rates of AD incidence.[182-184]

However, neuronal cholesterol content has also been shown to prevent AD. Neurons are comprised of, on average, 30% cholesterol,[185] which has been displayed to prevent peptide insertion, reduce fibrillization, and prevent membrane disruption.[186-188] These results are in direct conflict with that discussed above in terms of dietary and plasma cholesterol. The difference here may be that cellular cholesterol, rather than dietary cholesterol, has a protective effect toward AD, whereas dietary cholesterol has a damaging effect.[189-190] This connection requires further research, and remains a flashpoint for AD studies.

5.4 The TK9 Anti-Amyloid-β Peptide

Given the support for the amyloid hypothesis, it is only natural that investigations into the prevention of Aβ aggregation have been performed. A class of these anti-amyloid therapeutic agents is self-assembling peptide chains, which have inherent amyloidogenic properties themselves.[191] These peptides have been studied as models for amyloid aggregation, in order to simplify the analysis as well as probe specific interactions between peptides in assemblies before translation to the larger amyloid system.[192-193] Self-assembling peptides from amyloids themselves, such as NFGAIL in human islet APP (hIAPP) and KLVFF in Aβ have shown to have inhibitory pathways, affording valuable information as to the aggregation pathway and acting as potential therapeutic agents.[194-195]

Another characterized agent is TK9, a nine residue peptide taken from the carbonyl terminus of the SARS corona virus envelope protein (Figure 5.7).[196] This enveloped region is

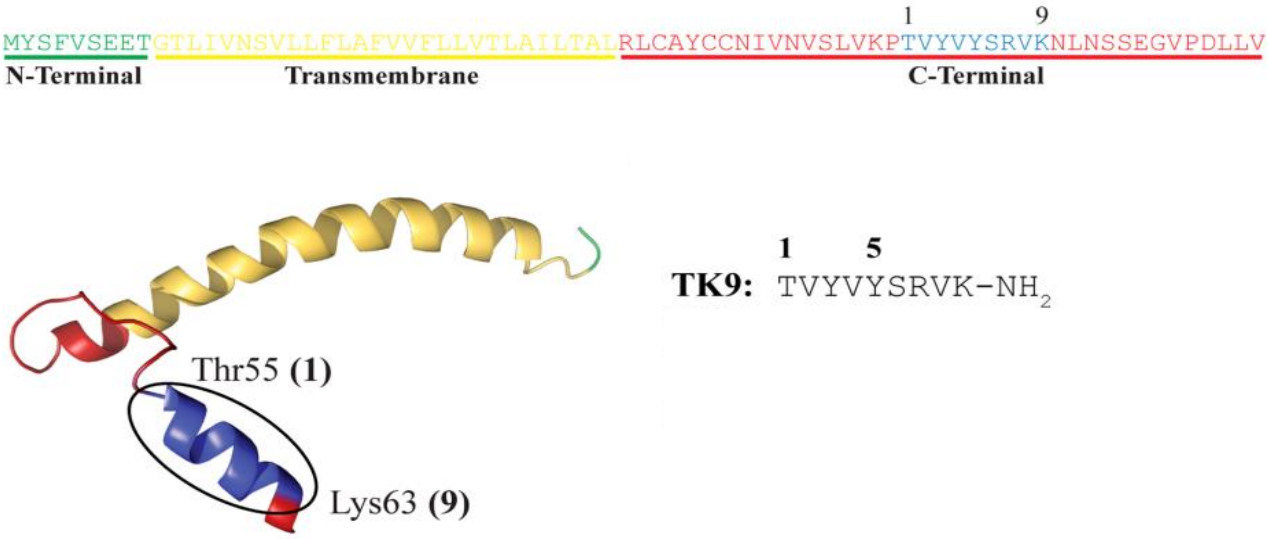

Figure 5.7. The SARS corona virus sequece and cartoon, with the TK9 sequence highlighted in both. Reprinted with permission from Ghosh, A.; Pithadia, A. S.; Bhat, J.; Bera, S.; Midya, A.; Fierke, C. A.; Ramamoorthy, A.; Bhunia, A. *Biochemistry.* 2015, *54*, 2249-2261.

thought to be the cause of such diseases as the common cold, severe acute respiratory syndrome (SARS, caused by the source virus), bronchitis, and others.[197-198] This envelope is a long peptide chain of up to 108 amino acids with flexibility at both ends and an α-helix in its transmembrane region, with known changes to its secondary structure upon contact with a membrane, as demonstrated previously with hIAPP.[196]

This peptide has established self-assembly characteristics, forming a β-sheet structure in solution. It has also been demonstrated to inhibit the aggregation of APP, which, as discussed above, is the source of Aβ.[196] The effect of TK9 on Aβ aggregation has been demonstrated previously (Figure 5.8), but further study on this initial peptide could open up the field of self-assembly proteins as therapeutic agents. A positive result would not only give great insight into the mechanism of Aβ aggregation but also allow for further testing of other peptides, such as NF11, leading to the customization of these peptides and pharmaceutical development for the eventual treatment of amyloidogenic diseases.

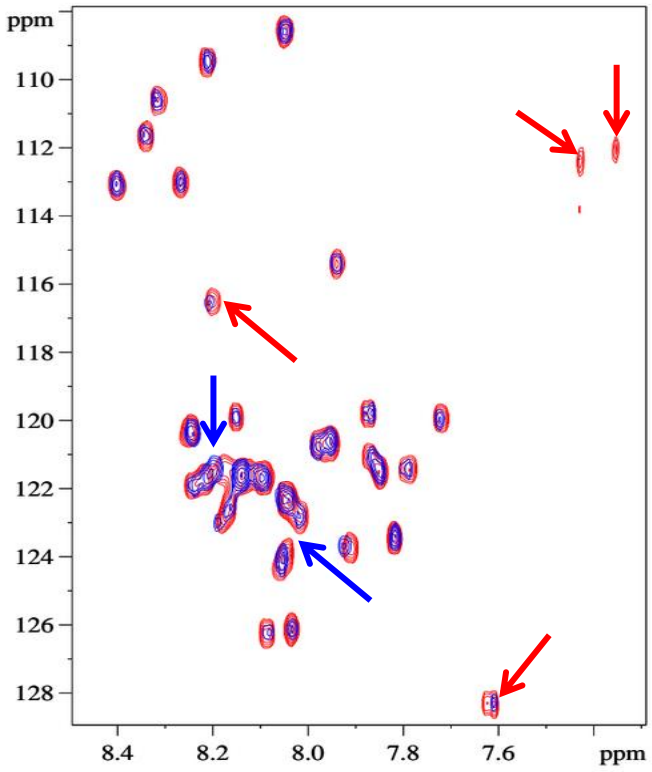

Figure 5.8. To the left is a two-dimensional heteronuclear single quantum coherence spectrum of amyloid-β(40) and TK9 (red peaks), overlaid with a spectrum of simple amyloid-β(40) (blue peaks). The red arrows indicate peaks that appeared or greup only upon the introduction of the TK9 spectrum. Blue arrows indicate those that disappeared or receded upon the addition of TK9.

5.5 Methodology of Analysis

5.5.1 Sample Preparation

5.5.1.1 Amyloid-β Preparation

In order to prevent aggregation before introduction to sample conditions, the Aβ peptide must be prepared carefully. The peptide is normally stored as a powder under -20 °C conditions.[128] Upon thawing, it is then dissolved in a solution of 190 µL millipure water and 10 µL 1% ammonium hydroxide, to break up any preformed aggregated. The concentration is then standardized through measuring the absorbance of Aβ at 280 nm.[128] Absorbance spectroscopy is a form of ultraviolet-visible spectroscopy in which the amount of photons absorbed by a molecule is measured through determining the difference in incident and transmitted light intensity.[199] This absorbance value is proportional to the concentration of the analyte via the Beer-Lambert law.[200] Absorbance is greatly affected by the physical nature of the sample,

including various excitation modes available, as well as the chemical environment. After determining the concentration of peptide in the sample, it is set aside until introduction into the analysis as needed.

Generally, investigations are done with the 40-residue peptide, rather than the 42 residue one. This is due to the increase in aggregation rate seen with the 42-residue peptide.[101-104] Its faster oligomerization rate obscures many details of Aβ interaction evident with the 40-residue one. Furthermore, the 40-residue peptide is more common in the body, and thus is more physiologically relevant.[103] Thus, the 40-residue peptide was used for all investigations herein.

5.5.1.2 Lipid Membrane Composition

Various choices exist for the model membrane used in analysis. A common combination used is 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS). This lipid system affords data that models eukaryotic cell composition, as opposed to the phosphoglycerols (PG) and the phosphoinositols (PI), as well as the dimyristoyl (DM) or dilauroyl (DL) lipids.[128,201-203] Furthermore, these lipids can be modulated to include gangliosides and cholesterols. The GM1 source generally used is porcine, due to the eukaryotic cell and the close resemblance to human gangliosides. Cholesterol is the general molecule, synthesized as such. Furthermore, studies can be performed on brain extract lipids, which are lipid membranes formed from neurons of an animal, generally pigs or cows.[204] These contain a full complement of gangliosides, sphingomyelin, cholesterol, and other compounds that would be expected in a neuron, and afford the best model for true interaction in neuronal cells possible. Lipids are all purchased from Avanti Polar Lipids, Inc (Athens, GA), which is the literature standard for such research.[128]

5.5.1.3 Extrusion and Membrane Formation

Lipid samples are kept in chloroform at -20 °C when not in use. When prepared for membrane formation, the desired lipids are chosen and mixed in the appropriate ratio. The solvent is then removed under nitrogen gas, and the resulting film is lyophilized to remove any extraneous solvent molecules.[128] The sample is then reconstituted in the buffer or dye-solution, if dye encapsulation is desired, of choice. Upon dissolution in an aqueous solution, the lipids spontaneously form multilamellar vesicles, which are essentially globular structures with many layers of lipid, like an onion.[205] This structure does not accurately represent the phospholipid bilayer in a cell, and thus the lipids are passed through a membrane filter in a process known as extrusion, where the excess layers are stripped off of the vesicle until one remains, forming a large unilamellar vesicle (LUV), which is a lipid vesicle with a single bilayer.[206-207] At this point, the lipid is ready for further experimentation.

5.5.1.4 Dye-Filled Vesicle Experiments and the Stewart Assay

It is possible to secure dye molecules within a membrane if extrusion is done with a dye solution, rather than a dye-free buffer. With the dye encapsulated in the membrane, characteristics of membrane integrity can be analyzed in dye-filled vesicle experiments. However, dye still remains in the vesicle solution, which must be removed.[128] This is done through the use of gravity-based column chromatography, in which the dye-filled vesicles come off the column first, due to their large size, in a solution free of external dye molecules.

To assess the concentration of the lipid obtained off the column, the Stewart assay is employed.[208] This assay uses ammonium ferrothiocyanate to bind to phospholipid head groups.

As this metal complex absorbs light at 461 nm, the absorbance can be used as a measure of the amount of ammonium ferrothiocyanate bound to the lipid head groups, and thus a representation of the amount of lipid present in the sample, given known values. At this point, with the concentration known, the sample is ready for further investigations.

5.5.2 Fluorescence Experiments

5.5.2.1 Introduction to Fluorescence

Fluorescence is another form of visible spectroscopy used in amyloid analysis. It is based on the interconversion of states upon the absorption of a photon.[209] Once a molecule absorbs a photon, it can relax in a variety of forms. The excited state can relax directly to the ground state, emitting radiation at the same energy as that which was absorbed.[210] Molecules also release heat after excitation via nonradiative relaxation processes, within the first excited state manifold.[211] If this were to be followed by radiative relaxation, the photon emitted would be slightly lower in energy as compared to that absorbed, affording a redshifted spectrum. This process is fluorescence. Common fluorophores are conjugated molecules that are easily excited, with many relaxation modes.[212] The redshift that the photon observes is known as a Stokes shift, which tends to be nonradiative relaxation within the excited state manifold, but depends on the environment, fluorophore, and wavelength of excitation and emission.[213-214] It is generally characteristic of the fluorophore and sample conditions.[215] Furthermore, the amount of photons emitted, and thus the intensity of the signal seen, is proportional to the concentration of the fluorophore in the solution, and thus is a quantitative measure of the relative amount of fluorophore.[215]

The quality of a fluorophore, and its fluorescence, is measured in quantum yield, a measure of the efficiency of the fluorescence process.[216] This quantum yield can be affected by nonradiative forms of relaxation that return the fluorophore to the ground state, known as quenching. This can be due to various interactions, such as collisions, energy transfer, or reactions, between the fluorophore and another element in the sample, known as the quencher, or even itself, at high enough concentrations.[217] Due to quenching, fluorescence intensity and quantum yield decrease. Intermolecular quenching, due to collisions, is the dominant form, and is dependent on fluorophore and quencher concentration.

Fluorescence is analyzed via fluorescence spectroscopy techniques, and related procedures. Typically, excitation wavelengths are scanned in order to give the maximum absorption.[218] After determination of the maximum absorption, the emission spectrum is scanned to determine the optimal fluorescent wavelength for detection. This procedure is used to maximize the quantum yield of the fluorophore detected, improving signal intensity and sensitivity. For many commonly used fluorophores, however, these values are known and standardized across research groups. Fluorescence is the main means by which amyloid aggregation and membrane interaction is studied, being the quickest and most effective characterization of qualitative Aβ mechanisms.[128]

5.5.2.2 Thioflavin-T

The most common tool used to study amyloid aggregation is the Thioflavin-T (ThT) assay.[219-220] ThT (Figure 5.9) is a fluorophore of the thioflavin family used to identify amyloid fibers. The maximum absorbance of ThT in Aβ-free solution is at 340 nm, with an emission wavelength at 445 nm. However, upon binding to β-rich amyloid sheets, the fluorescent nature of

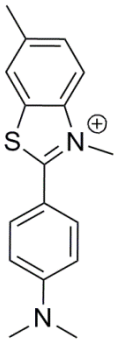

Figure 5.9. Thioflavin-T as used in fluorescence experiments

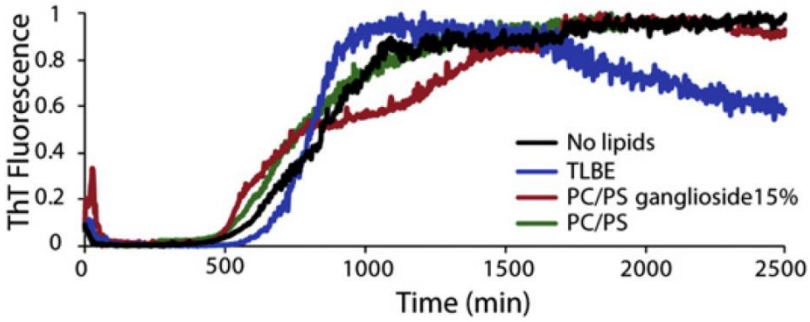

Figure 5.10. Example thioflavin-T spectra, as used in fluorescence experiments. Reprinted with permission from Sciacca, M. F. M.; Kotler, S. A.; Brender, J. R.; Chen, J.; Lee, D.-k.; Ramamoorthy, A. *Biophys. J.* 2012, *103*, 702-710. Copyright 2012 Elsevier.

the dye changes. Instead of the spectrum discussed above, the absorbance maximum shifts toward 440 nm, and the emission maximum to 480 nm. Furthermore, the quantum yield of this dye increases greatly upon binding to an amyloid-like structure. Monitoring this shift in the emission spectrum allows for the use of ThT as an indicator of the presence of aggregated amyloids, and thus is a useful monitor of Aβ fibrilization. As the intensity of the signal seen is proportional to the amount of dye bound to fiber, the signal intensity is actually a measure of the amount of fibrilization, and gives the clear sigmoidal curve, with distinct lag, elongation, and plateau phases characteristic of Aβ aggregation, as shown in Figure 5.10.[128]

5.5.2.3 Carboxyfluorescein

Another dye commonly used in Aβ experiments is carboxyfluorescein (Figure 5.11).[221-222] A larger dye, carboxyfluorescein has an excitation maximum at 494 nm and an emission wavelength of 520 nm. As a member of the fluorescein family of dyes, carboxyfluorescein has the distinct quality of self-quenching, in which high local concentrations of the dye causes the quantum yield to go down drastically. Thus, the dye changes color depending on its concentration from a bright green when dilute to a damp, dull orange when concentrated. Thus, carboxyfluorescein is used in amyloid analyses as a measure of membrane integrity in a dye-

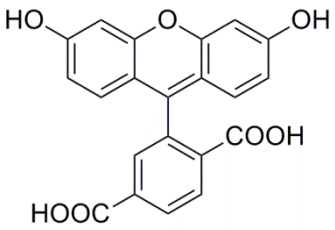

Figure 5.11. The 6-carboxyfluorescein dye as used in fluorescence experiments

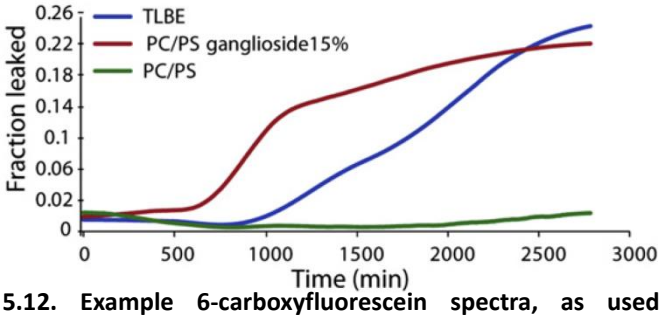

Figure 5.12. Example 6-carboxyfluorescein spectra, as used in fluorescence experiments. Reprinted with permission from Sciacca, M. F. M.; Kotler, S. A.; Brender, J. R.; Chen, J.; Lee, D.-k.; Ramamoorthy, A. *Biophys. J.* 2012, *103*, 702-710. Copyright 2012 Elsevier.

filled vesicle experiment. Trapped within a membrane, the dye exhibits a low level of fluorescence. However, if the membrane were to be degraded, then the dye would be released out into solution, lowering the local concentration, and thus increasing the quantum yield and fluorescence signal. Therefore, monitoring the change in signal at the 520 nm peak allows for a characterization of the integrity of the membrane being studied, and is a good indicator of the second stage of the two-step mechanism. An example spectrum is in Figure 5.12.

5.5.2.4 Fura-2

Much like carboxyfluorescein, Fura-2 is a dye used to probe the integrity of the membrane studied (Figure 5.13).[223-224] A large, acidic dye, Fura-2 has an excitation peak at 380 nm, with an emission spectrum at its maximum at 510 nm. However, upon binding to calcium or zinc ions, the excitation spectrum shifts its maximum to 340 nm, while not affecting the emission spectrum. The 340:380 ratio of fluorescence intensity is proportional to the amount of calcium or zinc binding to the dye. Thus, this dye can be used in dye-filled vesicle experiments to monitor the entrance of these ions via the monitoring of the change of this ratio. Upon pore formation, as in the first step of the two-step mechanism, calcium influx would cause the ratio to shift greatly. In fact, given the changes in size between calcium and zinc, this dye can be used to determine if the membrane disruption is due to pore formation or gross disruption. If zinc does not cause any

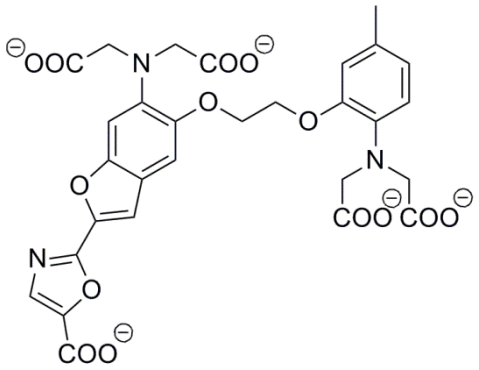

Figure 5.13. The fura-2 dye as used in fluorescence experiments

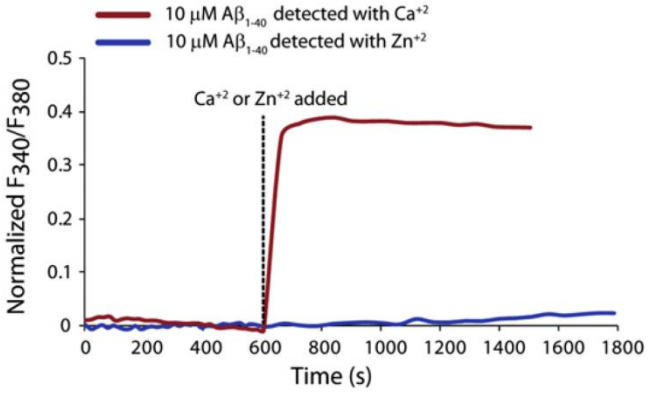

Figure 5.14. Example fura-2 spectra, as used in fluorescence experiments. Reprinted with permission from Sciacca, M. F. M.; Kotler, S. A.; Brender, J. R.; Chen, J.; Lee, D.-k.; Ramamoorthy, A. *Biophys. J.* 2012, *103*, 702-710. Copyright 2012 Elsevier.

change, then it is a simple pore formation, as hypothesized by the two-step mechanism. An example spectrum appears in Figure 5.14 for both calcium and zinc.

5.5.3 Nuclear Magnetic Resonance Experiments

5.5.3.1 Nuclear Magnetic Resonance and Amyloids

A full discussion of nuclear magnetic resonance (NMR) can be found in Chapter 1.4.3: Nuclear Magnetic Resonance and Metabolomics. It is sufficient here to state that NMR is a technique that exploits the inherent spin state of nuclei through the application of magnetic field,[225] and has been demonstrated to be highly useful in analyzing Aβ in both the solution and the solid state.[226-228] Upon excitation of the spin states, the relaxation time is measured, which is converted to frequencies normalized for the instrument into the chemical shift.[229] This shift gives information on the environmental conditions of the nucleus studied. Important nuclei for amyloid analysis are ^{1}H, ^{13}C, ^{15}N, ^{19}F, and ^{31}P, though the first and the last are the only to be discussed in great detail.[227] Furthermore, amyloid data benefits from both one-dimensional and especially two-dimensional data, as the detail and specifics afforded therein are highly useful.[226-228]

NMR is not the only spectroscopic tool available for amyloid analysis, however, as two-dimensional infrared spectroscopy is gaining popularity in this field.[230-233] However, this

technique remains laborious, preventing its implementation on a wider scale, and the theoretical implications of amyloid analysis are not fully characterized. Furthermore, it is less sensitive than NMR, and affords more complex spectra, requiring a skilled interpreter. NMR remains the superior and the widely used technique. Other options, such as mass spectrometry[234-235] and atomic force microscopy,[236-237] exist, but come with flaws such as limited interpretation and experimental flexibility, and destruction of sample, preventing an accurate time-course measurement. All discussed experiments can be done in the solution- or solid-state, as needed.

5.5.3.2 1D and 2D ^{1}H-NMR and Amyloids

Amyloid analysis via ^{1}H-NMR is done from many angles. One such form of analysis is a simple one-dimensional proton analysis, which has been demonstrated specifically with APP studies.[196] This technique, however, has the drawback of affording highly complex spectra. Though easy to acquire, interpretation remains a challenge in the one-dimensional field. It is best used for amyloids on its own to track aggregation rates through the appearance of new and broader peaks, rather than in contact with lipids, cholesterol, gangliosides, or other compounds.[196]

Two-dimensional spectroscopy, on the other hand, overcomes many of these limitations. In 2D spectroscopy, correlations between nuclei are analyzed, whether ^{1}H-^{1}H, ^{13}C-^{1}H, or ^{15}N-^{1}H. These are probed through complex pulse sequences and methods such as spin magnetization energy transfer, in which the excitation energy of one sample is transferred to another, coupled nucleus via quantum interactions, causing that interaction to be measured.[238-240] This is analogous to saturation transfer difference NMR spectroscopy, another useful amyloid analysis technique, in which certain protons can be saturated such that they give no signal in the

spectrum. The coupled protons to the initial ones will also have depressed signals, indicative of some sort of interaction, especially between receptors and ligands. Essentially, 2D spectroscopy comes down to the determination of various coupling modes between nuclei in a sample.[238-240]

Common ^{1}H-^{1}H 2D techniques used in protein analysis include correlation spectroscopy (COSY), in which α and β protons that are coupled via bonds are detected. Detection is seen via cross-peaks on the 2D spectrum, where excitation at the α proton causes a signal to be seen at both the α and the β proton, resulting in the α-α diagonal peak and the α-β cross peak.[241-242] COSY can be expanded to total correlation spectroscopy (TOCSY), where the coupled α, β, γ, δ, ε, and beyond protons are all detected upon excitation of the α proton.[243-244] Another one used is nuclear Overhauser effect spectroscopy (NOESY), in which protons related through space, rather than bonds, are detected.[245-256]

More important to amyloid analysis is heteronuclear correlation spectroscopy, such as heteronuclear single quantum coherence (HSQC), performed with ^{15}N or ^{13}C. The HSQC magnetization is prepared on the proton and subsequently transferred to the bound heteronucleus, after which the magnetization is allowed to evolve in the heteronuclear frequency, and transferred back to the bound proton for measurement. [247-248] Thus, the direct measurement of the proton is coupled with the indirect measurement of the heteronucleus and a single peak in a two-dimensional spectrum is indicative of a single ^{1}H-^{15}N or ^{1}H-^{13}C bond.. Spectra are plotted such that one axis is the proton and the other either nitrogen or carbon, and peaks thus appear in two dimensions. This allows for the separation of similar proton peaks from each other based on the differences in heteronuclear environments. An example spectrum of amyloid-β(40) with and without membrane binding is in Figure 5.15.[249]

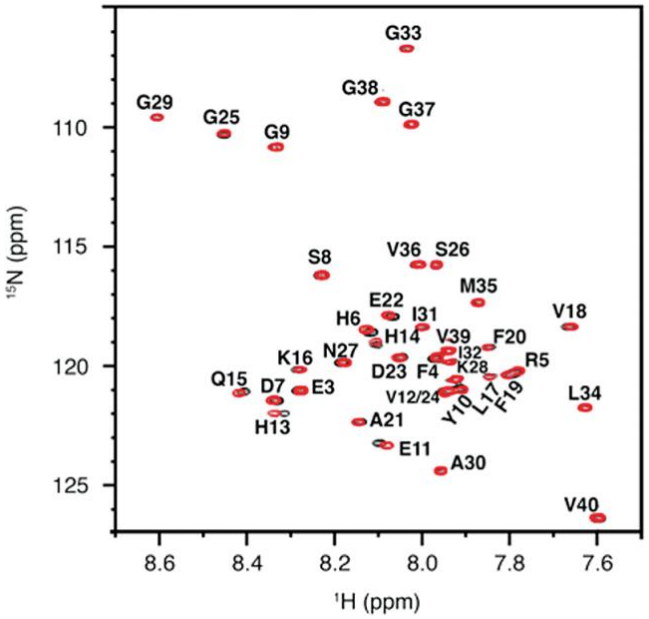

Figure 5.15. To the right is an example two-dimensional heteronuclear single quantum coherence spectrum of amyloid-β(40) (black) and amyloid-β(40) with a potential anti-oligomerization compounds (red). Reprinted with permission from Hindon, S. S.; Mancino, A. M.; Braymer, J. J.; Liu, Y.; Vivekanandan, S.; Ramamoorthy, A.; Lim, M. H. *J. Am. Chem. Soc.* 2009, *131*, 16663-16665.

NMR experiments utilizing proton magnetization are quite useful in tracking aggregation rates of the amyloid, as well as determining binding of the amyloid to a membrane, other proteins or components in the sample, and so on. In fact, HSQC data can reveal, with residue specificity in the amyloid, the formation of distinct binding sites through the depletion of peak intensity and changes in chemical shift. [247] Thus, important structural and kinetic information is obtained from these spectra in a time course fashion, without degradation of the sample or an overly complex setup.

5.5.3.3 ^{31}P-NMR and Amyloids

One-dimensional spectroscopy is not limited to hydrogen, however, but can also be performed on phosphorus nuclei. The principle is much the same as with hydrogen nuclei, but attuned to a different characteristic frequency. As ^{31}P is a component of membrane head groups, analysis of it can be used to study membrane quality. Thus, changes in the 1D spectrum are representative of different lipid membrane structures, such as the formation of micelle-like structures or interaction with peptide.[128,250] Furthermore, paramagnetic ions such as Mn^{2+} ions,

with its five unpaired electrons, can give rise to an effect known as paramagnetic relaxation enhancement when in close proximity to the lipid head group and the ^{31}P nucleus.[128] When a nucleus is exposed to a paramagnetic ion it causes quenching of the signal, and can be used as an indicator of phospholipid head groups are exposed to the environment.[128] Thus, this is a diagnostic technique for the integrity of a membrane. If the membrane is intact, then only the outer surface would be exposed and quenched, and the inner membrane would supply a signal as before. If the membrane was not intact, then the entire membrane would be quenched, and the phosphorus signal would not be detected.[128, 250-251]

5.6 Areas of Potential Growth

As well-demonstrated above, there is a wealth of information on the aggregation of amyloid-β; yet, for every fact known, there is a corresponding unknown fact. Thus, Aβ studies can go in any desired direction, probing any mechanism or hypothesis. As all techniques are well established, little methodology analysis can be done. It is the actual mechanism of action of the sample that needs further study. With the importance of membranes in the mechanism of interaction, that idea was pursued in this investigation.

The two moieties chosen to probe membrane analysis were cholesterol and anti-amyloid peptides, due to their controversial nature, in the case of cholesterol, and their potential benefits, in the case of TK9. Though they represent two different aspects of Aβ-membrane interaction, they both afford important data toward the full understanding of the mechanism of Aβ-membrane interactions, and potentially give insight into the pathophysiology of Alzheimer's disease.

Chapter 6:

Effects of Membrane Compositions on Amyloid-β Aggregation

6.1 Abstract

Alzheimer's disease is one of the most prevalent terminal conditions in the world. Though the exact mechanism remains unknown, a prevailing theory is the amyloid cascade hypothesis, where the aggregation of amyloid-β causes membrane disruption and cell death. This is purported to occur via the two-step mechanism, with initial pore formation followed by gross membrane fragmentation. The nature of amyloid-β binding to the membrane was investigated through the modulation of membrane composition via cholesterol concentration, and the offering of another platform for amyloid-β binding. It was shown that cholesterol prevented gross fragmentation of the membrane without ganglioside and that the peptide preferred binding to the membrane than to the inhibitory platform provided via the introduction of a self-assembly protein sequence, though the effects of ganglioside remains to be seen.

6.2 Introduction

As discussed above, Alzheimer's disease (AD) is a devastating neurodegenerative disease.[1-3] Though the exact mechanism of the disease is unknown, a prevailing theory of AD onset is the amyloid cascade hypothesis, in which the formation of fibrils and oligomers of the amyloid-β (Aβ) peptide is the source of apoptosis and neural degeneration in brains, leading the memory loss and cognitive impairment.[86-127] The exact mechanism of this interaction is unknown, but is believed to involve the influx of calcium and potential membrane disruption. This ion channel hypothesis has been widely supported via atomic force and electron

microscopy, as well as conductance experiments, though it does not characterize the membrane fragmentation seen.[116-119]

Recently, Sciacca, et al, proposed a two-step mechanism by which Aβ can cause membrane disruption and cell death.[128] In this mechanism, initial interaction of small Aβ monomers and oligomers interact with the membrane to form pores, through which calcium influx can occur. As the peptide oligomerizes further, a fragmentation pathway occurs, causing gross membrane disruption. Thus, this mechanism encompasses both the ion channel hypothesis and the flaw within that analysis, as discussed above. It was also demonstrated in this work that the presence of ganglioside was essential for this process to happen, due to the orientation effects of gangliosides in the lipid membrane inducing Aβ insertion.[128-145]

However, it was still unresolved as to the effects of other components of the membrane, namely cholesterol. The effect of cholesterol in Aβ aggregation and membrane disruption is still controversial, though recent studies seem to support the idea that membrane cholesterol has a protective effect against pore formation and membrane disruption.[150-190] Furthermore, as cholesterol is a major component of lipid rafts, it would be present near the ganglioside site in the membrane, potentially countering the effect of the sugar. [146-149]

Likewise, the binding affinity of Aβ to ganglioside is unknown. Self-assembling peptides, such as TK9, have shown some inhibitory effects toward Aβ aggregation through insertion within the oligomers formed.[191-195] Thus, the inherent binding of TK9 to peptide is more preferential than Aβ binding to itself. Therefore, TK9 can be used as a means by which to monitor the binding strength of Aβ to the membrane, as binding to the membrane would elicit changes in membrane structure, as shown in the two-step mechanism, whereas inhibition would not.[128, 194-195]

Knowledge of the membrane-peptide interaction would afford insight into the binding mechanism of Aβ and the pathophysiology of AD. Defining the specific roles of cholesterol in membrane binding, as well as fully understanding the kinetics and binding strength of Aβ to the membrane would help elucidate key factors in the two-step mechanism, and shedding more light on the cause of AD. It was thus proposed to study the aggregation rates of Aβ in the presence of cholesterol- and ganglioside-containing membranes, in the presence of TK9, via the standard thioflavin-T experiment, as well as analyzing the membrane integrity of these membranes through a measurement of the amount of dye that leaks through the membrane. It was hypothesized that TK9 would prevent Aβ aggregation and dye leakage, with the effect increasing at higher concentrations, while cholesterol would not have any effect on the aggregation overall, or the dye leakage in the presence of ganglioside.

6.3 Experimental

6.3.1 General Considerations

All conversions and manipulations were done to the open air. Peptide samples were purchased from Genscript. Extrusion equipment, total lipid brain extract, 1-palmitoyl-2- oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-Lserine sodium salt (POPS), cholesterol, and ganglioside extract were purchased from Avanti. The TK9 sample was provided by Dr. Anirban Bhunia. Ammonium thiocyanate, ferric chloride hexahydrate, Sephadex, Triton-X, and thioflavin-T were purchased from Sigma-Aldrich. The 6-carboxyfluorescein was purchased from Fluka. Lyophilization and absorbance measurements were done on the instruments provided by Biophysics at the University of Michigan. Fluorescence measurements were done on a Biotek Synergy 2 plate reader with 96 well Corning

non-binding surface plates. Solvents were either purchased from Sigma-Aldrich or provided by the University of Michigan solvent line.

6.3.2 Amyloid-β Preparation

A 0.1 mg aliquot of Aβ was dissolved in 190 μL of millipure water and 10 μL of 1% ammonium hydroxide. Next, the concentration was standardized through measurement of the absorbance of the peptide at 280 nm, given an extinction coefficient of 1490 1/(M*cm). The sample was then stored at 4 °C until use.

6.3.3 TK9 Preparation

TK9 was dissolved in millipure water in 2.5 mM aliquots, which were diluted in millipure water to a concentration of 500 μM, and stored at 4 °C until use.

6.3.4 Lipid Preparation

To perform assays, large unilamellar vesicles were prepared. Chloroform samples of lipids were taken to form the following ratios: 7:3 POPC/POPS and 7:3:3 POPC/POPS/cholesterol, along with a sample of total lipid brain extract (TLBE). These samples were dried under nitrogen gas and lyophilized overnight to remove any residual solvent. The resultant film was rehydrated with a buffer solution (10 mM phosphate buffer, pH 7.4, 100 mM NaCl for thioflavin-T experiments, with the addition of 10 μM of carboxyfluorescein for dye-leakage experiments), to a concentration of 4 mg/mL for thioflavin-T experiments, and 10 mg/mL for dye-leakage. The solution was then passed through a 100 nm polycarbonate membrane on an extruder to obtain vesicles with an average size of 100 nm.

6.3.5 Thioflavin-T Experiments

The 4 mg/mL lipid solution of choice was diluted to 0.2 mg/mL in the same buffer solution as before, along with thioflavin-T at a final concentration of 20 μM. To this solution was added amyloid-β such that the concentration was 10 μM. If desired, TK9 was added in concentrations of 10 μM, 50 μM, and 100 μM. Each condition was plated in triplicate, with peptide-free samples being used as controls. Aggregation was measured via fluorescence, with an excitation wavelength of 440 nm and an emission wavelength of 480 nm. Data was collected over 48 hours at 30 °C, with measurement every 3 minutes and 30 seconds of sample agitation before each measurement.

6.3.6 Dye-leakage experiments

After preparation of the dye-filled vesicles, external dye molecules were removed via gravity column chromatography through the use of a Sephadex G50 gel exclusion column, with the collection of the first visibly colored band. The concentration of the lipid was then assessed via the Stewart assay. A 10 μL sample of lipid was diluted in 1 mL of chloroform and 1 mL of the Stewart buffer, containing ammonium thiocyanate (400 mM) and ferric chloride hexahydrate (170 mM). After vigorous shaking, the chloroform layer was allowed to settle and was collected for absorbance measurements at 461 nm. Comparison to a previously made calibration curve was used to determine the final concentration of lipid.

After, the lipid was diluted to 0.2 mg/mL, and to this solution was added amyloid-β such that the concentration was 10 μM. If desired, TK9 was added in concentrations of 10 μM, 50 μM, and 100 μM. Each condition was plated in triplicate, with peptide-free samples being used as controls. Dye leakage was measured via fluorescence, with an excitation wavelength of 494 nm

and an emission wavelength of 520 nm. Data was collected over 48 hours at 30 °C, with measurement every 3 minutes and 30 seconds of sample agitation before each measurement. After collection, a 10% Triton-X solution in water was used to lyse the vesicles, and a separate measurement was taken as the maximum value for normalization purposes. All dye leakage data was collected on the same plate as the aggregation data.

6.4 Results and Discussion

6.4.1 Effects of Cholesterol on Amyloid-β Membrane Interactions

Initial samples were made with the following concentrations of lipid: 7:3 POPC/ POPS, and 7:3:3 POPC/POPS/cholesterol. The POPC and POPS lipids were chosen due to their applicability in eukaryotic investigations. The concentration of cholesterol chosen was 30% of the total membrane, representative of the average concentration of cholesterol in human neuronal cells. Both thioflavin-T (ThT) and dye leakage studies were done.

At first, the ganglioside free samples were measured to determine the effect of cholesterol on Aβ aggregation and dye leakage. As seen in Figure 6.1, the ThT data was unaffected by the introduction of cholesterol into the model membrane. This is to be expected, as cholesterol in the membrane has had no previously displayed effect on Aβ aggregation.[168-169] Though there were slight changes in the ThT spectrum for each condition, the overall spectrum still showed sigmoidal aggregation. Thus, these variations are not important, and the depression seen with the introduction of membrane is expected. A discussion on the calculation of the various spectra can be found in the supplementary information for all samples.

Next, a dye leakage experiment was done on the samples. This data appears in Figure 6.2. Based on the graph in the figure, it can be stated that the cholesterol actually acts as a protector

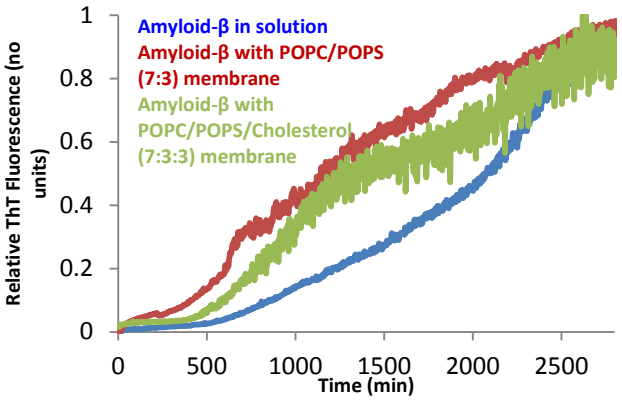

Figure 6.1. Kinetics of amyloid formation measured by ThT fluorescent emission. The graph shows 10 µM peptide in buffer in the absence of membranes (blue line) and in the presence of 0.2 mg/mL POPC/POPS 7:3 LUVs (red line), and 0.2 mg/mL POPC/POPS/Cholesterol 7:3:3 LUVs (red line). Experiments were performed at room temperature in 10 mM phosphate buffer, 100 mM NaCl, pH 7.4. Results are the average of three measurements. Data was normalized to the maximum value for each sample. Data was obtained at an excitation of 440 nm and an emission of 480 nm.

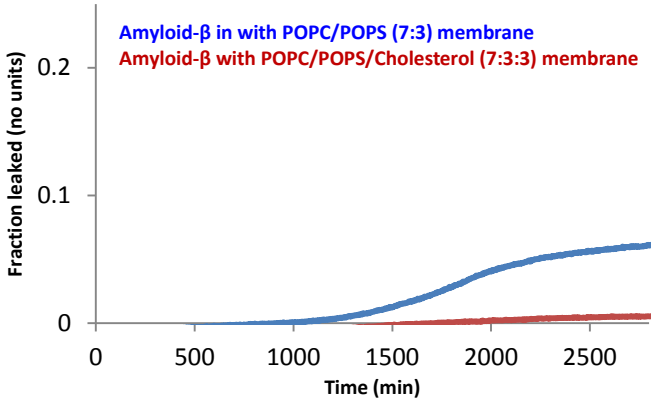

Figure 6.2. Membrane disruption induced by amyloid-β as measured by the 6-carboxyfluorescein dye leakage assay. The graph illustrates the release of 6-carboxyfluorescein induced by 10 µM peptide from 0.2 mg/mL POPC/POPS LUVs 7:3 (blue line) and 0.2 mg/mL POPC/POPS/cholesterol LUVs 7:3:3(red line). Dye leakage occurs only after a lag period and is detected at a negligible level in these samples due to the lack of ganglioside. Experiments were performed at room temperature in 10 mM phosphate buffer, 100 mM NaCl, pH 7.4. Results are the average of three measurements. Data was normalized to the total dye in solution as determined by obtaining a spectrum after membrane lysing with Triton-X. Data was obtained at an excitation of 494 nm and an emission of 480 nm.

against membrane disruption. Figure 2 displays the cholesterol and ganglioside free membrane against only the cholesterol-free membrane. Based on the aggregation rate of Aβ, the increase in dye leakage seen in the phospholipid-only membrane was due to the effects of fibrilization. This effect is greater than that of the cholesterol and phospholipid membranes, though both are quite small compared to that seen in previous studies with total lipid brain extract (TLBE).[128]

To fully understand the conclusions from this data, further studies need to be undertaken. These include studying the effects of gangliosides on membrane interactions, as this has been previously demonstrated to increase the amount of leakage seen.[128] Furthermore, Fura-2 studies to determine the appearance of pores in the membrane as a function of TK9 concentration, as well as ^{1}H-^{15}N HSQC NMR and ^{31}P NMR, to fully understand the physical nature of the membrane and oligomers over time. These studies are currently ongoing.

6.4.2 Effects of TK9 on Amyloid-β Membrane Interactions

After conclusion of the membrane studies, the effect of TK9 was investigated. Initially, the effect of TK9 on Aβ in solution was probed. TK9 has been demonstrated to be inhibitory in relation to the amyloid precursor protein,[196] but its effect on Aβ in solution is unknown in the literature. Therefore, initial samples of 10 μM Aβ were placed in solution for a ThT assay, along with varying concentrations of TK9. This data is presented in Figure 6.3. As shown in the graph, as the amount of TK9 increased, the resultant fluorescence value decreased. This fluorescence intensity is directly correlated to the amount of Aβ fibril present in the solution; thus, this lowered signal intensity is indicative of inhibitory properties of the TK9 peptide. Furthermore, the lag phase of Aβ aggregation increased with the increase in TK9 concentration, again a function of the inhibitory properties of the peptide. TK9, though self-assembling, did not afford any native ThT fluorescence; therefore, it was ignored as a contributor to ThT data.

Given this inhibitory nature, the data suggested that Aβ preferentially binds to TK9 rather than itself, causing the lack of aggregation seen. With this in mind, studies of the effect of TK9 in the presence of membrane were carried out. As TK9 is known to have self-assembly characteristics, forming β-sheets, it was thought that it may interact with membranes, causing disruption in dye leakage studies. Therefore, control studies were done with TK9 alone as well as Aβ. The ThT data is in Figures 6.4, and the 6-carboxyfluorescein data are Figures 6.5 and 6.6.

Based on the data presented, TK9 did not inhibit the aggregation of Aβ in the presence of membrane to the extent that it did in solution. As seen in Figure 6.3, the lag phase greatly increased as a function of TK9 concentration, along with a corresponding decrease in fluorescence intensity. Though this effect is seen in the presence of lipid, it is not as dramatic. Furthermore, binding with the lipid was not inhibited by the presence of TK9. As shown in

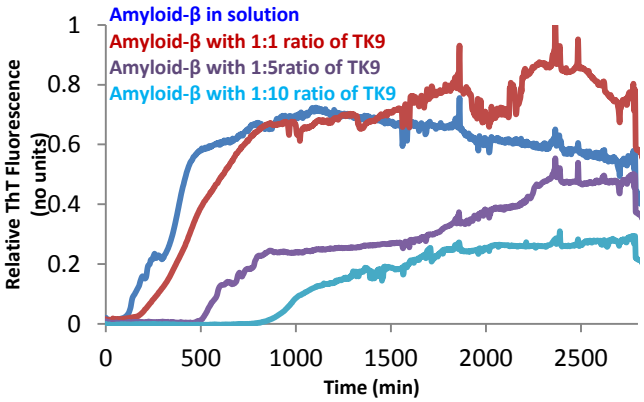

Figure 6.3. Kinetics of amyloid formation measured by ThT fluorescent emission. The graph shows 10 µM peptide in buffer in solution (dark blue line) and in the presence of 10 µM TK9 (red line), 50 µM TK9 (purple line), and 100 µM TK9 (light blue lien). Experiments were performed at room temperature in 10 mM phosphate buffer, 100 mM NaCl, pH 7.4. Results are the average of three measurements. Data was normalized to the maximum value for each sample. Data was obtained at an excitation of 440 nm and an emission of 480 nm.

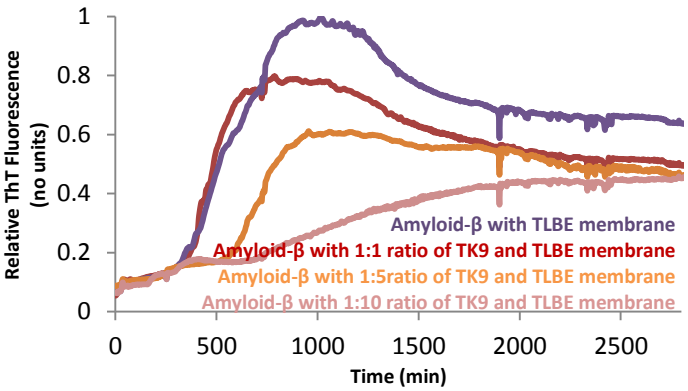

Figure 6.4. Kinetics of amyloid formation measured by ThT fluorescent emission. The graph shows 10 µM peptide in buffer in the presence of 0.2 mg/mL TLBE (purple line), 0.2 mg/mL TLBE and 10 µM TK9 (red line), 0.2 mg/mL TLBE and 50 µM TK9 (brown line), and 0.2 mg/mL TLBE and 100 µM TK9 (pink line). Experiments were performed at room temperature in 10 mM phosphate buffer, 100 mM NaCl, pH 7.4. Results are the average of three measurements. Data was normalized to the maximum value for each sample. Data was obtained at an excitation of 440 nm and an emission of 480 nm.

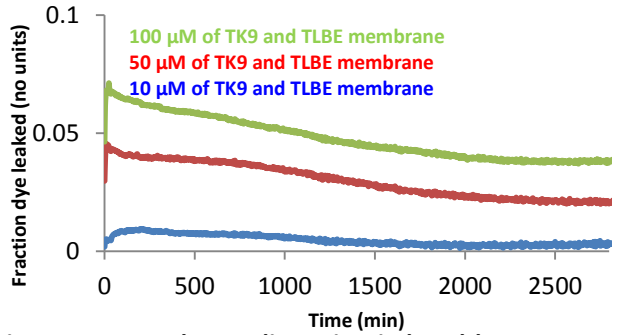

Figure 6.5. Membrane disruption induced by TK9 measured by the 6-carboxyfluorescein dye leakage assay. The graph illustrates the release of 6-carboxyfluorescein induced by 10 µM TK9 (blue line), 50 µM TK9 (red line), and 100 µM TK9 (green line) in 0.2 mg/mL of TLBE. Dye leakage occurs only after a lag period and is detected at a negligible level in these samples due to the lack of ganglioside. Experiments were performed at room temperature in 10 mM phosphate buffer, 100 mM NaCl, pH 7.4. Results are the average of three measurements. Data was normalized to the total dye in solution as determined by obtaining a spectrum after membrane lysing with Triton-X. Data was obtained at an excitation of 494 nm and an emission of 480 nm.

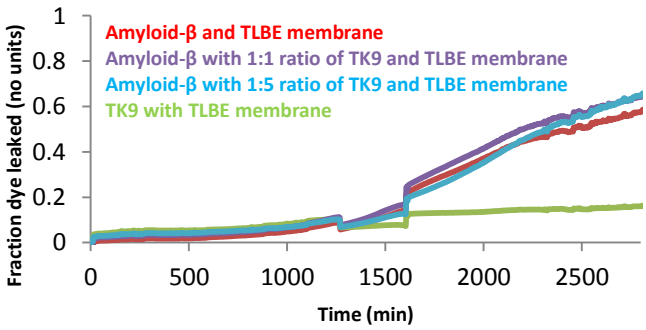

Figure 6.6. Membrane disruption induced by TK9 measured by the 6-carboxyfluorescein dye leakage assay. The graph illustrates the release of 6-carboxyfluorescein induced by 10 µM TK9 (green line), and 10 µM peptide with 10 µM TK9 (purple line), 50 µM TK9 (light blue line), and no TK9 (red line), all in 0.2 mg/mL of TLBE. Dye leakage occurs only after a lag period and is detected at a negligible level in these samples due to the lack of ganglioside. Experiments were performed at room temperature in 10 mM phosphate buffer, 100 mM NaCl, pH 7.4. Results are the average of three measurements. Data was normalized to the total dye in solution as determined by obtaining a spectrum after membrane lysing with Triton-X. Data was obtained at an excitation of 494 nm and an emission of 480 nm.

Figure 6.6, the dye leakage curves were essentially unchanged when TK9 was introduced. Though TK9 has its own basal leakage value (Figure 6.7), it is not large enough to cause the

leakages seen in Figure 6.5 to imply inhibition of Aβ membrane binding. Thus, Aβ must interact with the membrane at the same rate with and without TK9, though aggregation is inhibited.

Many factors could explain this confusing data. The lipid used for this investigation was bovine total brain extract lipid (TLBE), which contains cholesterol and ganglioside and models humans well.[204] As discussed above, ganglioside is a known promoter of Aβ aggregation and membrane disruption, as measured by ThT and dye leakage.[129-131] Therefore, it is likely that Aβ has a binding preference to the membrane ganglioside rather than the TK9 peptide. Though aggregation itself is inhibited, or at least slowed, this could be because of the effect on free peptide, rather than bound. In fact, it could be that the fluorescence shown from Figure 6.4 indicates that fibrilization still occurs on the membrane surface, but solution values are inhibited. Thus, the membrane and the peptide have a favorable interaction than the peptide as TK9, as inhibition is decreased. This could be a thermodynamic effect, in that the binding strength of the Aβ-ganglioside interaction is simply much stronger than the TK9-Aβ interaction. On the other hand, it could be a kinetic interaction, in that the Aβ-ganglioside interaction simply happens on a faster time scale than the effects of TK9, such that the Aβ peptide is already inserted within the membrane before TK9 can even affect aggregation.

To fully understand this data, further studies need to be undertaken. These include Fura-2 studies to determine the appearance of pores in the membrane as a function of TK9 concentration, as well as ^{1}H-^{15}N HSQC NMR and ^{31}P NMR, to fully understand the physical nature of the membrane and oligomers over time. It is to be thought that the cholesterol would show protection against pore formation in Fura-2, while these would still be present with all concentrations of TK9. NMR would confirm the binding to membrane in presence of cholesterol and TK9. These studies are currently ongoing, and will be completed as soon as possible.

6.5 Conclusion

In this study, the effect of membrane composition on the aggregation rate of amyloid-β, as well as its effects on membrane integrity was investigated, with the goal of gaining insight into the mechanism of amyloid-β oligomerization in the context of Alzheimer's disease. It was shown that cholesterol inhibits membrane disruption if alone in a lipid membrane, though this remains unproven in the presence of aggregation promoters such as gangliosides. Further studies were done with the peptide TK9, known to inhibit the amyloid precursor protein, to determine its effect on amyloid-β both with and without the presence of a membrane. Though this sequence prevented amyloid-β aggregation without the presence of a membrane, introduction of lipids removed all inhibitory effects of TK9. This was most likely due to the kinetic or thermodynamic preference of amyloid-β for the gangliosides present in the membrane. However, there remains many question marks about the interaction of amyloid-β with these membranes, as Fura-2 studies, to analyze the formation of pores in the membrane upon introduction of peptide, as well as one- and two-dimensional nuclear magnetic resonance procedures, to study the aggregation rate and gain a physical picture of the interaction, are ongoing. In conclusion, it can be stated that the mechanism of amyloid-β aggregation in the presence of various membrane compositions has been probed, underscoring important conclusions of the two-step mechanism, and lending support to that formulation. Eventual consolidation of that mechanism would allow clinical applications in the analysis of the pathophysiology of Alzheimer's disease and the eventual development of pharmaceuticals to combat it.

Chapter 7:

Concluding Remarks and Future Directions

The attention paid to Alzheimer's disease and amyloid-β, as the main aggressor in the amyloid cascade hypothesis, has never been higher. A full understanding of the physiological and biochemical methods of this disease has the potential to improve the quality of life of millions of future Alzheimer's patients. With the aging of the baby boomer generation, Alzheimer's disease is going to become more socially and economically cumbersome. Already the most expensive disease in the developed world, causing endless stress for caregivers, the imminent increase in cases threatens to upend the current health care system. Therefore, there is an even greater need for the development of better theories to explain how this disease actually functions. Furthermore, the implications of the amyloid cascade hypothesis and amyloid-β aggregation extend well beyond the confines of Alzheimer's disease. The amyloid hypothesis is implicated in diseases as varied as Parkinson's disease, Huntington's disease, and even type II diabetes, increasing the scope of the impact of potential amyloid-β mechanisms.

However, as displayed in this analysis, there still remains much work before the mechanism is fully determined for this disease. Though changing the moieties of membrane composition, as done above, have given some insight into how amyloid-β interacts with the membrane, and its preference for gangliosides over all else, the exact nature of this preferential binding still is not understood. Furthermore, many more components of the membrane, such as sphingomyelin and other glycophospholipids, remain untested. Even a full characterization of the two-step mechanism in the context of the above mechanism, through the use of Fura-2 assays and nuclear magnetic resonance studies remains to be seen, though experiments are ongoing.

Likewise, the difference between *in vivo* and *in vitro* data is striking. No data presented above can be taken as a true representation of the human body, as factors such as genetics, local environmental stress, bodily injury, diet and others may impact the progression of this disease. The studies done above are isolated from effects that may be as important as those analyzed.

Amyloid research, however, is benefitting from an increase in attention. Two-dimensional infrared spectroscopy and newly developed nuclear magnetic resonance pulse sequences are being applied to amyloid data, giving insight into previously unknown aspects of aggregation. Though theoretical obstacles remain before the full implementation of these techniques, the potential improvements in data suggested by initial results could revolutionize the field.

These improvements in amyloid analysis and mechanistic understanding, however, do not lose sight of the overall goal: to treat Alzheimer's disease, among others. All amyloid studies are done with one eye toward the pharmaceutical applications of this technique, as the elucidation of the mechanism of amyloid-β aggregation affords therapeutic targets. The improvements to the understanding of the mechanism discussed above represent steps toward reaching that potential. Being able to isolate gangliosides as the driving force in amyloid-β and membrane interactions highlights aspects of the potential mechanism of action that can be a target for the eventual synthesis of therapeutics, while simultaneously disavowing self-assembling peptides as a potential therapeutic agent. Once the mechanism is determined, clinical applications will follow.

This is an exciting time to be involved in amyloid-β research. With the new technologies discussed above, as well as potential explanations for membrane interaction and disruption hypothesized, the five-year outlook is extremely bright. Though many questions remained, the progress recently made in leaps and bounds suggests that those questions will not remain unanswered for long.

Appendix A.2:
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Appendix B.2:
Supplementary Information

Mathematical formulation of Thioflavin-T data (Figures 6.1, 6.3, 6.5, and 6.6)

Samples were taken for each condition, with and without amyloid-β, in triplicate. The baseline value was taken to be that without amyloid-β, and the three values were averaged at each time point. These were then subtracted from each individual sample with amyloid-β value, at the corresponding time point. The amyloid-β data was then normalized to the highest fluorescence value for each sample, and each condition was averaged to give relative fluorescence values. These were then plotted as above.

Mathematical formulation of 6-Carboxyfluorescein data (Figures 6.2, 6.4, 6.7, and 6.8)

Samples were taken for each condition, with and without amyloid-β, in triplicate. Each sample was measured in fluorescence units, over the total time of the experiment. These were then normalized to the value obtained after vesicle lysing using a Triton-X solution, done for each sample. Then the samples without amyloid-β were averaged and subtracted from each sample of the amyloid-β conditions. These conditions were then averaged and plotted as above, to give fraction of dye leaked.

Details of data acquired available upon request.

FINAL REMARKS

BIOPHYSICS REVISITED

As displayed in the above investigation, biophysical techniques are useful in investigating the nature of Alzheimer's disease and cancer. Furthermore, it is easily seen that the techniques used are in fact as chemical as they are biophysical, being at the interface of the two sciences. Through the use of spectroscopic techniques such as nuclear magnetic resonance and fluorescence, improvements to current methodologies and a better understanding of the mechanism of disease progression have been reached. Though metabolomics has a ways to go before truly becoming a science able to act in a diagnostic fashion, the simplification and standardization of spectra delineated above allows it to afford more accurate data, an important step toward clinical applications. Likewise, though the two-step mechanism and the amyloid hypothesis have not been, by any means, definitively proven, the studies done above give insight into the nature of amyloid-membrane binding, and its potential implications for the onset of Alzheimer's disease. Future directions for this research abound, and do not need to be discussed here. It is evident that the contribution above represents only a small step toward the full understanding of these terminal diseases, and leaves much to be learned in its wake. These diseases may never be solved, but that is not important. What is important is that they have been fought, they are fought, and they will be fought, as long as the scientific spirit lives on.