Development of Biosensors to Monitor the Interaction of Small Molecules with Amyloidogenic Proteins using Optical and Electrochemical Methods

by

Xavier Xin Ran Cheng

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Chemistry
University of Toronto

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Abstract
Amyloidogenic protein fibrils are well known pathological hallmark of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). The Amyloid Cascade Hypothesis attributes the onset and progression of AD to an imbalance in amyloid-beta (Aβ). In PD, a definitive diagnosis of Parkinson's disease can be confirmed only by post mortem examination of the patient's substantia nigra for the presence of Lewy bodies, mainly comprised of α-synuclein (α-S). As the toxicity in these neurodegenerative diseases is highly correlated with the formation of soluble oligomers from their corresponding proteins, a strategy to inhibit the aggregation of Aβ and α-S may help to ameliorate AD and PD respectively (Chapter 1). Herein, we review the fundamentals of electrochemistry (Chapter 2) before demonstrating the use of electrochemical techniques, acoustic wave sensor and BiacoreX™ surface plasmon resonance (SPR) to characterize the aggregation of Aβ (Chapter 3). We have shown that amyloid aggregation could be monitored through these label-free methods and clioquinol (CQ) inhibits the progression of aggregation.
We further increased the throughput of monitored small molecules and Aβ interactions through the use of SPR imaging (SPRi) (Chapter 4) and LED-interferometric reflective imaging sensor (LED-IRIS) (Chapter 5). These studies showed that epigallocatechin gallate (EGCG) modulates the Aβ aggregation pathway to form β-sheet absent aggregates while certain metal ions generally accelerate the Aβ aggregation process to form thick mature fibrils. These results are supported by Thioflavin T (ThT) and transmission electron microscopy (TEM) studies.

We then studied the effects of CQ on α-S (Chapter 6) using electrochemical techniques and spectroscopic dyes such as ThT and Congo Red. Both electrochemical and spectroscopic studies showed that Cu(II) accelerated the fibril formation of α-S, while CQ inhibited such activity. This electrochemical analysis was further modified to include an optical screening test on the same transduction platform by utilizing nanosphere lithography (NSL) (Chapter 7). Complemented by Localized-SPR, SPRi, TEM and ThT studies, it was found that dense and unstructured amorphous αS aggregates were induced by EGCG, while β-sheet-rich and compact α-S mesh-networks were promoted by Cu(II) ions, in agreement with previous results.
Acknowledgments

Completing my PhD degree has been one of the most rewarding activity of my life thus far. It has been a great privilege to spend several years in the Department of Chemistry at University of Toronto. Over the past four years, I have gained not just tremendous amount of knowledge about my research topics, but also lots of transferrable skills that make me a more matured and professional individual. I have also made many friends along the way, both locally and internationally. These would not have been possible without the support and guidance of many people.

The first person that I would like to express my most sincere gratitude to is my supervisor, Professor Kagan Kerman. His insightful advice has helped me grow as a student as well as a person. He would always put his students' best interest at heart. I had the opportunity of travelling to cities like Florence, Tokyo and Boston because of his recommendations and support. I feel really blessed to be able to enjoy these unique and precious PhD experiences; they have taught me to work with a foreign team and manage my time effectively to make impactful research while establishing long-lasting relationships. Professor Kerman is definitely my role model when it comes to working hard but smart.

I would also like to thank my supervisory committee members, Professor Aaron Wheeler and Professor Xiao-an Zhang for their patience and advice during my committee meetings and during the preparation of my thesis. I also thank Professor Andre Simpson for serving as my examiner for my oral comprehensive examination and final oral defense.

To all of our collaborators at the University of Toronto Scarborough, I am so proud to have been a part of this wonderful research community. Thank you to Professor Heinz-Bernhard Kraatz, Dr. Selena Martic for all the advice on our collaborations. I am also very thankful to our international collaborators, such as Professor Selim Unlu and Dr. George Daaboul, about our highly successful work on the optical sensing of amyloid aggregation. I had a great time at Boston University and I sincerely appreciate the hospitality I received from the entire lab. I am also grateful to Professor Maria Minnuni, Professor Marco Mascini and Dr. Simona Scarano for teaching me about quartz crystal microbalance and surface plasmon resonance and providing me
with much advice on our research. Even though we may have minute language barriers, I could feel the genuine vested interest from the lab in seeing me succeed during my trip. I also thank Professor Tatsuro Endo and Ben Hau for their valued input and help in our optical studies with indium tin oxide substrates. Many thanks to Bob Temkin for his help in my electron microscopy studies.

I would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC), the Ontario Ministry of Training, Colleges and Universities as well as the University of Toronto for their graduate research fellowships and travel grants.

To the members of the Kerman Group, your companionship and support mean a lot to me and I am profoundly grateful. I would especially like to thank Dr. Anthony Veloso for helping me learn the ropes in the lab. Without your advice and help, I know my PhD would have been a much rougher journey. I would also like to specially thank Vinci Hung, Han Su, Amy Liu and Nan Li for going through many unforgettable moments with me. I am continually energized and motivated during my graduate experience because of all of you. Thank you also to Tiffiny Chan, Amina Zaheer, Simon Biao Zhang, Daniel Fiorella, Kathy Qi Li and David Wu for their help over the years.

I would especially like to thank my brother Weiran Cheng for being so accommodating and patient with me over these years. To my father, Ying Cheng, and mother, Qian Wang- I would not have gotten so far without your support and sacrifices made for us. I know that you have endured extreme hardships and stress during our childhood to provide us with the best education possible. I am deeply grateful for the belief you had in me. Lastly, I would like to dedicate this work to my mother who left us quietly during my hectic PhD application period, I hope I have made you proud.
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<th>Description</th>
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<tbody>
<tr>
<td>α-S</td>
<td>Alpha synuclein</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>AC</td>
<td>Alternating current</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ACV</td>
<td>Alternative current voltammetry</td>
</tr>
<tr>
<td>ASV</td>
<td>Anodic stripping voltammetry</td>
</tr>
<tr>
<td>AWS</td>
<td>Acoustic Wave Sensor</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge coupled diode</td>
</tr>
<tr>
<td>CE</td>
<td>Counter electrode</td>
</tr>
<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
</tr>
<tr>
<td>CR</td>
<td>Congo red</td>
</tr>
<tr>
<td>CPE</td>
<td>Carbon paste electrode</td>
</tr>
<tr>
<td>CQ</td>
<td>Clioquinol</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CT</td>
<td>Computerized tomography</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic voltammetry</td>
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<tr>
<td>DME</td>
<td>Doping mercury electrode</td>
</tr>
<tr>
<td>DPV</td>
<td>Differential pulse voltammetry</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>EDL</td>
<td>Electrical double layer</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin gallate</td>
</tr>
<tr>
<td>EIS</td>
<td>Electrochemical impedance spectroscopy</td>
</tr>
<tr>
<td>GCE</td>
<td>Glassy carbon electrode</td>
</tr>
<tr>
<td>GE</td>
<td>Gold electrode</td>
</tr>
<tr>
<td>GPES</td>
<td>General purpose electrochemistry software</td>
</tr>
<tr>
<td>HMDE</td>
<td>Hanging mercury drop electrode</td>
</tr>
<tr>
<td>IHP</td>
<td>Inner Helmholtz plane</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>IRIS</td>
<td>Interferometric reflective imaging sensor</td>
</tr>
<tr>
<td>ISE</td>
<td>Ion-selective electrode</td>
</tr>
<tr>
<td>ITO</td>
<td>Indium tin oxide</td>
</tr>
<tr>
<td>LSPR</td>
<td>Localized surface plasmon resonance</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NPV</td>
<td>Normal pulse voltammetry</td>
</tr>
<tr>
<td>NSL</td>
<td>Nanosphere lithography</td>
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<tr>
<td>OCP</td>
<td>Open circuit potential</td>
</tr>
<tr>
<td>OHP</td>
<td>Outer Helmholtz plane</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PGE</td>
<td>Pencil graphite electrode</td>
</tr>
<tr>
<td>PIB</td>
<td>Pittsburgh compound B</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>RE</td>
<td>Reference electrode</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence unit</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RU</td>
<td>Response unit</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SERS</td>
<td>Surface enhanced Raman spectroscopy</td>
</tr>
<tr>
<td>SPCS</td>
<td>Screen-printed carbon strip</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SWV</td>
<td>Square-wave voltammetry</td>
</tr>
<tr>
<td>TCh</td>
<td>Thiocholine</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>TSM</td>
<td>Thickness shear mode</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UME</td>
<td>Ultramicroelectrode</td>
</tr>
<tr>
<td>WE</td>
<td>Working electrode</td>
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<td>Appendix D</td>
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Preface

The work presented in this thesis has been adapted from 3 published and peer-reviewed articles and 2 submitted manuscripts. All studies were designed through the collaborative efforts of XRC and KK. The manuscripts presented here have been written by XRC, with critical comments and revisions provided by KK. The specific contributions of ancillary authors is outlined in detail below:

Chapter 3


XRC, MMinnuni and KK designed the experiments, performed the experiments and wrote the manuscript. VWSH and SS assisted in performing the experiments. MMascini revised the manuscript.

Chapter 4


XRC and KK designed the experiments. XRC, BYH, AVJ and SM performed the experiments. XRC and KK wrote the manuscript. HBK revised the manuscript.

Chapter 5


XRC and KK designed the experiments. XRC and GGD performed the experiments and analyses. XRC, GGD and KK wrote the manuscript and MSU revised the manuscript.
Chapter 6


XRC and KK, designed the experiments and wrote the manuscript. XRC performed the experiments and analyzed the results.

Chapter 7


XRC, GQW and KK, designed the experiments and performed the experiments and analyses. XRC and KK wrote the manuscript and FLL revised the manuscript.
Chapter 1

1 Amyloidogenic Proteins

1.1 Introduction

In 1854, German physician scientist Rudolph Virchow, introduced the term amyloid to describe the abnormal appearance of an iodine stained cerebral corpora amylacea. When he discovered that the tissue stained blue on addition of iodine and violet upon addition of sulfuric acid, he deduced that the macroscopic abnormality observed was cellulose and named it amyloid, derived from the Greek ‘amylon’ and Latin ‘amylum’. With advanced technology, investigations of these structures utilizing microscopy and histopathologic dyes such as Congo Red and Thioflavin T (ThT) were conducted. It was initially thought that amyloid of various sources was structurally amorphous; Subsequent polarization light microscopic studies demonstrated amyloid accumulations in a variety of tissues display positive birefringence with respect to the long axis of the deposits.

Several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and prion-associated spongiform encephalopathies have been associated with the formation of fibrillar aggregates also known as amyloid. In each of these diseases, a particular protein misfolding event can lead to its aggregation, causing monomers to form dimers, trimers, oligomers and eventually insoluble fibrillar deposits. For the purpose of this thesis, Alzheimer's and Parkinson's disease will be discussed.

1.2 Alzheimer's disease and amyloid-β

Alzheimer's disease has recently been labelled the '21st century plague' that will affect more than 70 million patients in the next 10 years. First identified by Alois Alzheimer in 1906, Alzheimer's disease is a neurodegenerative disease characterized by the presence of amyloid deposition and neurofibrillary tangles. The disease is divided into two categories, early and late onset. The former accounts for only ~2% of all cases and it develops between 30-60 years old. In most of these cases, the cause is due to genetic issues with a strong Mendelian inheritance pattern. Late onset also has a genetic predisposition but it involves gene polymorphisms as well. Both stages have similar disease progression which is split into early, moderate and severe cases.
Some symptoms of Alzheimer's disease are the loss of memory, disorientation and misinterpreting spatial relationships, growing difficulty in thinking and reasoning, etc.

Microscopically, the Alzheimer brain is characterized by amyloid plaques and neurofibrillary tangles. These plaques are comprised of primarily amyloid-β (Aβ) protein. Aβ is a ~4 kDa protein with a homogenous core sequence but differing N- and C- termini. The most common form of Aβ has 40 amino acids (Aβ1-40). Aβ1-42, a less abundant form of Aβ having two additional amino acids at the C-terminus, has been particularly associated with the disease. Neurofibrillary tangles are not specific to Alzheimer's disease but evidence suggests that abnormal Aβ accumulation triggers tau pathology\(^7\)\(^8\) and tau has also been proposed to be involved in Aβ induced neurotoxicity.

Aβ comes from the amyloid precursor protein (APP) after cleavage by β and γ secretases. APP is first cleaved by β-secretase, leaving a 99-amino acid stub that is then cleaved by γ secretase, releasing Aβ thereafter. Depending on the cleavage point, either Aβ1-40 or Aβ1-42 is formed. The longer form of Aβ is particularly prone to oligomerize and form fibrils. When there is an overproduction Aβ, especially that of the 42-amino acid form, it causes early-onset Alzheimer's disease.

Aβ is a naturally present in the cerebrospinal fluid of normal subjects. However, when these protein self-assOCIate, they form amyloid plaques that characterize Alzheimer's disease. In these plaques, Aβ are in the form of fibrils ~10 nm in diameter. In vitro, many studies have shown that when Aβ is incubated to aggregate in the presence of neurons, toxicity to the neurons is observed\(^9\)\(^10\). Initially, the characterization of such amyloid assemblies was limited and it was thought that because the fibrils were detectable, it was the mature fibrils that resulted in the observed toxicity. It was later found that there was relatively weak correlation between the extent of dementia in dying Alzheimer's disease patients and the amount of fibrillar amyloid found in their brains.\(^11\) Instead, robust correlations have been observed between the concentrations of soluble Aβ and the severity of memory loss and degree of synaptic loss.\(^12\)\(^13\) Soluble Aβ is defined here as all forms of Aβ in aqueous phase after high speed centrifugation of brain extracts.

Researchers at University of Pittsburgh previously developed a ThT analog called Pittsburgh compound B (PIB) that binds β-sheet rich fibrils.\(^14\) This carbon-11 labelled compound is blood
brain barrier permeable and can be detected by PET imaging. This in vivo imaging provides promise for Alzheimer's disease diagnosis through amyloid monitoring. There is currently no effective treatment that can curb the progression of Alzheimer's disease. Current approved drugs can only ameliorate some of its symptoms. Cummings and coworkers\textsuperscript{15} recently published that the overall success rate of Alzheimer's drugs from 2002 to 2012 is merely 0.4% with one effective drug, memantine, being approved in 2004. This shows that the Alzheimer's disease clinical trials success is highly limited and an urgent need exists to increase the number of drugs entering the pipeline. The Aβ hypothesis states that the increased levels of Aβ aggregation is the main driver of Alzheimer's disease. Because Aβ will not oligomerize at low concentrations, reduction of Aβ levels is particularly interesting. Inhibition of the formation of toxic oligomeric Aβ also seems to be a promising strategy in Alzheimer's therapeutics.

1.3 Parkinson's disease and α-synuclein

Parkinson's disease is named after the English physician who first wrote "An Essay on the Shaking Palsy" published in 1817.\textsuperscript{16} It is a progressive and irreversible neurodegenerative disease that impairs movement. The occurrence of the disease increase significantly with age with 95% of diagnosed cases occurring after age of 40, 0.6% of the population affected between 65-69 years old and 2.6% of those between ages 85-89.\textsuperscript{17} It is the second most common neurodegenerative disease after Alzheimer's disease. Parkinson's disease is marked by resting tremor, bradykinesia and rigidity.

A definitive diagnosis of Parkinson's disease can be confirmed only by post mortem examination of the patient's substantia nigra for the presence of Lewy bodies and loss of neurons. The major protein constituent of Lewy bodies is thought to be α-synuclein (α-S).\textsuperscript{18} After examination by immuno-electron microscopy, α-S is observed to exist as long filaments in these Lewy bodies. It was demonstrated that dopamine worked as the neurotransmitter in neurons of the substantia nigra pars compacta, controlling voluntary movement. Substantia nigra derived its name from neuromelanin, the black pigment byproduct that is produced from the metabolic synthesis of dopamine. As the dopamine-containing neurons die, the loss of these neurotransmitter producing cells enhances the inability to coordinate movement. Simultaneously, the reduction in neuromelanin causes the tissue to be less black.\textsuperscript{19}
In 1997, a single amino acid substitution in the α-S gene in a family with an early-onset autosomal dominant form of Parkinson's disease\textsuperscript{20} led scientists to search for and found α-S in Lewy bodies. This implied that α-S plays a significant role in Parkinson's disease pathogenesis. This hypothesis was further supported when two other disease causing amino acid substitution mutations were discovered in the α-S gene.\textsuperscript{21-22} The exact function of α-S remains unclear but it is thought to belong to the family of natively unfolded proteins and act as chaperones.\textsuperscript{23-24}

Analogous to the case of Alzheimer's disease, α-S is thought to be crucial in the pathogenesis of Parkinson's disease. This is supported by various genetic animal modeling and biochemical data. For example, synthetic α-S fibrils resembles that of fibrils purified from Lewy bodies,\textsuperscript{25-26} α-S oligomers are toxic to dopaminergic SH-SY5Y human neuroblastoma cell,\textsuperscript{27} increase human α-S expression in transgenic flies\textsuperscript{28} and mice\textsuperscript{29} is associated to the formation of lesions similar to those found in Parkinson's disease brain and motor abnormalities, mutant forms of α-S in cells promote cell death, mitochondrial defects and oxidative stress effects whereas mice deficient in α-S are resistant to other mitochondrial toxins, etc. Recent evidence also implies that small aggregates of α-S rather than mature fibrils are responsible for neurotoxicity.\textsuperscript{30-31}

Currently it is challenging to diagnose for Parkinson's disease. Traditional blood, CSF and urine tests attempts are normally futile. CT and MRI scans of the head could not detect abnormalities in idiopathic Parkinson's disease. However, fluorodopa PET could detect the loss of striatal dopamine that is characteristic of Parkinson's disease.\textsuperscript{32} At present, the most advanced imaging biomarker for Parkinson's disease is fluorodopa; The accuracy of clinical diagnosis during life is merely 75\% of the time.\textsuperscript{33} PIB used in Aβ in vivo detection has been shown to work on α-S fibrils \textit{in vitro} but it is not detectable in the Lewy bodies of Parkinson's brains.\textsuperscript{34}

The first treatment for Parkinson's disease resulted from the realization that Parkinson's disease is related to low dopamine production levels. Because synthetic dopamine cannot cross the blood brain barrier, its precursor, L-dopa, is introduced as an effective treatment for Parkinson's patients. L-dopa is administered with carbidopa to prevent metabolism of L-dopa before crossing the blood brain barrier to reach its target tissue, thereafter converting into dopamine by neuronal decarboxylase. Another therapy tackles the high metabolism of dopamine in Parkinson's brains; monoamine oxidase B inhibitors reduce dopamine metabolism. L-dopa currently remains the gold standard for Parkinson's treatment. Because toxicity results from the formation of
amyloidogenic protein oligomers, strategies to prevent the formation of these aggregates are employed in therapies. For example, simvastatin has been reported to reduce $\alpha$-S aggregation, suggesting a possible treatment to reduce the incidence of Parkinson's disease.\textsuperscript{35} The flavonoid compound, baicalein has also been shown to inhibit $\alpha$-S aggregation with the capability to disintegrate existing fibrillar structures.\textsuperscript{36}
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Chapter 2

2  Fundamentals of Electrochemistry

2.1  Introduction

Electrochemistry is the branch of chemistry concerned with the interrelation of electrical and chemical effects, studying chemical changes resulting from the transfer of charges and generation of electrical energy by chemical reactions.\(^1\) Electrochemistry is a branch of chemistry that investigates chemical reactions that occur most commonly at the interface of electrodes and solution in contrast to homogenous bulk solutions.\(^2\) Measured electrical quantities include potential, current, charges and how they are correlated with chemical parameters. These measurements can be applied in various ways by tailoring electrode reactions such that the required reactions are enhanced and unwanted reactions are inhibited by modification of electrode materials or generating new ones. Electrochemistry once understood, could also be used to study complex systems in which many reactions occur consecutively or simultaneously as in bioelectrochemistry. Another major application of electrochemistry is the measurement of electroactive species concentrations by utilizing their unique redox properties.\(^3\) There are also investigations whereby the electrical properties of the systems themselves are of considerable interest, for example, for the electrosynthesis of a new product or in the fabrication of a new energy source.\(^2\) Because of these vast applications, electrochemical probes are receiving growing attention in the field of sensors. This chapter will address some prominent electrodes and electrochemical techniques and some detailed use of them in experiments.

The two major types of electrochemical measurements are potentiometric or potentiostatic, where at least two electrodes and an electrolyte are required to form the electrochemical cell. The electrode surface provides the interface between the ionic conductor and the electronic conductor. In most research efforts, we are interested in investigating the electrode processes at the indicator or working electrode, through potentiostatic or galvanostatic control.\(^4\) In the two-electrode systems, auxiliary electrode had the dual function of passing current and acting as a stable and reproducible potential, independent of the sample composition, with respect to the working electrode. However, such passing current would bring about potential stability issues and species in its vicinity would have their activities altered and resulting in large variance of
In such two-electrode systems, when a large current passes through an electrode, the potential applied can change due to an iR drop, where i is the passing current and R is the resistance of the solution, resulting in inaccurate measurements. In order to solve this major issue, three-electrode systems\textsuperscript{1} were developed and are used widely today for electroanalysis.

In the three-electrode systems, a counter electrode usually made of platinum or graphite is dedicated to pass the current, while a reference electrode is connected with a constant reference potential. The counter electrode takes into account overpotential issues and minimizes noise and overloading events from damaging the working electrode. The counter electrode provides a large electrode surface to balance the one occurring at the surface of the working electrode. To support the current generated at the working electrode, the surface area of the auxiliary electrode must be equal to, or larger than, that of the working electrode. Reference electrodes are able to maintain its stable potential. To prevent contamination of the reference electrode, it is usually insulated from the sample solution via an intermediate salt bridge.\textsuperscript{4} For biological measurements, it is popular to use the reference electrode encased in a tube that has a semi-permeable membrane (Fig. 1).

\textbf{Figure 1.} Components of three-electrode system.

The membrane allows the exchange of potassium and chloride ions. Since chloride can damage the working electrode surface, the reference electrode is usually kept soaked in 3 M KCl solution to protect the membrane. Typically, open circuit potential (OCP) should be between 0.1 V and 0.4 V vs. Ag/AgCl reference electrode. This is the commonly observed potential showing the natural diffusion of analytes to the working electrode surface with respect to the reference electrode. It refers to the difference that exists in electrical potential between two device terminals.
Potentiometry is the field of electrochemistry, where potential is measured under the conditions of little or no current flow. Because of these static conditions, the measured potential can be useful for quantitative purposes, for example, the determination of concentration of target analyte in solution. The potential measured in an electrochemical cell are based on free energy change that occurs, if the chemical reaction were to proceed to equilibrium (no net flow of charges). The first quantitative potentiometric applications appeared soon after the formulation, in 1889, of the Nernst equation relating the potential of the electrochemical cell to the concentration of an electroactive species in the cell. Therefore, it is important to understand the derivation of the Nernst equation.

An electrochemical sensor measures the changes in electrical quantities of potential, charge and current during a reaction and requires the ability to distinguish Faradaic processes from non-Faradaic ones. A Faradaic process is one that operates in agreement with Faraday's first law:

$$nf = \frac{q}{z}$$

**Eqn 1.1**

Where \( n \) is the number of moles of analyte undergoing reaction at the electrode surface, \( n \times F \) (Faraday's constant 96485 C.mol\(^{-1}\)) describes the change in quantity of electrical charge and \( z \) is the number of electrons transferred per ion. Non-Faradaic response describes other current contributions that do not participate in the transfer of electrons across the electrode surface and solution interface boundary. Consider the reaction:

$$aA + bB + ne^- \leftrightarrow cC + dD$$

**Eqn. 1.1**

and defining the reaction quotient, \( Q \), with activity \( a \) as follows:
The change in the thermodynamic potential of Gibb's free energy, $\Delta G$, may equivalently be written as the maximum negative value of electrical work of the reaction:

$$\Delta G = -W$$

where work is the energy required to move a charge, $q$, against an electric field with a potential of $E$:

$$\Delta G = -qE$$

By applying Faraday's first law where $z=1$, where in this case, $E$ is stored electrical potential between two electrodes, the way standard free energy change relates to the standard potential is defined by:

$$\Delta G^0 = -nFE^0$$

where $n$ is the number of exchanged electrons, $F$ is the Faraday’s constant and $E^0$ is the standard potential. By analogy the more general equation,

$$\Delta G = -nFE$$

Expresses the change in free energy for any extent of reaction, that is for any value of the reaction quotient $Q$. Substituting $\Delta G^0$ and $\Delta G$ into the free energy equation where $Q$ is the reaction quotient, $R$ is the ideal gas constant ($8.31451 \text{ J.K}^{-1}.\text{mol}^{-1}$) and $T$ is temperature in K,
\[ \Delta G = \Delta G^\circ + RT \ln Q \]

**Eqn 1.8**

That is, substituting Eqn 1.6 and Eqn 1.7 into Eqn 1.8,

\[ -nFE = -nF E^\circ + RT \ln Q \]

**Eqn 1.9**

Rearranging, we express the equation in terms of electrical potential,

\[ E = E^\circ - \frac{RT}{nF} \ln Q \]

**Eqn 1.10**

where \( E^\circ \) is the standard reduction potential and \( n \) is the number of electrons transferred in the half reaction. Because we know that \( \ln = 2.303 \log_{10} \), standard temperature is 298.15 K, ideal gas constant is 8.314 J.K\(^{-1}\).mol\(^{-1}\) and Faraday’s constant being 96485 C.mol\(^{-1}\), we would get the Nernst equation,

\[ E = E^\circ - \frac{0.05916}{n} \log_{10}(Q) \]

**Eqn 1.11**

The Nernst equation is very important for the fundamental studies of electrochemistry. The equation also describes that 59 mV can be calculated for every factor of 10 change in the reaction quotient \( Q \).
2.2 Cottrell equation

The heterogeneous transfer of an electron across the electrode-solution interface facilitates the formation of either a reduced (R) or oxidized (O) species:

\[
O + ne^- \xrightarrow{k_f/k_b} R
\]

Eqn 1.12

If the reaction is charge-transfer limited (reaction rates determined solely by the rate of electron transfer), the relationship between the sum of cathodic and anodic currents and the electrode potential could be described using the Butler-Volmer equation:

\[
i = nFAD_k_0 \left( C_{ox}e^{-\frac{anF}{RT}(E-E_0)} - C_{red}e^{\frac{(1-\alpha)nF}{RT}(E-E_0)} \right)
\]

Eqn. 1.13

where \( C_{ox} \) and \( C_{red} \) represent the concentration of oxidized and reduced species respectively and \( \alpha \) is the transfer coefficient associated with the symmetry between forward and reverse reactions. 

\( k_0 \) is the heterogeneous rate constant. The use of Butler-Volmer equation is limited to low-current density systems, because, at high overpotential, current would saturate. In high current densities, the current would become limited by the transport of ions to the electrode surface, termed as “concentration polarization”. The limiting current is given by the following equation:

\[
i = i_o \frac{nF}{RT}(E - E_o)
\]

Eqn. 1.14

Conversely, if the rate of reaction is mass-transport limited, meaning it is controlled only by the rate at which reactant diffuses to the surface, it is defined by reversible Nernstian behavior (i.e. in agreement with the Nernst equation) and can be described by the Cottrell equation.
The Cottrell equation describes the change in electric current with respect to time in a controlled potential experiment. For a simple redox event, the current measured is proportional to the rate at which the analyte diffuses to the electrode surface. Such a diffusion-controlled process is described by the Cottrell equation for planar surfaces using Equation 1.15. The equation can also be modified for spherical or cylindrical geometries.

In Equation 1.15, \( i \) represents current while \( n \) refers to number of electrons for the redox process, \( F \) is the Faraday constant, \( A \) is the area of the planar electrode, \( C \) is the initial concentration of the analyte, \( D \) is the diffusion coefficient and \( t \) represents time. The denominator of the equation \( (\pi D_o t)^{1/2} \), is often termed the Cottrell behavior, this decay factor indicates the diffusion-controlled nature of the rate of electrolysis.

From the equation, it is apparent that current is proportional to the number of electrons, area of the electrode, diffusion coefficient and concentration of the analyte, but it is inversely proportional to the diffusion layer thickness. The Cottrell equation can only be applied under some experimental limitations; the major one being its relevance only in non-convective solutions with a planar electrode surface. Furthermore, some of these parameters in the equation related to current signals are hard to control. For example, the surface area can change easily especially with carbon paste electrodes, species can form clusters such that number of electrons transferred per measurement can vary, and concentration of the analyte can also vary based on homogeneity of the solution. Even the diffusion coefficient would be hard to control perfectly with slight changes in temperature. Despite these factors that may contribute to irreproducibility, electrochemistry is still highly valued, because it has fast and cost-effective instrumentation capabilities that can be readily mass-produced and miniaturized.
2.3 Modes of mass transport

Mass transport is the movement of substance from one location in solution to another. Electrochemical processes are made up of two steps: 1) transportation of target analyte to the electrode surface and 2) flowing of electrons across the electrode-solution interface. These two processes occur one after the other and therefore, the slower step is rate limiting. Transport can occur by various pathways:

1) Diffusion- It is important to consider the movement of ions in electrolyte solutions between cathode and anode and some of the general properties of electrolytes. Electrochemistry is a process of diffusion. Molecules and ions travel at different velocities according to their charge and size. Diffusion is a process of such movements due to a concentration gradient from an area of high to low concentrations. It is this transport process that would result in a measurable electrochemical signal. The measured current at any time is proportional to the concentration gradient of the electroactive species according to Fick’s laws.\(^4\)

2) Migration- Whilst diffusion occurs for any species, migration affects only charged species. This is owing to the existence of dipoles and induced dipoles in neutral species, where a small electric field is observed. The charged particles are carried through the solution according to their transference number.

3) Convection- Other than diffusion and migration, convection is a form of transport to the electrode by a gross physical movement. Such fluid flow can occur via stirring or flow of the solution and with the rotation or vibration of the electrodes or even the natural density gradient of the electrolyte. A stirrer can be used to cause convection current to push analytes to the diffusion layer. This is usually used when the analytes are in low concentrations and a low scan rate is required to detect them. A stirrer helps to push the analyte molecules into the diffusion layer, but this might compromise the reproducibility of measurements. This is partly due to the disruption of the double-layer at the electrode surface that would result in currents larger than those predicted by the Cottrell equation.\(^1\) When the electrolyte does not allow stir bars to be introduced, ring disk electrodes could be used. Ring/rotating disk electrodes comprise rings on electrode surface that can rotate in different directions, generating a convection force that brings analyte to its surface for detection.\(^5\)
2.4 Diffusion layer and electrical double layer

Nernst diffusion layer is a predicted double-layer (also called as Helmholtz layer) corresponding to the dotted straight lines of the diagram below (Fig. 2), which shows the concentration profile along the direction perpendicular to an electrode surface. The thickness, d, of this layer is called the effective (or equivalent) thickness of the diffusion layer. Nernst diffusion layer is characterized by the thickness of the layer, which the diffusion layer would have, if the concentration profile were a straight line coinciding with the tangent to the true concentration profile at the interface, and that straight line were extended up to the point where the bulk concentration could be reached\(^6\) (Fig. 2).

Figure 2. Nernst diffusion layer definition by IUPAC.

The diffusion layer is approximately 10 times smaller than hydrodynamic boundary layer, defined by the region, where the fluid velocity increases from zero to the constant bulk concentration value.\(^4\) By increasing the scan rate, the diffusion layer would widen leading to high sensitivity, as analytes can easily diffuse into it, but this would be obtained at the cost of low reproducibility.

In electrochemistry, the electrical double-layer depicts the ionic region formed in the solution in response to the charge on the electrode. It is made up of charged particles or oriented dipoles that
are present at the interface. A negatively charged electrode will therefore attract a layer of positive ions and *vice versa*, since the interface charge must be neutral. Usually solid electrodes prefer to have positive charges while the electrolyte tends to have negative charges. The double-layer can be manipulated by using different buffers. For example, Tris buffer would be positively charged, while phosphate buffer have dominant negatively charged species that can attract ferri-ferro cyanide species. Modifying the surface with DNA can also introduce negative charge to the electrode surface due to the negatively charged phosphate backbone of DNA. In contrast to the conventional positively-charged surface, positively charged species, such as methylene blue which can intercalate into the double helices of DNA molecules, could be easily detected.\(^7,^8\)

### 2.5 Faradaic and non-Faradaic processes

\[
\begin{align*}
O + ne^- & \leftrightarrow R \\
\text{Eqn. 1.16} \\
O + ne^- & \rightarrow R \\
\text{Eqn. 1.17}
\end{align*}
\]

Considering \(O\) as the oxidizing species and \(R\) as the reducing species, reversible reactions described in Equation 1.16 and irreversible reactions described in Equation 1.17 are Faradaic reactions. Faradaic processes comprise reactions, in which charges are transferred across the electrode-solution interface. Since these reactions are governed by Faraday’s law that the amount of reaction caused by the current flow is proportional to the amount of electricity passed, they are termed Faradaic processes.\(^1\)

Under certain conditions, a given electrode-solution interface will show a range of potentials, where no charge-transfer processes occur, because such reactions would be thermodynamically or kinetically unfavorable. These are called as non-Faradaic processes. Although charge does not cross the interface, external currents can flow and may result in background charging of the electrode. Even though Faradaic processes are usually of primary interest in the investigation of an electrode reaction, the effects of the non-Faradaic processes should be taken into account, when interpreting electrochemical data regarding the chemical reactions.
The separation between the peak potentials for a reversible redox couple is given by the following formula:

$$\Delta E_p = E_{p,a} - E_{p,c} = \frac{0.059}{n} \text{ V}$$

Eqn. 1.18

where $E_{p,a}$ and $E_{p,c}$ are anodic and cathodic peak potentials of the corresponding species respectively. For a reversible reaction, according to the Nernst equation, the peak separation theoretically should be 59 mV for a fast one electron process (Fig. 3).

![Cyclic voltammogram](image)

**Figure 3.** A typical cyclic voltammogram of a reversible redox process. (Reproduced with permission from Ref. 4.)

However, in practical experiments, peak separation can sometimes be up to 100 mV, when a one-electron process is investigated. This may be attributed to varying conditions of the electrochemical cell that may alter this potential separation value, for example pH, temperature and ionic strength of the electrolyte.
For irreversible systems, the individual anodic and cathodic peaks are reduced in size and widely separated. For quasi-reversible systems, the current is controlled by both charge transfer and mass transport processes. Overall, the voltammograms of a quasi-reversible system are more drawn out and exhibit a larger separation in peak potentials compared to reversible voltammograms (Fig. 4).

Figure 4. Cyclic voltammograms of A) irreversible and B) quasi-reversible system. (Reproduced with permission from Ref. 4.)

2.6 Working electrodes

The characteristics of voltammetric procedures are strongly affected by the type of working electrode used. The working electrode should ensure high reproducibility and signal-to-noise ratio. Its selection is based on the redox behavior of target analyte and background current over the potential region required for the measurement.

2.6.1 Carbon paste electrode (CPE)

CPE comprises graphite powder mixed with water-miscible organic pasting liquids to generate low cost, easily renewable and modifiable surfaces with low background contributions. Pasting liquids are usually of low volatility and high purity. For example, Nujol and paraffin oil are popular choices.
The increase in proportion of these pasting liquids results in slow electron transfer rates and a decrease in signal-to-noise ratio.\textsuperscript{11} An appropriate composition of graphite to Nujol by mass would be 3:7 or 4:6. A pretreatment step of applying 1.7 V for 1 min to the electrode in phosphate buffer is necessary to smoothen the surface of the electrode. Because the carbon paste is created by physical mixing of the two components, it is convenient to incorporate modifying moieties into the matrix.\textsuperscript{12} Modifiers like enzymes or carbon nanotubes could be simply mixed together with the graphite and binder paste for the desired surface functionalizations. However, a significant disadvantage of CPE is that the organic binder could potentially dissolve in solutions containing an appreciable amount of organic solvent. Also, the manual surface renewal process could not be fully replicated despite limited control over the geometric area of the exposed electrode. Thus, this could result in reproducibility problems. Data within ±10\% error is acceptable for CPEs as working electrodes. A highly reproducible carbon paste-based electrode would be the screen-printed carbon electrodes that would produce data within ±5\% error.

2.6.2 Glassy carbon electrode (GCE)

The most widely used carbon based electrode is probably GCE because of its wide potential window and excellent electrical properties. The manufacturing of GCE requires the heating of phenol/formaldehyde polymers between 1000\degree C to 3000\degree C under high pressure, also known as carbonization.\textsuperscript{1} Unlike CPE, GCE is usually acquired commercially because of its tedious fabrication process. Because of its high density and small pore size, no impregnating procedure is required. Surface pretreatment is usually employed to create active and reproducible surfaces and to enhance their analytical performance.\textsuperscript{13} Such pretreatment requires the polishing of the electrode surface to a shiny mirror-like finish using successively smaller alumina powder. The surface should then be rinsed with deionized water before use. In practice, the alumina slurry is made on a rough pad by mixing water and the alumina powder. Smaller alumina powder size is used for detailed cleaning while bigger size is for more general qualitative experiments. It is important to use the alumina powder with care, as it may cause lung-related health problems, if inhaled.\textsuperscript{14} Electrochemical pretreatment of GCEs would be performed using cyclic voltammetry from 0.5 V to 1.0 V for 5 to 10 min in phosphate buffer at pH 7. This pretreatment process is useful to turn ketones, aldehydes, and hydroxyls on the surface into carboxyl groups, making the surface available for further modifications. The pretreatment step also oxidizes non-specifically adsorbed biomolecules and desorbs them from the surface. In general, the improved electron
transfer has been attributed to an increase in the density of surface oxygen groups, removal of surface contaminants and exposure of fresh carbon surfaces.

2.6.3 Pencil graphite electrode (PGE)

PGEs offer many attractive features; they are cost-effective and readily available. They also have a large electroactive surface area-to-volume ration. They are therefore suitable to be used to detect low concentrations of analytes.\(^{15}\) This is particularly significant when only small amounts of analytes are available. Furthermore, disposable PGEs significantly reduce total analysis time by bypassing the cleaning and pretreatment processes, which are essential when using GCEs. The major drawback of using PGE is its fragility. Carbon nanowires and carbon fiber electrodes work on similar principles, while the latter could be used for neurological measurements.

2.6.4 Gold electrode (AuE)

Various noble metals are used to construct metal working electrodes, such as platinum, gold and silver. They are commonly used as redox (‘inert’) electrodes to study electron transfer kinetics and mechanism, and to determine thermodynamic parameters. However, it should be remembered that at certain values of applied potentials, bonds could be formed between the metal and oxygen or hydrogen in aqueous solutions. Platinum also exhibits catalytic properties. The advantage of gold electrodes (AuEs) is their high conductivity resulting in low background noise. Because thiol groups have a strong affinity for gold surfaces, AuEs are widely used as substrates for self-assembled organosulfur monolayers or for stripping measurements in trace metals analyses. Due to the formation of surface oxide or adsorbed hydrogen layers, high background currents may be observed if cleaning-reactivation were not performed prior to experiments. AuEs are more inert than platinum electrodes, and are, thus, less prone to the formation of stable oxide films or surface contamination.\(^4\) In practice, to clean AuEs, cyclic voltammetry scans (Section 2.7.2) are performed in 0.1 M sulfuric acid from -0.3 V to 1.5 V for 100 to 200 scans at 100 mV/s vs. Ag/AgCl reference electrode. Sonication could enhance the cleaning process, but should not be performed for long durations. Pulsing the electrode in deionized water for 10 s would usually be sufficient.
2.6.5 Dropping mercury electrode (DME)

Professor Jaroslav Heyrovsky was awarded with the Nobel Prize “for his discovery and development of polarographic methods of analysis” in 1959.\textsuperscript{16} Polarography utilizes a dropping mercury electrode (DME) because of its easily renewable surface and wide cathodic potential range. As the name implies, it is a technique whereby the potential on the mercury drop could be switched easily. However, an extremely positive potential can result in the formation of mercury(I) oxide that is irreversible and could destroy the electrode. A DME is constructed by connecting a reservoir of high purity mercury through a tube of plastic to a fine capillary. Mercury drops are formed at the bottom of the capillary and fall due to gravity, as they reach a certain size. This is followed by a new drop coming from the Hg reservoir as illustrated in Fig. 5.

![Diagram of DME setup](image.png)

**Figure 5.** A typical DME set up in an electrochemical cell.

DME allows the continuous measurement of kinetic experiments. A modification of the DME results in a constant surface. This is called the hanging mercury drop electrode (HMDE) and is used often in anodic stripping analysis.\textsuperscript{17}

A big disadvantage of DME is that the surface area of a drop of mercury is never constant. This might be due to the changes in mercury surface tension caused by the applied voltage. Another
important drawback of DME is its ease of oxidation. It undergoes anodic dissolution at +0.25 V vs. a saturated calomel electrode (SCE) as the reference electrode and at 0 V vs. SCE in the presence of chloride ions. The biggest advantage of HMDE is its renewable characteristics, whereby one can simply release the used mercury drop and grow a new drop between each experiment, evading polishing and pretreatment steps in most solid electrodes. Also, the renewable characteristics greatly reduce contamination issues that would result in decrease in current response due to the decrease in electroactive surface area of the mercury drop caused by surface contamination. The radius of the droplet can easily be controlled by the changing the capillary size or instrument settings. For mercury electrodes, it is common to use them at potentials more negative than 0.2 V vs. Ag/AgCl by having a nitrogen gas purging the electrochemical cell. While carbon electrodes are highly suitable for positive potential measurements, mercury electrodes can be used to perform measurements in the potential regions as low as -2.5 V vs. Ag/AgCl.

2.6.6 Boron-doped diamond electrode

Diamond is normally an electrical insulator and cannot be used as an electrode material because of its large band gap of more than 5 eV. However, diamond can be made conducting by doping it with certain elements like boron, nitrogen or phosphorus. Boron-doped diamond can be used as a p-type semiconductor while phosphorus and nitrogen results in an n-type semiconductor. Diamond particle electrodes are produced by immobilizing conducting diamond particles into a substrate. For example, boron-doped high pressure-high temperature diamond particles are used to produce boron-doped diamond electrodes. Nowadays, low pressure chemical vapor deposition (CVD) diamond growth can be realized under appropriate conditions. The grain size, surface morphology and roughness of deposited diamond particles depend highly on film thickness, where generally, a thicker film possesses large grain sizes with high surface roughness.\(^\text{18}\)

Diamond is well known for its unique material properties, such as its hardness, thermal conductivity, and charge carrier mobilities. Doped diamond would display a very high overpotential for both oxygen and hydrogen evolution. This would lead to a very wide working potential window of about 3.5 V for electrochemical reactions in aqueous electrolytes.\(^\text{19}\) Doped diamond electrodes work exceptionally well for potentials larger than 2.0 V vs. SCE.
2.7 Voltammetric Techniques

2.7.1 Normal pulse voltammetry

Normal pulse voltammetry applies a series of square pulses of increasing amplitude to the working electrode over time at preset time-points (Fig 6). Between pulses, the electrode is kept at base potential, where no reaction of the analyte occurs. The pulse lasts for about 40 ms each. Because of the short pulse durations, the diffusion layer is thin, resulting in large Faradaic currents. However for this technique, it does not allow the analyte of interest to progress through the reaction as the potential applied repeatedly bring the sample back to its original potential state within short durations.

![Potential versus time graph of normal pulse voltammetry](image)

**Figure 6.** Potential versus time graph of normal pulse voltammetry, showing the applied potential to working electrode over time. (Reproduced with permission from Ref. 4.)

2.7.2 Cyclic voltammetry

Cyclic voltammetry is a widely used technique to obtain qualitative data from electrochemical reactions. Because of its ability to provide rapid qualitative information on the redox process, it is usually the first experiment performed in an electroanalytical study.
CV is characterized by the ramping of the working electrode potential linearly versus time (Fig. 7). This is different from NPV, as this ramping is much slower than the pulses applied in NPV. Depending on the potential sweep, the potentiostat measures the current resulting from the applied potential. Assuming a reduced species in solution, the linear sweep voltammetry would involve the potential scan starting from a potential, where no oxidation occurs towards a positive direction. An anodic current would start to increase until a peak is reached where maximum amount of species are in the diffusion layer. Then, the measured current drops as the applied potential moves away from the oxidizing potential of the analyte species. The rest of the current signals would comprise non-Faradaic response. After that, the potential sweep is reversed and the oxidized species would be reduced back to their original state, and a cathodic peak would be observed, assuming the electron transfer is electrochemically reversible.

**Figure 7** a) Cyclic potential sweep. b) Resulting cyclic voltammogram. (Reproduced with permission from Ref. 1.)

Repeating the CV scans may result in progressively smaller current signals. This is because once the species gets oxidized, its oxidized complex may be adsorbed to the surface of the working electrode, causing a decrease in its electroactive area, which would lead to a significant decrease in the measured Faradaic current. The Randles-Sevcik equation describes the relationship between Faradaic current and scan rate ($v$) and also the surface area ($A$):

$$i_p = (269,000)n^{3/2}AD^{1/2}Cv^{1/2}$$

Eqn. 1.19
where \( i_p \) is the current maximum in amps, \( n \) is the number of electrons transferred in the redox event, \( A \) is the electrode area in \( \text{cm}^2 \), \( F \) is the Faraday constant in \( \text{C.mol}^{-1} \), \( D \) is the diffusion coefficient in \( \text{cm}^2.	ext{s}^{-1} \), \( C \) is the concentration in \( \text{mol.cm}^{-2} \) and \( \nu \) is the scan rate in \( \text{V.s}^{-1} \). From the equation, the peak current which comprises the Faradaic current, would be proportional to the electrode surface area and square root of scan rate. The scan rate dependence test is commonly performed by plotting the \( i - \nu^{1/2} \) graph for analyte of interest. If the graph is linear, it implies that the electrode process was diffusion controlled. Increasing the scan rate can not only substantially increase the Faradaic peak, but also the non-Faradaic region. This would increase the sensitivity of the electrochemical system, but reproducibility could be compromised.

2.7.3 Square wave voltammetry

![Square wave voltammetry diagram](image)

**Figure 8** Square-wave waveform showing the amplitude \( E_{sw} \), step height \( \Delta E \), square-wave period \( T \), delay time \( T_d \), and current measurement times 1 and 2. (Reproduced with permission from Ref. 22.). b) The net current would be calculated as \( I(1) - I(2) \) from two points of measurement for the anodic response \( I(1) \) and cathodic response \( I(2) \).

SWV was invented in 1952 by Barker,\(^{21}\) but was uncommonly used then due to difficulties controlling the electronics. In SWV, a waveform composed of symmetrical square-wave is superimposed on a base staircase potential, which is applied to the working electrode (Fig 8).\(^{22}\) The current is measured twice for every square wave cycle, at the ends of the forward and reverse pulse. The difference between the two samples is plotted against the base staircase potential. The peak current is proportional to the concentration of analytes. SWV provides
excellent sensitivity, because the pulses are so fast, the oxidation and reduction are measured in consecutive waves, and thus, the net current is larger than either the forward or reverse components. SWV are about four times more sensitive than DPV (refer to 2.7.4) when compared. However, because of its high frequency that can go up to 200 Hz, reproducibility becomes an issue. Therefore, for reproducible results, lower frequency up to 20 Hz should be used. Because of the fast scan rates, kinetic studies can also benefit from the rapid scanning capability and the reversal nature of SWV.

2.7.4 Differential pulse voltammetry

DPV is similar to NPV, but the base potential is incremented between pulses and the increments are equal. The pulse amplitudes are also kept constant. The current measured in DPV is measured immediately before the application of the pulse and at the end of the pulse, so that the difference between these two currents is recorded (Fig 9.).

**Figure 9.** Waveform of DPV is similar to SWV, but the time interval between each pulse is significantly longer in the former. a) Application of potential waves vs. time for DPV with current measurement times at 1 and 2 (Reproduced with permission from Ref. 4.) b) I-E profile of a typical DPV measurement.

The DPV peak (Fig 10.) has a much higher resolution than that of NPV because the same potential is revisited 3 to 4 times at a slow scan rate (this ensures slowly diffusing analytes to be detected). Sensitivity in DPV is also markedly improved as compared to NPV or DC polarography.
Figure 10. Polarograms of a) NPV and b) DPV showing superior peak separation in DPV. (Reproduced with permission from Ref. 4.)

In many cases, peaks separated by 50 mV could be measured using DPV. Unlike CV, the area under DPV is not related to charge measurements. Instead, Faradaic response depends both on the height of corresponding peak potentials and the widths of the peak. The width of the peak at half height is related to electron stoichiometry:

\[ W_{1/2} = \frac{3.52 RT}{nF} \]

**Eqn. 1.20**

Ideally, the peak width at half height is 30.1 mV for n=1 (at 25°C) but in practice, any width of peak between 20 mV to 50 mV can be observed for a one-electron process. The non-Faradaic response observed at the region under the baseline of DPV i-E graphs is caused by charging of the electrical double layer.
2.7.5 Anodic stripping voltammetry

![Diagram of anodic stripping voltammetry]

**Figure 11.** a) Potential-time waveform of anodic stripping voltammetry, b) resulting voltammogram corresponding to the potential waveform. (Reproduced with permission from Ref. 4.)

ASV is commonly used for stripping analyses. In the case of metal detection, metal ions are cathodically deposited at a controlled potential about 0.3-0.5 V more negative than the standard reduction potential of the least easily reduced metal ion to be determined. The metals then reach the surface of mercury electrode by diffusion and convection, where they get reduced to their solid state. After the adsorption of the metal ions, the stripping step is performed by scanning the potential anodically either linearly or in more complex pulses (square-wave or differential pulse ramps). During this anodic scan, amalgamated metals are reoxidized, stripped off the electrode.
surface and a stripping current can be measured. This current is plotted on an I-E graph to show the peak potentials of different metals (Fig. 11).

2.7.6 Alternating current voltammetry

![Potential-time waveform of ACV](image)

**Figure 12.** Potential-time waveform of ACV. (Reproduced with permission from Ref. 4.)

ACV comprises the superimposition of small amplitude AC voltage on a linear ramp. (Fig. 12) Usually the frequency of the AC potential is 50-100 Hz at an amplitude of 10-20 mV. The height of the AC voltammetric peak is proportional to the concentration of the analyte. If the reaction is reversible, the height of the ACV peak is also proportional to the square root of frequency ($\omega$):

$$i_p = \frac{n^2 F^2 A \omega^{1/2} D^{1/2} C \Delta E}{4RT}$$

**Eqn. 1.21**

The peak width is independent of the AC frequency and is 90.4 mV per electron exchange.

2.8 Electrochemical impedance spectroscopy

Impedance describes the ability of a circuit to resist or impede charge without being confined to the descriptive limitations of an ideal resistor. EIS is a useful analytical tool for material science research, because it offers information about the dielectric properties, mass transport rates, rates of chemical reaction, surface composition, and steric at a liquid electrolyte and solid electrode interface, which would reveal information about defects or surface roughness. EIS is
particularly useful for probing the complex physiochemical behavior of materials and systems by directly relating output impedance data to an idealized, equivalent model circuit. This equivalent model circuit can be isolated into its individual circuit components and provide corresponding values that can be simulated and fitted from the impedance data. In turn, this helps characterize electrochemical processes and properties that occur at interfacial surfaces. It is important to note that EIS is a non-destructive technique, and thus, the given ‘spectroscopy’ name.

In order to investigate the electrochemical properties of an interfacial surface, an alternating potential is applied to an electrode resulting in an alternating current and the electrode becomes polarized. Upon electrode polarization, a potential difference exists between the surrounding electrolyte solution and the electrode, allowing for current to flow. Electrons supplied by the electrode can flow through electrochemical reactions that occur at the electrode surface. In turn, the flow of electrons is controlled by the diffusion of reactants and products towards and away from the electrode and the kinetics of the electrochemical reduction-oxidation reaction. For example, equation 1.22 shows the Faradaic process of the negative charge reduction-oxidation indicator, Fe$^{3+}$ that occurs at the electrode surface [b]:

$$\text{Fe}^{3+} + e^- \rightarrow \text{Fe}^{2+}$$

**Eqn. 1.22**

For this reduction reaction to occur, the ferric ion must diffuse to the surface and an electron charge must be transferred, and the ferrous ion formed must diffuse away from the electrode. The electron in this redox reaction is supplied by the electrode, which is part of an electrical circuit whose properties can be monitored. Figure 13 shows the Randles equivalent model circuit used to fit and describe the impedance data that is then presented in a Nyquist plot (Fig 14) with imaginary impedance against real impedance.
This model includes the double-layer capacitance $C_{dl}$, the ohmic resistance of the electrolyte solution $R_s$, the electron transfer resistance $R_{ct}$, and the Warburg impedance $W$ resulting from the diffusion of ions from the bulk solution to the electrode surface. Thus, for kinetically favored reactions, charge transfer resistance tends to be 0 Ohm, while Warburg impedance predominates, showing a linear Nyquist plot (Fig. 14). More components could be added to the circuit to fit the impedance data towards a lower error. This modification thus results in variance in data interpretation at the discretion of the researcher. Usually, the circuit is kept as simple as possible as long as the error of results falls within ±5%.
There is also an impedance measurement for solid state boundaries. By applying the alternating potentials to the solid boundaries, oxidation and reduction of internal elements within the metal would be activated. EIS has been widely applied to a variety of electrochemical systems, including those involved in biosensors, corrosion, electrodeposition, and polymer films.\textsuperscript{29,30}

2.9 \textbf{Chronoamperometry and Chronocoulometry}

Chronoamperometry is the study of the current response with time under potentiostatic control and chronopotentiometry is the study of potential response with time under galvanostatic control.\textsuperscript{31} Both processes involve the stepping of potential or current from a value where no Faradaic reaction occurs to one that the electroactive species is effectively zero.\textsuperscript{4} A stationary working electrode and an unstirred solution are used and the resulting current-time dependence is monitored. Faradaic current decreases as oxidizing species get depleted at the electrode surface obeying Cottrell equation (equation 7), where the Faradaic current is inversely proportional to the square root of time. The oxidizing species is only replenished by diffusion. The capacitive current (non-Faradaic) resulting from the charging of the electrode capacitive layer also decays for a constant applied potential. Because the decay for non-Faradaic current is more exponential than the Faradaic current, it decays faster than the Faradaic current. This is then shown on
current versus time graph of the measurement. Chronoamperometry is commonly used for measuring the diffusion coefficient of electroactive species or the surface area of working electrodes. Chronoamperometry has been widely applied in commercial test kits for blood glucose detection. This could be achieved by designing a calibrated electrochemical cell on a screen-printed electrode with glucose oxidase-modified working electrode that would measure the oxidation of glucose by the enzyme resulting in a measurable change in current, when blood is sampled. The electrochemical signal is shown as a quantitative read-out by interpolating the measured current from a calibration curve for that sensor.

The potential step experiment can also be used to record electrical charges versus time dependence. This could be accomplished by integrating the current signal since current is the rate of flux of charges. This measurement procedure is known as chronocoulometry. It is particularly useful for quantitative measurements of adsorbed analytes. The advantages are that the signal increases with time, which facilitates measurements towards the end of the transient, when the current is almost zero; a better signal-to-noise ratio can be achieved through integration and it is easy to separate capacitive charge from diffusing electro-reactants.
2.10 References


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Chapter 3

3 Label-free methods for probing the interaction of Clioquinol with Amyloid-β

3.1 Preface to Chapter 3

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http://pubs.rsc.org/en/content/articlepdf/2012/ay/c2ay25123j

Author Contributions

XRC, MMinnuni and KK designed the experiments, performed the experiments and wrote the manuscript. VWSH and SS assisted in performing the experiments. MMascini revised the manuscript.
3.2 Abstract

The presence of amyloid-β (Aβ) fibrils is characteristic of Alzheimer’s disease (AD) patients and aggregation of these amyloidogenic proteins is a nucleation process. Surface Plasmon Resonance (SPR) and Acoustic Wave Sensor (AWS) were used as major transduction principles for the monitoring of monomer elongation. Subsequent modulation by flavonoid clioquinol (CQ) was tested to observe inhibitory effects. Additional techniques like fluorescence and electrochemistry were also employed to support the inhibitory effect of CQ on β-amyloid aggregation.

3.3 Introduction

Alzheimer’s disease (AD) is a neurodegenerative disease marked by clinical symptoms such as a decline in cognitive ability, alterations in behaviour, irreversible memory loss, and language impairment. The onset of the disease is not considered a natural pathway of aging and is the most prevalent cause of dementia with about 18 to 30 million patients worldwide. AD is one of the most common neurodegenerative diseases including Parkinson’s, Huntington’s, and Creutzfeld Jacob disease, in which symptom progression is correlated to the development of neural fibrillary tangles and neuritic plaques deposited within the limbic and association cortices of the brain. One of the major neuropathological hallmarks of AD is the deposition of β-amyloids on the walls of cerebral and meningeal blood vessels as senile plaques. Amyloid is the generic term describing abnormally fibrillated proteins possessing a β-pleated sheet conformation. In AD, the central core of SPs comprise insoluble amass of fibrillated aggregates of amyloid beta (Aβ) peptides. The cytotoxic effect of these conformers had been correlated to the onset and progression of permanent cognitive impairments to memory, learning, and language. Aβ 1-42 in AD patients are formed when amyloid precursor protein gets cleaved by β-secretase to produce Aβ 1-40 or Aβ 1-42. Aβ 1-42 is known to have a higher propensity to form fibril-like structures and also possesses more toxic properties. It is being increasingly accepted that oligomeric Aβ 1-42 is neurotoxic and that this neurotoxicity leads to the neurodegenerative changes in AD patients. However, this hypothesis is still a controversial issue. There is accumulating evidence that monomeric form of Aβ seems to mediate the growth of AD amyloid plaques in human brain rather than mature fibrils. Therefore, the study of monomer elongation might provide clues to therapeutic interventions.
Aggregation of Aβ is a nucleation dependent process, which means that aggregation formation is dependent on monomer concentration. This dependence is nonlinear and is independent of the addition of preformed aggregates. Nucleation is followed by accelerated aggregate growth by incorporation of soluble aggregates (intermediate species) before forming mature fibrillar networks. Several advanced analytical techniques such as circular dichroism spectroscopy, high resolution atomic force microscopy and nuclear magnetic resonance have been applied to study the mechanisms of amyloid formation. However, most of these techniques require complex instrumentation and tedious effort. As such, more robust and simple techniques are greatly desired to develop accurate models for amyloid nowadays considering recent aging population crisis in developed countries.

Recently, reports have been published about the applications of thickness shear mode acoustic wave sensors (TSM-AWS) and surface plasmon resonance (SPR) sensors to monitor Aβ aggregation. Hasegawa and coworkers used SPR to characterize the effect of monomeric and fibrillar Aβ concentrations on formation and extension of Aβ fibril and calculated that the critical monomer concentration to be 20 nM and equilibrium binding constant of monomers to fibril of 5 x 10^7 M⁻¹. A range of plasma proteins was also studied to explore the mechanism by which these proteins may control Aβ polymerization, and it was found that albumin accounted for 60% of the inhibitory activity found.

Alkaloids have also been tested on SPR for their effects on Aβ1-40 aggregation inhibition. Some TSM-AWS studies have proposed a “dock and lock” mechanism to describe rapid binding of monomers to fibril aggregates occurring rapidly in a reversible fashion, which was then followed by a conformational change in structure that stabilizes the aggregates. The stabilization process generates new binding sites to allow further docking process of new monomers to already formed aggregates. Buell and coworkers studied the interactions between amyloidophilic dyes and Aβ on a TSM-AWS sensor, and observed the binding of these dyes to the fibrils. They proposed that TSM-AWS can elucidate the mode of action of potential inhibitors of Aβ. It is important to study these soluble aggregation intermediates as recent revisions to the amyloid cascade implicate their capability of inducing synaptic loss and impairing hippocampal long term potentiation.
Clioquinol (CQ, 5-chloro-7-iodo-8-hydroxyquinoline) is known to significantly reduce Aβ plaque deposits, and improve cognitive behaviour in humans.\textsuperscript{20} CQ, like other flavonoids, has metal chelation properties, and this is important as metals such as zinc and copper are known to accelerate AD progression. This hydrophobic molecule has the ability to cross the blood brain barrier, and is hypothesized to have the ability to solubilize Zn/Cu-induced amyloid aggregates and inhibit Aβ from participating in redox reaction.\textsuperscript{21} It had been shown that oral administration of CQ significantly hinders Aβ accumulation in the brain of Tg2576 mice, and in clinical studies, patients also exhibited slower cognitive decline after CQ treatment.\textsuperscript{21, 22} Therefore, it is a model drug to be tested.

In this study, novel biosensor designs were developed to evaluate the Aβ1-42 monomer aggregation in real time, starting from fibrils immobilized on the sensing surface to act as seeds for nucleation. This system was then later on used to study the inhibitory activity of CQ on Aβ 1-42 aggregation. The real-time data were supported by electrochemical and fluorescence data.

### 3.4 Experimental Methods

#### 3.4.1 Chemical reagents

Amyloid-β (Aβ) peptide Aβ1-42 trifluoroacetic salt was purchased from EMD Chemicals Inc. (Gibbstown, NJ) (Peptide sequence: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIG LMVGGVVIA). Thioflavin T (ThT, 4-(3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl)-N,N-dimethylaniline chloride), multiwall carbon nanotubes (MWCNT, diam.= 110–170 nm, length= 5-9 μm, 90+% purity), graphite powder (<20 μm, synthetic), mineral oil, ammonium iron (II) sulfate hexahydrate and copper (II) chloride dihydrate were purchased from Sigma Aldrich (Oakville, ON). Zinc chloride was purchased from Fluka Analytical Sigma-Aldrich (St.Louis, MD). 11-Mercapto-1-undecanoic acid (MUA) was purchased from Sigma (Milan, Italy), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) from Calbiochem (Slovakia) and N-hydroxysuccinimide (NHS) from Fluka (Milan, Italy). The buffer used was 50 mM PBS, 100 mM NaCl pH 7.4. All solutions were made from double distilled MilliQ water, unless otherwise stated. Ammonia (28%) was purchased from VWR. Hydrogen peroxide (35%) was obtained from Merck (Germany). Ethanol and all the reagents for the buffers were purchased from Merck (Italy). Ethanolamine hydrochloride (EA) from Sigma (Milan, Italy) was prepared to a 1 M
solution at pH 8.6 in ultrapure water. Clioquinol (CQ) was purchased from Sigma-Aldrich (St. Louis, MO).

For SPR, HBS-EP was used as the running buffer that contained 5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 5 mM HEPES-Na, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% Surfactant P20. The buffer was filtered and degassed before use. EDTA was purchased from Merck (Milan, Italy); all the other reagents for the buffers were obtained from Sigma (Milan, Italy). Immobilization buffer contained 10 mM acetic acid and 10 mM sodium acetate (trihydrate) before adjusting pH using NaOH to pH 5. PBS buffer with 100 mM NaCl was also used. All chemicals were of analytical grade and used without further purification.

2.4.2 Apparatus

Quartz crystals (9.5 MHz AT-Cut, 14 mm) with gold evaporated (42.6 mm² area) onto both sides were purchased by International Crystal Manufacturing (Oklahoma, USA). The measurements were conducted in methacrylate cells where only one side of the crystal was in contact with the solution. The quartz crystal analyzer used for the measurements was the Model QCA922 (Seiko EG&G, Chiba, Japan) for piezoelectrical studies. The frequency shifts reported here are the differences between two stable frequency values (±1 Hz). TSM-AWS measurements were performed in batch mode where injection of sample and buffer were in static conditions.

For SPR experiments the Biacore X™ (Biacore AB Uppsala, Sweden) equipped with research grade Au chip (SIA kit Au) was used to monitor the fibrillation process. Thermomixer Comfort (Eppendorf, Hamburg, Germany) was used for the incubation of fibrils. The change in Resonance Units (RU) was recorded in real time on a sensorgram where 1 RU is equivalent to one picogram per square millimeter on the sensor surface. All these experiments were conducted at room temperature 25°C±2°C.

3.4.3 Sample preparation for acoustic wave sensor

Aβ1-42 peptides were dissolved in DMSO to create a stock solution of 553.7 μM and stored at -20°C. The peptide was diluted to 40 μM using PBS and then subjected to 24 h incubation at 37°C with shaking at 800 rpm in order to produce fibril seeds. A concentration of 40 μM was
chosen because it is well above the critical concentration for fibril formation determined by Hasegawa and coworkers\textsuperscript{15} and it was also shown by Ryu and coworkers\textsuperscript{26} that 30 \( \mu \text{M} \) was sufficient for A\( \beta \)1-42 fibril formation in a cuvette based SPR system. The monomer was obtained from the stock solution by diluting the peptides to 15 \( \mu \text{M} \) with PBS and then sonicated for 5 min before storing at -20\( ^\circ \text{C} \) until use.

All fibril seeds and monomers were sonicated for at least 5 min before use in attempts to obtain a homogeneous seed solution. It had also been shown that sonicated fibrils supported elongation better.\textsuperscript{23, 24}

Cleaning of the quartz crystal was done by boiling it in a 5:1:1 by volume of water: ammonia: hydrogen peroxide solution respectively for 10 minutes. The crystal was then incubated in 95\% ethanol for 1 h before immersing in a 1 mM ethanol solution of MUA overnight to allow self-assembled monolayer (SAM) formation. The immobilization chemistry was the classical NHS/EDC covalent peptide bond forming process. Activation of the carboxylic groups of MUA was done by adding on the crystal an aqueous solution containing 5.8 mg/mL NHS and 38.3 mg/mL EDAC mixture and allowed to react for 15 min. After that, the fibril seeds were further diluted in PBS to 15 \( \mu \text{M} \) and then sonicated before 100 \( \mu \text{L} \) of this solution was introduced to the cell for 30 min. The gold surface was then rinsed with PBS to remove non-specific binding before adding 1 M EA to block any active sites available for primary amine binding for 15 min. 100 \( \mu \text{L} \) of 2 \( \mu \text{M} \) of A\( \beta \)1-42 peptide monomers with or without 10 \( \mu \text{M} \) of CQ was then introduced onto the seeded surface for 50 min before a subsequent PBS wash. The change in frequency before and after monomer incubation was recorded.

### 3.4.4 Sample preparation for surface plasmon resonance (SPR)

For the SPR measurements, the cleansing procedure and self-assembly of MUA for the gold chip is identical to that of TSM-AWS. For the EDC/NHS activation of the SAM surface, the mixture was added at 5 \( \mu \text{L/min} \) for 7 min followed by 15 \( \mu \text{M} \) or 30 \( \mu \text{M} \) solution of fibril seeds (dissolved in 1:1 v/v of 50 mM PBS: 10 mM Acetate buffer injection) for 30 min at 3 \( \mu \text{L/min} \). Eventually, EA was added for 10 min at 5 \( \mu \text{L/min} \). The monomer and inhibitors were injected at 2 \( \mu \text{L/min} \) for 50 min. The difference in RU before injection and after injection was recorded. Then, CQ was introduced with the monomer to observe inhibitory effects on elongation by the compound.
3.4.5 Sample preparation for electrochemical analysis

Solutions were prepared in 50 mM Phosphate Buffer Saline (PBS) with 100 mM NaCl at pH 7.4 with ultrapure water using a Cascada LS (Pall Co. NY) water purification system at 18.2 MΩ and stored at 4°C. ThT stock solutions (10 mM) were prepared daily with ultrapure water and stored at room temperature while shielded from light to prevent photodegradation. Aβ1-42 stock solutions (553.7 µM) was prepared in DMSO and stored at -20°C to prevent aggregation. A working solution of 250 µL was prepared containing 50 µM of Aβ1-42 alone and with 50 µM CQ incubated at 37°C with shaking to induce spontaneous aggregation of Aβ peptides.

3.4.5.1 Preparation of MWCNT-modified carbon paste electrode

Composition of the CNT-CPE consisted of 10% MWCNT, 60% graphite powder and 30% mineral oil by weight. Constituents were mixed thoroughly until a thick paste was created. The paste was then forced into a carbon paste working electrode body with 3.0 mm internal diameter. Surfaces of the paste electrodes were smoothed between experiments on weighing paper (VWR Scientific Products, West Chester, PA).

3.4.5.2 Square wave voltammetry (SWV)

The use of SWV has several advantages including faster analysis time, less amount of electroactive species required and lowered possibility of poisoning the electrode surface. Pretreatment was done each time before a measurement for 60 s at 1.7 V then rinsed with ultrapure water. A 15 µL droplet of sample was placed on an inverted CNT-CPE and allowed to adsorb for a duration of 15 min at 25°C. After, a final rinsing step was done for 5 s using 10 mL of PBS. Measurements were done in 10 mL of solution of PBS using SWV with a potential range from 0 to 1.3 V at a frequency of 230 Hz.

3.4.6 Sample preparation for fluorescence analysis

Dilutions of the samples were made in 50 mM PBS with 100 mM NaCl at pH 7.4. Using a black 96-well plate from BD Biosciences (Franklin Lakes, NJ), 10 µM of ThT, 10 µM of Aβ1-42 10 µM of CQ in a total volume of 180 µL was added to each well. ThT fluorescence was read at 440 nm (30 nm bandwidth) excitation and 485 nm (20 nm bandwidth) emission. All samples
were incubated at 37° C with shaking. Data collected was analyzed with the associated Gen5 Microplate Data Collection and Analysis Software.

3.5 Results and Discussion

3.5.1 Thickness shear mode acoustic wave sensor

For an effective quantitative analysis of Aβ 1-42 elongation, it was crucial to avoid nonspecific binding of Aβ 1-42 monomers to the biochip. With this objective in mind, a self-assembled monolayer surface of MUA was first formed on the quartz crystal surface and then activated with EDC/NHS chemistry. The surface was directly saturated with EA, by-passing the immobilization of seeds, to obtain a control surface. In this way unreactive ethyl hydroxyl groups would be bound to active carboxylic ends of MUA. On this surface, a 2 μM monomer solution of Aβ1-42 and subsequently, a 10 μM CQ solution were tested, both showing negligible signal. (Fig. S1A and B).

This demonstrates that the passivated sites were resistant to non-specific adsorption, indicating that any significant change in signal from their incubation after peptide seed immobilization was attributed to the interaction of these peptides with the sensor surface.

After confirming that the monomers and CQ do not cause non-specific adsorption, the immobilization of peptide fibril seeds was initiated. After completion of 24 h incubation at 37°C, mature fibrils formed in the 40 μM Aβ1-42 monomer solution, and were observed as white deposits present at the bottom of the microcentrifuge tube (Fig. S2A). These formed fibrils were sonicated to form fibril fragments (also coined here as ‘seeds’), and the solution became clear to the eye (Fig S2B). 15 μM of the seeds were then added to the covalently activated surface so the amino terminals to bind the seeds to the active carboxyl groups of the MUA. The surface was then blocked with EA, so that the unreacted carboxyl groups would not create further binding sites during the addition of Aβ1-42 monomers (Scheme 1).
Scheme 1. Schematic of simple fibril seed immobilization chemistry and elongation process.

Measuring cycle of peptide-based piezoelectric biosensor (frequency (Hz) vs time). Step a, baseline signal in buffer, before Aβ1-42 monomer addition (only immobilized fibril fragments on surface); step b, deposition and elongation of Aβ1-42 monomers shown as binding curve; step c, washing the surface to remove the unbound Aβ 1-42 monomers; The analytical net frequency decrease was taken as the difference (c-a) values in Hz.

The serial introduction of 2 µM of Aβ1-42 monomers on the seed immobilized surface for 50 min induced a decrease in the measured frequency of 46±1 Hz (Table 1 and Fig. 2) after a PBS washing step. The net decrease in frequency is correlated to the mass increase on the TSM-AWS. This showed that the monomers were physically attached to the seeded surface, and this was indicative of the Aβ 1-42 elongation process. This supports previous hypotheses that fibril formation initiates from nuclei sites.30-32

When 10 µM CQ was simultaneously introduced with 2 µM monomer solution for 50 min to the surface, the net decrease in frequency becomes 6.0 Hz, which was significantly smaller than the monomer incubation on its own. During the serial injections, the presence of CQ with monomers even induced a net increase in frequency (Fig. 3). This implied that the immobilized seeds were lifting off from the sensor surface. Recently, Bieschke and co-workers showed the β-sheet breaking properties of polyphenol (-)-epi-gallocatechine gallate (EGCG).33 It is hypothesized here that CQ might have a similar disintegration effect on the elongated fibrils, but the exact mechanism is unclear.
Table 1. Net change in frequency corresponding to sample injection for 50 min.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface</th>
<th>Net ΔFrequency (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10μM CQ</td>
<td>EDAC/NHS activated - EA passivated</td>
<td>-1 ± 5</td>
</tr>
<tr>
<td>2μM Aβ 1-42 +10μM CQ</td>
<td>EDAC/NHS activated - EA passivated</td>
<td>-3 ± 7</td>
</tr>
<tr>
<td>10μM CQ</td>
<td>Seed immobilized - EA passivated</td>
<td>-3 ± 10</td>
</tr>
<tr>
<td>2μM Aβ 1-42</td>
<td>Seed immobilized - EA passivated</td>
<td>-46 ± 7</td>
</tr>
<tr>
<td>2μM Aβ 1-42 +10μM CQ</td>
<td>Seed immobilized - EA passivated</td>
<td>-6 ± 9</td>
</tr>
</tbody>
</table>

Figure 1. Typical frequency (Hz) against time (min) graph showing decrease in net frequency change (ΔF) after 50 min of incubation of 2 μM Aβ1-42 monomers on immobilized seed surface.

To show that the small change in frequency was not due to the saturation of the immobilized surface, another incubation of 2 μM monomers alone was incubated with the surface for 50 min and the net decrease in frequency returned to 41 Hz. (Fig. 4) This recovery of the drop in frequency might indicate that CQ inhibited the elongation by interacting with monomers but not fibril seeds on the surface as a sustained inhibition effect would be expected if CQ was to physically interact with the immobilized seeds preventing further elongation by monomers.
Figure 2. Serial injection of 2 μM Aβ1-42 monomers with 10 μM CQ showing initial decrease in ∆F (white arrows) and then gradual increase in ∆F. Subsequent injection of 2 μM Aβ1-42 monomers injected to observe elongation effect again.

CQ alone was also introduced to the seed immobilized surface, but little change in frequency (Fig. S3) was observed showing that the CQ did not bind physically to the elongated seed surface. This might be due to the hydrophilicity of its halogen and hydroxyl groups, and therefore CQ was unable to interact with the hydrophobic core of the fibrils.

3.5.2 Surface plasmon resonance

Peptide immobilization chemistry and elongation were similar to that described for the TSM-AWS method. SPR sensorgrams showed different characteristics of binding to the surface in both cases. (Fig. 4SA & B) As in the case of TSM-AWS sensors, preliminary assays were carried out to verify the absence of non-specific signal due to the sole addition of Aβ 1-42 and/or CQ on a negative reference surface. When Aβ 1-42 monomers in the presence and absence of CQ were introduced to the passivated gold surface, RU signals of about 162±22 and 276±20 were observed, respectively (Fig. S5). The monomers in the presence of CQ resulted in a consistently smaller RU increase than in its absence because of the intrinsic effect of CQ on Aβ 1-42 that hindered the binding potential of these soluble peptides.34 When compared with 2 μM Aβ1-42 monomers introduced to a 15 μM seed immobilized surface that showed a response of 541±61 RU, the control RU signals were drastically lower. This indicated more monomer deposition onto the seed immobilized surface, and likely related to the elongation of fibrils. CQ alone was also introduced to the passivated chip surface to test for non-specific adsorption of
CQ, but only a negligible signal was observed (data not shown).

To ensure that the A\(\beta\) 1-42 is ‘growing’ on the biochip, four subsequent injections were also performed (Fig. S6). The results obtained showed stepwise increase in RU after every injection. A total of about 2000RU was observed over the injections which corresponded to 32 ng of sample being deposited on the biochip for a channel of 16 mm\(^2\) area (1000RU=1 ng/mm\(^2\)).

To increase the SPR signal for fibril elongation, the concentration of seeds was doubled to 30 \(\mu\)M. According to Canon and coworkers\(^{23}\), the increase in seed concentration would result in an increase in the elongation rate. Our results were in consensus with those previously reported values, as the RU increase was almost doubled in the time span of 50 min. Injection of monomers together with 10 \(\mu\)M CQ, caused an almost 5-fold decrease in RU. CQ alone was also introduced to the sensor surface, and it was shown that CQ had negligible affinity to the immobilized seeds. (Figure)

![Figure 3. SPR sensorgram of RU against time showing the inhibitory effects of 10 \(\mu\)M CQ, when injected together with 2 \(\mu\)M A\(\beta\)1-42, and CQ alone did not change the RU on the seed-immobilized sensor surface.](attachment:image)

### 3.5.3 Electrochemical analysis

To confirm the results obtained from QCM and SPR studies of A\(\beta\) 1-42 peptide aggregation in-vitro, electrochemical and fluorescence measurements were done in the presence and absence of CQ. Electrochemical monitoring of A\(\beta\) aggregation interactions were observed through the well-established oxidation peak of a lone tyrosine residue (Tyr-10) intrinsic to human A\(\beta\) 1-42\(^{35,36}\).
The magnitude of the oxidation signal at approximately 0.7 V (vs. Ag/AgCl) was recorded over a 24-h time span. As Aβ1-42 peptides aggregated over time and formed large, ordered, fibrillar species, Tyr-10 became more shielded within the peptide, therefore less available to be oxidized at the electrode surface. This caused the observed oxidation signal to decrease as the aggregation continued.

It was expected that in the absence of aggregation modulators, Aβ1-42 alone would show the fastest decrease in Tyr-10 oxidation signal, while after the addition of CQ, the time required to reach the same extent of aggregation would increase. As shown in Fig. 5, electrochemical assessment of Aβ1-42 aggregation kinetics supports this hypothesis, as the decrease was most significant for the incubation with Aβ1-42 alone, and plateaued shortly after 200 min passed. The observed phenomenon had been reported previously, when baicalein was incubated with α-synuclein, another amyloidogenic protein, and thus, confirmed the validity of our electrochemical analysis.37

![Figure 4](image_url)

**Figure 4.** The dependence of Tyrosine (Tyr-10) oxidation signal (50 μM Aβ1-42) on time in the absence of CQ (dotted line) and in the presence of 50 μM CQ (dashed line). Error bars are represented by the standard deviation of triplicate measurements (n=3).

In the presence of CQ, the decrease in the oxidation signal of Tyr-10 progressed slowly. This indicated that aggregation of Aβ1-42 was modulated by the flavonoid. As well, the overall
current intensity recorded for Aβ1-42 in the presence of CQ was higher than that of Aβ1-42 alone, suggesting that the aggregates formed in the presence of CQ were less densely packed, and as such allowed for more Tyr-10 residues to be oxidized.

3.5.4 ThT fluorescence analysis

A similar trend was observed in our fluorescence analysis as depicted in Fig. 6. The use of Thioflavin T (ThT) as a fluorescent dye to monitor β-sheet formation during peptide aggregation had been described extensively in the past, and as such provides another method to validate the results obtained from our label-free experiments. In the absence of CQ, Aβ1-42 alone showed the most significant increase in fluorescence intensity, as well as the highest values of relative fluorescence unit (RFU), as expected. Fluorescence of CQ alone was also monitored to make sure that there were no significant RFU contributions that could complicate the values recorded for Aβ1-42 in the presence of CQ.

![Figure 5. The dependence of ThT fluorescence signal on time with 50 μM Aβ1-42 in the absence (dotted line) and the presence (dashed line) of 50 μM CQ. Solid line demonstrates that 50 μM CQ alone did not show significant fluorescence activity. Error bars are represented by the standard deviation of triplicate measurements (n=3).](image)

Upon addition of CQ, the first notable difference was the slower rate of increase in fluorescence. This corresponds to what had been observed in the previously described label-free methods in
this report, further supporting the ability of CQ to modulate the progression of Aβ1-42 fibril formation.

### 3.6 Conclusions

The modulation effects of CQ were observed on the aggregation of Aβ1-42 peptides. To the best of our knowledge, this is the first report that describes three label-free methods (TSM-AWS, SPR and SWV) for studying peptide aggregation. These label-free methods show high promise in screening for aggregation modulators, which would be highly valuable in developing new therapeutic agents against AD. High-throughput, multi-array label-free analysis of other aggregation modulators is under progress in our laboratory.
3.7 References


Chapter 4

4 Surface Plasmon Resonance imaging of Amyloid-β Aggregation kinetics in the presence of Epigallocatechin gallate and Metal Ions

4.1 Preface to Chapter 4

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**Author Contributions**

XRC and KK designed the experiments. XRC, BYH, AVJ and SM performed the experiments. XRC and KK wrote the manuscript. HBK revised the manuscript.

**Acknowledgements**

The authors gratefully acknowledge the financial support from the Biomedical Young Investigator Award of the Alzheimer Society of Canada and NSERC Discovery Grant.
4.2 Abstract

A number of human protein misfolding disorders, including Alzheimer’s disease (AD), are closely related to the accumulation of β-sheet-rich amyloid fibrils or aggregates. Neuronal toxicity in AD has been linked to the interactions of amyloid-β (Aβ) with metals, especially Zn$^{2+}$, Cu$^{2+}$ and Fe$^{3+}$, which leads to the production of reactive oxygen species. Nucleation-dependent Aβ aggregation, or “seeding”, is thought to propagate fibril formation. In this Surface Plasmon Resonance Imaging (SPRi) study, we have shown that the fibril seeds formed with the incubation of Aβ in the presence of metals are better at promoting monomer elongation compared to Aβ alone or in the presence of a well-described polyphenol, (-)-epigallocatechin-3-gallate (EGCG). This is a novel attempt to simultaneous monitor the effects of multiple modulators on fibril elongation using a single chip. EGCG was shown in transmission electron microscopy (TEM) and Thioflavin T (ThT) studies to promote the formation of off-pathway, highly stable unstructured oligomers, supporting the SPRi results. These findings suggest that SPRi provides a promising platform as a screening tool for small molecules that can affect the aggregation pathways in neurodegenerative diseases.

4.3 Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by clinical symptoms such as a decline in cognitive ability, alterations in behaviour, irreversible memory loss, and language impairment. AD is marked by the accumulation of amyloid deposits, the major component of which is an approximately 4 kDa polypeptide known as amyloid-beta (Aβ) protein. Amyloid precursor protein is cleaved by β-secretase to produce Aβ$_{1-40}$ or Aβ$_{1-42}$, where the former is the most abundant species in AD. There is accumulating evidence that monomeric forms of Aβ mediate the growth of amyloid plaques in the human brain. Soluble α-helical or random-coil structures can convert into β-sheet containing protofibrils or oligomeric intermediates, which act as seeds for plaque precipitation and amyloid formation. Formation of soluble β-sheet oligomers has been reported to be essential for neurodegeneration processes and is suspected to play a principal role in the Aβ-mediated toxicity. Thus, targeting the inhibition of Aβ random-coil structures’ transition into β-sheet containing species during elongation could prevent or delay the onset of AD.
Aggregation of Aβ is a nucleation-dependent process, which is dependent on monomer concentration. This dependence is nonlinear and is independent of the addition of pre-formed aggregates. Nucleation is followed by accelerated growth by incorporation of soluble aggregates (intermediate species) before forming mature fibrillar networks. Several advanced analytical techniques such as circular dichroism spectroscopy, high resolution atomic force microscopy and nuclear magnetic resonance had been applied to study the mechanisms of amyloid formation. However, these techniques require long preparation time, skilled technicians and sophisticated instrumentation. Therefore, more robust and simple techniques are in demand for monitoring the growth of monomers into aggregates.

In this report, surface plasmon resonance imaging (SPRi) technology was used for monitoring the elongation process of Aβ. SPRi has become an established tool for monitoring the biomolecular interactions in an array-based format used in numerous studies. SPRi technology uses an expanded beam of parallel light to illuminate the entire sensing area covering several square centimeters on a chip. In contrast to SPR, where either the wavelength or the angle is fixed and the reflected light is detected by a single element transducer, SPRi is performed with both parameters fixed, measuring the change in reflectance across the sample surface area. A CCD camera then captures the reflected light. Recently, a plethora of reports have been published about the application of well-established SPR technology to study amyloids. In particular, Hasegawa and coworkers showed using atomic force microscopy, that the fibrils immobilized on the biosensing chip surface had the appropriate structure such that monomer addition extended the length of the fibrils.

The fibrillization of Aβ is strongly dependent on the external factors such as small molecules and metals. Flavonoids such as clioquinol and green tea extract, (-)-epigallocatechin gallate (EGCG), have been shown to inhibit Aβ deposition in the brain. Earlier studies have shown substantial evidence that EGCG reduces amyloid plaques either by indirectly chelating metal ions resulting in the removal of accelerating factors for disease progression or by directly remodeling large and matured fibrils of Aβ into smaller, amorphous amyloid aggregates that are non-toxic to cells by relieving oxidative stress. Specifically, recent studies have shown EGCG’s affinity for metal ions such as Fe$^{3+}$, Cu$^{2+}$, Zn$^{2+}$, and Al$^{3+}$. In these articles, neuroprotection property was correlated with the EGCG’s metal chelation property. These metals are known to affect the rate of Aβ aggregation as well as its morphology, which in turn would
influence the progression of AD significantly.\textsuperscript{41-43} Flavonoids\textsuperscript{44}, alkaloids\textsuperscript{23} and metals\textsuperscript{22,25} have been studied on conventional SPR systems for their effects on Aβ aggregation. From SPR studies, it has been widely observed that flavonoids did not promote the growth of amyloid, while on the other hand, metals such as Fe\textsuperscript{3+}, Cu\textsuperscript{2+} and Zn\textsuperscript{2+} accelerated Aβ aggregation.

In the present study, we demonstrated the development of a high-throughput SPRi sensor for monitoring Aβ fibril elongation on different immobilized ‘seed’ conditions. Various incubation conditions gave insight to the effect of EGCG and various metals (Fe\textsuperscript{3+}, Cu\textsuperscript{2+} and Zn\textsuperscript{2+}) on the growth of Aβ fibril (Scheme 1). Moreover, to the best of our knowledge, this is a novel attempt to study EGCG and its interaction with Aβ in the presence of metals on array array-based SPRi platform.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Scheme1.png}
\caption{Experimental flow for monitoring Aβ fibril elongation on ‘seed’-immobilized gold spots of an SPRi array.}
\end{figure}

\subsection*{4.4 Experimental Methods}

\subsubsection*{4.4.1 Chemical reagents}

Human β-amyloid peptide\textsubscript{1-40} Gln11 (Aβ\textsubscript{1-40}; trifluoroacetate salt) <H-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Gln-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-OH> was obtained from EMD Biosciences (Gibbstown, NJ). Dimethyl sulfoxide (DMSO; 99.99\%), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 99.0\%), thioflavin T (ThT, 4-(3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl)-N,N-dimethylaniline chloride; ~75\%), sodium phosphate monobasic (NaH\textsubscript{2}PO\textsubscript{4}; 99.0\%), sodium phosphate dibasic (Na\textsubscript{2}HPO\textsubscript{4}; 99.0\%), copper(II) chloride, zinc(II) chloride, iron(III) chloride, (}
-epigallocatechin gallate (EGCG) and ethanolamine were purchased from Sigma-Aldrich (Oakville, ON). Electrochemical analysis was performed using a microAutolabIII potentiostat and operated with the General Purpose Electrochemistry Software (GPES) (EcoChemie; Kanaalweg, The Netherlands). Lipoic acid N-hydroxysuccinimide (NHS) ester was synthesized in the laboratory as described previously. All samples were of analytical grade and prepared in phosphate buffer saline (50 mM, PBS), sodium chloride (NaCl, 100 mM) at pH 7.4 using 18.2 MΩ analytical grade water obtained from a Cascada LS water purification system (Pall Co., Mississauga, ON).

4.4.2 Peptide pretreatment

Aβ1-40 was pretreated by dissolving in neat HFIP at a ratio of 1 mg: 1 mL. Resulting suspensions were sonicated for 15 min until samples became clear. Aβ1-40/HFIP solutions were agitated at 400 rpm for 2 h at 4±1°C. The samples were then left in HFIP and sealed overnight. HFIP was then removed by passing a stream of nitrogen gas across solvent surface, leaving a clear, thin film of amyloid at the bottom of the sample vial. The thin film of peptides were reconstituted in DMSO and mixed by vortex, followed by dilution to the appropriate concentrations with 50 mM PBS, 100 mM NaCl pH 7.4. Peptide concentrations were determined by measuring the OD at 280 nm (ε280 = 1280 M⁻¹) using a NanoDrop 2000 (ThermoScientific, Mississauga, ON).

4.4.3 Surface modification of an SPRi array

Aβ1-40 monomers were incubated under different conditions at 37°C for 75 h to form fibrils (as confirmed by ThT and TEM studies). The varying conditions include incubating 50 μM of Aβ1-40 with 5 μM of Cu²⁺ (or two other metals, Fe³⁺ and Zn²⁺), 50 μM of Aβ1-40 with 5 μM of EGCG, Aβ1-40 alone and 50 μM of Aβ1-40 with 5 μM each of EGCG and Cu²⁺ (or two other metals, Fe³⁺ and Zn²⁺). After incubation, the amyloid fibrils would be formed in various morphologies. The solutions were all sonicated for 5 min before mixing to obtain fibril ‘seeds’. Gold spots of an SPRi array (GWC SpotReady 16 chip) were activated with 2 mM ethanolic solution of lipoic acid NHS ester for 48 h at 4°C. After rinsing the surface with ethanol, 0.5 μL of Aβ and Aβ with metals and EGCG variants were spotted onto the gold surface, and allowed to incubate for 4 h (Figure S-1). The remaining active esters on the gold spots were quenched by incubating the array in 100 mM ethanolamine solution for 1 h at room temperature. Finally, the array was rinsed.
with ethanol before mounting it in the SPR imager II system (GWC Technologies). Three spots were used for each incubation condition for statistical relevance (n=3).

### 4.4.4 SPRi of seed-modified arrays

The SPRi using near-infrared excitation from an incoherent white light source was used for all in situ measurements. In brief, p-polarized light was generated by passing the white light beam through a polarizer, and then used to illuminate the prism/thin gold film assembly at a fixed incident angle slightly smaller than the SPR angle. The plane face of SpotReady chip was coupled, via index matching fluid (Cargille, RI = 1.72) to the prism. A liquid flow cell was attached to the other side of the glass slide and sealed with a rubber O-ring (Viton). The light was directed incident onto the gold film to excite the surface plasmons. The reflected light then passed through a narrow band-pass filter centered at 830 nm, and was collected using a CCD camera in connection with V++ 4.0 (Digital Optics, NZ). All the experiments were measured at 37°C (controlled by a thermostat in the instrument) to simulate physiological temperature. After the spotted chip was inserted into the instrument, the spots could be imaged and regions of interests (ROI) were selected for the software to detect changes. The gold spotted films were located inside the sealed cell, through which Aβ1-40 monomers in PBS were circulated for 15 min after stable SPR signal was observed with PBS alone. The increase in pixel intensity in the ROI was then observed as a difference image and plotted on a graph (Figure S-2). The changes in intensity of all spots were normalized with the Aβ1-40 control spot on every chip. The PBS wash was repeated after monomer flow to remove non-specific binding.

A cross-seeding experiment was performed to determine if elongation of various seed conditions was affected by the different monomer mixture. Aβ1-40 (30 μM) was incubated in the presence and absence of 5 μM modulators (Zn²⁺ or EGCG) for 72 h. Then, the seeds were immobilized on the lipoic acid activated chips for 4 h. The chips were then equilibrated with PBS buffer in the SPRi instrument for ~1 h to determine if the stability of the immobilized fibrils differed from various seed conditions. Aβ1-40 (5 μM) monomers were then introduced to the chip surface for ~500 s. PBS was used to rinse the surface for ~200 s to remove non-specific binding before introducing various incubation conditions such as Aβ1-40 with 5 μM EGCG and then subsequently Aβ1-40 with 5 μM Zn²⁺. A flow rate of 100 μL /min was maintained throughout these cross-seeding measurements.
4.4.5 ThT fluorescence and electrochemistry

ThT stock (10 mM) was prepared in 18.2 MΩ water and stored in aluminum foil. Fluorescence measurements were conducted in black optiflux fluorescence 96-well plates (BD Biosciences, Mississauga, Canada) using a Synergy HT Multimode Microplate reader (BioTek, Winooski, VT). Each sample well contained: ThT (50 µM), Aβ_{1-40} (50 µM), and the respective 5 µM metals and/or EGCG. Conditions were replicated for n = 3. Control experiments were also performed for each condition (incubations containing all compounds except Aβ_{1-40}) and ThT results were normalized with their respective control fluorescence values to prevent any complications from the absorbance of the fluorescence by modulators. Spontaneous aggregation of amyloid samples was induced by incubation at 37±1°C with shaking at 300 rpm. Fluorescence (λ_{ex} 440 nm, λ_{em} 485 nm) was recorded at various time intervals over 120 h. Graphite electrodes were used for the EGCG and Cu(II) interaction study. The pencil graphite was used as a working electrode together with a Ag/AgCl reference electrode and Pt counter electrode. Square wave voltammetry was performed at a frequency of 20 Hz with a 5 mV step potential and 10 mV amplitude, scanning from -0.35 to 0.8 V.

4.4.6 Transmission electron Microscopy

An aliquot (6 µL) of Aβ_{1-40} samples was spotted onto nickel Formvar mesh grids (Electron Microscopy Sciences, Hatfield, PA) for 1 min and blot dried. The TEM grids were subsequently stained using 6 µL of 1% uranyl acetate for 1 min followed by blot drying. Samples were imaged using a Hitachi H-7500 transmission electron microscope.

4.5 Results and Discussion

Incubating Aβ_{1-40} in various conditions allowed the aggregation state of the polypeptide to differ among individual samples. The amyloid proteins with varying morphologies were then spotted on different gold spots (Figure S-1). These gold spots were utilized for the covalent binding of Aβ_{1-40} peptide seeds. The immobilization procedure is shown in Scheme 1. When the monomer amyloid was incubated by flow injection over the gold spot surface on the array, the pixel intensity first increased due to the switch in buffer conditions from PBS solution to amyloid containing buffer solution, followed by the increase in signal resulting from elongation of the fibrils. The spatial contrast in the SPRi image was derived from the heterogeneity in the complex dielectric resulting from the variations in refractive index near the surface, which in turn shifted...
the resonance angle. Since the monomers elongated from fibril seeds that were immobilized, there was a resultant increase in SPRi intensity at the pixels of interest (i.e. ROI after buffer rinse in the 300-450 s range). This difference (final v.s. initial equilibrium SPRi intensity) was the net increase in SPR signal caused by the elongation of fibril seeds by monomer addition on each gold disc. Figure 1A showed a representative image of SPR pixel intensities that could be obtained simultaneously (Figure 1B) with one run of the high-throughput instrument. The net increase in SPRi intensities (compared to the Aβ control), which demonstrated the variation in elongation rate, is shown in Figure 2. The SPRi intensities before Aβ injection was subtracted from those after buffer wash. Then, the differences were divided by the difference observed for the control Aβ experiments and multiplied by 100% to derive the values plotted in Figure 2.

Figure 1. A) SPRi output of pixel intensities against time. Each graph represents the elongation on a gold spot immobilized with different fibril seeds under varying incubation conditions, subsequent gradual increase in pixel intensity was caused by the growth of the monomers on the surface as observed in the part of the curve from 400 to 1300 s. B) SPRi image taken by CCD camera of the gold spots, where pixel intensities were simultaneously monitored in the ROIs (dotted boxes).

After normalizing the varied conditions of Aβ1-40 immobilization, it was observed that the relative pixel intensity changes for Aβ1-40 with Fe3+, Zn2+ and Cu2+ were 156.8±8.8%, 137.9±8.0% and 127.2±3.6%, respectively (Figure 2). This showed that all the metals incubated with the Aβ1-40 peptide resulted in a higher elongation rate as observed with a steeper slope in the curve of the immobilized fibril seeds. Using the software from GWC that simulated and fitted these SPRi curves, the association constants of Aβ1-40 peptide to these fibril seeds were
calculated (Table S-1) and found to be within the range of previous reports. With more fibril seeds immobilized on the gold spots, more sites for elongation were exposed, and thus caused a larger elongation effect manifested in the SPRi pixel intensity increase. However, it was observed that Cu\(^{2+}\) accelerated the elongation process to a smaller extent compared to the other two metals. A possible explanation of this phenomenon was that the fibril seeds induced by the copper ions were unstable and may be easily disrupted, during fibril elongation and after PBS rinse. It was previously reported that copper accelerated A\(\beta\)\(_{1-40}\) aggregation to a smaller extent compared to zinc and iron ions in a turbidimetric analysis of metal ions on A\(\beta\) seeding. The pixel changes after the introduction of EGCG to these metals in the presence of A\(\beta\)\(_{1-40}\) were 56.1±5.6\%, 60.3±17.8\%, and 68.4±8.1\% for Fe\(^{3+}\), Zn\(^{2+}\) and Cu\(^{2+}\) respectively (Figure 2). These results showed that in the presence of EGCG, the resultant fibrils immobilized were not readily available for elongation with A\(\beta\)\(_{1-40}\) monomers. This was probably due to the chelation of metals with EGCG, which prevented the metals from interacting and accelerating fibril formation.

These results were supported by electrochemical data, which showed that the typical oxidation peak of Cu\(^{2+}\) at ~0.15 V diminished in a concentration dependent manner, as EGCG was titrated into the Cu\(^{2+}\) solution. This was a result of EGCG chelation with Cu\(^{2+}\) ions and the reduced availability of the Cu\(^{2+}\) ions to be oxidized at the electrode surface. It was also noteworthy that when equimolar EGCG was introduced, a very small copper peak was observed, showing that free Cu\(^{2+}\) ions were still available to the electrode surface for oxidation (Figure S-3) This control experiment showed that Cu\(^{2+}\) interacted with EGCG. However at a 1:1 ratio used in the experiment, there were still free Cu\(^{2+}\) ions that could modulate the amyloid aggregation process. This result supported our SPRi and TEM results. Similar diminishing oxidation signal trend was observed for the titration of other metals with EGCG (not shown). An experiment was also performed with the incubation of A\(\beta\)\(_{1-40}\) with EGCG only and it was found that only 24.1±4.4\% of the control pixel intensity was observed. This might imply that EGCG had interacted with the A\(\beta\)\(_{1-40}\) and prevented its aggregation into a mature fibril that allowed monomer extension. In the presence of metals, chelation to EGCG caused a decrease in free EGCG available to interact with the peptide monomers and thus, moderate inhibition was observed during elongation processes. It could also be seen that negative control experiments (incubations of metals, EGCG in PBS) did not show a significant increase in pixel intensities of 9.1±4.0\% after monomer injection in the absence of fibril seeds. It was therefore demonstrated that these modulators affected the nucleation process of A\(\beta\)\(_{1-40}\). A cross-seeding experiment was also performed to
determine the effects of modulators on the elongation process. It could be seen from Figure S-4A that the SPR pixel intensity dropped drastically for $\text{A}\beta_{1-40}$ in the presence of EGCG in $\sim1$ h. This implied that fibril seeds in the presence of EGCG had lower stability as compared to fibril seeds incubated with $\text{Zn}^{2+}$ or just $\text{A}\beta_{1-40}$ alone. When the SPR sensograms of all the seed conditions were normalized, and overlayed as shown in Figure S-4B, we could observe that the increase in pixel intensities for $\text{Zn}^{2+}$ modulated fibril seeds was the greatest for all monomer conditions followed by $\text{A}\beta_{1-40}$ alone and then, EGCG modulated fibril seeds. The resultant pixel intensity change was graphed in Figure S-4C, and it could be observed that inhibition of EGCG and acceleration of $\text{Zn}^{2+}$ on $\text{A}\beta_{1-40}$ aggregation were in agreement with Figure 1. It was interesting to note a decrease in SPRi intensity for EGCG-modulated seeds when interacting with monomers in the presence of EGCG. This implied once again that EGCG-modulated fibril seeds were unstable, and were susceptible to rapid disintegration.
Figure 2. SPRi pixel intensities of peptide seeds under various conditions (in the presence and absence of 5 μM Fe³⁺, Zn²⁺ and Cu²⁺ ions and EGCG with 50 μM Aβ₁₋₄₀) immobilized on gold spots on the array. The intensities were expressed as a percentage against Aβ₁₋₄₀ control measurement. The negative controls contained the buffer, EGCG and metals in the absence of Aβ₁₋₄₀.

The several orders of magnitude increase in ThT fluorescence intensity upon fibril-binding makes it an unusually sensitive and efficient indicator. ThT experiments were carried out to determine the formation of β-sheets in the incubation processes (Figure 3). It was observed that during the incubation over 120 h, Aβ₁₋₄₀ peptides incubated in the presence of Fe³⁺, Zn²⁺ and Cu²⁺ showed higher average fluorescence intensities as compared to controls with Aβ₁₋₄₀ alone. Fe³⁺ and Zn²⁺ containing samples showed higher final fluorescence intensities while Cu²⁺ incubated with Aβ₁₋₄₀ had the smallest fluorescence intensity increase. It was also observed that
at 120 h, Cu$^{2+}$-containing samples had average fluorescence intensities that seemed to decrease. This was attributed to the unstable nature of Cu$^{2+}$-induced fibrils as discussed earlier.$^{25}$

\[ \text{Figure 3. Average relative fluorescence intensity of ThT monitored over 120 h under various conditions (in the presence and absence of 5 \textmu M Fe}^{3+}, \text{Zn}^{2+} \text{and Cu}^{2+} \text{ions and EGCG with 50 \textmu M A\textbeta}^{1-40}. \text{Triplicate measurements were performed for each sample, and error bars were less than 20 a.u. (not shown for clarity) } \]

For samples that contained EGCG, in the absence and the presence of metals, the fluorescence intensities were negligible (under 20 a.u.). In the presence of metals and EGCG, a higher fluorescence was observed as compared to A\textbeta$^{1-40}$ peptides with EGCG alone. This implied that ThT was not able to bind with A\textbeta$^{1-40}$ aggregates formed in the presence of EGCG. $^{48}$ The low ThT intensities obtained for incubations with EGCG indicated that unstructured complexes were formed. This would result in the immobilization of ‘seeds’ with low contents of active nucleation sites and thus, adversely affect the elongation of monomers. ThT fluorescence therefore, supported the SPRi results, which showed negligible increase in pixel intensities for samples containing EGCG. A recent report by Hudson and coworkers suggested that small molecules could affect the results of indicator molecules such as ThT and Congo Red. They showed that fluorescence intensity of ThT at emission wavelength of 485 nm dropped drastically in the presence of 5 \textmu M curcumin and quercetin after fluorescence was re-acquired.$^{49}$ To ensure that the EGCG results were not interfering with the ThT fluorescence, we have also formed fibrils
from 50 μM Aβ for 70 h and 5 μM EGCG were spiked into the solution to see if quenching from EGCG would be observed. From Figure S-5, it was observed that after a slight fluctuation, the fluorescence intensity returned to that of the amyloid control, and reached equilibrium in 1 h, showing negligible influence of EGCG on ThT fluorescence.

TEM images were taken with Aβ1-40 after incubation under various conditions. It was shown in Figure 4B that, in the presence of Fe³⁺ ions, long (up to several μm) and thick (~10 nm) Aβ1-40 fibrils were formed after 75 h incubation. These fibrils were denser and longer than the Aβ1-40 fibrils incubated in the absence of metals (Figure 4A). When EGCG and Fe³⁺ were incubated with Aβ1-40 peptides, amorphous aggregates were formed (Figure 4C). They were observed to be dense structures chelated within the fibril network. However, it was apparent that the defined and lengthy fibril forms disappeared and more scattered peptides were observed. The TEM of Aβ1-40 incubated with EGCG alone showed even more pronounced, dense amorphous aggregates where little signs of fibril like structures remained (Figure 4D). TEM images of Aβ1-40 fibrils formed in the presence of Cu²⁺, Zn²⁺ and EGCG were given in the Supporting Information (Figure S-6). In incubations with Aβ1-40 alone, some fibril clusters were observed. However, in the presence of metals in the Aβ1-40 incubation, complex fibril networks were formed. By contrast, samples containing EGCG formed amorphous aggregates after incubation. It could be inferred from the SPRi results that these amorphous aggregates immobilized on gold spots did not promote elongation of monomers, unlike fibrils formed in the absence of EGCG.
Figure 4. TEM images of 50 μM Aβ1-40 in the absence (A), and presence of (B) 5 μM Fe³⁺; (C) 5 μM Fe³⁺ and 5 μM EGCG; and (D) 5 μM EGCG. All incubations were kept at 37⁰C for 75 h. (each scale bar represents 200 nm)

According to Cannon and co-workers,⁴⁸ it was reported that sonicated fibrils contained free ends available for monomer addition and supported fibril growth better than the nonsonicated ones. TEM images of sonicated fibril seeds after incubation were taken (Figure S-6) to demonstrate the effect of sonication. We have observed that the sonicated samples contained shorter fibrils and therefore, possessed more free ends compared to non-sonicated fibrils, in agreement with the previous reports.⁵⁰-⁵²

4.6 Conclusions

In conclusion, we demonstrated the feasibility of SPRi as a multiplexed system to screen for modulators of protein misfolding and formation of β-sheet-rich amyloid fibrils. TEM images showed that denser and more abundant fibril networks were formed for Aβ1-40 in the presence of
metals. While in the presence of a metal-chelating EGCG, amorphous aggregates were observed. ThT results implied that these amorphous aggregates did not contain β-sheets during seed formation. The TEM and ThT results showed varying degree of aggregation, which explained the different elongation rates in the SPRi experiment. Even though the exact inhibition mechanism of EGCG is unknown, it has been suggested in a recent report that EGCG disrupted oligomer formation and caused the aggregate to be unrecognized by an established Aβ oligomer antibody. To the best of our knowledge, this is the first attempt to test multiple modulators of Aβ on an array using the SPRi platform. The reported procedure would be highly promising in the screening of small molecules that can affect the protein aggregation pathways in neurodegenerative diseases.
4.7 References


5 LED-based Interferometric Reflectance Imaging Sensor for the detection of Amyloid-β Aggregation

5.1 Preface to Chapter 5

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**Author Contributions**

XRC and KK designed the experiments. XRC and GGD performed the experiments and analyses. XRC, GGD and KK wrote the manuscript and MSU revised the manuscript.

**Acknowledgements**

XRC and KK gratefully acknowledge the financial support from the Alzheimer Society of Canada and NSERC Discovery Grant. GGD and MSU gratefully acknowledge the financial support from the Center for Integration of Medicine and Innovative Technology (CIMIT) in Boston University.
5.2 Abstract

Self-aggregation of amyloid-β (Aβ) plays an important role in the pathogenesis of Alzheimer’s disease (AD). Small molecule inhibitors of Aβ fibril formation reduce the Aβ-mediated neuroticixity. In this report, the interaction of amyloid-β (Aβ) with well-described modulators, (-) epigallocatechin-3-gallate (EGCG) and Zn(II), was detected using a LED-based interferometric reflectance imaging sensor (LED-IRIS) in a high-throughput and real-time format. Nucleation-based fibril growth strategy was employed, as the “seeds” of Aβ were prepared in the presence of EGCG and Zn(II). The seeds were then covalently immobilized on the chip surface. Using microfluidics, Aβ oligomers were exposed onto the seeds resulting in the elongation of fibrils, which was detected as the increase in the spot height. Monitoring the changes on the chip surface enabled to detect the efficacy of modulators to inhibit or facilitate the growth of Aβ fibrils. The proof-of-concept study reported here introduces a novel platform to facilitate the screening of small molecules towards the discovery of promising AD therapeutics.

5.3 Introduction

Amyloid-β (Aβ) peptide is the major component of amyloid deposits and senile plaques of Alzheimer’s disease (AD). AD is a complex neurodegenerative condition characterized by decline in cognitive ability, irreversible memory loss and impairment of judgment and reasoning. The neuropathological hallmarks of AD, other than the neurotransmitter depletion of acetylcholine, also include the deposition of two abnormal protein aggregates: intracellularly occurring neurofibrillary tangles composed of Tau protein and extracellular insoluble Aβ aggregates. The spontaneous conversion of oligomeric Aβ into fibrillar aggregates is associated with the development of AD. Numerous genetic, biochemical and transgenic animal tests supported the “amyloid hypothesis”. Currently, more than a hundred drugs are in clinical trials for AD treatment. These mainly consist of neuromodulatory signaling and Aβ-related therapies. Out of seven that went to Phase III trials, five of them were Aβ aggregation/clearance targeting drugs. It is therefore feasible to develop novel techniques to study the interaction of Aβ with small molecules in the attempt to facilitate drug discovery efforts.

The molecular mechanism of Aβ aggregation has been extensively studied, but the exact pathway of misfolding and elongation is still somewhat unclear. It has been hypothesized that the formation of small protein oligomers, which act as nuclei or ‘seeds’, seems to be a crucial step in the aggregation pathway. This elongation is known as the nucleation-dependent
polymerization model,\textsuperscript{50-51} which describes that initial unfavorable interactions between oligomers will result in a lag phase, in which stable oligomers are formed. These oligomers then provide nuclei to catalyze further growth of the amyloid, determining a growth phase until the equilibrium between aggregates and oligomers is reached (steady phase).

One strategy to identify promising candidates as amyloid inhibitors is the high-throughput screening of small chemical or natural compounds. Small molecules such as curcumin,\textsuperscript{52} rosmarinic acid\textsuperscript{53} and various polyphenols\textsuperscript{54} have been reported as promising compounds for the inhibition of Aβ aggregation. Among these small molecules, (-)-epigallocatechin-3-gallate (EGCG), which is the main polyphenol in green tea, has been reported to modulate the misfolding of prion proteins.\textsuperscript{19-22} In the presence of EGCG, the assembly of a new type of unstructured, non-toxic protein aggregate was observed, suggesting that it promoted the off-pathway oligomeric formation.\textsuperscript{55-56} EGCG was therefore, used in this report as a model inhibitor. On the other hand, metal ions such as Fe(III),\textsuperscript{57-58} Cu(II),\textsuperscript{59} Zn(II)\textsuperscript{60} and Al(III)\textsuperscript{61} were shown to generally accelerate the formation of amyloid plaques and reactive oxygen species. It has been found through several reports that Zn(II) could promote amyloid formation under certain conditions.\textsuperscript{62-64}

A plethora of techniques have been used to study the aggregation of Aβ such as acoustic wave sensors,\textsuperscript{65} electrochemistry,\textsuperscript{66} atomic force microscopy,\textsuperscript{67} total internal reflection fluorescence microscopy,\textsuperscript{68} surface plasmon resonance (SPR),\textsuperscript{68-69} etc. Flavonoids, alkaloids\textsuperscript{69} and metals,\textsuperscript{70-71} have also been studied using SPR for their effects on Aβ aggregation.\textsuperscript{72}

We applied the LED-Interferometric Reflectance Imaging Sensor (LED-IRIS) for the first time to study the interaction of Aβ with small drug candidates and metal ions (Scheme 1). The technique utilizes common-path interferometry through a Si/SiO\textsubscript{2} layered substrate as the sensing surface to detect local path length changes as a result of mass accumulation with an increase in the spot height at the surface (Fig. S-1).\textsuperscript{73} LED-IRIS is a versatile platform, which has been recently applied to detect single nucleotide polymorphisms, DNA hybridization, viral particles and antigen-antibody interactions.\textsuperscript{73-77} LED-IRIS provides a high-throughput platform to monitor biomolecular interactions with a low noise floor in real-time.\textsuperscript{78}
**Scheme1.** Schematic representation of LED-IRIS system showing the CCD camera, light source and optical components used for illumination of the chip surface. The illumination path consists of two sets of lenses to collect the LED light. The layered Si-SiO₂ substrate was spotted with seeds (incubated under varying modulator conditions) before monomers were introduced to monitor the changes in the spot height in real time.

## 5.4 Experimental Methods

### 5.4.1 Chemical reagents

Human Amyloid-β peptide 1-40 Gln11 (Aβ; trifluoroacetate salt) <H-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Gln-Val-His-His-Gln-Lys-Leu-Val-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-OH> was obtained from EMD Biosciences (Gibbstown, NJ). Dimethyl sulfoxide (DMSO; 99.99%), 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, 99.0%), Thioflavin T (4-(3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl)-N,N-dimethylaniline chloride; ~75%), sodium phosphate monobasic (NaH₂PO₄; 99.0%) and sodium phosphate dibasic (Na₂HPO₄; 99.0%) were purchased from Sigma-Aldrich (Oakville, ON). Zinc (II) chloride, (-)epigallocatechin-3-gallate (EGCG) and ethanolamine were all obtained from Sigma-Aldrich Inc. (St. Louis, MO). LED-IRIS chips were prepared on 15x15 mm square silicon
substrates with 500 nm thermally grown oxide that were purchased from Silicon Valley Microelectronics (Santa Clara, CA). All samples were prepared in phosphate buffer saline (PBS, 50 mM) with 100 mM NaCl at pH 7.4 using 18.2 MΩ Milli-Q water. Unless stated otherwise, all reagents were of HPLC quality and purchased from Sigma-Aldrich (Oakville, ON).

5.4.2 Amyloid pretreatment

\( \text{Aβ}_{1-40} \) was pre-treated by dissolving in HFIP at a ratio of 1 mg: 1 mL. Resulting suspensions were sonicated for 15 min until sample solutions became clear. Aβ/HFIP solutions were shaken at 400 rpm for 2 h at 4±1°C. The samples were then left in HFIP and sealed overnight. HFIP was then removed by passing a stream of nitrogen gas across solvent surface, leaving a clear thin film of peptides at the bottom of the sample vial. The thin film of peptides were re-constituted in DMSO and mixed by vortex, followed by dilution to the appropriate concentrations with 50 mM PBS with 100 mM NaCl, pH 7.4. Peptide concentrations were determined by measuring the OD at 280 nm using a NanoDrop 2000 (\( \varepsilon_{280} = 1280 \text{ M}^{-1} \)) from ThermoScientific (Mississauga, ON).

5.4.3 Surface functionalization

LED-IRIS chips were cleaned by washing extensively using acetone, methanol followed by deionized water prior to functionalization with a coating of N,N-dimethylacrylamide -acryloyloxy succinimid-3(trimethoxysilyl)-propylmethacrylate polymer.\(^79\) The chips were first activated with 0.1 M NaOH before immersing in the polymer coating (1% w/v polymer in 20% saturated ammonium sulfate solution) for 30 min, then dried with argon before being baked for 15 min at 80°C. The chips were then kept dry in a dessicator until use. The immobilization chemistry utilizes the N-hydroxysuccinimide (NHS) functional groups of the polymer reacting with primary amines on Aβ peptides to form covalent bonds.

5.4.4 Real time monitoring of amyloid aggregation

The pretreated Aβ peptides were incubated under different conditions at 37°C for 3 h with 300 rpm shaking. The varying conditions included incubating Aβ with Zn(II), Aβ with EGCG and Aβ alone. After incubation, the fibrils were formed in different morphologies. These solutions were sonicated for 5 min to obtain seeds.\(^80\) The NHS-functionalized chips were used directly and the seeds were dispensed by using a desktop spotting unit at 55% chamber humidity (Odyssey Calligrapher microarrayer, BioRad). An aliquot (~0.1 µL) of seed solution was dispensed on the
LED-IRIS chip in an array format containing 42 spots (Fig. 1). To allow complete immobilization of seeds, the chip was incubated at 37°C in a custom made 75% humidity chamber overnight. The LED-IRIS chips were then incubated in 50 mM ethanolamine adjusted to pH 7.4 with shaking for 30 min to deactivate any unreacted NHS groups on the surface. The chips were then washed extensively with PBS-Tween and rinsed briefly with water to remove salts. The chips were then dried under an argon stream.

**Fig. 1.** Image of 42 seed spots on LED-IRIS chip (A) before and (B) after the exposure to Aβ oligomers. The spots were automatically detected by a custom-made software to calculate the changes in the spot heights (shown in nm).

To measure the amyloid growth, each chip was secured in a custom-made flow-cell sealed with a glass window that enabled the optical measurements. Solutions were driven through the flow-cell by using a peristaltic pump at a rate of 100 μL/min. PBS was initially flowed through the system to clean and condition the associated tubing and the flow-cell for 30 min. This process also removed any weakly bound seeds before any tests were performed. Aβ oligomer solution (5 μM) was then introduced over the seed spots for ~5 h. The chip surface was scanned and monitored continuously during the flow of oligomers. All flow-cell experiments were performed at 37°C, which was controlled by a mini-handheld thermo-coupler (Thermoworks, UT) that was in contact with the metallic flow cell. Each spot in the images was then analyzed using the custom-made software to obtain the optical thickness information (see supplementary information) The changes in spot height at each spot was compared to the background by averaging optical thickness information for each pixel within a spot and an annulus outside of the spot and making a direct comparison between those two regions (Fig. S-2). The analysis of fibril formation was performed by subtracting post and pre-incubation images. The accumulation of Aβ fibrils was
detected as a change in optical path length at those spots indicating an increase of surface thickness. To find the relative change in the spot height, the following formula was used:

$$\text{Relative height change} = \frac{S_{\text{final}} - S_{\text{initial}}}{S_{\text{initial}}} \times 100\%$$

where $S_{\text{initial}}$ and $S_{\text{final}}$ represented the initial and final measured spot height (nm) of each spot respectively. LED-IRIS has also been calibrated to allow conversion of spot height to mass density of proteins. Therefore, in this study, LED-IRIS signal could also be converted from height (nm) into ng/mm$^2$.

5.4.5 Cross-seeding study

A cross-seeding study was performed to determine, if fibril accumulation was affected by various oligomer seeding conditions. Aβ (50 µM) was incubated in the presence and absence of modulators (100 µM Zn(II) or EGCG) for 0, 1, 3 or 5 h to produce the seeds. The seeds were spotted on three LED-IRIS chips using an identical array setup, and left overnight in a humidity chamber as described before. The chips were then deactivated and washed with PBS buffer. Aβ (5 µM) oligomers in the presence and absence of modulators (10 µM of Zn(II) and EGCG) were then introduced to their corresponding chip surfaces. Modified chips were incubated with shaking in a small petri-dish. PBS-Tween was used to rinse the surface twice to remove the non-specifically bound species before a subsequent water rinse to remove salt debris on surfaces. Then, the measurements were performed using the LED-IRIS system as described in the previous section.

5.4.6 Scanning electron microscopy (SEM)

SEM of Aβ samples on LED-IRIS chip surfaces was performed using a Hitachi S530 scanning electron microscope (Fig. S-3). Aβ samples (100 µM) were incubated at 37°C with 300 rpm shaking in the presence and absence of EGCG (200 µM) or Zn(II) (200 µM). After 3 h of incubation, samples were exposed to the LED-IRIS chips. The spots were left to sit overnight to ensure immobilization before rinsing with PBS-Tween and then water as described before. Aβ (10 µM) oligomer solution was then incubated on each chip for 16 h before washing with PBS-Tween followed by a quick water rinse. The chips were dried under a stream of nitrogen gas. All LED-IRIS chips were sputtered with Au using the SEM coating unit PS3 (Agar Scientific) at 19
mA plasma current for 100 s. The chips were then electrically connected to the sample stub by smearing graphite paste dissolved in acetone from the sample to the metallic stub. The surfaces of the chips were imaged at an acceleration voltage of 20 kV with a working distance of 5.0 mm.

5.5 Results and Discussion

5.5.1 Label-free monitoring of amyloid growth

Aβ incubated under different conditions resulted in the formation of seeds with varying morphologies. The seeds were then immobilized onto the LED-IRIS chips. The presence of preformed seeds significantly shortened the lag phase for fibril growth. When the oligomers were flowed across these spots at a rate of 100 µL/s, they were captured by the immobilized seeds, increasing the spot height. Fig. 2A shows the average response of various seed spots after oligomers were exposed to them. The increase in the signal (optical path length) indicated the rate of fibril growth over time (~5 h). A classical sigmoidal curve was observed for the amyloid aggregation process (Fig. 2A). The lag phase, however, was shortened to ~1 h due to the presence of seeds. This was significant, compared to the 40-h lag phase of amyloid growth, typically observed in solution based fluorescence studies using Thioflavin T as reported previously. This was attributed to the fibril formation being a nucleation-dependent process. Therefore, the presence of immobilized seeds accelerated the fibril growth to a great extent.
Fig. 2. (A) Real-time monitoring of spot intensity at each seed spot with equimolar concentration of Aβ and modulators at a flow rate of 100 µL/min. (B) The signal of each seed spot condition was compared for high (1:2) and equimolar (1:1) amyloid:modulator relative concentrations. Other conditions were as described in the Experimental section.

Fig. 2B shows that the signals (relative height change) for equimolar concentrations (1:1) of 100 µM Aβ with 100 µM Zn(II) and EGCG were 331±18% and 221±21%, respectively. Aβ alone displayed a signal of 267±23%. The data showed that the seeds formed in the presence of Zn(II) resulted in a significantly more fibril growth. This was due to the acceleration effect of Zn(II) in amyloid formation that resulted in compact seeds being immobilized on the chip surface compared to Aβ alone. When EGCG was incubated with Aβ before immobilization, the lowest signal was obtained after the flow of oligomers. This result was attributed to the interaction of EGCG with Aβ, which inhibited its aggregation with oligomers. The effects of modulators could be observed from the signal changes at the spots in real-time. To determine if this effect was concentration-dependent, high concentrations (200 µM) of Zn(II) and EGCG were used during seed incubation. Aβ (200 µM) alone was also used in this study as the positive
control. Fig. 2B shows that an increase in Aβ concentration alone (red) did not significantly change the signal (283±27%) after the flow of oligomers. However, when the modulator concentrations were doubled, the signal of Zn(II)-modulated growth increased significantly (376±20%), while that of EGCG decreased (154±21%) (Fig. S-4). It is also noteworthy that the entire signals recorded using modulator concentrations group were more statistically significant relative to each other. Therefore, the high concentration ratio was used throughout the subsequent experiments.

5.5.2 Effect of cross-seeding

To determine if the amyloid growth under different seed conditions was affected by different oligomer and modulator conditions, a cross-seeding experiment was performed, where the signals of the spots were monitored intermittently over ~15 h (Fig. 3). In this case, varying oligomeric conditions were used, as well. As shown in Fig. S-5, the Aβ oligomers with Zn(II) displayed the highest signal followed by Aβ oligomers alone and the lowest signals were obtained from the seeds prepared in the presence of EGCG.

Fig. 3. (A) Array design for LED-IRIS chip in a cross-seeding experiment. The blue, green, red and yellow zones correspond to Aβ seeds incubated in the presence and absence of modulators for 0, 1, 3 and 5 h, respectively; (B) A representative image of the chip after 16 h of incubation under various conditions.

The results implied that the growth of fibrils was significantly accelerated at Zn(II)-containing seed spots. Negligible fibril growth was detected at EGCG-containing seed spots. This was
consistent with previous literature,\textsuperscript{55} which suggested that amorphous aggregates formed in the presence of EGCG inhibited the aggregation and caused no cytotoxicity, because they were off-pathway aggregates.\textsuperscript{48} Fig. 4 shows the representative changes in signals under various conditions, and it was observed that different conditions exhibited significant effects on the rate of Aβ aggregation.

It was observed that EGCG inhibited the formation of Aβ fibrils both in the presence and absence of Zn(II) in seeds (Fig. 4A).

Fig. 4. Time-dependence of LED-IRIS signals with the exposure of Aβ alone (green line), Aβ in the presence of Zn(II) (blue line), and Aβ in the presence of EGCG (orange line) at seed spots modified with (A) 50 μM Aβ with 100 μM EGCG, (B) 50 μM Aβ and (C) 50 μM Aβ with 100 μM Zn(II). Other conditions were as described in the Experimental section.

This was attributed to the weakening of the cross-β structure by the interaction of EGCG with the β-sheets of fibrils.\textsuperscript{83} Interesting to note, the signal decrease was significant, when Aβ with EGCG were exposed to the EGCG-containing seed spots. This implied that the seeds prepared in the presence of EGCG had lower stability compared to other seed conditions, leading to disintegration from chip surface. This was in agreement with the recent literature, where EGCG was shown to break down amyloids into non-toxic off-pathway aggregates.\textsuperscript{52-54}
When the Aβ oligomer incubation contained Zn(II), elongation seemed to persist for the seeds formed in the presence of EGCG (although at a significantly lower response level compared to other seed conditions) as shown in Fig. 4C. It was proposed by Miller and coworkers\textsuperscript{55} that two Zn(II) ions coordinated with residues of two different Aβ peptides, facilitating the elongation of fibrils. Zn(II) binding also decreased the solvation energy for tightly packed oligomers, which stimulated amyloid aggregation.\textsuperscript{56} The accelerated formation of amyloids induced by Zn(II) was observed in previous studies.\textsuperscript{57,58} It was also reported that Zn(II) physically bound to Aβ peptides by coordinating with histidine residues as confirmed by NMR studies.\textsuperscript{59,60} In parallel to these previous reports, the addition of Aβ oligomers in the presence of EGCG to the Zn(II)-bound seeds resulted in a slow increase in fibril height, demonstrating that the Zn(II)-containing seeds were not easily disintegrated (Fig. 4C).

5.6 Conclusions

The experiments detailed here indicate that the LED-IRIS platform is a promising platform for high-throughput and real-time detection of protein-small molecule interactions. The high-throughput capability of LED-IRIS is suitable for the screening of novel Aβ aggregation modulators that would significantly accelerate the drug discovery efforts towards AD therapy.
5.7 References


6  Electrochemical Detection of Interaction between Clioquinol and α-synuclein

6.1  Preface to Chapter 6

This chapter has been adapted with permission from Electroanalysis, submitted:


Author Contributions

XRC and KK, designed the experiments and wrote the manuscript. XRC performed the experiments and analyzed the results.

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Acknowledgements

This work was supported by NSERC Discovery Grant program. We appreciate the help of Anthony Veloso and Tiffiny Chan in troubleshooting the electrochemical and UV-vis experiments. We would also like to acknowledge Qi Li for her help with ThT assays and Biao Zhang for his assistance with the CQ and Cu(II) binding studies. We are very grateful to Raymond Or for his advice and troubleshooting with the SEM instrumentation at the Center for Neurobiology of Stress in UTSC.
6.2 Abstract

α-Synuclein (α-S) protein is expressed in presynaptic terminals in the central nervous system. The aggregation of α-S is implicated in the pathogenesis of Parkinson’s disease (PD). In this report, the interaction of α-S with a flavonoid and metal chelator, clioquinol (CQ), was investigated in the presence of Cu(II) ions using electrochemistry, Thioflavin T-based fluorescence and Congo Red-based UV-Vis spectroscopy. In the presence of CQ, the α-S aggregation rate was observed to decrease. The preliminary results showed promise in the future development of CQ derivatives as novel small molecule therapeutic agents with potential efficacy targeting α-S in PD therapy.

6.3 Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disease after Alzheimer’s disease (AD), which are both associated with the aggregation of amyloidogenic proteins in the brain. Clinically, it is a movement disorder that is characterized by rigidity, resting tremors and bradykinesia. PD has received much attention, as it has an alarming occurrence rate of 1% of the population by age of 65, and it severely reduces patients’ quality of life and increases their expenses. Pathologically, PD is marked by a progressive loss of dopamine producing cells in the substantia nigra in the brain.

α-Synuclein (α-S) is a 140-aa highly conserved presynaptic protein, and is natively unfolded at neutral pH, as demonstrated by circular dichroism spectroscopy, small angle X-ray scattering, Fourier transform infrared spectroscopy and NMR. In vitro experiments had shown that α-S undergoes aggregation via a pathway of intermediates that include soluble oligomers, protofibrils and mature fibrils. α-S is found in high concentrations at presynaptic terminals and the aggregation of α-S in these regions is the most compelling observation that characterizes PD. Evidence suggests that the formation of Lewy bodies and Lewy neurites is caused by the accumulation of α-S fibrils.

Currently, there is no preventive therapy for PD. However, biometals such as Zn(II), Cu(II) and Fe(II) are known to accelerate the formation of α-S oligomers, which are speculated to be more toxic than mature fibrils. Atomic force microscopy and scanning electron microscopy have shown these fibrous aggregates in the presence of Fe(II) and Cu(II). Particularly, the
binding of Cu(II) to α-S has been associated with its ability to aggregate.\textsuperscript{15} The general mechanism for the metal-induced aggregation process was thought to be caused by the binding of metal cations with the negatively-charged carboxylates of the polypeptides, and thus masking the natural electrostatic repulsion within the protein, facilitating the misfolding process.\textsuperscript{8} Furthermore, there seems to be specific affinity sites on α-S for metals.

Flavonoids are a group of polyphenolic compounds that have important medicinal applications mainly due to their antioxidant activity.\textsuperscript{16} Intake of antioxidant flavonoids was also reported to have a dementia lowering effect.\textsuperscript{17} It was reported that flavonoids may have excellent radical scavenging ability attributed to their power to chelate to metals.\textsuperscript{18} Numerous flavonoids have been studied recently to test their effect on amyloidogenic protein aggregation inhibition.\textsuperscript{9, 19-21}

CQ (5-chloro-7-iodo-8-hydroxyquinoline) is an anti-fungal compound, which was reported to significantly reduce amyloid-β (Aβ) plaque deposits, that resulted in improved cognitive behavior.\textsuperscript{22} CQ, like other flavonoids, has metal chelation properties.\textsuperscript{23, 24} Long term oral use of CQ may be limited due to its adverse side effect of subacute myelo-optic neuropathy.\textsuperscript{25, 26} However, it was also reported that, episodes of subacute myelo-optic neuropathy may be avoided, if CQ was taken in controlled dose and with supplementary vitamins.\textsuperscript{27} CQ binds Cu(II) and Zn(II) (2:1 ratio) in a square, planar arrangement\textsuperscript{28} with moderate affinity (log $K_1$(Cu)=12.5, log $K_2$(Cu)=10.9, log $K_1$(Zn)=8.5 and log $K_2$(Zn)=7.6)\textsuperscript{29}. CQ has shown to reduce toxicity of the PD-inducing agent, 1-methyl-4-phenyl-1,2,3,6-tetra-pyridine, by ameliorating neurodegenerative effects in mice models.\textsuperscript{30} PBT2 is an 8-hydroxyquinoline derivative like CQ but lack the iodine atom.\textsuperscript{31} Currently, PBT2 is utilized in the Phase II IMAGINE trial for AD and the Phase II REACH2HD trial for Huntington’s disease. Both trials reported promising findings in February and March 2014.\textsuperscript{32} PBT2 prevents production of Aβ oligomers, breaks down the existing Aβ oligomers, and enhances cognitive function in transgenic mice, while exhibiting higher safety and efficacy than CQ.\textsuperscript{33} Lindquist \textit{et al.}\textsuperscript{34} reported that these 8-hydroxyquinolines were also able to rescue toxicity caused by the expression of αα-S and polyglutamine proteins in yeast models for PD and Huntington disease respectively. Our group has previously reported interactions between baicalein\textsuperscript{1}, dopamine\textsuperscript{35} and α-S using electrochemical and optical techniques. Palecek and co-workers\textsuperscript{36-38} reported the electrochemical techniques to detect the changes in interfacial properties of α-S preceding its aggregation using mercury and carbon electrodes. Osmium tetroxide, 2,2′-bipyridine was also utilized as an electro-active marker for probing the
accessibility of tryptophan residues in various proteins including α-S. Recently, Lopes et al.\textsuperscript{40} reported the electrochemical analysis of α-S fibrillation using the tyrosine (Tyr) oxidation signal. Zhou \textit{et al.}\textsuperscript{41} also utilized electrochemical techniques to study the production of reactive oxygen species by α-S. The redox behavior of CQ was also characterized by Ghalkhani and coworkers\textsuperscript{42} using various voltammetric techniques. To the best of our knowledge, the \textit{in vitro} interaction of CQ with α-S is reported for the first time using electrochemistry here. Studying the aggregation of α-S in the presence of CQ and Cu(II) may aid in the rapid characterization of novel therapeutic candidates for PD.

6.4 Experimental Methods

6.4.1 Chemicals and reagents

Congo Red (94%), poly-L-lysine solution (0.1%) and Cu(II) chloride dihydrate (ACS reagent, P99.0%) were purchased from Sigma–Aldrich (Oakville, ON). Human α-S, (recombinant, \textit{Escherichia coli}) was purchased from EMD Chemicals (Gibbstown, NJ). All solutions were prepared with ultrapure water using a Cascada LS (Pall Co., NY) water purification system at 18.2 MΩ, unless otherwise stated.

CQ stock solution (1 mM) was prepared in 95% ethanol and stored in 1.5 mL vials at 20°C. Stock solution of Cu(II) (2 mM) was prepared in ultrapure water, and stored at 4°C, until required for analysis. All solutions were equilibrated to room temperature before analysis. Aliquots of CQ and Cu(II) solutions were diluted with 50 mM phosphate buffer saline (PBS, pH 7.4 with 100 mM NaCl). Other reagents were of analytical grade and used as received.

6.4.2 Apparatus

Synergy HT plate reader (Bio-Tek, Bad Friedrichshall, Germany) was used to perform Thioflavin T (ThT) experiments. UV–vis spectroscopy was performed using Nanodrop 2000c spectrophotometer (Wilmington, DE) with cuvette capability. Scanning electron microscopy was performed with a Hitachi-S350 microscope. Cyclic voltammetry (CV) and square-wave voltammetry (SWV) were performed with Autolab-III electrochemical analyzer (Metrohm, Switzerland) operated in conjunction with its three general-purpose electrochemistry software
(GPES). The planar screen-printed carbon strips (SPCSs) were kindly donated by Professor Eiichi Tamiya (Osaka University, Japan) and Biodevice Technology Ltd. (Ishikawa, Japan). The three-electrode system on SPCS comprised of a carbon working electrode (geometric working area: 2.64 mm²), a carbon counter electrode and Ag/AgCl reference electrode. All measurements were taken at room temperature (23 ± 1°C).

6.4.3 Electrochemistry

Solutions of α-S (50 µM) in 50 mM PBS were incubated with 100 µM CQ and 50 µM concentrations of Cu(II) at 37±1°C using a thermal blockα-Shaker (Fisher Scientific) with constant shaking at 300 rpm. The samples were then diluted to 500 nM using PBS. Aliquots (20 µL) of samples were spotted onto the working electrode area of SPCS. Electrochemical signals were measured using square-wave voltammetry (SWV) from 0.5 V to 1.2 V with a step potential of 5 mV and an amplitude 25 mV at the frequency 50 Hz. The anodic current peak at ~0.55 V was monitored for the oxidation of Tyr residues in α-S.

The interaction of Cu(II) with CQ was also studied using differential pulse voltammetry (DPV). DPV conditions were set to pulse periods of 0.2 s, with pulse amplitude of 50 mV and a step potential of 4.0 mV scanning from 0 to 1.0 V, and reversed for reduction. Pencil graphite electrode (Pentel Lead Ref 50-HB) was used as the working electrode and immersed in 100 µM CQ in PBS containing various concentrations of Cu(II) before the measurement with a Ag/AgCl reference electrode and a platinum counter electrode. The anodic and cathodic peaks of CQ at ~0.35 V and ~0.30 V were monitored, respectively.

The raw SWV and DPV voltammograms were treated with the Savitzky–Golay level-3 smoothing feature of GPES and baseline corrected to a peak width of 3 mV. The measurements were repeated in replicates (n=3) for statistical relevance.

6.4.4 Aggregation studies and ThT fluorescence assays

α-S (1 mg/mL) was centrifuged at 14000 rpm with Eppendorf Centrifuge 5418 to remove any aggregated material. Aliquots of α-S (20 µM) were prepared in 50 mM PBS (pH 7.4, 0.1 µM NaCl). The protein was incubated in the absence and presence of 10 µM CQ and 20 µM Cu(II), respectively at 37±1°C using a thermal blockα-Shaker (Fisher Scientific) with constant shaking
at 300 rpm for desired periods of time. Aliquots of 5 µL samples were removed from the incubated solutions and added to 145 µL 10µM ThT solution in PBS in a 96-well microplate. ThT fluorescence was recorded at 485 nm with excitation at 440 nm using the Biotek Synergy HT multimode Multiplate reader. Repetitive measurements (n=3) were made at each time point for statistical relevance.

6.4.5 Congo Red-based UV-Vis spectroscopy assay

Solutions of α-S (50 µM) at 50 mM PBS were incubated with 100 µM CQ and 50 µM of Cu(II), respectively in the presence of 10 µM Congo Red at 37±1°C in the cuvette section of Nanodrop 2000c with stirring at 300 rpm. The software of the instrument was programmed to take the absorbance measurements at 541 nm in every 10 min intervals for 48 h.

6.4.6 Scanning electron microscopy (SEM)

Coverslips underwent acid wash in 1 M HCl for 4 h at 55°C with occasional agitation, and then washed stringently with distilled water and dried with 100% ethanol. The coverslips were then incubated in 0.1% Poly-L-lysine for adhesion purposes. After incubation of the α-S samples at 37°C, aliquots of sample solutions were fixed in 2.5% glutaraldehyde on the coverslips in 0.1 M PBS (pH 7.4) for 1 h, followed by wash with PBS. The material was then dehydrated in graduated ethanol steps: 50%, 70%, 95%, 100% and then critical point dried with liquid CO₂ and lightly coated with gold-palladium. The samples were observed with a Hitachi S530 scanning electron microscope (SEM) at acceleration voltage of 20 kV and a working distance of 5.0 mm.

6.5 Results and Discussion

The four Tyr residues in α-S protein were the direct source of electrochemical oxidation signal. Our group has previously shown that Cu(II) ions resulted in a decrease in electrochemical oxidation signal of α-S.¹ This was attributed to the association of Cu(II) ions with the electroactive Tyr residues of α-S. Also, the binding of Cu(II) to the protein resulted in the rapid aggregation of the peptide monomers, forming a hydrophobic core that would make the Tyr residues inaccessible to the electrode surface, causing a decrease in electrochemical signal over time.
SWV was used in this study to monitor the interaction of CQ with α-S using planar screen-printed carbon strip (SPCS). In Fig. 1, it was shown that there was a significant increase in current signal after the introduction of CQ as compared to α-S incubated in PBS alone. The initial fluctuation was caused by the dynamic rearrangement process of the protein. The oxidation current in the presence of CQ seemed to be similar to that of α-S alone for the first 3 h, but later differentiated itself at 5 h (Fig. 1A). Since CQ (anodic peak potential at ~0.35 V vs. Ag/AgCl) did not oxidize in the Tyr oxidation potential range, the increase in the anodic peak current (at ~0.55 V) was attributed to the conformational change of the α-S protein. Bil et al.\textsuperscript{43} confirmed that CQ induced an ordered structure alpha-helix from its native form as a random coil in α-S, as studied by circular dichroism and fluorescence spectroscopy. This might have resulted in more Tyr residues being exposed to the electrode surface for oxidation and thus, the slight increase in anodic peak current was observed.

**Figure 1.** A) Plot for the electrochemical oxidation of Tyr residues in 50 μM α–S in the presence (square) and absence (diamond) of 100 μM CQ. A control sample of 100 μM CQ (triangle) in PBS was also analyzed at the same time periods. The anodic current peak intensities for the electrochemical oxidation of Tyr residues were measured at 0.55 V vs. Ag/AgCl. B) SWV of a) 100 μM CQ alone in PBS, b) 50 μM α–S alone in PBS and c) 50 μM α–S after 9 h of incubation with 100 μM CQ. Other conditions were as described in the Experimental section.

To observe the electrochemical effect of CQ in the presence of Cu(II) on the α-S aggregation, they were concurrently introduced to α-S and aliquots were measured using SWV. It was shown
in Fig. 2 that the electrochemical signal remained low at 5 h, but then gradually increased over time. This difference was attributed to the presence of CQ chelating to Cu(II), reducing the acceleratory effect of Cu(II) on α-S aggregation.

The interaction of Cu(II) with CQ was also studied using electrochemistry at SPCSs (Fig. S1). The incubation of Cu(II) ions with CQ resulted in a lower electrochemical current signal at 0.35 V (vs. Ag/AgCl) compared to CQ alone at 0 h. The low current intensity was attributed to the rapid formation of Cu-CQ complex preventing the oxidation of phenolic group in CQ, in agreement with Ghalkhani et al.³⁹

![Figure 2. Plot for the electrochemical oxidation of Tyr residues in 50 μM α-S alone (diamond) and in the presence of Cu (II) (square) or 100 μM CQ with 50 μM Cu(II) (square). The anodic current peak intensities for the electrochemical oxidation of Tyr residues were measured at 0.55 V vs. Ag/AgCl. Other conditions were as described in the Experimental section.](image)

To determine whether Cu(II) ions affected the α-S aggregation, we utilized ThT fluorescence assays. Aqueous solutions of α-S formed β-sheet rich fibrils over a period of days to a couple of weeks, depending on the solvent conditions. Under the conditions used in our experiment, the fluorescence of ThT increased over time, taking the form of a sigmoidal shape, with a lag phase of ~28 h. During the lag phase, entropically unfavorable process of initial aggregation occurred, once the association process began after the formation of a critical nucleus, the aggregation advanced promptly to form protofibrils before more monomers incorporated into the fibrils.⁴⁴ α-
S samples were treated as described by Nilsson to disintegrate any preformed nuclei or oligomers to ensure that only monomer aggregation was monitored.\(^4^5\) In the presence of Cu(II), the lag phase was significantly reduced to 5 h. The maximum ThT fluorescence intensity was also increased by more than 2-folds compared to that of α-S alone. (Fig. 3) The reduced lag-phase and greater fluorescence intensity were attributed to the binding of Cu(II) to the active sites in the protein that facilitated the aggregation process. This accelerated aggregation was in agreement with data reported by by Bharathi \textit{et al.}\(^4^6\) In the presence of CQ, no aggregation was observed. This result was in agreement with Zhu \textit{et al.},\(^1^9\) where it was shown that the flavonoid baicalein inhibited the aggregation of α-S in a similar manner.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Time dependence of ThT fluorescence upon the interaction of α-S with Cu(II) and CQ. 10 μM ThT (diamond), 20 μM α-S alone (square) and in the presence of 20 μM of Cu(II) (triangle) or 10 μM of CQ (cross) were tested. All standard deviations were less than 3 a.u. (not shown for clarity). Other conditions were as described in the Experimental section.}
\end{figure}

ThT fluorescence results were also supported with UV-Vis spectroscopy of Congo Red with α-S (Fig. S2). The increase in the absorbance peak at 541 nm implied β-sheet formation.\(^1\) This is because Congo Red displays green birefringence under polarized light and in a spectral shift assay, the maximum absorbance would display red-shift and show an increase in absorbance at 541 nm.\(^4^7\) In particular, the cross-β-sheet structure found in fibrils is thought to be the key structure the dye binds to. The absorbance gradually increased with time for the α-S sample, indicating that Congo Red was binding to aggregates that were slowly forming in solution. The
gradual increase in absorbance of the shoulder peak at 541 nm was attributed to the fibril formation. In the presence of Cu(II), an abrupt increase in absorbance at 541 nm was observed. This was due to the accelerating effect of Cu(II) on the aggregation of α-S. In the presence of CQ, the initial absorbance at 541 nm was lower than both α-S in the presence and absence of Cu(II). Despite the initial increase in the first 15 h to the absorbance level of the α-S control, the absorbance was then suppressed by the effects of CQ, inhibiting the formation of fibrils. Instead of complete inhibition of fibril formation, CQ was observed to form disintegrated amorphous structures as observed in the cuvette (Fig. S3) where a chalky and dispersed staining of the protein was observed after 48 h. Cu(II) induced aggregation resulted in fibrillar staining of the Congo Red dye (Fig. S4). The control sample of α-S had a homogeneous orange-red color even after 48 h (not shown), but had a higher absorbance at 541 nm compared to α-S with CQ sample probably due to the aggregate formation in solution, while CQ could disintegrate the Congo Red-bound α-S aggregates.

It was crucial to monitor the conformational changes of α-S over time to visualize its progression from soluble monomers to mature fibrils. Here, we imaged α-S samples in the absence and presence of Cu(II) and CQ at various time points. To evaluate if Cu(II) and CQ were capable of modulating the aggregation process of α-S, upon 24 h incubation of Cu(II) or CQ with freshly prepared α-S, the resulting protein species were monitored using SEM. As depicted in Fig. S5, SEM images of α-S species generated from 24 h incubation resulted in protein clumps that had no distinct fibril structures. The α-S monomers started to aggregate to form those visible dispersed protein lumps (Fig. S5B), which were absent in the initial fresh α-S monomers fixed at 0 h incubation (Fig. S5A). Similar amorphous aggregates with irregular shapes were also observed by Hoyer and co-workers.\textsuperscript{48} In the presence of Cu(II) ions, defined and mature fibrils were observed (Fig. S5C). From TEM studies, the width of a single α-S fibril was found to be 10 nm,\textsuperscript{49} but the SEM images displayed fibrils with greater width (Fig. 4C). This indicated that the Cu(II) had accelerated the fibril formation such that bundles of mature fibrils were formed within 24 h. This supported our ThT and UV-Vis data, where there was rapid fibril formation for α-S in the presence of Cu(II) within 24 h. In the case of α-S alone, there was an approximate lag phase of about 30 h before ThT fluorescence increased significantly, coherent with the SEM results. In the presence of CQ, there was no α-S fibril formation as shown in Fig. S5D. The solid structures observed in Fig. 4A and D were salt crystals present in PBS.
After 1 week of incubation at 37°C, it was observed that the α-S samples formed fibril like aggregates (Fig. 4A). They were less extensive as compared to the large fibril networks formed in the presence of Cu(II) ions (Fig. 4B). However, in the presence of CQ, a mesh-like structure was formed (Fig. 4C, D). This chalky texture of α-S did not seem to contain mature fibrils. This result was attributed to the disintegration effect of CQ on α-S fibrils.

**Figure 4.** SEM images depicting the influence of Cu(II) and CQ on α–S aggregation. A) 50 μM α–S alone after 1 week of incubation at 37°C with agitation and in the presence of B) 50 μM Cu(II) or C)100 μM CQ at 5000 X magnification. D) depicted 100 μM CQ incubation with α–S at 8000 X magnification. Other conditions were as described in the Experimental section.

### 6.6 Conclusions

We describe here the effects of Cu(II) ions and CQ on the aggregation of α-S using electrochemical techniques. Well-established optical techniques were utilized to support the electrochemical data. It is important to know the effects of such metals on the physical morphology of the protein in order to further study metal chelators (e.g. PBT2) as promising
therapeutic agents for PD. Both electrochemical and spectroscopic studies showed that Cu(II) accelerated the fibril formation, while CQ inhibited such activity. The simple operation and rapid detection capabilities of our electrochemical system enabled the study of protein-small molecule interactions at the interface. The electrochemical assay provides a promising platform to monitor the interaction of small molecules with α-S, which may be useful in the rapid screening of future PD therapeutics.
6.7 References


7 Electrochemical Monitoring of Metal and Polyphenol-induced Conformational Changes in α-synuclein on Au Nanostructured Sensors

7.1 Preface to Chapter 7

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**Author Contributions**

XRC and KK, designed the experiments and performed the experiments and analyses. XRC and KK wrote the manuscript and FLL revised the manuscript.

**Acknowledgements**

The authors gratefully acknowledge the financial support from the Biomedical Young Investigator Award of the Alzheimer Society of Canada and NSERC Discovery Grant. We also appreciate the help of Brandon Bernal and Vithien Nguyen for performing some of the experiments.
7.2 Abstract

In this proof-of-concept study, the fabrication of novel Au nanostructured indium tin oxide (Au-ITO) surfaces is described for the development of a dual-detection platform with electrochemical and localized surface plasmon resonance (LSPR)-based biosensing capabilities. Nanosphere lithography (NSL) was applied to fabricate Au-ITO surfaces. Oligomers of α-synuclein (αS) were covalently immobilized to determine the electrochemical and LSPR characteristics of the protein. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed using the redox probe [Fe(CN)₆]³⁻/⁴⁻ to detect the binding of Cu(II) ions and (-)-epigallocatechin-3-gallate (EGCG) to αS on Au-ITO surface. Electrochemical and LSPR data were complemented by Thioflavin T (ThT) fluorescence, surface plasmon resonance imaging (SPRi) and transmission electron microscopy (TEM) studies. EGCG was shown to induce the formation of amorphous aggregates that decreased the electrochemical signals. However, the binding of EGCG with αS increased the LSPR absorption band with a bathochromic shift of 10-15 nm. The binding of Cu(II) to αS enhanced the DPV peak current intensity. NSL fabricated Au-ITO surfaces provide a promising dual-detection platform to monitor the interaction of small molecules with proteins using electrochemistry and LSPR.

7.3 Introduction

Nanosphere lithography (NSL) is a low-cost nanofabrication technique capable of producing well-ordered 2D arrays of periodic nanostructures over large surfaces.¹ NSL has also been applied for the synthesis of size-tunable noble metal nanoparticles organized onto surfaces.²⁻⁵ This characteristic of NSL has been especially valuable for investigating the size-dependent optical properties towards the development of chemical and biological nanosensors.

In general, NSL makes use of transferring a monolayer of nano- or microspheres onto a surface forming a tightly packed pattern, which is ultimately used as a mask for pattern transfer. By removing the spheres after metal thin film deposition, the remaining 2-D nanostructures on the substrate displays nanoscale triangular or pyramidal features arranged in a hexagonal pattern. Au and Ag nanopyramid and nanotriangle arrays that were prepared using NSL, have been extensively characterized using extinction measurements to identify the dipolar and quadrupolar resonances of the localized surface plasmon (LSPR)¹ while surface-enhanced Raman spectroscopy (SERS) was demonstrated onto platforms functionalized with organic molecules.⁴.
LSPR occurs when the free electrons of noble metal nanoparticles resonate in response to an optical excitation. The resonance wavelength of such NSL platforms can be finely tuned by modifying the chemical nature of the material as well as the geometric parameters of the individual structure. Once the excitation wavelength matches the LSPR of the metallic nanostructure, large enhancement of the confined electromagnetic field can be used to detect molecules located in its vicinity. It is known that the uniformity of Au nanostructures on surfaces as well as the opto-geometric parameters of the metallic structures are critical and ultimately affect the width and the position of the LSPR bands as well as the sensitivity of the optical measurements. To the best of our knowledge, this is the first report about the application of NSL-based Au nanostructures on ITO surfaces (Au-ITO) for the electrochemical and LSPR-based detection of small molecule-protein interactions.

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the world after Alzheimer’s disease (AD). PD is more common in the elderly, and it imposes a significant social and economic burden on society. It is pathologically characterized by a loss of dopaminergic neurons in the substantia nigra and aggregated α-synuclein (αS) protein deposits in the peripheral of intraneuronal inclusions called Lewy bodies. Several studies have shown that αS misfolding and its dysfunctional regulation in Lewy bodies are key factors in the pathogenesis of PD. Oligomers of αS are viewed as the neurotoxic species that are responsible for neuronal death in the early stages of PD. There is compelling evidence that the loss of transition metal homeostasis results in oxidative stress and toxicity in PD. Such toxicity has been shown to be caused by αS oligomers that have undergone morphological changes in the presence of metal ions. In particular, αS was reported to bind strongly to the Cu(II) ions. Cu(II) was found highly effective in inducing αS aggregation. Circular dichroism analysis of the interaction between Cu(II) and αS suggested an increase in helical content. Structural studies based on nano-electron-spray ionization mass spectrometry also showed that Cu(II) induced the formation of highly compact fibrils of αS at pH 7.4.

On the other hand, EGCG, a green tea polyphenol, has been found to possess anti-amyloidogenic properties. It has been shown to inhibit the misfolding of both αS and amyloid-β (Aβ) (protein associated with the progression of AD) by directly binding and preventing their conversion into toxic aggregates. Instead of β-sheet-rich amyloid fibrils, non-toxic and unstructured forms of αS and Aβ were promoted in the presence of EGCG. Mechanistic studies
revealed that EGCG mediated the amyloid conformational change without their disassembly into monomers or small diffusible oligomers.\textsuperscript{34}

Due to the hypothesis that there may be independent, competing aggregation pathways in amyloidogenic proteins that can be specifically targeted with chemical compounds, the effects of these small molecules on amyloid aggregation have been of great interest to researchers.\textsuperscript{35-38} These anti-amyloidogenic compounds have been extensively studied using fluorescence spectroscopy,\textsuperscript{39} electron microscopy\textsuperscript{40} or SPR.\textsuperscript{35} Many of these techniques require labeling and expensive instrumentation. Our group has previously reported the label-free detection of aggregation in Aβ and αS by monitoring the electrochemical oxidation signal of electro-active Tyrosine residues found in these proteins.\textsuperscript{41-43} An indirect method to monitor the amyloid aggregation has also been developed in our laboratory using benzothiazole dyes, which had specific affinity to β-sheet structures.\textsuperscript{44} In this report, a novel approach to the electrochemical and LSPR-based detection of αS aggregation is demonstrated using Au-ITO with the well-described interaction of αS with EGCG and Cu(II) ions. The effects of Cu(II) and EGCG on immobilized αS oligomers were detected using the redox probe [Fe(CN)\textsubscript{6}]\textsuperscript{3-/4-} in connection with cyclic voltammetry (CV) and differential pulse voltammetry (DPV).

Our electrochemical and LSPR measurements were confirmed using conventional Thioflavin-T fluorescence, SPR imaging and TEM studies. Electrochemical and LSPR-based analyses of proteins on surfaces can be critical techniques to understand and provide insight into the effects of metals and small molecules on proteins. The label-free approach, nanofabrication and mass production capabilities, together with the quick response time may aid in the high-throughput screening of therapeutic candidates for PD.

7.4 Experimental Methods

7.4.1 Chemicals and reagents

Recombinant human α-synuclein (αS) was purchased from Anaspec Inc. (Fremont, CA). Thioflavin T (ThT, 4-(3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl)-N,N-dimethylaniline chloride; \textquotedblleft75\%\textquotedblright), 11-unmercaptodecanol (MU), sodium phosphate monobasic (Na\textsubscript{2}HPO\textsubscript{4}; 99.0%), sodium phosphate dibasic (Na\textsubscript{2}HPO\textsubscript{4}; 99.0%), sodium bicarbonate (NaHCO\textsubscript{3}), copper(II) chloride, 3,3'-dithiobios (sulfosuccinimidil-propionate) (DTSSP), sodium dodecyl sulfate (SDS) and
epigallocatechin gallate (EGCG) were purchased from Sigma-Aldrich (Oakville, ON). Hydrogen peroxide (30% v/v) was obtained from EMD Inc., (Mississauga, ON). Polystyrene microspheres (10% w/w, 1.00 μm i.d.) were purchased from ThermoScientific Co (Mississauga, ON). All samples were of analytical grade and prepared in phosphate buffer saline (50 mM, PBS), with 100 mM NaCl at pH 7.4 using 18.2 MΩ ultra-pure water obtained from a Cascada LS water purification system (Pall Co., Mississauga, ON), unless stated otherwise.

7.4.2 Fabrication of Au-ITO chips

Au-ITO surfaces were fabricated following a previously described protocol.\textsuperscript{45-46} Briefly, ITO glass substrates were sonicated in acetone for 5 min followed by cleaning with copious water several times. ITO substrates were then sonicated in a 5:1:1 mixture of ammonium hydroxide/hydrogen peroxide/ultrapure water for 1 h before the glass substrates were further sonicated for 15 min in water. Polystyrene beads of 1 μm were equilibrated to room temperature before an aliquot (30 μL) was mixed with 100% (v/v) ethanol in a 1:1 ratio. An aliquot (20 μL) was then deposited on the dried ITO surface before immediately introducing it to the air-water interface of a Petri-dish filled with ultrapure water. ITO glass floated on the air-water interface as the solution spread out. After the microsphere solution was dispersed, a drop of 2% (w/v) SDS solution in water was added to the substrate to promote the formation of an ordered monolayer. ITO substrate was then left to dry. After samples were dried, 3 nm of Ti and 30 nm or 400 nm of Au were deposited using an electron beam evaporator (Hoser, Ottawa, ON). The beads were then removed by sonicking the samples in ethanol for about a minute before drying with nitrogen (Scheme S1).

7.4.3 Electrochemistry

Electrochemical analysis was performed using a PGSTAT302N Metrohm Autolab potentiostat (Metrohm, Switzerland) and operated with the General Purpose Electrochemistry Software (GPES). Au-ITO surface was activated using 2 mM DTSSP in 100 mM Na\textsubscript{2}CO\textsubscript{3} (pH 8.5) by leaving it overnight at 4°C.\textsuperscript{47} Unbound DTSSP was later removed by stringent rinsing with water. After the immobilization of 10 μM αS oligomers in a droplet of 35 μL overnight, the unreacted DTSSP groups on the surface were blocked by incubating with 100 mM Tris-HCl (pH 7.5) at room temperature for 1 h. Backfilling of the non-coated Au surfaces was achieved by the immobilization of 1 mM MU (in 40% (v/v) ethanol) for 10 min, followed by ethanol rinsing
Aliquots (20 µL) of 50 µM EGCG and Cu(II) were applied at to the predetermined spots on the chip surface and allowed to incubate over 48 h, while electrochemical measurements were intermittently performed. Control experiments were also performed by incubating EGCG and Cu(II) on solely MU-modified surfaces to determine the non-specific adsorption of those molecules.

**Scheme 1.** Illustrative representation of αS immobilization onto Au-nanopyramids on ITO surfaces. a) DTSSP was immobilized as a linker for the covalent attachment of b) αS. The unmodified DTSSP was quenched using c) Tris-HCl and the pinholes on the Au-nanopyramids were backfilled using d) MU. Other conditions were as described in the Experimental section.

CV and DPV measurements were performed using a three-electrode system that consisted of the Au-ITO surfaces as the working electrode, a leak-free miniature Ag/AgCl reference electrode (2 mm i.d., eDAQ Inc., Colorado Springs, CO) and a Pt wire as the counter electrode. An aliquot (35 µL) of 10 mM [Fe(CN)₆]³⁻/⁴⁻ solution was dispensed onto the predetermined spots on the surface. CV measurements were taken at various scan rates from -0.25 V to 0.65 V (vs.
Ag/AgCl). DPV was performed under the step potential of 5 mV with an amplitude 50 mV. The nanostructured surfaces were stored in a fridge at 4°C for 14 days, while CV measurements were intermittently performed at the various scan rates (10, 25, 50, 100, 250 and 500 mV/s) to monitor the changes in the electrochemical characteristics of the surfaces over time. Raw voltammograms were treated with Savitzky-Golay smoothing and baseline correction with a moving average peak width of 4 mV. All measurements were repeated (n≥3) to ensure statistical relevance.

7.4.4 Localized surface plasmon resonance (LSPR)

The optical system was comprised of a spectrophotometer (USB-4000-UV-Vis), a tungsten halogen light source (LS-1-LL, wavelength range 200-1100 nm), a microfiber probe bundle (fiber core diameter 400 μm, wavelength range 300-1100 nm) and WS-1 diffuse reflectance standard, that were obtained from Ocean Optics. (Dunedin, USA) The diffuse reflectance standard was used as a Lambertian reference surface. The microfiber probe was placed close to the working electrode surface so that incident light was reflected upon hitting the surface and then backed in the detector situated in the light probe. The absorbance mode of the Spectra Suite software was used to measure the spectral characteristics of the samples using 400 nm Au-pyramids coated ITO surfaces. The intensity and wavelengths of spectral peaks were recorded as modifications were made to the surfaces. The probe height was held constant (~1 mm above sample surface) throughout study. All experiments were performed at room temperature. (23 ± 2°C).

7.4.5 ThT fluorescence

The stock solution of 10 mM ThT was prepared in 18.2 MΩ water and protected from light. Fluorescence measurements were conducted in 96-well plates (BD Biosciences, Mississauga, ON) using a Synergy HT Multimode Microplate reader (BioTek, Winooski, VT). Each sample well contained: 50 μM ThT, 10 μM αS in the presence or absence of Cu(II) and EGCG (50 μM each). All experimental conditions were repeated in triplicates (n=3). Control experiments were also performed for each condition Results were normalized with their respective control fluorescence values to prevent any complications from the quenching of fluorescence by the small molecules. Spontaneous aggregation of αS samples was induced by incubation at 37±1°C.
with shaking at 300 rpm. Fluorescence (\( \lambda_{ex} 440 \text{ nm}, \lambda_{em} 485 \text{ nm} \)) was recorded at various time intervals for over \(~10\) days.

### 7.4.6 Surface plasmon resonance imaging (SPRi)

\( \alpha S \) samples were incubated at 37\(^{\circ}\)C for 6 days to form oligomers (as confirmed by ThT and TEM studies). Au spots of an SPRi array (GWC SpotReady-16 chip) were activated using 2 mM DTSSP that was deposited onto Au spots, and left overnight at 4\(^{\circ}\)C. Non-specifically adsorbed DTSSP was later removed by rinsing with water. After the immobilization of 10 \( \mu \)M \( \alpha S \) overnight, the unreacted functional groups were blocked using 100 mM Tris-HCl (pH 7.5) incubated at room temperature for 1 h. Additional blocking of exposed Au surfaces was achieved by incubating with MU as described for the Au-ITO surfaces before. Control experiments were performed on five Au spots (processed with all previous steps except the protein immobilization). Finally, the SPRi array was mounted in the SPR imager-II system (GWC Technologies, Madison, WI). The SPRi-based detection principals were described in detail in our previous work.\(^{35}\) In brief, p-polarized light, passing through a prism, was used to illuminate the Au surfaces at a fixed incident angle slightly smaller than the SPR angle. The reflected light then passed through a narrow band-pass filter centered at 830 nm, and was collected using a CCD camera in connection with V++ 4.0 (Digital Optics, NZ). All the experiments were measured at 37\(^{\circ}\)C. The Au array was located inside the sealed flow cell, through which EGCG solution was circulated at varying concentrations at a flow rate of 100 \( \mu \)L/min. PBS wash was performed after EGCG exposure to remove non-specific binding. The increase in pixel intensity was then observed as a difference image. After converting to% reflectivity, the signals were plotted on a graph against time. The changes in reflectivity of all spots were normalized with the Au control spots on the array to account for the non-specific binding events. SPR data was analyzed using the Langmuir model in a customized software (GWC Technologies, Madison, WI).

### 7.4.7 Transmission electron microscopy (TEM)

An aliquot (6 \( \mu \)L) of 10 \( \mu \)M \( \alpha S \) oligomers was spotted onto nickel Formvar mesh grids (Electron Microscopy Sciences, Hatfield, PA) for 1 min and blotted dry. TEM grids were subsequently stained using 6 \( \mu \)L of 1% uranyl acetate for 1 min followed by blot drying. Samples were imaged using a Hitachi H-7500 TEM, which was operated at a range between 2 and 200 kV depending on the magnification required. Similar imaging experiments were performed using the \( \alpha S \)
oligomers that were exposed to 50 µM Cu(II) and EGCG after 48 h of incubation.

7.4.8 Scanning electron microscopy (SEM)

A thin layer of Os was deposited and the samples were imaged using an SEM system (LEO Zeiss 1530, Oberkochen, Germany). The electron gun voltage was set at 5.0 kV and the software used to view the SEM images was Quartz PCI (Quartz Imaging Corp., Vancouver, BC). SEM of microspheres on ITO surfaces was performed using a Hitachi S530 scanning electron microscope (Hitachi, Japan). All ITO surfaces were sputtered with Au using the SEM coating unit PS3 (Agar Scientific, Essex, UK) at 19 mA plasma current for 100 s. The Au-ITO surfaces were then electrically connected to the sample stub by smearing silver paste dissolved in acetone from the sample to the metallic stub. The surface was observed at an acceleration voltage of 20 kV with a working distance of 5.0 mm.

7.5 Results and Discussion

Initially, the interaction of αS oligomers with EGCG and Cu(II) was monitored using electrochemistry on Au-ITO surfaces. We hypothesized that the covalent immobilization of αS on the Au nanopyramids provided a large surface and flexibility for the dynamic aggregation of fibrils, while suppressing the steric hindrance. Using NSL on ITO surfaces, nanostructures resembling triangles were fabricated by depositing a 30-nm thick layer of Au (Figure 1A). When a 400-nm thick layer of Au was deposited on the surface, due to the large curvature of the polystyrene beads (inset of Figure 1B), the resulting shapes of the deposited Au resembled pyramid-like nanostructures (Figure 1B). When the Au-nanostructured ITO surfaces were used as a working electrode in CV measurements, the anodic and cathodic peaks appeared at approximately +0.27 and -0.15 V (vs. Ag/AgCl), respectively. The contribution of Au nanostructures to increase the electro-active surface area was studied using CV (Figure 2) and DPV (Figure S1). A significant increase in current signal was observed using the Au-nanopyramid modified ITO electrodes. From the DPV analysis, the relative increase in current signal for nanopyramid- and nanotriangle-modified ITO surfaces were determined as 26.6±5.5% and 3.9±1.3%, respectively. These results were attributed to the significantly larger electro-active surface area available on the nanopyramids as compared to the nanotriangles. Besides providing a larger electro-active surface area, the Au-nanopyramid modified ITO (Au-ITO) also allowed
more space for the immobilization of thiolated molecules. Thus, Au-ITO surfaces were utilized for subsequent measurements.

**Figure 1.** SEM images of (A) Au-nanotriangles and (B) Au-nanopyramids fabricated using NSL at 20,000 X and 30,000 X magnification, respectively. Inset depicts the SEM of nanospheres taken at 45° angle before removal by sonication. Other conditions were as described in the Experimental section.

**Figure 2.** Cyclic voltammograms of 10 mM [Fe(CN)₆]³⁻/⁴ at (A) nanotriangle-modified ITO (grey line) and (B) nanopyramid-modified ITO (grey line) electrodes at a scan rate of 50 mV/s. Black line displays the CV response recorded for the blank ITO electrodes before Au nanostructure modification. Other conditions were as described in the Experimental section.
Incubation of αS at 37°C with shaking for 6 days promoted the formation of oligomeric species as described by Danzer et al.\textsuperscript{16} After activating the Au-ITO surfaces with DTSSP, the oligomers were spotted on the chip surface for covalent immobilization, followed by the quenching of the remaining functional groups using Tris HCl as described in the Experimental section. Uncoated Au surfaces were also blocked by incubating with MU. After these modifications, a 55.8±12.3% decrease in current signal was observed (Figure 3A and B). This implied that the protein and blocking layer contributed a significant resistance to the diffusion of [Fe(CN)\textsubscript{6}]\textsuperscript{3-4} to the electrode surface. CV at varying scan rates were performed as shown in Figure S2. The inset of Figure S2 shows the plot of peak current (i) vs. (scan rate)\textsuperscript{1/2}. The linear correlation demonstrated the reversible and diffusion-controlled characteristics of the redox processes on Au-ITO surfaces. A stability experiment was also performed over 2 weeks to ensure that the current signal did not fluctuate over time. Figure S3 shows that the CV characteristics were similar within ~5% error over time.

Cu(II) and EGCG were also individually incubated with the protein immobilized Au-ITO surfaces for 48 h to determine their effects on αS aggregation kinetics. As shown in Fig. 3C, in the presence of EGCG, the current signal decreased over time, while in the presence of Cu(II), the current signal increased. Control experiments that were performed in the absence of αS, displayed negligible current signal changes over time (not shown), indicating that the changes in current were not contributed by non-specifically adsorbed Cu(II) or EGCG on surfaces. The electrochemical results were in agreement with the TEM images (Figure S4). TEM images were taken to observe αS aggregation in 20% (v/v) ethanol (Figure S4A and B). We observed that after two days of incubation, short nuclei of about ~50-100 nm were formed, and in 6 days, oligomeric forms of αS were formed. The αS oligomers were up to several μm in length, collectively forming a mesh-network. Since previous reports indicated that αS aggregation was a seed/nucleation-dependent process,\textsuperscript{48-49} the strand-like structures might have elongated from the ends of the short aggregates (formed in 2 days). After incubating the αS oligomers with Cu(II) for 48 h, a more compact network was formed (Figure S4C). On the other hand, when EGCG was incubated with αS oligomers for 48 h, we observed the formation of optically dense amorphous aggregates (Figure S4D), that were similar to those formed in our previous report about amyloid-β peptides.\textsuperscript{35} Natalello and co-workers\textsuperscript{28} reported that Cu(II) binding to αS induced extensive structural rearrangement of the protein, where αS was found in a highly
compact state. We hypothesized that the dynamic rearrangement of αS oligomers into compact fibrils could have induced an increase in current signal by exposing more electro-active surface enabling the diffusion of $[\text{Fe(CN)}_6]^{3/-4}$ to the surface. There may also be important effects of metal binding on the enhanced electro-activity on Au-ITO. Since Cu(II) binds tightly to αS, the presence of metals embedded in the αS fibrils could have facilitated the charge transport to the Au-ITO surface.

**Figure 3** Representative differential pulse voltammograms of αS-immobilized Au-ITO surfaces, displaying the effect of (A) Cu(II) and (B) EGCG incubation on the anodic peak current signals at $t= 48$ h. (C) Electrochemical analysis of EGCG and metal interactions with αS oligomers showing time dependence study of DPV anodic peak current of 10 mM $[\text{Fe(CN)}_6]^{3/-4}$ after incubating 50 μM EGCG (light grey) and Cu(II) (dark grey) on corresponding immobilized αS oligomers on Au-ITO surfaces. Other conditions were as described in the experimental section.
The Au nanostructured surface had a uniformly repetitive pattern, thus generating an LSPR effect upon excitation by light. The surface showed an LSPR absorbance peak at about 606 ± 0.6 nm with 0.446 ± 0.008 a.u. in intensity (Figure 4A). Au-ITO surfaces modified with αS, MU and EGCG were utilized for LSPR measurements (Figure 4B). The LSPR peak intensities and associated λ_{lspr} increased with the addition of αS and MU layers. Formation of these layers altered the electron density of the Au nanostructures, which then directly affect the surface plasmon absorption band and caused a bathochromic shift. Upon the interaction of EGCG with αS on the surface, the absorption of the plasmon mode increased and displayed a further bathochromic shift of 10-15 nm. This was in agreement with previous LSPR-based studies^{50-51} and electrochemical data that supported the potential of using Au-nanopyramid coated ITO surfaces for future LSPR applications.

**Figure 4.** (A) Plot for the variations in LSPR absorption band peak wavelengths and intensities (B) LSPR spectra observed on 400 nm pyramid Au-ITO surfaces as different target biomolecules interacted with αS. Other conditions were as described in the Experimental section.

In order to support our electrochemical and LSPR data, the aggregation of αS in the presence of Cu(II) and EGCG was analyzed using the well-described ThT fluorescence assay (Figure 5).^{52} The several orders of magnitude increase in ThT fluorescence intensity upon fibril-binding makes it an especially sensitive indicator to detect the formation of β-sheets.^{53} Following the protocol by Danzer *et al.*16, the polypeptide was incubated in the presence and absence of 20% ethanol with perturbation by shaking at 300 rpm in order to produce αS oligomers. The
oligomeric forms of αS were used in this report, as they were reported to be the toxic species leading to neuronal death. It was found that αS, in the presence of ethanol, significantly promoted aggregation and increased ThT fluorescence over ~10 days; the signal increase was more than 10-fold smaller in the absence of ethanol (Inset of Figure 5). The increase in ThT fluorescence induced by the formation of β-sheets reached a plateau in ~7 days, in agreement with a previous report. Cu(II) and EGCG were also separately incubated with oligomeric αS in the presence of ethanol. It was observed that αS incubated in the presence of Cu(II) displayed higher ThT fluorescence as compared to the αS alone (Figure 5), indicating that Cu(II) promoted the formation of β-sheets. This was consistent with the comparable ThT studies performed in the absence of ethanol (Figure S5), as well as, in the previous literature that reported the acceleration effect of Cu(II) on αS aggregation.

![Graph](image_url)

**Figure 5.** Average relative fluorescence intensity of ThT incubated with 10 µM αS and 20% ethanol monitored over 10 days in the presence and absence of 50 µM Cu(II) and EGCG. Triplicate measurements were performed for each sample (n=3), and error bars were less than 50 a.u. (not shown). Other conditions were as described in the experimental section.
In the presence of EGCG, the formation of αS fibrils were suppressed as observed from negligible ThT fluorescence (under 60 a.u.) recorded in over 10 days, indicating that the amorphous aggregates observed in TEM did not contain β-sheets. The low ThT fluorescence indicated that unstructured aggregates were formed.\(^{34, 37}\) We have also shown in our previous report\(^{35}\) that there was negligible influence of EGCG on ThT fluorescence in the presence of amyloid-β peptides related to AD.

To verify the formation of αS-EGCG complexes on surfaces, SPRi studies were also performed. The binding affinity of EGCG was determined using αS oligomers that were formed in 6 days of incubation. αS oligomers were first immobilized on the Au surfaces of the array using the DTSSP-based method as described in the Experimental section. EGCG samples at varying concentrations were then exposed to the array at a flow rate of 100 µL/min. As the EGCG interacted with the immobilized αS, the reflectivity ratio increased as shown in Figure S6). After \(~150\) s, PBS buffer was flown across the chip surface to remove non-specifically bound EGCG, causing a drop in the reflectivity ratio. Using the Langmuir model to fit the different concentration curves (inset of Figure S6), the equilibrium dissociation constant, \(K_D\) between EGCG and αS oligomers was calculated to be \(2.71 ± 0.54\) µM. A similarly strong affinity between EGCG and αS was also observed by Bieschke \textit{et al.}\(^{34}\) in their recently published report.

**7.6 Conclusions**

We demonstrated the preliminary data from a promising dual detection platform for monitoring the morphological changes of αS oligomers upon small molecule interactions using electrochemistry and LSPR. The changes in the structure of αS oligomers were manifested as fluctuations in DPV peak current and LSPR absorption band signals. To the best of our knowledge, this was a novel attempt to monitor structural changes of immobilized αS oligomers by following the changes in the well-described electrochemical properties of \([\text{Fe(CN)}_6]^{3-/4}\). Our preliminary electrochemical and LSPR results were complemented by established techniques for the detection of protein misfolding. ThT fluorescence and TEM imaging studies suggested that dense and unstructured amorphous αS aggregates were induced by EGCG, while β-sheet-rich and compact αS mesh-networks were promoted by Cu(II) ions. SPRi studies also confirmed the strong binding affinity of EGCG to αS aggregates on Au surfaces. The reported electrochemical system is a promising platform for the low-cost and high-throughput screening of small drug
candidates that target αS, and would accelerate the drug discovery efforts towards the therapy of PD.
7.7 References


Chapter 8

8 Summary and Conclusions

Misfolded proteins are usually insoluble and tend to form fibrillar aggregates known as amyloid deposits. Amyloidogenic protein fibrils are well known pathological hallmark of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). AD and PD are the most common neurodegenerative diseases but there is currently no cure for these multifactorial diseases. Alzheimer's disease is the most prevalent form of progressive dementia characterized by severe impairments to judgement, decision making, language and memory. PD is the second most common neurodegenerative disorder, characterized by resting tremor, rigidity and bradykinesia. Pathologically, there is a loss of neurons in mainly the substantia nigra. In AD, Aβ protein are found mostly extracellularly while in PD, α-S are mostly found in Lewy bodies and neurites, located in neurons. Current diagnosis and therapeutics have umpteen room for improvement as there is currently few successfully established pre-mortem diagnosis or therapeutics for these diseases. The marginal benefits provided by current drugs emphasize the urgent need to develop and screen for effective AD and PD drugs.

As the toxicity in these neurodegenerative diseases is highly correlated with the formation of soluble oligomers from their corresponding proteins, a strategy that may help curb the progression of AD and PD is to inhibit the aggregation of Aβ and α-S respectively or to alter their aggregation pathways. In the case of AD, many types of small molecules could be promising in the alteration of Aβ. Researchers could target the γ and β secretases, but there are toxicity and size (too large to pass through blood brain barrier) challenges respectively. Another approach is to remove Aβ altogether in the brain by introducing immunotherapy. An example is AN1792 vaccine designed to sequester Aβ; It has failed in Phase II clinical trials due to the development of meningoencephalitis, suggesting Aβ may be important for physiological health and inhibiting Aβ may not be a sound strategy. Recent research has led scientists to believe that more specifically, Aβ's aggregation into prefibrillar oligomers is the key pathogenic event in the onset of AD. Therefore, blocking Aβ aggregation into oligomers has emerged as a valid disease modulating therapy for AD. Interestingly, similar small molecules also inhibit the formation of toxic α-S oligomers in the case of PD. Besides inhibiting the aggregation of these amyloidogenic proteins, they are also known to have neuroprotective effects in a number of paradigms. It is also generally know that metals such as Cu²⁺, Fe²⁺ and Zn²⁺ modulates
these protein aggregation to induce higher toxicity in cells. It is probable that metal chelators and beta-sheet breakers are potential small molecules that could reduce amyloid mediated cell toxicity.\textsuperscript{7} Therefore it is promising to generate a library of compounds based on rational design\textsuperscript{8-12} to tackle the oligomer toxicity in these neurodegenerative diseases. In this PhD thesis, many novel analytical methods were developed and tested as a screening tool for these protein misfolding inhibition screening, compared with traditional detection methods.

There is an ongoing demand for fast and simple analytical methods for the point of care diagnostics of many biomarkers. Electroanalytical techniques utilize electrical quantities like current, charge or potential to measure chemical parameters. These methods have been applied in biomedical analysis, environmental monitoring, quality control and many other fields. Electrochemical detections are commonly known for its high sensitivity, fast analysis, portability and real-time monitoring capabilities.

While the use of electrochemical detection in protein aggregation studies is an emerging field, there are many established methods used in the monitoring of protein interactions. For example, Thioflavin T and Congo Red have been known to intercelate beta sheet structures and alter their fluorescent activities. These changes in fluorescence of the dyes provide us with insights into the process of amyloid aggregation. We have used Thioflavin T and Congo Red to characterize the amyloid protein (prepared under various conditions) aggregation and juxtaposed these trends against those measured by our novel analytical methods.

In this PhD Thesis research, many non-conventional methods- acoustic, optical and electrochemical, were used in the characterization of amyloid aggregation as well as amyloid interactions with small molecules. Aβ aggregation was monitored using electrochemistry, acoustic wave sensor and BiacoreX™ surface plasmon resonance (SPR). Because there are electro-active tyrosine (Tyr) residues in Aβ peptides, electrochemical monitoring of aggregation state utilizes the decrease in Tyr access to the electrode surface during misfolding to track the progress of fibril formation. Acoustic wave sensor and SPR utilizes the real mass and optical mass of Aβ respectively to determine if elongation of monomeric Aβ has occurred on immobilized Aβ nuclei sites on the transducer surface.

We have shown in these studies that 1) aggregation could be monitored through these label-free methods and 2) Aβ interaction with clioquinol (CQ) greatly hampers the aggregation/elongation of Aβ in all three analytical methods. This effect of CQ is supported by previous literature.\textsuperscript{13} Thus,
these label-free methods show high promise in screening for other aggregation modulators, which would be highly valuable in developing new therapeutic agents against AD.

Even though BiacoreX™ SPR could monitor the aggregation process, it has only two flow channels. The previous experiments demonstrated a good proof of concept that SPR is highly promising as a technique in the screening of small molecules that inhibits amyloidogenic proteins. However, BiacoreX™ can only monitor the effects of one drug per test. This low throughput led to our next test of GWC™ SPRimaging (SPRi) as a platform to screen multiple drugs in one test. Epigallocatechin gallate (EGCG) and metals were used as model modulators since they inhibit and promotes the aggregation process of Aβ respectively. We demonstrated the feasibility of SPRi as a multiplexed system to screen for modulators of protein misfolding and formation of β-sheet-rich amyloid fibrils. Transmission electron microscopy (TEM) images showed that denser and more abundant fibril networks were formed for Aβ1-40 in the presence of metals. While in the presence of a metal-chelating EGCG, amorphous aggregates were observed. Thioflavin T (ThT) results implied that these amorphous aggregates did not contain β-sheets during seed formation. After testing multiple modulators of Aβ on an array using the SPRi platform, the effects of these modulators on Aβ were supported by TEM and ThT fluorescence results. Therefore, SPRi would be highly promising in the screening of small molecules that can affect the protein aggregation pathways in neurodegenerative diseases.

In an effort to further increase the throughput of screening, LED-Interferometric Reflectance Imaging Sensor (LED-IRIS) was utilized for the first time to study the interaction of Aβ with small drug candidates and metal ions. The technique utilizes common-path interferometry through a Si/SiO2 layered substrate as the sensing surface to detect local path length changes as a result of mass accumulation with an increase in the spot height at the surface. LED-IRIS could measure up to hundreds of samples in one experimental run to monitor biomolecular interactions with low noise floor in real-time. We successfully demonstrated that the LED-IRIS could monitor the different effects of EGCG and Zn2+ on Aβ aggregation, holding promise for this technique to screen other novel Aβ aggregation modulators to accelerate the drug discovery efforts towards AD therapy.

In future work, we hope to quantify the aggregation inhibition potential of novel drugs that were screened by these biosensors mentioned above. To do this, additional analyses of larger inhibitor
libraries and varying classes of amyloid modulating agents are cardinal to further validate the utility of these methods.

Because PD is an important dementia that is prevalent in the older population, its associated protein, α-S, was also studied. We demonstrated the effects of Cu(II) ions and CQ on the aggregation of α-S, characterized using ThT fluorescence and electrochemical techniques. It is important to know the effects of such metals on the physical morphology of the protein in order to further study metal chelators as promising therapeutic agents for PD. Both electrochemical and spectroscopic studies showed that Cu(II) accelerated the fibril formation of α-S, while CQ inhibited such activity. The simple fabrication and rapid detection nature of our electrodes enabled the study of protein morphology changes at the carbon/aqueous interface. It is promising to apply this platform to monitor effects of other modulators on α-S aggregation which may be useful in the screening of future PD therapeutics.

The electrochemical method was further evolved to include an optical screening test on the same transduction platform by utilizing nanosphere lithography (NSL). NSL is attractive for its low-cost fabrication of well-ordered 2D arrays of periodic nanostructures over large surfaces, which is crucial in localized surface plasmon resonance (LSPR) effects when probed with light. LSPR is a phenomenon that occurs when noble metal nanoparticles resonate in response to optical excitation. The intensity of the LSPR absorption peak increases with binding of biomolecules to the surface. It is known that the uniformity of Au nanostructures on surfaces affected the width and sensitivity of the LSPR band. Our electrochemical and LSPR results were complemented by established techniques for the detection of protein misfolding. ThT fluorescence and TEM imaging studies suggested that dense and unstructured amorphous αS aggregates were induced by EGCG, while β-sheet-rich and compact αS mesh-networks were promoted by Cu(II) ions. SPRi studies also confirmed the strong binding affinity of EGCG to αS aggregates on Au surfaces. In light of all the above results, electrochemical biosensors pose as highly promising tools to study the effects of different modulators on amyloidogenic protein aggregation. Apart from high sensitivity, rapid detection and real-time monitoring capabilities, these electrochemical detection platforms have the potential to be miniaturized and mass produced at low cost. These benefits warrant further investigation into the development of these systems to be used as point-of-care devices in clinical settings.
8.1 References


Appendix A - Supporting Data for Chapter 3

Figure S1. Typical frequency against time graphs of PBS incubated quartz crystal being perturbed by A) 2μM Aβ 1-42 monomers and B) 10μM CQ only, showing little or no net frequency changes after PBS wash. The two vertical lines indicate the injection of the samples onto the quartz crystal surface and subsequent PBS wash after 50 min.

Figure S2. A) Fibril aggregates formed at the bottom of micro-centrifuge tube. B) Solution turned clear after sonication, indicating the breaking up of fibrils into soluble seeds.
Figure S3. 10μM CQ introduced across the immobilized seed surface to show no changes in frequency values.

Figure S4. SPR sensorgram of resonance units (RU) against time showing a (1) sudden increase in RU due to the switch of buffer due to the injection of samples and subsequent (A) stepwise increase in RU during fibril fragment immobilization and (B) smooth increase in RU for Aβ 1-42 monomer injection. The (2) drop in RU was due to the injection of running buffer.
Figure S5. Graph to show RU increase of various conditions on controlled passivated surface and on 15μM immobilized seed surface.

Figure S6. SPR sensorgram of RU against time showing serial injections of 2μM Aβ 1-42 monomer causing a consistent increase in RU and 10μM CQ injection resulting little or negligible increase in RU on a 15μM fibril seed immobilized surface.
Appendix B - Supporting Data for Chapter 4

Table S-1. Association constants of Aβ monomers binding to different fibril seed spots calculated using GWC imager II V++ software. Errors indicate the standard deviations of replicate measurements (n≥3)

<table>
<thead>
<tr>
<th>Fibril Seed Spots</th>
<th>$K_a \times 10^6 \text{ M}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>1.65 ± 0.03</td>
</tr>
<tr>
<td>$\alpha + \text{Fe(III)}$</td>
<td>1.71 ± 0.04</td>
</tr>
<tr>
<td>$\alpha + \text{Zn(II)}$</td>
<td>1.72 ± 0.01</td>
</tr>
<tr>
<td>$\alpha + \text{Cu(II)}$</td>
<td>1.70 ± 0.03</td>
</tr>
<tr>
<td>$\alpha + \text{Fe(III)} + \text{EGCG}$</td>
<td>1.54 ± 0.04</td>
</tr>
<tr>
<td>$\alpha + \text{Cu(II)} + \text{EGCG}$</td>
<td>1.55 ± 0.02</td>
</tr>
<tr>
<td>$\alpha + \text{Zn(II)} + \text{EGCG}$</td>
<td>1.54 ± 0.02</td>
</tr>
<tr>
<td>$\alpha + \text{EGCG}$</td>
<td>1.44 ± 0.05</td>
</tr>
</tbody>
</table>

Figure S-1. GWC SpotReady 16 chip spotted with 0.5 μL of fibril seed sample on each gold spot.
Figure S-2. Image of spots of one SpotReady chip in V++ software A) before immobilization of peptides, B) after immobilization and ROI selected, C) during monomer flow and D) expressed as pixel intensity against time graph of a particular spot.

**Fundamentals of SPR:**
When the light travels from the higher refractive index medium 1 to the lower refractive index medium 2, total internal reflection (TIR) can occur when the incident angle, $\theta$, is greater than the critical angle, $\theta_c$, where $\sin(\theta_c) = n_2/n_1$.

Evanescent waves are formed in the medium 2 during TIR. The amplitude of evanescent waves decays exponentially with the distance from the interface of the two media. When a gold film coats the interface, the evanescent wave is enhanced in medium 2. The magnitude of the parallel wave vector of the evanescent wave, $k_{\text{evan},||}$, can be expressed as
\[ k_{\text{evan.}} = \frac{2\pi}{\lambda} n_1 \sin(\theta) \]  

where \( \lambda \) is the wavelength of the incident light, \( n_1 \) is the refractive index of the higher refractive index medium 1, and \( \theta \) is the incident angle.

Surface plasmons are confined to the metal–dielectric interface. The magnitude of the wave vector of the surface plasmon \( (k_{SP}) \) is determined by the dielectric constants of the two media and the gold film. \( k_{SP} \) is determined by \( n_2 \) and \( n_g \) according to

\[ k_{SP} = \frac{2\pi}{\lambda} \sqrt{\frac{n_2^2 n_g^2}{n_2^2 + n_g^2}} \]  

where \( n_2 \) is the refractive index of medium 2 near the interface and \( n_g \) is the refractive index of the gold film.

The surface plasmon can be excited by the evanescent wave through wave vector coupling and this phenomenon is called surface plasmon resonance (SPR). During this event, the intensity of the reflected light decreases sharply. One requirement for the SPR is that \( k_{SP} \) equals to \( k_{\text{evan.}} \).

Thus, using eqs 1 and 2 gives the SPR angle \( (\theta_{SPR}) \), required for resonance,

\[ \theta_{SPR} = \sin^{-1} \left( \frac{1}{n_1} \sqrt{\frac{n_2^2 n_g^2}{n_2^2 + n_g^2}} \right) \]  

\( \theta_{SPR} \) is related to \( n_2 \) when \( n_1 \) and \( n_g \) are fixed. During molecular binding events near the gold surface, the refractive index of medium 2 is altered and the resonance angle changes accordingly. Therefore, the monitoring of the \( \theta_{SPR} \) shift can be used to analyze the association–dissociation events on the gold surface.
Figure S-3. Square wave voltammogram showing Cu\textsuperscript{2+} oxidation signal at -0.15 V, EGCG oxidation peaks at 0.05 and 0.15 V. The oxidation peak of Cu\textsuperscript{2+} decrease dramatically in the presence of EGCG.
Figure S-4. Cross seeding experiments to show A) stability of fibril seeds after ~1 h equilibration of chip surface with PBS buffer, B) normalized and overlayed SPR curves for all seed conditions and C) resultant pixel difference of each monomer injection in the presence and absence of modulators over all seed conditions. Errors indicate the standard deviations of replicate measurements (n≥3)
Figure S-5. ThT fluorescence of 50 μM Aβ over 80 h in the presence and absence of 5 μM EGCG and 50 mM PBS introduced into the wells at ~70 h. The fluorescence was re-acquired immediately after spiking and monitored for another 10 h. At least 5 replicates were performed for each condition and the error bars were within ± 25 a.u. (not shown for clarity)
**Sonicated**

\[ A\beta_{1-40} \]

![Sonicated Image](image1)

**Non-Sonicated**

\[ A\beta_{1-40} \]

![Non-Sonicated Image](image2)

\[ A\beta_{1-40} + Fe^{3+} \]

![Sonicated with Iron Image](image3)

![Non-Sonicated with Iron Image](image4)
$A\beta_{1-40} + Cu^{2+}$

$A\beta_{1-40} + Zn^{2+}$
$A\beta_{1-40} + \text{EGCG} + \text{Fe}^{3+}$

$A\beta_{1-40} + \text{EGCG} + \text{Cu}^{2+}$

$A\beta_{1-40} + \text{EGCG} + \text{Fe}^{3+}$

$\beta_{1-40} + \text{EGCG} + \text{Cu}^{2+}$
Figure S-6. TEM images of fibrils after 75 h incubation with shaking, showing the differences before and after sonication of fibrils. Measurement conditions are as described in the experimental section.
Appendix C - Supporting Data for Chapter 5

Fig. S-1 Schematic illustration of optical setup. The focal lengths of all lenses are given. Achromatic lenses are shown in blue; IRIS, the beam splitter, and the CCD (Retiga200R, Qimaging) are shown in black. ACULED VHL surface-mount LED package (Perkin-Elmer), which has four independently driven LEDs with peak emission wavelengths of 455 nm, 518 nm, 598 nm, and 635 nm was used.

Data Acquisition and Analysis:

We use the Fresnel reflections to calculate the amount of biomass added on top of the sensor surface by assuming it is an added thickness to the oxide top layer. The wavelength dependence of the Fresnel reflection is used to measure the thickness of the top layer of the sensor which consist of the silicon dioxide and biomass. Referring to equations 1, 2 and 3, shown below, we can determine 'd', the thickness of the top layer when 'R' the reflection coefficient is measured.
Equation 1:

\[ R = |r|^2 = \frac{r_1^2 + r_2^2 + 2r_1r_2\cos(2\phi)}{r_1^2r_2^2 + 2r_1r_2\cos(2\phi)} \]

where \( r_1 \) and \( r_2 \) are the Fresnel reflection coefficients of the air-SiO\(_2\) (or buffer-SiO\(_2\)) and Si-SiO\(_2\) interfaces respectively. The reflection coefficients can be calculated by

Equation 2:

\[ r_1 = \frac{n_{ox} - n_1}{n_{ox} + n_1} \quad \text{and} \quad r_2 = \frac{n_{Si} - n_{ox}}{n_{Si} + n_{ox}} \]

where \( n_1, n_{ox}, \) and \( n_{Si} \) are the refractive indices of air (or buffer), SiO\(_2\), and Si respectively. The optical path difference is described by the phase difference, \( \phi \), from equation 1, which is given by:

Equation 3:

\[ \phi = \frac{2\pi d}{\lambda} n_{ox} \cos\theta \]

Here, \( d \) is the thickness of the layer (SiO\(_2\) or SiO\(_2\) + biomass), \( n_{ox} \) is the refractive index of SiO\(_2\), \( \lambda \) is the wavelength of the incident light, and the \( \theta \) is the angle of incidence. IRIS uses low angles of illumination and collection; therefore, the angle of incidence can be assumed to be near zero (\( \cos\theta = 1 \)).

\( R \) are measured using the intensity measurements made by the CCD camera when illuminated with the 4 different LEDs sequentially. These data points, \((R, \lambda)\), are used to fit curves based on Equation 1 for each pixel. Spectral shifts in these Fresnel curves from pixel to pixel are used to determine the corresponding changes in surface thickness.
To monitor changes in biomass on the surface like fibril formation/growth the optical path difference at a region of interest in compared to the background where we expect no change. Fig. S-1 shows how circular spots are analyzed.

All the spots in the field of view (6 mm by 4.5 mm) can be detected/monitored. In the current study, we assumed all fibril growth are only occurring in the seed spots. The software used in this experiment was used to detect circular spots and the detection parameters can be adjusted based on the size of seeds spotted. The average measured height on the spot is subtracted from the average height over an annulus region that is sufficiently far away from the spot such that even if some fibrils formed out of the central spot, they will not affect the spot height measured. Dust and large contaminants (micron sized objects) will also not fit the Fresnel model; they are rejected because the residual to the fitting surpass a set threshold.

**Fig. S-2.** Absolute height of each spot was calculated by subtracting the average spot intensity of the background (red region) from that of the spot (green region). Dust particles were detected automatically by the developed MATLAB software as shown in yellow spots, and disregarded in the height calculation.

*Scanning Electron Microscopy*

In order to monitor the elongate fibril morphology, Aβ samples incubated in the presence and absence of EGCG or Zn(II) were imaged. As shown in Fig. S-2, it was observed that the Zn(II)-containing seeds showed denser fibril networks compared to those found on Aβ alone. EGCG-containing seeds displayed amorphous aggregates of dispersed networks, which did not seem to contain mature fibrils. These images supported the LED-IRIS results, which showed EGCG
inhibiting the fibril formation on seed spots, while the presence of Zn(II) facilitated its aggregation.

Fig. S-3. Representative SEM images of Aβ, at the surface of A) an unmodified LED-IRIS chip surface, and modified with B) Aβ alone, C) Aβ with Zn(II), and D) Aβ with EGCG. Other conditions were as described in the Experimental section.
Fig. S-4. Real-time monitoring of signal intensity for high (1:2) amyloid:modulator relative concentrations with monomers flowing at 100 µL/min through the microfluidic channel. The concentration of Aβ alone was 200 µM. Other conditions were as described in the Experimental section.

Fig. S-5. Compilation of cross-seeding experimental data using different seed conditions including A) 50 µM Aβ alone, and Aβ in the presence of 100 µM B) EGCG and C) Zn(II). Other conditions were as described in the Experimental section.
Appendix D - Supporting Data for Chapter 6

**Figure S1.** Presence of Cu(II) significantly decreases CQ oxidation signal over time. The graph shows the electrochemical signal anodic peak at ~0.54 V and cathodic peak of ~0.45 V of CQ over 3.5 h.

**Figure S2.** Plot for the relationship between the absorbance of Congo Red at 541 nm and time for 50 μM α-S (triangle) alone and in the presence of 100 μM CQ (diamond) or 50 μM Cu(II) (square) for over approximately 48 h.
**Figure S3.** Chalky appearance of Congo Red dye for α–S incubated with CQ over 48 h.

**Figure S4.** Fibrillar appearance of Congo Red dye for α–S incubated with Cu(II) over 48 h.
Figure S5. SEM images for the interaction of Cu(II) and CQ with α–S fibrillation. A) Fresh α–S fixed at 0 h. and after 24 h incubation at 37°C with agitation of B) 50 μM α–S alone and in the presence of C) 50 μM Cu(II) or D) 100 μM CQ. All images were taken under 6000 X magnification. (Scale bars represent 5 μm) Other conditions were as described in the experimental section.
Scheme S-1. Fabrication of Au-ITO process by NSL. Au deposition of different thickness on polystyrene beads results in the formation of nanotriangles or nanopyramids after the removal of beads by sonication from the ITO surfaces. Other conditions were as described in the experimental section.

Figure S-1. Differential pulse voltammograms of (A) 30 nm and (B) 400 nm Au nanostructures on ITO surfaces. Other conditions were as described in the Experimental section.
Figure S-2. Scan rate dependence of (10, 25, 50, 100, 250 and 500 mV/s) anodic peak current signal using 10 mM $[\text{Fe(CN)}_6]^{3-/4}$ performed on αS immobilized Au-ITO surfaces. Other conditions were as described in the Experimental section.

Figure S-3. Electrochemical stability test for the αS-immobilized Au-ITO surfaces that were stored at 4°C for two weeks. CV scans were measured on day 0, 3, 7, 10 and 14 at 25 mV/s. Other conditions were as described in the Experimental section.
Figure 4. TEM images of 10 μM αS in 20% ethanol after (A) 2 days and (B) 6 days incubation. Representative images of αS aggregates after incubation with (C) 50 μM Cu(II) and (D) 50 μM EGCG. All incubations were kept at 37°C with shaking at 300 rpm (each scale bar represents 500 nm). Other conditions were as described in the Experimental section.

Figure S-5. Average relative fluorescence intensity of ThT with 10 μM αS and 20% ethanol monitored over 10 days in the presence and absence of 50 μM Cu(II) and EGCG. Triplicate measurements were performed for each sample (n=3), and error bars were less than 50 a.u. (not shown for clarity). Other conditions were as described in the Experimental section.
Figure S-6. SPRi analysis for the interaction of αS with EGCG interaction. Representative sensorgrams were derived from the injection of different concentrations of EGCG over immobilized αS oligomers on the microarray. Kinetic data were fitted using a Langmuir binding model. Inset shows the Langmuir fit of the representative SPR sensorgram corresponding to 5 µM EGCG injection. Other conditions were as described in the Experimental section.