NANOFLOWIDIC BIOSENSING FOR β-AMYLOID DETECTION
USING SURFACE ENHANCED RAMAN SPECTROSCOPY (SERS)

A Thesis
by
I-HSIEN CHOU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2007

Major Subject: Biomedical Engineering
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Approved by:
Chair of Committee, Gerard L. Coté
Committee Members, Jun Kameoka
Kenith Meissner
Head of Department, Gerard L. Coté

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ABSTRACT

Nanofluidic Biosensing for β-amyloid Detection

Using Surface Enhanced Raman Spectroscopy (SERS).

(December 2007)

I-Hsien Chou, B.S., Tatung University, (Taiwan)

Chair of Advisory Committee: Dr. Gerard L. Coté

A nanofluidic biosensor using surface-enhanced Raman scattering (SERS) was developed to detect the β-amyloid (Aβ) protein, one of the biomarkers of Alzheimer’s disease (AD). Recent studies have indicated that investigating changes in relative concentrations of structure specific Aβ oligomers in cerebral spinal fluid (CSF) during the progression of AD could be important indicators for diagnosing AD pre-mortem. However, there is no definitive pre-mortem diagnosis of AD thus far because of the lack of technology available for sensitive Aβ detection. Hence, the development of a system for detecting the structure specific Aβ oligomers, along with the concentrations of these oligomers in CSF, would be useful in the investigation of the molecular mechanisms of Aβ cytotoxicity associated with AD.

In this thesis, a nanofluidic trapping device trapping system for detecting biomolecules at sub-picomolar concentrations was developed for using SERS. The device, with a microchannel leading to a nanochannel, carries out dual functions: encouraging size-dependent trapping of gold nanoparticles (60nm) at the entrance of the nanochannel as well
as restricting the target molecules between the gaps created by the aggregated nanoparticles.
Initially, the trapping capability of the nanofluidic device was tested using fluorescent polystyrene and gold nanoparticles. UV-vis absorption spectroscopy was used to characterize the gold nanoparticle clusters at the entrance to the nanochannel. The device established controlled, reproducible, SERS active sites within the interstices of gold nanoparticle clusters and shifted the plasmon resonance to the near infrared, in resonance with incident laser light.

Two strongly Raman active molecules, adenine and Congo red, were used to test the feasibility of the SERS nanofluidic device as a platform for the detection of multiple analytes. The results showed that strong SERS signals were obtained from the nanoparticle clusters at the nanochannel entrance.

Once the feasibility of the approach was determined with strong Raman molecules, Aβ was detected using this nanofluidic SERS platform. Distinct surface-enhanced Raman spectra of Aβ was observed in different conformational states as a function of concentration and structure (monomer versus oligomer form) due to Aβ refolding from α-helical to a predominantly β-pleated sheet form. The sensor was also shown to potentially distinguish Aβ from insulin and albumin, confounder proteins in cerebral spinal fluid. Thus, a novel platform was developed to detect picomolar levels of Aβ with the ultimate goal of facilitating the diagnosis and understanding of Alzheimer’s disease by means of detecting structure specific oligomers of Aβ.
DEDICATION

This thesis is dedicated to my family who has supported me in all of my endeavors.
ACKNOWLEDGEMENTS

I am very thankful to my advisor Dr. Gerard Coté for his guidance throughout the course of my research and during my time in his lab group. He has been an outstanding mentor and a great friend throughout my stay at Texas A&M University. A special thank you goes to Dr. Theresa Good for giving me guidance and insight in this research. I also want to thank Dr. Jun Kameoka and Dr. Kenith Meissner for providing knowledgeable information fulfilling this thesis.

I would especially like to thank Melodie Benford who has been kindly helping me correct my paper and thesis and Hope T. Beier who has been consistently helping me understand the intricacies of chemistry during my time here. Also, I would like to thank the students in Optical Biosensing Laboratory (OBSL) for their numerous suggestions.

On a personal note, I would like to express my most sincere gratitude to my family whose love, sacrifice and dedication are the biggest reasons behind all my success. They will always be a source of great inspiration in my life.
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CHAPTER I

INTRODUCTION

1.1 Background and Significance

Alzheimer’s disease (AD), a progressive neurodegenerative disease and the leading cause of dementia in the aging population, affects 4.5 million people according to the 2000 US census\(^1\). One of the primary pathological hallmarks of Alzheimer’s disease is the presence of insoluble neuritic plaques, composed primarily of \(\beta\)-amyloid peptide (A\(\beta\)), in the cerebral cortex. A\(\beta\) peptide, a natural metabolic byproduct, results from the proteolytic cleavage of the amyloid precursor protein, either in the membrane or while undergoing endosomal processing. During progression of the disease, 39-42 amino acid long peptides are released into the extracellular fluids\(^2\)-\(^9\) and can be found in other parts of the body, such as cerebral spinal fluid. These species have a strong propensity to aggregate. Many hypothesize that aggregation of A\(\beta\) triggers a cascade of events that brings about neurotic dystrophy and neuronal death. Currently, Alzheimer’s disease can only be diagnosed definitively by post-mortem identification of neuritic plaques and neurofibrillary tangles in central nervous system tissue.

This thesis follows the style of *Journal of Analytical Chemistry*. 
While methods exist for probable pre-mortem diagnosis of AD, including in vivo imaging of the brain with magnetic resonance imaging or functional positron emission tomography, along with tests of cognitive and psychological function\textsuperscript{10, 11}, reliable methods of pre-mortem diagnosis are needed and may involve the use of biomarkers of AD such as Aβ.

The most prevalent species of Aβ present in people with AD are Aβ (1-40) and Aβ (1-42). Both forms of Aβ are found in cerebrospinal fluid (CSF) and blood plasma of all people, regardless of health\textsuperscript{2-9}. Aβ (1-40), the dominant peptide species, has a concentration of 5nM in CSF\textsuperscript{3}. The relative concentrations of different Aβ species including Aβ (1-40), Aβ (1-42), and their conformation/aggregation states change with the progression of disease\textsuperscript{12}.

Most investigators believe that the cytotoxic species of Aβ are one or more of the following species: soluble oligomers, Aβ derived diffusible ligands (ADDL)\textsuperscript{7}; protofibrils\textsuperscript{6, 13}, and/or heterogeneous globular species\textsuperscript{9}. These species arise during the misfolding and aggregation of the nontoxic, soluble Aβ monomer that is predominantly α-helical and random coil in structure to a conformational form of Aβ with abundant β-pleated sheets (i.e., Aβ fibrils and protofibrils)\textsuperscript{14-16}. However, the degree of neurotoxicity and relevance to disease progression of each conformational form of Aβ is still under debate\textsuperscript{17, 18}. Even the structure of the Aβ fibril, described by the best studies of aggregated Aβ species, has only recently been elucidated (and not without some disagreement about the exact structure)\textsuperscript{19-22}. Needless to say, the structure of soluble Aβ oligomers has not been determined\textsuperscript{13, 23-25}. The development of tools useful in probing the structure of disease associated Aβ species would
be useful in the investigation of the molecular mechanisms of Aβ cytotoxicity associated with AD.

The toxic form of Aβ is associated with the change in secondary and tertiary structures, which lead to the aggregation of the peptide. Current methods for probing the secondary and tertiary structure of proteins include Fourier transform infrared spectroscopy (FTIR), circular dichroism spectroscopy (CD), nuclear magnetic resonance (NMR) spectroscopy, and X-ray diffraction. X-ray diffraction and NMR have cumbersome preparation techniques. For FTIR, sampling is often difficult and it has strong absorption background due to water. X-ray diffraction and CD are limited by the state of the probed protein; molecules probed with X-ray diffraction must be crystallized, while the molecules must be in solution for CD. However, Aβ does not crystallize, and toxic forms of Aβ do not all stay in solution. Plus, CD lacks the sensitivity required to detect the lower range of toxic concentration of Aβ. Hence, development of a sensor platform that enables the detection of structure specific oligomers of Aβ along with conformational changes of the protein would be a valuable tool in aiding the understanding of AD pathology and the development of a pre-mortem diagnostics for this disease.

In the work presented here, the feasibility of using surface-enhanced Raman spectroscopy (SERS) to detect Aβ(1-40), one of the two most prevalent Aβ species, in CSF is addressed. Past protein studies involving Raman spectroscopy included probing particular amino acid residues in local environments, as well as structural investigation of proteins in biological samples. However, Raman spectroscopy alone is not an efficient detection tool because of its low sensitivity, small optical cross-section, and the effects of intrinsic
fluorescence and absorption of biomolecules on the spectra. Surface-enhanced Raman scattering can provide a significant enhancement of the Raman signal intensity of a molecule by several orders of magnitude (10^6 to 10^{12}) through electromagnetic field enhancements and chemical enhancements due to adsorption of molecules onto metal surfaces. The effect of the electromagnetic enhancements is believed to be the dominant mechanism responsible for the enhancements found in SERS. Extreme SERS enhancements of 10^{12}-10^{15} are observed when molecules of interest are adsorbed onto surfaces of gold nanoparticles, followed by electrolyte-induced aggregation. However, such enhancements are observed only on certain unknown active spots or “hot spots” that provide the appropriate electromagnetic nano-environment, which has not been reproducible in the past, a major hindrance of aggregated particle-based substrates for biological applications.

Recent advances in theory and computational techniques have shown that a strong electromagnetic field is invoked between solid nanoparticles, such as colloids, and at sharp boundaries of nanoscale geometries, such as on gratings and island films. Hence, many researchers have developed a well-ordered array of geometric nanoscale features on a substrate that can provide amplified electromagnetic fields on these surfaces, thereby, expanding the use of SERS in analytical applications. For example, Van Duyne et al. reported strong enhancement factors for pyridine molecules adsorbed on metal film over nanosphere (MFON) electrodes, with silver deposited between the interstices formed by the removal of the nanoparticle arrays from the substrate. However, random adsorption of molecules on the substrate is a critical drawback especially for the detection of biomolecules at trace levels.
To overcome the previously described limitations, we used a nanofluidic device that can significantly improve the reproducibility and sensitivity of SERS by exclusively localizing nanoparticles and increasing the concentration of target molecules at the entrance to the nanochannel. This nanofluidic device transports a mixture of target molecules and gold colloid (60 nm nanoparticles) down a microchannel to the entrance of a nanochannel. The nanometer scale of the nanochannel depth causes size-dependent trapping of the gold colloid particles, creating a high density of gold nanoparticle clusters. In a similar fashion, the capillary flow induced by the device transports the target molecules through the interstices between the clusters, the SERS active sites, increasing the concentration of the target molecule and nanoparticles at the nanochannel entrance. The feasibility of using the nanofluidic biosensor has been previously reported for detecting nonresonant biomolecules at a concentration as low as 10 pM. 

The use of a nanofluidic device is also advantageous because the aggregated nanoparticles, encouraged by the geometry of the device, result in a shift of the plasmon resonance to the near-infrared region (NIR). Hence, near-infrared excitation, selected in resonance with the plasmon resonance frequency, takes advantage of the low water absorption in this range and eliminates the possibility for interference due to autofluorescence from the protein samples. In addition to eliminating sample fluorescence by using NIR light, the proximity of the molecules to the gold nanoparticles can be useful in further diminishing fluorescence via quenching. Thus, a SERS platform using a nanofluidic trapping device is described in this paper for eventually detecting Alzheimer’s disease through Raman spectra that are conformation dependent and unique to Aβ refolding from α-
helical to β-sheet structure, with the ultimate goal of facilitating diagnosis and understanding of Alzheimer’s disease.

1.2 Raman Spectroscopy

When light is incident on biological samples, it can interact with atoms and molecular bonds in several different ways. The photons of the incident light can be either absorbed directly or scattered by the target. Absorption of light occurs when the incident radiation excites vibrational modes in the target molecule (IR-absorption) or causes excitation of the electronic transitions in the target molecules (UV-Visible Absorption). Sometimes electronic transitions events are followed by electromagnetic radiation by the target at longer wavelengths through radiative emission. This phenomenon is called fluorescence and exploited widely in biomedical research.36

Most photons are elastically scattered at the same wavelength as the incident light when light impinges upon a molecule. However in the 1920’s, C.V. Raman and Krishnan were the first observed that when monochromatic light passes through a liquid the wavelength of scattered light is not necessarily the same as the incident light.37 This small fraction of light is inelastically scattered at optical frequencies different from the frequency of the incident radiation. This inelastic scattering of photons is called Raman scattering. In contrast, the elastic scattering of photons or those that remain at the same energy as the incident photons are called Rayleigh scattered photon. Generally Rayleigh scattering has a much higher probability of occurring than Raman scattering because the most probable event is the energy transfer in which molecules in the ground state re-emit and return to the ground
state. A diagram of energy transfer pertaining to Raman and Rayleigh scattering is shown in Figure 1.1.

![Diagram of Raman and Rayleigh scattering](image)

Figure 1.1: Diagram description of Raman and Rayleigh scattering. The arrows in same color have the same frequency.

The thickest arrow (on the far left in Figure 1.1) depicts the energy change in a molecule when it interacts with an incident photon. The photon energy is depicted as follows:\(^\text{38}\)

\[ E(\nu_L) = h\nu_L \quad (1.1) \]

where \(\nu_L\) is the frequency of the incident photon, \(h\) is Planck’s constant \((6.62608\times10^{-34} \text{ J}\cdot\text{s})\), \(E(\nu_L)\) is the energy of the incident photon. However, this process is not quantized and depends on the frequency of radiation of the source. The vibrational energy of the molecule can assume an infinite number of values or virtual states between the ground state and first
excited electronic state. The second arrow, pointing upward on the left, depicts the energy change that occurs when the molecule encountered by the incident photon is already in the first vibrational level of the ground state. Generally, most of the molecules at room temperature are in their lowest vibrational state and are rarely in excited vibrational states. Hence the thin width of the arrow shows the low probability of occurrence for this event. The downward arrow in the middle shows the changes occurring in the target molecule that result in Rayleigh scattering. As shown in the diagram, there is no energy loss in Rayleigh scattering as a result of the elastic collision between the incident photons and target molecules. Hence the photon emitted by Rayleigh scattering or, elastic scattering, has the same frequency as incident photons.

The next two arrows on the right on Figure 1.1 show the changes occurring in the target molecule interacting with incident photons. That produce stokes and anti-stokes Raman scattering. The two differ from the Rayleigh scattered radiation by an energy difference of $\pm \Delta E$, which is the energy of the first vibrational level at ground state.

\[
\Delta E = h(\nu_{vib}) \quad (1.2)
\]
\[
E(\nu_s) = h(\nu_L - \nu_{vib}) = h\nu_s \quad (1.3)
\]
\[
E(\nu_{as}) = h(\nu_L + \nu_{vib}) = h\nu_{as} \quad (1.4)
\]

In the above equation, $h$ is Planck’s constant, $\nu_L$ is the frequency of the incident photon, $\nu_{vib}$ is the frequency of molecular vibration, $\nu_s$ is the frequency of Stoke Raman scattering and $\nu_{as}$ is the frequency of Anti-stoke Raman scattering. Anti-stokes Raman scattering has a photon
energy $E(\nu_s)$ higher than the energy, $E(\nu_a)$, of Stokes Raman scattering. In addition, the width of the arrows shows the probability of the event occurrence. It is important to note that the probability of Stokes Raman scattering is higher than Anti-Stokes Raman scattering. Generally at room temperature, the intensity of Stokes shifted Raman bands are higher than the Anti-Stokes ones due to the nature of molecular distribution, namely that there are more molecules in the lowest vibrational state than in the excited vibrational state. This ratio of intensities begins to rearrange in favor the anti-stokes bands with increasing temperature as a larger fraction of a target molecule are anticipated to be in the first excited vibrational state under a situation.

1.3 Surface Enhanced Raman Spectroscopy (SERS)

The Raman spectrum is considered as an optical fingerprint for analytical investigation of molecules since the spectral lines give optical information about the vibrational properties of molecules. However, the relative low efficiency of Raman scattering due to the small optical cross section of molecules (typical Rayleigh scattering cross sections of molecules are in the range of $10^{-26}$ cm$^2$ and typical Raman scattering cross sections are in the range of $10^{-29}$ cm$^2$)\textsuperscript{38} and small sample volume that can be probed impede the application of Raman spectroscopy in biological detection. Surface Enhanced Raman Spectroscopy (SERS) is a technique that has been used successfully to increase the Raman signals from a molecule by factors of $10^6$ to $10^{12}$\textsuperscript{30, 31} This great enhancement of Raman intensities is generally achieved by exciting vibrational transitions in molecules directly or in close vicinity to a roughened metal electrode. In general, the consensus for the enhancement of
Raman signals through SERS is a contribution from two contributing mechanisms, namely the electromagnetic mechanism and the chemical mechanism (CM).

The electromagnetic mechanism which is believed to be the dominant mechanism responsible for the enhancements found in SERS, explains enhancements primarily due to the collective electromagnetic resonance (or localized plasmon resonances), which refers to the excitation of collective oscillation of free electrons shared by the material in conduction bands. A strongly localized plasmon resonance, supported by a metallic nanostructure, is assisted by modifications in the local electromagnetic density.

This can occur, for example, when a small metal sphere is influenced by electromagnetic field provided that the radius of the sphere is much smaller than the wavelength of electromagnetic radiation. Figure 1.2 shows that a Raman active molecule placed at a distance, $d$, away from a metal nanoparticle of radius, $r$, will experience a total electromagnetic field ($E_M$) which is the superposition of the incoming field ($E_0$) and the electromagnetic field of dipole ($E_{sp}$) induced by the metal sphere. The electromagnetic field ($E_{sp}$) on the metal particle’s surface is a surface plasmon which is expressed as

$$E_{sp}(\nu) = r^3 \frac{\varepsilon - \varepsilon_o}{\varepsilon + 2\varepsilon_o} E_0 \left( \frac{1}{r + d} \right)^3$$  (1.5)

When the incident electromagnetic wave ($E_0$) resonates with the electromagnetic of the dipole field induced from the surface of the nanoparticle ($E_{sp}$), the incident field and the surface plasmon reinforce each other resulting in a large enhancement of $E_M$. This increase in
the field intensity experienced by the molecule will lead to an increase in its Raman scattered signals.

Figure 1.2: A simple schematic illustrating the concept of the electromagnetic SERS enhancement for a Raman active molecule at a distance \( d \) from surface.

In the following derivation, a small sphere approximation in which radius \( r << \lambda_{inc} \) is used with a complex dielectric constant \( \varepsilon(\nu) = \varepsilon' + i\varepsilon'' \), where \( \nu \) is the frequency and \( \varepsilon' \) and \( \varepsilon'' \) are the real and imaginary portions of the metal dielectric constant in a surrounding medium with a dielectric constant of \( \varepsilon_0 \). The field enhancement factor \( A(\nu) \) (at a specific frequency \( \nu \)), for molecule in the vicinity of the sphere at a distance, \( d \), is the ratio of the field at the position of the molecule and the incident field.

\[
A(\nu) = \frac{E_M(\nu)}{E_o(\nu)} \approx \frac{\varepsilon(\nu) - \varepsilon_o}{\varepsilon(\nu) + 2\varepsilon_o} \left( \frac{r}{r + d} \right)^3 \tag{1.6}
\]

\( A(\nu) \) in Equation 1.6 is strongest when the real part (\( \varepsilon' \)) of \( \varepsilon(\nu) \), which is frequency dependent, equals \(-2\varepsilon_o\). In addition, the imaginary part of \( \varepsilon(\nu) \) has to be small, which is
possibly achieved by modifying the nanoparticles to be optically highly scattered. This condition occurs at the resonance excitation wavelength of the surface plasmon and assumes that an embedding medium does not have an imaginary portion in its dielectric constant (i.e., the metal nanoparticle is embedded in a non-absorbing medium).

Since the Raman scattered light is of a different frequency than the incident laser, both incident and Raman scattered fields can be nearly resonant with the surface plasmons at small frequency shift with the above assumption the total electromagnetic enhancement factor for the SERS process for a single particle can be expressed as

\[ G(\nu_s) = |A(\nu_L)|^2 |A(\nu_S)|^2 \approx \left| \frac{\epsilon(\nu_L) - \epsilon_\infty}{\epsilon(\nu_L) + 2\epsilon_\infty} \right|^2 \left| \frac{\epsilon(\nu_S) - \epsilon_\infty}{\epsilon(\nu_S) + 2\epsilon_\infty} \right|^2 \left( \frac{r}{r + d} \right)^{12} \]

In equation 1.7 \( A(\nu_L) \) and \( A(\nu_S) \) are the electromagnetic enhancement factors for the incident laser light at a frequency \( (\nu_L) \) and for Raman Stokes scattered field at a frequency \( (\nu_S) \). This enhancement is strongest when the surface plasmons are in resonance with both the excitation and Raman-scattered fields; and this enhancement is dependent, to the fourth power, on the local nanostructure field of the metal. The decay of the total gain is strongly spatially dependent to \([1/d]^{12}\) as described in Equation 1.7. The electromagnetic field rapidly decreases with distance \((d)\) between the analyte molecule and the metal nanoparticle. Although, the distance \(d\) does not necessarily have to be zero for SERS enhancement to occur, the molecules in direct contact with the nanoparticle surface contributes most to the
SERS intensities. Those molecules in proximity to metal surface are exposed to a strongest 
clarified energy field, which is called the first layer effect.

These strong spatial and local nanostructure field dependencies may be producing the 
small, localized “hot spots” of extremely strong enhancements known to occur with SERS, 
which give the potential for trace analyte detection but also make the production of 
reproducible SERS substrates difficult.

Electromagnetic enhancement should non-selectively amplify all Raman scattered 
signals emitted by molecules adsorbed to the particular surface. However, experimental 
results observed varying enhancements by a factor of 200 for CO and N₂ molecules. This 
phenomenon is hard to explain by only electromagnetic enhancements, since the 
polarizabilities of the molecules are nearly identical. Even radical differences in orientation 
of the molecule-surface interactions could not produce such a great difference. The best 
electromagnetic SERS enhancement factors can vary by two order of magnitude which 
indicates another enhancement mechanism, independent from electromagnetic enhancement, 
is taking place. This additional enhancement factor can be explained, although inferentially, 
by a chemical enhancement due in part to electronic coupling, resulting in an increased 
Raman cross section as well as altering charge transfer, resulting in a new electronic 
transitions between molecules and the metal surface, creating a molecule-surface complex. In 
general, chemical enhancement is speculated to contribute to the total enhancement factors 
on the order of 10-10². ³¹

The Raman enhancement is speculated to be more of a “nanostructure effect” rather 
than a “surface effect” since the discovery of enhanced Raman signals from pyridine
molecules in aqueous colloidal solution made up of noble particles with a radius much smaller than the wavelength of the incident laser. These electromagnetic and chemical mechanisms are considered to represent the SERS stoke power $P_{\text{SERS}}^S(\nu_s)$ at a Raman-scattered frequency $\nu_s$ in Equation (1.8)\textsuperscript{30}

$$P_{\text{SERS}}^S(\nu_s) = N' \sigma_{\text{ads}}^R |A(\nu_L)|^2 |A(\nu_S)|^2 I(\nu_L) \quad (1.8)$$

In equation 1.8, $I(\nu_L)$ is excitation the laser intensity, $A(\nu_L)$ and $A(\nu_S)$ express enhancement factors for the incident frequency and for the Raman-scattered frequency. $\sigma_{\text{ads}}^R$ represents the increase in cross section of the adsorbed molecules on the nanostructure. $N'$ is the number of molecules involved in the SERS process and could be smaller than the amount of molecules that generally contribute to the Raman scattered signals because of the first layer effect described previously.

According to Equation 1.8, it is important to point out that the high SERS efficiency takes place (larger the value of $P_{\text{SERS}}^S$) when (1) a molecule is emerged in the enhanced energy field supported by metallic nanostructures (i.e., electromagnetic enhancement) and (2) a molecule in direct contact with the metal surface forms a molecule-surface complex which increases the Raman cross section larger than the cross section of a free molecule involved in the traditional Raman process (i.e., chemical enhancement).
1.4 Alzheimer’s Disease and Beta–Amyloid Related to Neurotoxicity

Alzheimer’s disease (AD) is a neurodegenerative disease that gradually destroys a person’s memory and their ability to learn, make judgments, communicate, and conduct daily activities. Currently, this senile dementia affects 4.5 million Americans. This figure doubled between 1980 and 2000, according to the 2000 US census. At the present time, there is no single premortem test that definitively answers the question whether an individual has AD or only cognitive impairment due to the normal aging process. The full understanding of this progressive disease is still ongoing.

AD can only definitively be diagnosed by identifying amyloid plaques and neurofibrillary tangles surrounding dead or dying neurons in postmortem central nervous system tissue. The main constituent of the neuritic plaques associated with Alzheimer’s disease is 39-43 amino acid residues. Thesis originate in vivo from the proteolytic cleavage of a longer amyloid precursor protein (APP), a trans-membrane protein that is produced throughout the entire human body. These APP fragments, Aβ peptides, are then released into extracellular fluids and subjected to further conformational changes.

The most prevalent species of Aβ peptides present in people with AD are Aβ (1-40) and Aβ (1-42). Both forms of Aβ are found in cerebrospinal fluid (CSF) and blood plasma of all people, regardless of health. Aβ (1-40), the dominant peptide species, has a concentration of roughly 5nM in CSF. The relative concentrations of different Aβ species including Aβ (1-40), Aβ (1-42), and their conformation/aggregation states change with the progression of disease. Figure 1.3 shows the structural scheme of Aβ (1-40).
Figure 1.3: Aβ amino acid sequence and designated areas of hydrophobicity. The peptide has two hydrophilic regions located from 1-16 and 22-28 as well as two hydrophobic regions located from 17-21 and 29-42. The turn structures are located at 6-8 and 23-27 eventually allowing for the β-pleated sheet structure associated with the toxic state of Aβ.\textsuperscript{41,42}

The toxicity of Aβ is associated with the change in secondary and tertiary structures, which lead to the aggregation of the peptide. Aβ has the propensity to accumulate and aggregate itself into insoluble structural conformations. Hypothesized aggregation pathway of Aβ is shown in Figure 1.4. Initially, the monomeric form of Aβ is predominately α-helical with some random coil structure or maybe understood. During the Aβ aggregation, the monomeric form of Aβ peptide refolds itself into a β-strand, going from being a monomer to a dimer, which has mainly β sheet structure.

Figure 1.4: Hypothesized aggregation pathway of Aβ. The protein monomer is in a soluble α-helical form, refolds to β-sheet strands, and eventually shifts to a β-sheet structure. The β-sheet structure helps to form nucleation sites, which soon form protofibrils. Multiple protofibrils form a fibril. Toxicity observed in Aβ is from the intermediate steps in the aggregation pathway.\textsuperscript{43}
These sheets then form protofilaments that form protofibrils, which have a diameter around 25-30Å. Finally, the protofibrils form the full fibril structure with a range of diameter from 60-100Å. Once the protein adopts a β-sheet structure, it becomes resistant to degradation. This leads to the deposition of Aβ in the form of senile plaques within the brain. Although the cytotoxic species of Aβ responsible for the majority of the toxicity associated with AD have been narrowed down to soluble Aβ oligomers (i.e., ADDLs and protofibrils), the degree of neurotoxicity of each form is still under debate.\textsuperscript{17, 18}

\textit{In vitro} studies show that aggregated Aβ, when added to cells in culture, become neurotoxic when either aggregated Aβ that forms outside of the that cell may later interact with the outer cell membrane or Aβ aggregates within or in association with the lipid bilayer of the cell. These protein-neuronal membrane interactions that damage the cell membrane will lead to cell apoptosis. Although Aβ may interact with the cell in different fashions, the kinetics of Aβ misfolding that result in β-sheet conformations and intrm cause aggregation is most likely the key toxic effect. A recent study pointed out that Aβ fibrillogenesis involves the soluble Aβ monomer en route from α-helices to a conformational structure with predominant β-pleated sheets. Hence, the development of tools useful in probing the structure specific conformation of the Aβ species would be useful in the investigation of the molecular mechanisms of Aβ cytotoxicity associated with AD.

1.5 Nanofluidic Device Fabrication

The nanofluidic channel device was fabricated by conventional top-down microfabrication techniques on a UV grade 500 μm-thick fused silica wafer (Mark Optics,
Inc., Santa, Ana, CA). The detection region is a step structure which connects a deep microchannel with a shallow nanochannel. The dimension of this nanochannel is critical for the particle trapping mechanism described in this article. Since we are using nano-particles with diameter of 60nm, the shallow nanochannel is designed to have a depth of 40nm. Contact photolithography was first used to transfer the nanochannel pattern onto the fused silica wafer and CF4 reactive ion etching was subsequently performed to etch the fused silica to a depth of 40nm using standard photoresist as masking material. To connect the nanochannel with sample inlets, microchannel was carefully aligned with the previous nanochannel and again patterned by photolithography. Concentrated (49%) hydrofluoric acid was then used to etch the microchannel to a depth of 2.5 $\mu$m using electron-beam-evaporated Cr/Au as masking materials. This microchannel–nanochannel junction (i.e. entrance to the nanochannel) will serve as a trapping site for the 60nm nanoparticle used in this experiment. Finally, the sample inlet holes were drilled through the substrate wafer by a high-speed sandblaster tool followed by thoroughly cleaning the wafer. A fused silica cover wafer is used to carefully bonded to the substrate wafer to create an enclosed fluidic channel. The schematic diagrams of the nanofluidic channel with top and lateral views are shown in Figure 1.5 (A) and (B). The configuration encourages size-dependent trapping of the gold nanoparticles and target molecules at the entrance to the shallow nanochannel, forming dense clusters with high local concentrations of target molecules. This mechanism is shown in Figure 1.5 (C).
Figure 1.5: Schematic diagram of a nanofluidic biosensor: (A) lateral view, (B) top view, and (C) lateral view of a nanofluidic biosensor with aggregated nanoparticle SERS active clusters formed at the step structure. The depth of the nanochannel is smaller than the diameter of the nanoparticles, trapping the nanoparticles, forcing aggregation of the particles and target molecules.
CHAPTER II

CHARACTERIZATION OF NANOFLUIDIC SYSTEM FOR SERS

SERS is extremely sensitive to local variations in the nanoparticle surfaces and inhomogeneous surface roughness of SERS substrates. One alternative to SERS sensing is concentrating metal nanoparticles in one specific location in a device where the SERS signals from analyte molecules can be measured. (The schematic diagram of the nanofluidic device and fabrication procedure are illustrated in Nanofluidic Device section.) To determine whether our nanochannel meets this goal, we tested the trapping capability of the device to determine where the particle trapping occurs and if this trapping is in one specific and consistent location. In addition, we probed the extinction spectrum of aggregated nanoparticle cluster induced by the nanofluidic device for optimizing this nanofluidic based SERS platform for biomolecule detection.

2.1 Nanofluidic Trap Test Using Fluorescent Beads

To confirm the trapping capability of this device, fluorescent polystyrene (PS) nanoparticles in aqueous solution (Spherotech Inc., IL) with a size ranging from 40–90 nm were introduced into the optofluidic device. Since the diameter of the fluorescent nanoparticles is larger than the depth of the shallow nanochannel, they were trapped at the entrance to the nanochannel. The solution of PS beads was diluted to 5 mg/l using deionized
(DI) water and then introduced into the nanochannel from the reservoir. Due to capillary force, the solution was transported into the device within a few seconds. The fluorescent image of PS particles trapped at the entrance of the nanochannel, in Figure 2.1, was taken immediately after the dispensing of the solution. The PS particles emitted extremely high fluorescent signals around the entrance to the nanochannel obviously in contrast to the microchannel region. This strong fluorescent emission resulted from a high density PS nanoparticles aggregated at the nanochannel entrance. To investigate channel clogging by the aggregated nanoparticles, 12 mM Rhodamine B in DI water was dispensed into the channel after the PS nanoparticle cluster had been formed at the entrance of the nanochannel. The fluorescent signal from Rhodamine B was observed at both input and output sides of the microchannels. This showed that there were tiny interstices between the particle clusters at the entrance of the nanochannel and a weak capillary flow could be used to transport Rhodamine B molecules through the cluster and nanochannel site.

Figure 2.1: Fluorescent image of polystyrene nanoparticles trapped at the entrance of the nanofluidic device.
2.2 Gold Nanoparticle Test and Nanoparticle Cluster Analysis

Gold nanoparticle clusters at the nanochannel entrance are responsible for the SERS enhancement for our system. To investigate the trapping properties of aggregated nanoparticles in the channel, gold colloid (Polysciences, Inc, PA), with an average diameter of 60nm and concentration of 0.01% in water, were introduced into the nanofluidic device from the plastic reservoir covering the inlet holes. Due to capillary forces, the nanoparticles in solution were transported through the deep microchannel within a few minutes. Since the diameter of the gold nanoparticle is larger than the depth of shallow nanochannel, they were trapped at the nanochannel entrance and formed nanoparticle clusters. After loading the sample, the reservoir was covered with Parafilm to reduce evaporation of the solution. The device was kept at room temperature and bright field images of the nanoparticle clusters at the nanochannel entrance were taken every 24 hours over a three day period using Leica DMLM microscope with 20x (NA = 0.40) air objective and a digital video camera.

Figure 2.2 (A) shows the bright field image of an empty microchannel-nanochannel junction. Figure 2.2 (B) and (C) show nanoparticle clusters at the entrance to the nanochannel after loading the device with colloidal gold and taking images after 24 hours and 72 hours, respectively. Forming the clusters is reproducible and easily seen at the entrance to the nanochannel. The expansion of the aggregate size over time, as seen in a comparison of Figure 2 (B) to (C), indicates that the nanofluidic device maintains a capillary force over the 72 hours with only 120 µL of solution. Even though aggregation is not necessarily required for observing the SERS signals of target molecules, aggregation is employed to observe stronger Raman enhancements from target molecules with a low optical scattering cross
section, such as the proteins investigated here. In previous work from this laboratory, Aβ could not be detected using SERS without the aid of a secondary detection molecule of higher optical scattering cross section than most proteins. Plus, our approach takes advantage of the enhancements while also providing consistent and reproducible aggregates in a specific location. The aggregates provide an environment for the target molecules to be concentrated in close proximity to active sites (e.g., between adjacent nanoparticles or local resonant nanostructures) and exposed to the induced electromagnetic field simultaneously. The resulting platform overcomes the problems of random localized “hotspots” persistent in previous approaches to SERS platforms due to uncontrollable aggregation of nanoparticles on substrates.

Figure 2.2 Brightfield microscope image of nanoparticle clusters at the entrance to the nanochannel. This image depicts (A) An empty micro/nanochannel, (B) the micro/nanochannel 24 hours after being loaded with 120 μL of gold colloid solution, and (C) on day 3. The red circles in the images show an increase in aggregation size of the nanoparticles over time. The arrows indicate the entrance to the nanochannel.
2.3 UV-Vis Absorption Experiments

After dispensing gold colloid into the channel on the third day, the device was subjected to UV-Vis absorption studies performed using a converted CCD based spectrometer (Spectra Pro 150 Imaging Dual Grating Monochromator/Spectrograph containing a Roper Scientific/Princeton Instruments HAM 256 × 1024 CCD camera). The extinction spectrum of aggregated gold nanoparticles at the nanochannel entrance was taken using a “knife edge” approach. A tungsten halogen lamp light source (Oriel Systems) delivered incident light via a 1mm core fiber optic (Thor Labs, Newton NJ) through a 20x (NA=0.4) air objective, providing a 35 μm spot size onto the wafer mounted on a microscope stage and the wafer surface was orthogonal to the axis of incident light. The experimental setup is shown in Figure 2.3. This setup allowed light transmission along the whole length of the wafer. The focal area of the objective was carefully aligned close to the microchannel–nanochannel junction to illuminate fully the nanoparticle aggregation. The transmitted light was back collected by a 10x (NA=0.25) objective lens coupled to a 400 μm core fiber to deliver the collected light to a CCD spectrometer. The distance between the incident core fiber, 1um in diameter, and the objective lens and the objective lens to the nanoparticle aggregation was at a ratio of 30:1. The extinction spectrum was collected with an integration time of 0.5 seconds and wavelength between 400 – 900 nm. A clean borosilicate wafer was used as a blank for the study. The extinction spectrum of gold colloid, at the same concentration used in the nanofluidic device, was taken in a standard 1 cm path length quartz cuvette and characterized using the same set-up described above.
Figure 2.3: UV-Vis absorption experimental setup for the collection of extinction spectra of gold nanoparticle clusters in the nanochannel.

In this approach, the aggregated particles caused a shift in the Plasmon resonance frequency to the near-infrared region, in resonance with our excitation wavelength of 785nm. The use of near-infrared excitation is advantageous because water has low absorption in this range and this NIR range reduces native fluorescent background inherent to CSF. To confirm that the aggregated gold nanoparticles shift the plasmon resonance, light focused to a 35 μm spot size was used to probe the nanoparticle clusters at the nanochannel entrance over five days. Typical UV-Vis absorption and transmission spectra of gold colloid and gold nanoparticle clusters are shown in Figure 2.4. Gold colloid, extinction spectra shown in Curve B of Figure 2.4, exhibits a relatively sharp absorption band at 540 nm with a long tail trailing out toward longer wavelengths. Upon aggregation (Curve A in Figure 3), the 540 nm band broadens and another large band appears around 800nm. The changes of the spectra, from minimal absorption and scattering properties to high extinction at 800 nm, indicate that
gold nanoparticles are clustered at the nanochannel entrance. Actually, the gold nanoparticle clusters at the entrance could have 1500 times the optical density than those featured in the spectrum shown in Figure 2.4, according to Beer-Lambert’s law \( A = abc \) where \( A \) is absorbance, \( l \) is the distance that the light travels through the material and \( c \) is the concentration of the absorbing species). Since the microchannel has a depth of 6 μm, the distance of light traveling through the nanoparticle clusters formed at the micro-nanochannel junction, is on the order of several micrometers. In contrast, the path length of the cuvette that we used to measure the extinction spectrum filled of the gold colloid solution is 1 cm, about 1500 times longer than the microchannel depth. Furthermore, we were unable to detect the extinction spectrum of gold colloid in the middle of microchannel, away from the aggregation at the nanochannel entrance (Curve C in Figure 2.4). This lack of signal is consistent with the observation that high-density gold nanoparticle clusters were formed only at the entrance to the nanochannel.
Figure 2.4: Extinction spectra of gold nanoparticle cluster and gold colloid. (A) Extinction spectra of gold nanoparticle clusters at the entrance of the nanochannel, (B) gold colloid extinction spectra of gold colloid in a cuvette, and (C) gold colloid extinction spectra collected in the middle of the microchannel channel. Note the broadening and shift in the 550 nm gold colloid peak upon aggregation at the nanochannel entrance. No extinction was detected away from the nanochannel entrance.
CHAPTER III

NANOFLUIDIC CHANNEL BASED BIOSENSOR USING SERS

In this chapter, we tested the feasibility of using the nanofluidic channel as a SERS platform for detecting multiple molecules. Two molecules were mixed with gold colloid and dispensed into the channel at the same time and then SERS spectra were collected at the entrance to the nanochannel. Adenine, one of the testing molecules, is a non-resonant molecule with a characteristic ring breathing mode at the spectral region $730 \pm 5 \text{ cm}^{-1}$, which can be useful to estimate the SERS enhancement factor. Congo red, which exhibits fluorescent activity when bound to amyloid fibrils, tends to be used as a sensitive diagnostic tool for staining Aβ fibrils, and also shows five characteristic bands in the Raman spectra. We found that the nanofluidic channel significantly improves the robustness and reproducibility of the SERS enhancement environment. The target molecules are temporarily restricted in the interstices between the aggregated nanoparticles, since the trapping of the nanoparticles limits their overall mobility and allows the target molecules to pass through the gaps between the nanoparticles. This effect produces a high density of aggregated nanoparticles at a precise region and trapping-induced concentration of target molecules in the gap between the nanoparticles to render a extremely high SERS signal. Thus, by exploiting this nanofluidic device, we have developed a reliable and repeatable SERS substrate with preliminary results that are promising for label-free detection of single molecules and a multi-analyte investigation in biological applications.
3.1 Experiment Methods

In our SERS experiments, gold nanoparticles with a diameter of 60nm (Polysciences, Inc, PA) are used as provided. To determine the SERS signal using a traditional gold colloid solution, 1 M sodium chloride was mixed with colloidal gold at the volume ratio of 1:10. The mixture sat for 15 minutes before adenine or Congo red was added to a final optimized concentration of 100 nM or 100 μM, respectively. For the nanochannel experiments, samples were prepared by adding 100 nM adenine and 100 μM Congo red to deionized water and colloidal gold at a volume ratio of 1:10, resulting in a final sample concentration 83 nM adenine (AD) and 83 μM Congo red (CR). These solutions were loaded into the nanochannel with a syringe pump connected to the reservoir to improve flow. The nanofluidic devices used in the experiment has a microchannel 6 μm in depth and 40 μm in width and a nanochannel 40 nm in depth and 5 μm in width. The fabrication of nanofluidic device is described in Chapter II. To characterize this device, a 12 mW, 785nm laser beam was focused on the entrance site of nanochannel by a 50X microscope objective lens and the back scattered Raman signal within the detection volume was collected by the same objective lens. The power delivered at the sample is 12mW and the integration time per spectra was 30 sec. The Raman Spectrometer used to obtain the spectra is discussed in the next section. For the traditional gold colloid approach, the spectra were acquired using a 63X water immersion objective with an integration time of 60 sec.
3.2 Raman Spectrometer Setup

All SERS spectra were collected using a Renishaw System 1000 Raman Spectrometer coupled to a Leica DMLM microscope. The laser source was a 785 nm GaAlAs diode laser (SDL Model XC30). A block diagram of the system is shown in Figure 3.1. The incident laser first passed through a beam expander (in order to fully illuminate the microscope objective lens for delivering the smallest spot size to the sample). The beam was reflected by a series of mirrors to the first notch filter (NF 1). The notch filter was designed to reject the incident laser line and served two purposes mainly to reflect the incident beam to the microscope and to reject the Rayleigh scattered light in the back reflected beam from reaching the detector. After reflection from NF 1, the incident beam traveled through a tunnel connecting the spectrometer to the microscope and hit the sample. The back reflected light that is scattered from the sample travels via the same path back to the spectrometer to hit NF 1 again. As mentioned earlier, the NF 1 rejected the Rayleigh scattered light. The rest of the back reflected beam then passed through a second notch filter (NF 2) which further rejected any Rayleigh scattered light that might have bled through NF 1. The remaining light then passed through a series of lenses and mirrors to strike a diffraction grating, which spatially separated the light into respective wavelengths, and then the diffracted light hit a thermoelectrically cooled CCD camera. The alignment data for the Raman Spectrometer is provided in Appendix A.
3.3 Results and Discussions

Adenine is a non-resonant molecule and shows Raman scattered activity only at high concentration due to a low optical cross section. The Raman spectrum of adenine has been well characterized for some of the vibrations, like the ring-breathing mode at 729 cm\(^{-1}\). In the SERS spectra, on the other hand, adenine bands are slightly shifted by up to 20 cm\(^{-1}\) when compared to regular Raman conditions. These shifts are most likely due to the interaction of adenine with the metal surfaces rather than the different states of aggregation. Adenine is advantageous in that a band should always be found around 730 cm\(^{-1}\)\(^{33,34}\). This distinct peak
differs from the Raman profile of either glass of quartz, the substrates for making the nanochannels.

Figure 3.2 provides the Raman spectra of adenine and Congo red (CR) as well as the SERS spectra of the two molecules using a traditional gold colloid and salt solution. The addition of the halide electrolyte (i.e., salt) into the gold colloid solution, can break the electron balance on the surfaces of the gold nanoparticles and result in particle aggregation. This aggregation of particles was critical for observation of the Raman spectrum since shifted the surface plasmon of the gold nanoparticles to the near infrared to achieve resonances with the 785 nm excitation wavelength. Since adenine is a non-resonant molecule and has low Raman cross section, the spectrum, Figure 3.2 (B), of adenine without the SERS enhancement shows no Raman activity. Figure 3.2 (A) shows the spectrum of 100 nM adenine in colloidal gold solution and exhibits Raman characteristic signatures of adenine at 735 cm\(^{-1}\) and 630 cm\(^{-1}\), assigned to ring breathing of the whole molecule and deformation of the five carbon ring. The 100 \(\mu\)M Congo red spectra in Figure 3.2 (D) shows weak Raman signals of Congo red that are too small to be able to justify clearly. While the SERS spectra in Figure 3.2 (C), Congo red has five identifiable peaks. The peaks at 1159 cm\(^{-1}\) and 1598 cm\(^{-1}\) are related to the N-C stretch and phenyl-ring mode respectively. The Congo red peaks around 1350~1450 cm\(^{-1}\) are due to the contribution of the N=N stretching modes. Table 3.1 provides information about the Raman characteristic signals of the two molecules.
Figure 3.2: Raman spectra of adenine and Congo red. SERS spectra of adenine and Congo red using conventional salt induced gold aggregation of gold nanoparticle as the agent. (A): SERS spectrum of 100nM adenine in an aggregated gold colloid solution, (B): Raman spectrum of 100nM adenine, (C): SERS spectrum of 100uM Congo Red with the same condition as (A), and (D): Raman spectrum of 100uM Congo red. Both adenine and Congo red were dissolved in DI water.

The samples, 83nM adenine and 83 μM Congo red with colloidal gold, as described in procedure, were loaded into the nanofluidic device reservoir and constantly pumped at a rate of 2 μl per minute. When the gold nanoparticle and target molecules are driven toward the nanochannel entrance using the fluidic force, the nanoparticles are trapped at the nanochannel entrance and the plasma frequency of the nanoparticles begins to red shift as aggregation commences. The aggregation was verified by optically probing the microchannel
region, the areas without nanoparticle aggregation provided no contributing Raman signal. We monitored the SERS signal at the entrance over time and determined that a significant trapping effect of both molecules and gold nanoparticles lasted up to 25 minutes. This amount of time can be varied by controlling the pumping frequency.

Figure 3.3 shows the Surface-enhanced Raman signals of the combined adenine and CR monitored over 25 minutes after introducing the samples. The Raman signals started increasing immediately after the pumping began. This phenomenon showed that first, the localized surface plasma resonance is red shifted to the infrared to resonate with the excitation laser and second, that the target molecules are temporary localized at the entrance site since the aggregated nanoparticles limit the overall flow velocity causing trapping of the target molecules. As time progressed, more characteristic Raman peaks of both molecules became visible, in addition to increases of the peak intensities. After 25 minutes, however, there was no obvious increase in Raman signals because the aggregation was completed. Several peaks besides those seen in the Raman signal of adenine and CR in the conventional colloidal gold SERS technique, Figure 3.2 (a) and (c), were observed. These peaks can be explained by the extreme sensitivity caused by this enhancement. Only slight deformations of molecules in the vicinity of the SERS substrate can produce changes in the enhancement factors by several orders of magnitude. Additionally, inter-molecular coupling can result in different enhancements that provide additional peaks.
Figure 3.3: SERS signal for 83nM adenine and 83 uM Congo red at different times using nanofluidic device. No attempt was made to optimize the method for increased sensitivity.
Table 3.1: The characteristic SERS bands of Adenine and Congo red. Congo red peaks from Mod 3 to Mod 7 are consistent with the previous literature.21

<table>
<thead>
<tr>
<th>Molecule</th>
<th>MOD</th>
<th>Wavenumber [cm(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>Mod 1</td>
<td>630</td>
</tr>
<tr>
<td></td>
<td>Mod 2</td>
<td>735</td>
</tr>
<tr>
<td>Congo Red</td>
<td>Mod 3</td>
<td>1156</td>
</tr>
<tr>
<td></td>
<td>Mod 4</td>
<td>1379</td>
</tr>
<tr>
<td></td>
<td>Mod 5</td>
<td>1402</td>
</tr>
<tr>
<td></td>
<td>Mod 6</td>
<td>1453</td>
</tr>
<tr>
<td></td>
<td>Mod 7</td>
<td>1597</td>
</tr>
</tbody>
</table>

To evaluate the Raman signal enhancement factor using the nanofluidic device, we compared the measured SERS intensities to that of traditional Raman scattering by using Equation 3.1:

$$EF = \left( \frac{I_{\text{SERS}}}{I_{\text{norm}}} \right) \left( \frac{N_{\text{bulk}}}{N_{\text{surf}}} \right) \quad (3.1)$$

where \(I_{\text{SERS}}\), \(I_{\text{norm}}\), and \(N_{\text{surf}}\) are the measured SERS intensity for probe molecules close to the gold nanoparticle surface. \(I_{\text{norm}}\) and \(N_{\text{bulk}}\) are the normal Raman intensities contributed from the probe molecules within the laser focal volume. The enhancement factor of the nanofluidic device was calculated to be \(10^8\), which is two orders of magnitude higher than the enhancement factor of the conventional method of \(10^6\), found using the same calculation method. It seems likely that more than one factor may be contributing to the overall enhancement (e.g., increased localized concentration of target molecules). It is important to point out that the molecules in close vicinity to the nanoparticle surfaces could be involved in
the surface enhancement process that contributes to most of the SERS signals. Since the actual amount of molecules that are close to the nanoparticle surface is not known, but could be less than the molecules in free solution, the estimated enhancement factor using the nanofluidic device for SERS may be higher than the value calculated previously.
CHAPTER IV

NANOFLUIDIC BIOSENSING FOR β-AMYLOID DETECTION USING SERS

Studies have indicated that there are significant changes in oligomeric Aβ structure and Aβ concentration in cerebral spinal fluid (CSF) as Alzheimer’s disease (AD) progresses.\textsuperscript{17, 18} Hence, it is possible to develop a biosensor to probe structure specific conformation of Aβ isoforms for early detection of AD. Work is currently underway to develop a definitive pre-mortem detection method for AD using a wide variety of modalities. For example, Haes et al. have instituted the use of localized surface plasmon resonance nanosensors to detect anti-ADDL antibodies and ADDLs.\textsuperscript{47} This approach was able to determine the concentration of ADDLs in the diseased brain to be around 1 pM. Georganopoulou et al. used bio-barcode technology for the specific detection of ADDLs in CSF.\textsuperscript{48} This approach was able to detect ADDLs into the femtomolar range and was important in demonstrating the correlation of elevated ADDL levels with AD. However, these two approaches were specific only to ADDLs, one form of the many oligomeric forms of Aβ that could be indicative of AD. Moreover, these technologies are strongly dependent on the specificity of the targeting agent (or antibody) and cannot provide any structural information about Aβ.

The toxic form of Aβ is associated with the change in secondary and tertiary structures, which lead to the aggregation of the peptide. Current methods for probing the secondary and tertiary structure of proteins include Fourier transform infrared spectroscopy
(FTIR), circular dichroism (CD) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and X-ray diffraction. X-ray diffraction and NMR have cumbersome preparation techniques. For FTIR, sampling is often difficult and has strong absorption due to water. X-ray diffraction and CD are limited by the state of the probed protein; molecules probed with X-ray diffraction must be crystallized, while the molecules must be in solution for CD. However, Aβ does not crystallize, and toxic forms of Aβ do not all stay in solution. Plus, CD lacks the sensitivity required to detect the lower range of the toxic concentration of Aβ. Therefore, using the nanofluidic device for the development of a sensor platform that is able to detect structure specific oligomers of Aβ (in addition to ADDLs) along with conformational changes of the protein, would be a valuable tool. Such a platform has a potential to help facilitate disease diagnosis and aid in understanding Aβ assembly.

4.1 Experimental Method

Aβ (1-40) was synthesized and purified by BioSource International (Camarillo, CA). The sequence is DAEFRHDSGYEVHHQKLVFFADVGSNKGAIIGLMVGGVV. Aβ was dissolved in DMSO to a concentration of 2.3 mM, aliquoted into micro-centrifuge tubes containing 5 µL of solution, and stored in a -80 °C freezer until use. Then, 95 µL of deionized water (DI water) was added to the Aβ to create a 115 µM stock solution. The Aβ was stepwise diluted using DI water to the concentrations of interest before adding the gold colloid. Aβ oligomer samples were prepared at concentrations ranging from 11.5 nM to 11.5 pM. The sample was observed at room temperature. Soluble Aβ monomers (1.15nM) were prepared using the method described above and stored at 6 °C throughout the observation
period. Insulin (FW 5734) from bovine pancreas was purchased from Sigma-Aldrich Co. in powder form and was diluted to a concentration of 115 µM in DI water then further diluted stepwise to a final concentration of 1.15 nM. Albumin (FW ≈66,120) from bovine serum was purchased and purified by SIGMA (≥99% agarose gel electrophoresis). The albumin sample was prepared using the same procedure mentioned previously to a final concentration of 1.15 nM. The 2 µm width nanochannel was used in the experiment comparing albumin, insulin, and Aβ.

All protein SERS samples were prepared by mixing the protein with gold nanoparticles in solution (volume ration 1:10). After mixing, 120 µL of the gold colloid-protein mixture was then loaded immediately into the reservoir and observed over five days. Raman spectra were taken of the nanoparticle clusters every 12 hours over the course of observation. The residual samples were stored in the micro-centrifuge tubes for later pH measurement, determined using Thermo Orion pH Meter (Model 420, Thermo Scientific, Waltham, MA) with an AquaPro Combination pH Electrode. The accuracy was ±0.5 pH units. A pH of 6.8 was measured immediately after mixing and 6.4 after 48 hours for Aβ samples, and insulin and albumin samples maintained a pH of 6.8 throughout the experiment. SERS spectra were collected using the Raman spectrometer and laser source, which have been described in the previous chapter. The fabrication of nanofluidic device has been presented in Chapter 1.5. The significance of Alzheimer’s disease (AD) and the information for the Aβ peptides associated with AD have been described in Chapter 1.4.
4.2 Results and Discussions

4.2.1 Concentration Dependence of Aβ SERS Spectra

We took SERS spectra of Aβ at three different concentrations in the spectral range of 500-1600 cm\(^{-1}\) (Figure 4.1). The SERS Raman bands were assigned based on existing literature pertaining to the spectra of amino acids and proteins\(^ {49-53}\). Table (4.1) illustrates the observed frequencies of several SERS bands. As expected, aromatic rings, amides, and carboxylic group vibrations dominate the SERS spectrum of Aβ. In fact, shifts in the Amide III region, 1200 – 1300 cm\(^{-1}\), reflect the most compelling changes in proteins and are widely used to quantitatively explore secondary structure\(^ {26, 27, 29, 54}\).

For a low concentration of Aβ (11.5 pM), we observed Raman bands associated with the aromatic side chains at 1000, 1187, and 1488 cm\(^{-1}\) (phenylalanine, tyrosine and phenylalanine, and histidine residues respectively), shown in Figure 4.1 (A). A small shoulder band at 1036 cm\(^{-1}\), besides 1000 cm\(^{-1}\), supports the presence of phenylalanine in each sample. The presence of these bands indicates that the aromatic side chains are in a favorable position with respect to the gold nanoparticle surface. The phenylalanines, at amino acid 19 and 20 in the Aβ sequence, are located in a region of the Aβ peptide thought to be critical for Aβ aggregation\(^ {55}\) and should only be available to interact with the metal surface in unaggregated forms of Aβ. Furthermore, the histidine band at 1488 cm\(^{-1}\) in Figure 4.1 (A) is shifted about 10 cm\(^{-1}\) from the assigned band in the literature\(^ {51}\), which may have been altered due to protein-metal interactions, implying Aβ interacts with the charged metal surface through its histidine molecules\(^ {56}\). This histidine band and a band at 1266 cm\(^{-1}\) (in the amide III region) suggests that Aβ has an α-helix structure.
At a higher concentration (1.15 nM), as seen in Figure 4.1 (B), the band at 1266 cm\(^{-1}\) diminishes while a new band appears at 1244 cm\(^{-1}\) at a higher intensity. The red-shift from 1266 cm\(^{-1}\) (assigned to \(\alpha\)-helix) to 1244 cm\(^{-1}\) (probably due to \(\beta\)-sheet), signifies that the proteins are in two different conformational states. With a concentration of 11.5 nM, Figure 4.1 (C), the 1266 cm\(^{-1}\) band is weak and hidden in the shoulder of the new broad band, a good indication that the polypeptide backbone of A\(\beta\) has taken on a different conformation in close vicinity to the nanoparticle surface. Plus, the increase of the 961 cm\(^{-1}\) mode (assigned to the C-C stretching mode in the hydrophobic segment of A\(\beta\) polypeptide backbone) and the decrease in intensity of aromatic side chain signals are most likely due to the same refolding transition, which suggests that in the more aggregated \(\beta\)-sheet structure of A\(\beta\), the aromatic residues are no longer available to interact with the gold surface.\(^{56}\) While most investigators would suggest that increasing the A\(\beta\) concentration would result in conformational changes associated with aggregation, few have examined A\(\beta\) aggregation at the concentrations used in these studies because of the limits of analytical tools used for structure determination.
Figure 4.1: SERS spectra of Aβ at (A) 11.5pM (B) 1.15nM (C) 11.5nM after 24 hours in the nanofluidic device. Aβ samples at three different concentrations were prepared in the monomer form and loaded into the nanofluidic device immediately. Seven consecutive scans were taken from the nanoparticle clusters at the entrance to the nanochannel using 50x (NA=0.75) air objective with excitation laser 785nm
Table 4.1: Assignment of bands in SERS spectra of Aβ

<table>
<thead>
<tr>
<th>Wavenumber [cm(^{-1})]</th>
<th>Assignment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>675</td>
<td>Tyr</td>
<td>49, 52</td>
</tr>
<tr>
<td>823</td>
<td>Tyr doublet</td>
<td>49, 52</td>
</tr>
<tr>
<td>856</td>
<td></td>
<td></td>
</tr>
<tr>
<td>963</td>
<td>(\nu(C-C))</td>
<td>49, 50, 52</td>
</tr>
<tr>
<td>1000</td>
<td>Phe ((\nu_{12}))</td>
<td>49-52, 57</td>
</tr>
<tr>
<td>1036</td>
<td>Phe ((\nu_{18a}))</td>
<td>49-52</td>
</tr>
<tr>
<td>1077</td>
<td>(\nu(C_{\alpha}-N))</td>
<td>50, 51</td>
</tr>
<tr>
<td>1144</td>
<td>(\nu(CCN))</td>
<td>50, 51</td>
</tr>
<tr>
<td>1187</td>
<td>Phe and Tyr</td>
<td>49, 51, 52</td>
</tr>
<tr>
<td>1244</td>
<td>Amide III ((\beta)-sheet)</td>
<td>50, 57, 58</td>
</tr>
<tr>
<td>1266</td>
<td>Amide III ((\alpha)-helix)</td>
<td>49-51</td>
</tr>
<tr>
<td>1350</td>
<td>(\nu(=C-N))</td>
<td>50, 53</td>
</tr>
<tr>
<td>1455</td>
<td>(\delta(CH2))</td>
<td>49-53, 57</td>
</tr>
<tr>
<td>1490</td>
<td>His</td>
<td>50, 51</td>
</tr>
<tr>
<td>1547</td>
<td>Phe, amide II</td>
<td>50, 51</td>
</tr>
<tr>
<td>1580</td>
<td>Tyr, (\nu(COO^-)) and/or</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phe</td>
<td></td>
</tr>
</tbody>
</table>

\(\nu = \) stretching \quad \(\delta = \) deformation

4.2.2 Conformation Dependence of SERS Spectra

To confirm that the shifts in the spectral modes observed in Figure 4.1 at elevated concentrations were due to conformational changes rather than the sole effect of changes in concentration, we performed SERS on Aβ as a soluble monomer and an insoluble oligomer. Figure 4.2 (A) and (B) illustrate the SERS spectra of soluble Aβ taken after residing in the nanofluidic device at 6 °C for 38 and 48 hours, respectively. Others have shown that maintaining Aβ at low temperatures prevents or significantly retards aggregation.\(^{59, 60}\) An increase in intensity over time of the SERS bands (except the band 1000 cm\(^{-1}\)) in Figure 4.2 (A) and (B) can be attributed to an increase in concentration of the target molecules and in
the density of the clusters due to the continuous flow of the device (as previously illustrated in Figure 2.2). This sharpening feature may be due to a longer interaction time, allowing more Aβ to bind to the nanoparticle’s surface. As also seen in Figure 4.1 (A), a sharp band at 1266 cm\(^{-1}\) is observed in Figure 4.2 (A) and (B); probably due to the α-helix structure adsorbing to the metal surface.\(^{51}\) These spectra also exhibit distinct bands (seen previously in Figure 4.1) assigned to phenylalanine (1000 cm\(^{-1}\)), histidine (1488 cm\(^{-1}\)) and the tyrosine doublet (823 and 856 cm\(^{-1}\)).

In Figure 4.2 (C) and (D), the spectra of Aβ oligomer, prepared by allowing Aβ to aggregate at room temperature, contain similar qualities which are in contrast to the soluble monomers of Figure 4.2 (A) and (B), such as a relatively strong band at 1244 cm\(^{-1}\) in (C) and 1266 cm\(^{-1}\) in (D). As explained in the previous section, the shift in the amide bands, from a mode characteristic of α-helix to β-sheet signifies conformational changes in the Aβ protein typical of the transition from soluble monomer to insoluble protofibrils or fibrils upon incubation.\(^{9,16,18,61}\) A strong band at around 1244 cm\(^{-1}\) associated with β-sheet structure has been observed.\(^{50,57,58}\) Furthermore, the presence of the amide III modes (1244 and 1266 cm\(^{-1}\)) in Figure 4.2 (D) confirms that the Aβ was probably in the midst of Aβ fibrillogenesis. If we further compare Figure 4.2 (C) and Figure 4.1 (C), spectral similarities attributed to β-sheets (675 and 1244 cm\(^{-1}\) modes) and phenylalanine suggest that Aβ at 11.5 nM had appreciable β-pleated sheets after 24 hours. It is difficult to predict \textit{a priori} the effect that the metal surface may have on protein structure or how the metal surface will alter the kinetics of a self-association process, such as the Aβ aggregation under investigation. Thus, while we cannot directly compare our experimental conditions to other Aβ aggregate experiments in solution,
the general conclusions of others regarding the factors affecting aggregation should still apply, such as aggregation should be the predominant intermolecular interaction at high concentrations and that reduced temperature should retard all intra- and intermolecular interactions.

Figure 4.2: SERS spectra of soluble Aβ (1.15nM kept at 6 °C) after (A) 48 hours and (B) 38 hours in the nanofluidic device. Insoluble Aβ oligomer (1.15 nM at room temperature) after (C) 48 hours and (D) 38 hours. A pH of value was effectively consistent throughout the experiment.
4.2.3 Detection of Aβ and Confounder Proteins Using SERS

To test the feasibility of detecting Aβ in the presence of confounder proteins, SERS spectra of insulin and albumin are shown in Figure 4.3. Figure 4.3 (A) is the insoluble Aβ oligomer. The 675 cm⁻¹ band has a stronger intensity in the spectra of the insoluble Aβ oligomer than in the spectra of insulin (B) and albumin (C). Albumin, in Figure 4.3 (B), is known to have a structure consisting of around 55% α-helices and 45% random coil. The amide III band at 1294 cm⁻¹, rather than at 1244 cm⁻¹, could be due to modified Raman selection rules due to the α-helix not directly adsorbed to the metal surface because of the folding complexity of the protein. Albumin also shows strong bands associated with tyrosine, phenylalanine and tryptophan (832, 856, 1000, 1030, 1185, and 1580 cm⁻¹), indicating that albumin is adsorbed onto the nanoparticle surfaces via its aromatic side chains. Insulin, Figure 4.3 (C), consists of two polypeptide chains joined with two cysteine disulfide bonds, with one disulfide bond involved in an intra-chain link. Although the C-S vibrations (654 cm⁻¹) are overlapped by the broad band at 675 cm⁻¹, the spectrum of insulin is still distinguishable from spectra of other proteins due to the S-S stretching mode at 549 cm⁻¹.

A method of quantitatively assessing the degree of the protein’s structural conformations involves using a radiometric measurement of the spectral height of amide III bands relative to some band (such as the CH₂ bending mode at 1455 cm⁻¹) whose intensity is independent of conformational content. Taking radiometric measurements of these bands gives ratios of 1.070, 1.223, 1.207, and 1.154 for Aβ monomer, insoluble Aβ oligomer, insulin, and albumin, respectively. The larger fraction indicates that more β-sheet and random coil structural elements are present in the protein. Consequently, we may be able to
distinguish confounder proteins from Aβ by their ratiometric spectral intensities and the absence and presence of certain Raman modes pertaining to amino acid residues in the protein available to interact with the metal surface.

Figure 4.3: SERS spectra of (A) insoluble Aβ oligomer (B) albumin and (C) insulin after 48 hours in the nanofluidic device. The three proteins were mixed with gold colloid at 0.1% v/v, bringing the proteins to a final concentration of 1.15 nM, and loaded into the channel immediately.
CHAPTER V

CONCLUSIONS AND FUTURE WORK

This thesis has demonstrated a novel nanofluidic trapping device with the ability to encourage aggregation of gold nanoparticles and circumvent the problem of random aggregation induced by electrolytes. Compared to other SERS techniques, the nanofluidic device is favorable for Aβ detection as well as molecule detection at trace levels for several reasons: (1) The SERS active environment is highly reproducible since nanoparticle-molecule clusters are always aggregated at the same, specific location, namely the entrance to the nanochannel. (2) Our device has a higher sensitivity than other SERS substrates because the target molecules become more concentrated over time at the entrance to the nanochannel. (3) The bioactivity of Aβ is preserved, allowing the protein to undergo conformational changes, which opens up the possibility of direct detection of Aβ in CSF.

Future works will focus on two aspects: (1) Optimization of the nanofluidic device for SERS applications. The dimension of the nanofluidic device must be tailored to improve flow in this application by embedding a larger microchannel, in terms of width and depth, where more nanoparticles and target molecules can be transported into the nanochannel. On the other hand, the length of nanochannel, connecting the two microchannels, should be as short as possible to reduce the fluidic resistance. Besides, varying the ratio of nanochannel and microchannel width is critical to affects the SERS signals because the flow rate and cluster size may be affected. In addition, the geometric structure of the nanochannel entrance
must be designed to eliminate the dead volume regions where nanoparticle clusters are formed away from detection area and cannot contribute to SERS signals. (2) For SERS detection of Aβ, we may functionalize the nanoparticle surface to specifically bind to oligomeric Aβ in CSF and provide further discernment from other potential confounders. The use of gold nanoparticles is advantageous since its surface, which provides hydrophobicity like the cell lipid bilayer, is easily modified through self-assembly of thiol functional groups. Hence, by modifying the gold nanoparticle surface with a cell surface rich in sialic acid residues, mimicking the cell membrane, which has a high affinity and specificity for the toxic form of Aβ. Along with the use of the nanofluidic device, we have the potential to develop a sensitive and reliable SERS platform for detecting Aβ in conformations associated with AD disease.
REFERENCES


(43) Cowan, C. B., University of Maryland Baltimore County Baltimore, Maryland, *Ph.D proposal*, **2006**.


APPENDIX A

ALIGNMENT DATA FOR RAMAN SPECTROMETER

Figure A: The Raman signals of a clean silicon wafer using 20x (top left) and 50x (top right) air objectives. The intensity of the laser source was measured to be 15mW. The images of focused laser spots on the silicon wafer of 20x and 50x objectives are shown on the bottom of Figure A (left and right respectively).
APPENDIX B

REPEATABILITY OF SERS SIGNALS USING NANOFLOWIDIC DEVICE

One of the important features of using the nanofluidic device is that SERS signals are reproducible and repeatable due to the molecule-nanoparticle clusters at the entrance to the nanochannel. Figure B (inset) shows the raw spectra of Aβ monomer taken for seven consecutive scans from seven random locations on the aggregation area within 30 um around the nanochannel entrance. There are minor differences in these spectra; however, when normalized by dividing the SERS band at 1245 cm\(^{-1}\) (which does not depend on conformational content), the spectra are very similar (Figure B main). The percentage of standard deviation of the five major peaks in peak height for the Aβ monomer were determined for each of the scans as shown in the inset table, with the largest percentage of standard deviation in peak height to be a maximum of 4.6%. This result indicates that the enhancement is consistent within the area close to nanochannel. It should be noted that the SERS signal falls off rapidly as we move away from the nanochannel entrance, in agreement with one of the advantages of using nanofluidic device, the high density of nanoparticle clusters are confined in the specific region accompanied with enriched target molecules in the clusters.
Figure B: Spectra of Aβ monomer (at 1.15nM) showing the reproducibility of the SERS signals from random locations on the aggregation area at the entrance to the nanochannel. The inset spectra are the raw spectra that show slight differences in background intensity. When the difference in background intensities are corrected by normalization, the spectra show at the largest 4.6% standard deviation in peak height.
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