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Investigating the Interactions of Tri-peptides on Gold Nanoparticles using Solid-state NMR Spectroscopy and the Development of ^1H - ^2H Dipolar Recoupling Experiments

by

Ichhuk Karki

Dissertation submitted to the
Eberly College of Arts and Sciences
at West Virginia University

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in
Department of Chemistry/ Physical Chemistry

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Morgantown, West Virginia
2014

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Abstract

Investigating the Interactions of Tri-peptides on Gold Nanoparticles using Solid-state NMR Spectroscopy and the Development of ^1H - ^2H Dipolar Recoupling Experiments

Ichhuk Karki

Solid-state NMR spectroscopy is a powerful technique to study the structure of biological and inorganic systems, especially non-crystalline and insoluble samples that are difficult to study for X-ray diffraction and solution NMR spectroscopy methods. We used solid-state NMR spectroscopy to elucidate the structure and interactions of tri-peptides on gold nanoparticles. Tri-peptides CysAlaAla (CAA) and AlaAlaCys (AAC) are the focus of this work. These peptides are incorporated on gold surfaces through thiol-gold interactions and their structures on gold surfaces are studied using ^1H and ^{13}C solid-state NMR spectroscopy. NMR spectroscopy shows that CAA forms a uniform monolayer on gold nanoparticles while a bilayer of AAC forms on gold nanoparticles. The evidence of monolayer and bilayer of CAA and AAC gold nanoparticles was also supported by TGA analysis by calculating the number density of CAA and AAC on gold nanoparticles. The number of CAA and AAC on AuNPs was found to be 6 and 16 peptides/nm² respectively.

^1H -observe Rotational-Echo Double Resonance (REDOR) NMR spectroscopy and rotary resonance recoupling experiments with fast sample spinning have potential for measuring ^1H - ^2H dipolar couplings. Both of these dipolar recoupling experiments were successful in measuring heteronuclear dipolar couplings of ^2H with ^1H nuclei. Potential limitations of the experiments include ^1H background signals arising from the probe and Bloch-Siegert effects.

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List of Abbreviations

AuNPs	Gold Nanoparticles
NMR	Nuclear Magnetic Resonance
SSNMR	Solid-state NMR Spectroscopy
MAS	Magic Angle Spinning
R ³	Rotary Resonance Recoupling
REDOR	Rotational Echo Double Resonance
CP	Cross Polarization
CSA	Chemical Shift Anisotropy
CysAlaAla	Cysteine-Alanine-Alanine (CAA)
AlaAlaCys	Alanine-Alanine-Cysteine (AAC)
TNF α	Tumor necrosis factor alpha
HPLC	High Performance Liquid Chromatography
SPR	Surface Plasmon Resonance
SPPS	Solid-phase Peptide Synthesis
2-ClTrt	2-Chlorotrityl Resin
HOBt	Hydroxybenzotriazole
HBTU	<i>O</i> -Benzotriazole- <i>N,N,N',N'</i> -tetramethyl-uronium-hexafluoro-phosphate
DIPEA	<i>N,N</i> -Diisopropylethylamine
Fmoc	Fluorenylmethyloxycarbonyl
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane

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Chapter 1: Introduction

The science of gold nanoparticles (AuNPs) started in 1857 when Faraday depicted AuNPs as being finely divided metallic gold in solution.¹ When Mie quantitatively explained the size dependent optical properties found in metal nanoparticles in 1908, interest in nanoparticles quickly took over the scientific community.² The pioneering works by Schmid et al.³ and Brust et al.⁴ to synthesize AuNPs had an immense impact in this field and as a result scientists became more interested in studying the physical and chemical properties of AuNPs.

In nanometer scale, materials exhibit many interesting phenomena that are often different compared to those observed from their respective bulk material.⁵ Larger bulk material or micrometer size substances have been heavily studied by chemists, physicists, and material scientists and their properties are relatively well understood.⁶ Asymmetry at the interface and non-linear electron confinement with different sizes on the nano-scale lead to unusual physical and chemical properties that are commonly attributed to ‘quantum size effects’.⁷

AuNPs have unique optical and electronic properties such as high extinction coefficients and distance-dependent optical emissions. AuNPs also have several biomedical and nanotechnological applications.⁸ Incorporation of ligands on surfaces of nanoparticles provides additional chemical properties.⁹ A wide range of organic or biological molecules can act as ligands offering selective binding and detection of small molecules and biological targets.¹⁰ Hence, the overall characteristics of a nanoparticle are dictated by the combined effect of size and shape of the inorganic core, type of ligands, inter-particle distance, and surrounding environment such as the dielectric constant of a solution.¹¹ Nano-carriers based on gold have emerged as attractive candidates in therapeutic applications because they are inert, non-toxic, conductive, and

catalytically active.¹² Mono-dispersed nanoparticles can be formed with excellent size and shape control.^{12b} AuNPs emerged as a bio-technological tool in 1996 when thiol-modified DNAs were used to functionalize AuNPs.¹³ Also, their physicochemical properties could trigger drug release at remote sites.¹⁴ AuNPs functionalized with proteins or specific polypeptides have shown promise in treatment of cancer and immunological diseases.¹⁵ A rapid increase in AuNP related publications in various fields ranging from nanotechnology to biomedical engineering has been seen in the past decade (Figure 1.1).

Figure 1.1: Number of gold nanoparticles papers published each year from 1991 to 2013. Source: Scifinder Scholar.

There are several examples of use of AuNps in biomedical applications. AuNPs functionalized with $\text{TNF}\alpha$, termed as CYT-6091, were successfully used in Phase I dose escalation

clinical trials in advanced stage cancer patients.¹⁶ TNF α was delivered successfully to the tumor site and the AuNPs were cleared by the liver in a slow phase after TNF α degradation as shown by the pharmacokinetic and bio-distribution results.¹⁷ Alkanethiol capped AuNPs can also offer several advantages in diagnostics and therapeutics applications such as organelle-specific delivery, cell targeting, and imaging.¹⁸ Thiols bound to the surface of AuNPs provide stability and control the shape and size of the particles. Compared to the extensively studied alkanethiols on AuNPs, cysteine has the additional potential for hydrogen bonding through amino and carboxyl groups, making it an interesting molecule to study on gold surfaces. Cysteine on gold surfaces has been studied in the past using NMR spectroscopy¹⁹ and Density Functional Theory (DFT) calculations.²⁰ ¹³C and ¹H NMR spectroscopy^{19, 21} have shown that cysteine forms two layers on AuNPs. The first layer of cysteine is made of cysteine forming a thiolate bond with the gold surface and with charged amino and carboxylate functional groups oriented away from the gold surface. The second layer of cysteine has amino and carboxylate functional groups oriented toward and interacting with the first layer and its thiol functional group oriented away from the gold surface.

Peptide-functionalized AuNPs can be used as molecular linkers with a wide variety of applications. Peptide-modified gold surfaces also influence cell attachment, shape, and function.²² Kumar et al. functionalized AuNPs with CRGDK peptide, which resulted in maximal binding interaction between the CRGDK peptide and targeted Nrp-1 receptor over expressed on MDA-MB-321 cell surface. This nano-carrier improved the delivery of therapeutic P12 peptide inside targeted cells.²³ Despite the importance of interactions between AuNPs and biological molecules, the major concern is the retention of biological specificity (structure and dynamics) after adsorption or conjugation of the biomolecules.

The structure and electronic properties of AuNPs can be characterized by various methods.^{3d, 24} Several sophisticated imaging techniques such as Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM), and Transmission Electron Microscopy (TEM) could be employed to determine the shape and size of the particles.²⁴⁻²⁵ The core structural properties and composition could be probed using X-ray photoelectron spectroscopy (XPS)²⁶ and X-ray diffraction (XRD) spectroscopy.²⁷ Solid-State NMR spectroscopy (SSNMR) is one of the best structural tools in characterizing the interactions of molecules bound to gold surfaces because internal interactions such as chemical shift, quadrupolar coupling, and Knight Shift are sensitive to the electronic environment of the nuclei being examined.²⁸ SSNMR can provide the local structure and dynamics of the immobilized molecules on gold surfaces where low solubility and lack of long range order preclude the use of more traditional methods such as solution NMR spectroscopy and X-ray crystallography.²²

This dissertation presents two projects. The first part of this dissertation is the development of tri-peptide functionalized AuNPs and subsequent examination of the interactions of peptide on gold nanoparticles using SSNMR spectroscopy. Two different tri-peptides CysAlaAla (CAA) and AlaAlaCys (AAC) were synthesized for this purpose. Figure 1.1 (a) is the tri-peptide AAC with cysteine on the C-terminus and alanine in the middle and at N-terminus. Figure 1.1 (b) shows CAA with cysteine on N-terminus followed by alanine and another alanine at C-terminus. CAA and AAC were designed to create a small hydrophilic peptide that would readily attach on AuNPs forming a stable, well-packed AuNP. Cysteine was incorporated on either N-terminus or C-terminus to have strong affinity for gold surfaces through thiol functional group and the presence of alanine promotes the self-assembly of the peptide due to its ability to self-assemble into a dense layer.²⁹ Both peptides were attached on AuNPs and ¹H and ¹³C NMR spectra were obtained to

understand the interactions between peptides and AuNPs. The second part of this thesis is the development of ^1H - ^2H REDOR and ^1H - ^2H rotary resonance recoupling experiments to determine ^1H - ^2H dipolar couplings. Dipolar recoupling experiments using ^1H as the observe nucleus are inherently difficult but have great potential to contribute to structural characterization of peptides on surfaces. Potential limitations of both experiments are identified and solved.

Figure 1.2: Structure of (a) AlaAlaCys (b) CysAlaAla, Grey: Carbon; Blue: Nitrogen; Yellow: Sulfur; Red: Oxygen

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Chapter 2: Solid –State NMR Spectroscopy

NMR spectroscopy is a powerful tool to characterize the molecular-level structure and dynamics irrespective of the sample's morphology.³⁰ In the early 1920's and 1930's, Stern and Gerlach discovered the space quantization of electron spin and nuclear spin with their atomic beam experiments.³¹ Their experiments on silver atoms involved sending a beam of atoms having no orbital angular momentum of the electron through an inhomogeneous magnetic field and observing their deflection. The observed deflection of the beam into two components implied a new type of angular momentum, which attributed to the intrinsic spin of the electron, by Uhlenbeck and Goudsmit. Stern and Gerlach later discovered the intrinsic nuclear spin using a beam of hydrogen molecules. From these types of measurements, the magnetic moments of the electron and proton nuclei were determined. In the 1930's Rabi discovered that nuclear spin state transitions could be induced using varying fields or by applying electromagnetic radiation.³²

The first successful NMR spectroscopy experiments were performed independently by Felix Bloch and Edward Purcell in 1946.³³ Bloch analyzed protons in liquid water while Purcell performed experiments on solid paraffin wax. Even though many nuclei are NMR active, most NMR experiments are focused on spin-half nuclei such as ^1H , ^{13}C and ^{19}F . Low natural abundance, small gyromagnetic ratios, long relaxation times, and large quadrupole moments can complicate NMR experiments.³⁴ Solution NMR spectroscopy is a standard tool to determine the structure and dynamics of small molecules and complicated systems such as partially disordered proteins.³⁵ However, solution NMR spectroscopy is limited by molecular size which affects the tumbling rate of a sample.³⁶ Therefore, experimental techniques like SSNMR can be used to study and

characterize the behavior of large molecules with solubility issues.³⁷ Additionally, many materials simply need to be examined in the solid-state because that is the phase of interest.

One of the reasons that NMR spectroscopy is so powerful can be attributed to the fact that there are many types of spin interactions that contain information on structural and dynamics of a sample. There are two different types of interactions in SSNMR spectroscopy: external spin interactions and internal spin interactions. The total Hamiltonian can be described as the sum of different interactions.

$$\mathcal{H}_{\text{NMR}} = \mathcal{H}_{\text{Z}} + \mathcal{H}_{\text{RF}} + \mathcal{H}_{\text{S}} + \mathcal{H}_{\text{J}} + \mathcal{H}_{\text{D}} + \mathcal{H}_{\text{Q}} \quad (2.1)$$

$\mathcal{H}_{\text{RF}}$ is the radio frequency Hamiltonian associated with radio-frequency rf excitations of the spin system. $\mathcal{H}_{\text{Z}}$ is the Zeeman energy, and $\mathcal{H}_{\text{S}}$, $\mathcal{H}_{\text{J}}$, $\mathcal{H}_{\text{D}}$, $\mathcal{H}_{\text{Q}}$ are all internal interactions associated with magnetic shielding, indirect couplings, dipolar couplings, and quadrupolar couplings, respectively. Each of these interactions is discussed below in detail.

Most of these internal interactions depend on the orientation of the molecule with respect to the magnetic field direction and are inherently anisotropic.³⁸ In solutions, molecules are rapidly tumbling due to Brownian motion, thus the relevant energies are averaged over all orientations. However, in solids, where motions are usually restricted, the internal interactions depend strongly on the orientation of the molecule with respect to the external Zeeman field.

2.1 The Zeeman Interaction

When a sample is placed in a magnetic field, $\vec{B}_0$, the nuclear magnetic moments, $\vec{\mu}$, interact with $\vec{B}_0$ to produce a net nuclear magnetization vector $\vec{M} = \sum \vec{\mu}$. This interaction is called the Zeeman interaction and is the largest contribution to the total energy of the spin system. The nuclear magnetic moment is related to the spin angular momentum operator $\hat{I}$ by $\vec{\mu} = \gamma \hbar \hat{I}$ where

$\vec{\mu}$ is nuclear magnetic moment operator, $\hbar$ is the Plank's constant divided by 2π , and γ is the gyromagnetic ratio. Accordingly taking $\vec{B}_0 = B_0 \hat{z}$, the Zeeman Hamiltonian, $\hat{H}_z$ is:

$$\hat{H}_z = -\vec{\mu} \cdot \vec{B}_0 \quad (2.2)$$

Since, $\vec{\mu} = \gamma \hbar \hat{I}$

$$\hat{H}_z = -\gamma \hbar B_0 \hat{I}_z \quad (2.3)$$

In the presence of a strong magnetic field the Zeeman interaction splits the degenerate spin energy levels with the energy spacing directly proportional to the $\vec{B}_0$ strength. For $I = 1/2$ nuclei there are two states represented as $|\frac{1}{2}, \frac{1}{2}\rangle$ and $|\frac{1}{2}, -\frac{1}{2}\rangle$, where the first number is the value of I and the second number is the value of magnetic quantum number, m_s . The energies of these two states are determined by the eigenvalue equation $\hat{H}\psi = E\psi$,

For $m_s = 1/2$,

$-\gamma \hbar B_0 \hat{I}_z |\frac{1}{2}, \frac{1}{2}\rangle = -\frac{1}{2} \gamma \hbar B_0 |\frac{1}{2}, \frac{1}{2}\rangle$, which gives an energy of $-\frac{1}{2} \gamma \hbar B_0$.

For $m_s = -1/2$, the energy is $+\frac{1}{2} \gamma \hbar B_0$

The difference in energy between the two states is $\Delta E = \frac{1}{2} \gamma \hbar B_0 - (-\frac{1}{2} \gamma \hbar B_0) = \gamma \hbar B_0$.

For simplicity, the two spin states for spin $1/2$ nuclei can be depicted as being parallel $|\frac{1}{2}, \frac{1}{2}\rangle$ or anti-parallel $|\frac{1}{2}, -\frac{1}{2}\rangle$ to the $\vec{B}_0$ direction. At thermal equilibrium, the population in each spin state, $n_{1/2}$ and $n_{-1/2}$, is given by Boltzmann distribution.³⁹

$$\frac{n_{-1/2}}{n_{1/2}} = e^{-\left(\frac{\Delta E}{kT}\right)} \quad (2.4)$$

where k is the Boltzmann constant, T is the absolute temperature, ΔE is the energy level

difference between the $|\frac{1}{2}, \frac{1}{2}\rangle$ and $|\frac{1}{2}, -\frac{1}{2}\rangle$ states. To give some idea of the size of this population difference, consider ^1H nuclei in a field of 7 tesla (~ 300 MHz for protons).

At room temperature:

$$\frac{n_{-1/2}}{n_{1/2}} = e^{-\left(\frac{\Delta E}{kT}\right)} = e^{\left\{-\frac{(26.75 \times 10^7) \left(\frac{6.626 \times 10^{-34}}{2\pi}\right)(7)}{1.38 \times 10^{-23} \times 300}\right\}}$$

= 0.99995. Hence, for every 1,000,000 nuclei in the upper energy state there are 1,000,050 nuclei in the lower energy state. The net magnetization is what is detected in NMR spectroscopy and as can be seen from this calculation the population difference is very small. As a consequence NMR signals are generally considered weak. Increasing the magnetic field strength and using isotopic enrichment for the rare spin nuclei are ways to increase NMR spectroscopy signals. Since, we cannot change our magnetic field strengths, ^{13}C labeling will be used in many of our ^{13}C -observe NMR spectroscopy experiments.

Figure 2.1: Energy levels for a nucleus with spin quantum number $\frac{1}{2}$.

2.2 The Effect of RF Pulses

Excitation of the nuclear spin system in NMR spectroscopy is done by applying a pulsed radiofrequency (rf) field with angular frequency ω_{rf} and amplitude B_1 . The frequency ω_{rf} of the

applied rf is close to the Larmor frequency, ω_0 . The Larmor frequency is $\omega_0 = \gamma B_0$ and is the energy difference (in frequency units) between spin states. The total external magnetic field is the combination of the Zeeman field and the rf irradiation. The time-dependence of the magnetic field in this case means that both the eigenstates of the spin systems and their energies are time-dependent, in contrast to the previous case considered of the nuclei in the static field B_0 . The oscillating field $B_1(t)$ mixes the Zeeman states. The Hamiltonian, $\hat{H}$, describing a single spin in this situation include the interaction of the nuclear spin with both the static B_0 field along z and the oscillating $B_1(t)$ field, which will be taken to oscillate along the x direction. The total field felt by the nucleus is then:

$$\vec{B}_{\text{total}}(t) = \hat{x}B_1\cos(\omega_{\text{rf}}t) + \hat{z}B_0 \quad (2.5)$$

Where, $\hat{z}$ and $\hat{x}$ are unit vectors along the z- and x-axes respectively.

Thus,

$$\hat{H}_{\text{rf}} = -\hat{\mu} \cdot \vec{B}_1 = -\gamma\hbar B_1\cos(\omega_{\text{rf}}t) \hat{I}_x \quad (2.6)$$

and the external Hamiltonian is

$$\hat{H} = -\gamma\hbar B_0 \hat{I}_z - \gamma\hbar B_1\cos(\omega_{\text{rf}}t) \hat{I}_x \quad (2.7)$$

2.3 Chemical Shift Anisotropy

An important interaction in both solid-state and solution NMR spectroscopy is the chemical shift interaction. The chemical shift is due to the change in the actual magnetic field at the nucleus because the applied magnetic field induces changes in electronic orbital about the nucleus that generate local fields. This is similar to Lenz's law in classical physics. Hence, the interaction between the nuclear spin and the field generated by the surrounding electrons is known as chemical shift or chemical shielding interaction. The corresponding Hamiltonian is

$$\hat{H} = -\gamma h \hat{I}_z \cdot (-\bar{\sigma}) B_0 = \gamma h \hat{I}_z \cdot \sigma \cdot \vec{B}_0 = \gamma h \hat{I}_z \cdot B_{\text{loc}} \quad (2.8)$$

$\bar{\sigma}$ is a second rank Cartesian tensor called the chemical shielding tensor, which is a 3×3 matrix with 9 components used to define the relative orientation of chemical shielding tensor with respect to the laboratory frame (Equation 2.9). The shielding tensor, $\bar{\sigma}$, links the magnetic field arising from shielding of the nucleus by the electrons, B_{loc} , to the applied magnetic field, B_0 , i.e. $B_{\text{loc}} = \sigma B_0$. However, tensors are often symmetric and the anti-symmetric contributions are usually ignored in NMR spectra. Therefore, it is possible to choose the tensors in its principal axis system (PAS).

$$\sigma = \begin{pmatrix} \sigma_{xx} & \sigma_{xy} & \sigma_{xz} \\ \sigma_{yx} & \sigma_{yy} & \sigma_{yz} \\ \sigma_{zx} & \sigma_{zy} & \sigma_{zz} \end{pmatrix}_{\text{sym}} \xrightarrow{L \rightarrow P} \begin{pmatrix} \sigma_{11} & 0 & 0 \\ 0 & \sigma_{22} & 0 \\ 0 & 0 & \sigma_{33} \end{pmatrix} \quad (2.9)$$

Figure 2.2 : (a) An ellipsoid represents the chemical shielding tensor oriented in the laboratory frame. (b) The orientation of the chemical shielding tensor with respect to the external magnetic field, where θ and ϕ represent the polar and azimuthal angles.

The isotropic chemical shielding σ_{iso} is described by equation 2.10.

$$\sigma_{\text{iso}} = \frac{1}{3} (\sigma_{xx} + \sigma_{yy} + \sigma_{zz}) \quad (2.10)$$

σ_{iso} is the quantity that would be observed in solution state NMR spectroscopy. The anisotropy Δ and asymmetry η of $\bar{\sigma}$ is defined by equation 2.11 and 2.12 respectively.

$$\Delta = \sigma_{zz} - \sigma_{\text{iso}} \quad (2.11)$$

$$\eta = \frac{\sigma_{xx} - \sigma_{yy}}{\Delta} \quad (2.12)$$

The anisotropy in solution is averaged out by the rapid tumbling of molecules and only isotropic chemical shift remains. However, in SSNMR spectroscopy the chemical shielding anisotropy gives rise to rich structural features in the spectrum.

2.4 Indirect Coupling

Indirect coupling or J coupling arises from the interactions between the nuclei and local electrons. This is also known as scalar coupling or spin-spin coupling. This coupling parameter is very important for solution NMR spectroscopy but somewhat less important in SSNMR. This is mainly because J coupling are the smallest contribution to the total energy obtained in SSNMR.

2.5 Dipolar Coupling

The dipolar coupling is a through-space coupling between two NMR active nuclei. When two spins are close to each other, the magnetic field generated by one nucleus can interact through space with the neighboring spin. Dipolar couplings between identical nuclei are called homonuclear dipolar couplings (i.e. ^{13}C - ^{13}C) and those arising between different nuclei are called heteronuclear dipolar coupling (i.e. ^{13}C - ^1H). Consider two spin nuclei I and S, the dipolar coupling is:

$$\hat{H}_{\text{Hetero}} = -d (3\cos^2\Theta - 1) \hat{I}_z \hat{S}_z \quad (2.13)$$

$$\hat{H}_{\text{Homo}} = -\frac{d}{2} (3\cos^2\Theta - 1) [3\hat{I}_z \hat{S}_z - (\hat{I}_z \cdot \hat{S}_z)] \quad (2.14)$$

where d is the dipolar coupling constant and Θ is the angle between the z-axis and the internuclear vector.

Figure 2.3: Dipolar coupling between heteronuclear spin pair I and S where r is the distance between I and S and θ and ϕ represent polar and azimuthal angles, respectively.

2.6 Quadrupolar Coupling

Nuclei containing spin number greater than $\frac{1}{2}$ have quadrupolar coupling interactions. Quadrupolar nuclei have an asymmetric distribution of nucleons giving rise to a non-spherical positive electric charge distribution that interacts with local electric field gradients. The quadrupolar interaction $\mathbf{Q}$ depends on the electric field gradient V at the nuclei as described by equation 2.15:

$$\mathbf{Q} = \frac{eQV}{2I(2I-1)h} \quad (2.15)$$

Where Q is the electric quadrupolar moment, e is the charge of an electron, I is the spin quantum number, and the electric field gradient V is given by:

$$V = \begin{bmatrix} V_{xx} & 0 & 0 \\ 0 & V_{yy} & 0 \\ 0 & 0 & V_{zz} \end{bmatrix} \quad (2.16)$$

$$\text{The principal axes being denoted by the order } |V_{zz}| \geq |V_{xx}| \geq |V_{yy}| \quad (2.17)$$

The nuclear quadrupolar coupling constant, χ , and quadrupolar asymmetry, η , are defined as:

$$\chi = \frac{e^2 Q V_{zz}}{h} \quad (2.18)$$

$$\eta = \frac{(V_{yy} - V_{xx})}{V_{zz}} \quad (2.19)$$

where V_{zz} corresponds to the largest principal component of the electric field gradient, V .

Figure 2.4: Graphical representation of the charge distribution between a spin $\frac{1}{2}$ and spin greater than $\frac{1}{2}$ nuclei. The non-spherical distribution of charge in these nuclei gives rise to the quadrupolar coupling.

2.7 Relaxation Processes

There are two main types of relaxation commonly encountered in NMR spectroscopy: spin-lattice and spin-spin relaxation.

Spin-lattice relaxation, also known as longitudinal relaxation, describes the process of equilibration of the z-component of the nuclear magnetization of a sample in a magnetic field. This process is generally exponential and is described by a time constant T_1 . Spin-spin relaxation reflects the lifetime of the observed NMR signal. This decay process is known as transverse relaxation and is described by time constant T_2 . The value of T_2 is directly related to the line width of resonances.

2.8 Magic-Angle Spinning

A High-resolution SSNMR spectrum is obtained by employing the magic angle spinning (MAS) technique. The most prominent internal interactions like dipolar coupling, chemical shift anisotropy, and first order quadrupolar coupling depends on the orientation of the molecule in the applied magnetic field. All of these NMR interactions have a common mathematical $(3\cos^2\Theta - 1)$ form. The rapid molecular motion in solution NMR spectroscopy averages this spatial component to zero. In solids, however, averaging of this term can be achieved by spinning the sample about an angle of 54.74° with respect to the applied magnetic field.⁴⁰ This technique was originally introduced by Andrew⁴⁰ and Lowe⁴¹ in 1958 and since then it has been used to obtain high-resolution NMR spectra of solids.

Figure 2.5: Magic Angle Spinning of the sample. B_0 represents the external magnetic field and θ_m is the angle between the external magnetic field and the sample rotor.

A manifold of spinning sidebands becomes visible if the sample is spun at a rate less than the magnitude of anisotropic interaction. The physical spinning of the sample is obtained via an air turbine mechanism. The compressed air is directed at a tiny, carefully crafted turbine attached to a sample rotor that makes the rotor spin. The bearing pressure helps the rotor float on a cushion of air.

2.9 Cross Polarization

Obtaining NMR spectra of dilute spins such as ^{13}C and ^{15}N tend to take a long time owing to their low sensitivity. Polarization from abundant spins such as ^1H can be transferred to dilute spins such as ^{13}C to enhance the sensitivity. This technique is called cross polarization, which exploits the fact that the dilute and abundant nuclei are in close proximity in many solids and are thus coupled via the magnetic dipolar interaction.⁴² There are two advantages of cross polarization:

1) Cross polarization enhances the dilute spin magnetization and 2) Spin-lattice relaxation times

of protons tend to be short compared to rare spin nuclei and hence signal averaging can take place at a faster rate. In order for the polarization transfer to be possible, the magnetization of dilute and abundant spins must fulfill the Hartmann-Hahn condition.⁴³

$$\gamma_{\text{H}}B_I(^1\text{H}) = \gamma_{\text{C}}B_I(^{13}\text{C}) \quad (2.20)$$

The cross polarization pulse sequence is shown in Figure 2.6. The initial ^1H 90° pulse directed along x rotates the ^1H magnetization onto the y axis. The second 90° phase-shifted ^1H pulse spin-locks the magnetization. During the spin-lock period, a pulse is applied simultaneously at the ^{13}C frequency. The time for which these two pulses are applied is called the contact time. Under the Hartmann-Hahn condition, magnetization will transfer from ^1H to ^{13}C , allowing ^{13}C magnetization will build up during the contact time. The NMR signal of the ^{13}C spins is then measured during the acquisition time.

Figure 2.6: Cross-Polarization pulse sequence

2.10 Rotational-Echo, Double-Resonance NMR

Internuclear distances between nuclei provide important structural information.⁴⁴ The internuclear distances can be measured by SSNMR by measuring the dipolar interaction between the coupled pairs because of the r^{-3} dependence of this interaction. The heteronuclear distance measurement was demonstrated by spin-echo double resonance, which measures weak dipolar couplings selectively in the presence of other large spin interactions. REDOR⁴⁵ is used to measure the dipolar couplings between heteronuclear pairs of spin under magic angle spinning. REDOR heteronuclear dipolar recoupling experiment has become a standard SSNMR method for determining internuclear distances. The dipolar interaction is suppressed in a coherent manner by MAS NMR.⁴⁶ The molecular tumbling in solution NMR spectroscopy averages the dipolar coupling in a non-coherent manner and makes it impossible to recouple,⁴⁷ while MAS only averages the spatial coordinates in a coherent manner. Therefore, by manipulating the spin coordinates through appropriate rf pulses, it is possible to measure the dipolar coupling. REDOR NMR utilizes rotor-synchronized π pulses to recover the heteronuclear dipolar interaction averaged away during the MAS.

Since the REDOR experiment is based primarily on trains of π pulses, it basically toggles the spin states synchronously with the sample rotation, counteracting the spatial averaging of the dipolar interaction caused by sample rotation. According to the average Hamiltonian theory, the spin system evolves with a nonzero average dipolar interaction. This causes the signal intensity to dephase and become attenuated. This dependency of signal reduction on the dipolar evolution time provides a direct way to determine the dipolar coupling and obtain internuclear distances. REDOR data analysis is done by comparing the normalized dipolar dephased signal to a universal dipolar dephasing curve.

Under MAS, the dipolar coupling between heteronuclei within a magnetic field is dependent upon both spatial and spin coordinates as described by Equation 2.21

$$\hat{\mathcal{H}}_D = \omega_D \mathbf{S}_z \mathbf{I}_z \quad (2.21)$$

where,

$$\omega_D = d[\sin^2\beta \cos 2(\omega_r t + \alpha) - \sqrt{2} \sin 2\beta \cos(\omega_r t + \alpha)] \quad (2.22)$$

$$\text{and } d = \frac{\mu_0 \hbar \gamma_I \gamma_S}{4\pi r^3}.$$

γ_I and γ_S are the gyromagnetic ratios for the I (observe spin) and S (dephase spin) spins, μ_0 is the permeability of free space, and r is the internuclear distance between I and S spins. The orientation of a vector directed between the I and S spins in the rotor frame is defined by azimuthal angle α and the polar angle β .

Under MAS condition, equation 2.22 shows that the average heteronuclear dipolar interaction is zero, and the consequence of this averaging is that the dipolar interaction has little effect on the observed spectra. It is possible to obtain a non-zero dipolar Hamiltonian by toggling the spin states of the S and I spin synchronously with the sample rotation. This is the principle behind the REDOR experiment.

REDOR consists of three parts as shown in Figure 2.7. They are preparation period, dipolar evolution period, and signal detection period. During the preparation period, the transverse magnetization of the observed spin is produced. Cross polarization is performed to enhance the signal of low sensitive nuclei. Dipolar evolution is performed in two parts: 1) With rotor-synchronized S-spin pulses to cause dipolar dephasing of the I-spins to obtain dephase (S) spectrum and 2) without the pulse yielding (S_0) spectrum that is used as a control experiment. Comparison of the two experiments yields the internuclear distances between dipolar-coupled spin pairs. Finally, the acquisition of time domain signal occurs during the signal detection period.

Figure 2.7: ^{13}C - ^{15}N REDOR pulse sequence.

2.11 NMR Spectroscopy Hardware

The major components for an NMR spectrometer consist of a transmitter, magnet, and a receiver.

Figure 2.8: Basic set-up of an NMR spectrometer

The magnet is made of superconducting wire and immersed in liquid helium surrounded by a liquid nitrogen dewar. The cold environment maintains the superconducting properties of the wire. Thus, the sample inside the bore of the magnet experiences a stable external magnetic field. Commercially, magnets producing 23.5 tesla fields (900 MHz) are available. Magnetic field inhomogeneities can be adjusted with shimming coils.

Probes in NMR spectrometer are used to hold and irradiate the sample. The probe consists of a stator with an NMR coil. The coil for the SSNMR probe is normally aligned at the magic angle to the external field. Other components of the NMR probe consist of circuits used to tune the probe and isolate the channels.

The high power amplifier and the preamplifier are separated by high power pass diodes, RF filters, and quarter-wavelength transmission lines to direct the signals appropriately. The amplifier is used to amplify the weak rf pulses from the transmitter to the probe. A pre-amplifier is required to amplify the subsequent NMR signal sent to the receiver. The computer is used to collect the information obtained from NMR console and process the data.

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Chapter 3: Experimental Methods

3.1 Peptide Synthesis

A revolutionary advance in the field of peptide chemistry occurred when Merrifield introduced the method of solid phase peptide synthesis (SPPS) in 1963.⁴⁸ As a result of which the 1984 Nobel Prize for chemistry was awarded to R.B. Merrifield for its significance and impact. In SPPS, the peptide is synthesized while it is covalently bonded to an insoluble polymeric support (resin). This enhances the final yield of the peptide compared to solution phase peptide synthesis methods since this method enables the resin bound products to be washed multiple times without product loss. Washing between each successive steps helps to get rid of unwanted byproducts that can complicate subsequent amino acids coupling steps. Figure 3.1 gives an overview of peptide synthesis.

The synthesis of the peptide is started with the amino acid that has on C-terminus. The first amino acid is bound to the resin through an ester linkage formed using the carboxyl group of the C-terminal amino acid. The amino functional group and heteroatom-based side chain groups are masked by protecting groups to avoid coupling of these functional groups during the reaction. The N-terminal protecting group of the amino acid is then removed without affecting any other functional groups and an excess of second amino acid is introduced to form an amide bond between carboxyl group of second amino acid and amino group of first amino acid in the presence of coupling reagent. The polystyrene beads are cross-linked and are therefore totally insoluble in all organic solvents. The deprotection of N-protecting group and coupling with other amino acid is repeated until the desired peptide is formed. During each step, excess reagents are removed by

multiple washing. At the end of synthesis the peptide is cleaved from the bead by cleaving the ester linkage and any side chain protecting groups in the peptide.

Figure 3.1: Generalized Solid-phase peptide synthesis method

Unlike the Merrifield approach, Carpino and Han described the use of the Fmoc group for the protection of amino function instead of Boc functional group.⁴⁹ This method (see figure 3.2) provides a simple, rapid and efficient alternative that brought about milder reaction conditions than the traditional Merrifield method. The Fmoc protecting group can be easily deprotected with piperidine solution that does not affect the acid labile linker between the peptide and the resin. Also, the fluorene derivative present in Fmoc group can be determined easily to verify complete deprotection during the synthesis since it has strong ultraviolet absorption. Fmoc SPPS is efficient and versatile with step wise assembly procedures and simple monitoring techniques.⁵⁰

Figure 3.2: Fmoc peptide synthesis with 2-Cltrt resin.

3.1.1 The Support for SPPS: Resin

The selection of proper resin is critical in SPPS. It is essential to choose an ideal support with required stability under the reaction conditions and one that can be functionalized easily. When cysteine is used as a first amino acid during the peptide synthesis, 2-Cltrt resins is commonly used instead of other resins to avoid racemization.⁵¹

Figure 3.3: Structure of 2-Cltrt Resin

3.1.2 First residue attachment

The first step in the process of SPPS is the attachment of the resin linker to the C-terminal amino acid (see Figure 3.4). This step should be executed properly as the extent of this reaction will determine the yield of the final product. Also, any resin sites left unattached can potentially be coupled in next loading steps leading to truncated peptide by-products.

Figure 3.4: First residue attachment to 2-Cltrt resin. The Fmoc protected amino acid is attached to the 2-Cltrt resin through the ester linkage formed using the carboxyl group of the C-terminus amino acid

3.1.3 Deprotection Step

The N-Fmoc group is removed by treating with piperidine in DMF 20% v/v before coupling with the next amino acid (see Figure 3.5). The nature of acylating agent and protected amino acid activated species determines the efficiency of the coupling reactions. The product of the deprotection reaction absorbs UV strongly and hence the reaction can be monitored with spectroscopic techniques.

Figure 3.5: Fmoc deprotection using piperidine

3.1.4 Coupling step

The coupling step shown in Figure 3.6 involves amide bond formation, which requires the chemical activation of the carboxyl component of the incoming amino acid.

Figure 3.6: Second amino-acid attachment

3.1.5 Monitoring the progress of amino acid couplings

The progress of amino acid couplings can be followed using the ninhydrin test for free amines, as shown in Figure 3.7.⁵² The ninhydrin solution turns dark blue (positive result) in the presence of free primary amine but is otherwise colorless (negative result).

Figure 3.7: Ninhydrin Test

2 drops of 40% phenol in ethanol, 2 drops of 0.014mol/L KCN in pyridine, and 2 drops of 5% ninhydrin in ethanol were added to a few beads and heated for 5 minutes at 100°C. A positive ninhydrin test was indicated by blue/purple resin beads, indicating that coupling was not complete and that there were still uncoupled amines left on the beads. A negative test was indicated by a colorless or yellow/brown color.

3.1.6 Cleavage of the peptide from resin

The final step in SPPS is the removal of the peptide chain from the solid support, as indicated in Figure 3.8. The most popular reagent for cleavage of peptides from N-Fmoc based resins is TFA. In order to prevent possible side reactions during the cleavage procedure, appropriate scavengers and reaction conditions must be chosen.

Figure 3.8: Cleavage of peptide from resin

3.1.7 Synthesis of CAA

250 mg (0.39mmol) of 2-Cltrt resin was placed in a 10 ml plastic syringe, shaken in 6 ml of dry DCM for 10 minutes, and drained. To the resin 180.9 mg of Fmoc-alanine (0.58 mmol) was dissolved in 6 mL DCM and 175 uL DIPEA was added and shaken for 45 minutes at room temperature. To chemically unreacted trityl groups, 0.2 mL methanol was added and shaken for 5 more minutes. The resin was washed with DMF (4 times at 2 minutes each) to remove any unbound alanine on the beads. In order to deprotect the Fmoc group the resin was treated with 5 mL of 20% piperidine/DMF (4 times at 5 minutes each). After complete removal of Fmoc group with piperidine, beads were washed multiple times with DMF to remove residual piperidine. To couple the second alanine, 603 mg of Fmoc-alanine (1.94 mmol) was dissolved in 3.88 mL HBTU/HOBT (0.45 M) and 675.9 uL DIPEA. This solution was added to the reaction vessel and the coupling reaction was shaken for 45 minutes at room temperature. The Fmoc group was deprotected with 20% piperidine and washed multiple times with DMF. Finally, 1.13 g of Fmoc-cysteine (U- ^{13}C , ^{15}N) was added to 3.88 mL HBTU/HOBT (0.45 M) and 675.9 uL DIPEA and coupled in the reaction vessel for 45 minutes; the fmoc group was deprotected again and washed with DMF and then with DCM. To obtain crude CAA a total of 3 ml of TFA/TIS/Water/EDT

(94:1:2.5:2.5) was added to the resin and shaken for 1.5 hours. This solution was then added to cold ether to precipitate glassy peptide films of crude CAA.

3.1.8 Synthesis of AAC

250 mg (0.39mmol) of 2-cltrt resin was placed on a 10 ml plastic syringe, shaken in 6 ml of dry DCM for 10 minutes, and drained. To the resin 340 mg of Fmoc-cysteine ($U-^{13}C$, ^{15}N) was dissolved in 6 mL DCM and 175 μ L DIPEA was added and shaken for 45 minutes at room temperature. To endcap any remaining trityl groups, 0.2 mL methanol was added and shaken for 5 more minutes. The resin was washed with DMF (4 times at 2 minutes each) to remove any unbound Fmoc-cysteine on the beads. In order to deprotect the Fmoc group the resin was treated with 5 mL of 20% piperidine/DMF (4 times at 5 minutes each). After complete removal of Fmoc group with piperidine, beads were washed multiple times with DMF to remove residue of piperidine. To couple the second alanine, 603 mg of Fmoc-alanine (1.94 mmol) was dissolved in 3.88 mL HBTU/HOBT (0.45 M) and 675.9 μ L DIPEA. This solution was added to the reaction vessel and the coupling reaction was shaken for 45 minutes at room temperature. The Fmoc group was deprotected using 20% piperidine as before. Finally, 603 mg of Fmoc-alanine was added to 3.88 mL HBTU/HOBT (0.45 M) and 675.9 μ L DIPEA and coupled in the reaction vessel for 45 minutes and the Fmoc group was deprotected again. To obtain crude AAC a total of 3 ml of TFA/TIS/Water/EDT (94:1:2.5:2.5) was added to the resin and shaken for 1.5 hours. This solution was then added to cold ether to precipitate glassy peptide films of crude AAC.

3.1.9 Purification and Characterization of Cleaved Peptides

High performance liquid chromatography (HPLC) was developed in the mid-1970s and has been an important tool to achieve peptide with high purity.⁵³ HPLC has gained importance as an analytical tool because of its high sensitivity, speed, and resolving power.⁵⁴ HPLC is the term

used to describe liquid chromatography in which the mobile phase is mechanically pumped through the column that contains the stationary phase. HPLC consists of an injector, a pump, a stationary column, a detector, and a sample collector. Stationary phases typically used in Reversed Phase-HPLC are silica-based supports modified by chemically bonded octyl (C8) and octadecyl (C18) functional groups. These allow for the hydrophobic surface where the separation takes place. The mobile phases used in reversed-phase chromatography are based on a polar solvent, typically water, to which a less polar solvent such as acetonitrile is added.

Analysis conditions

Two different buffer solutions were prepared. The aqueous solution was prepared by adding 0.1% Trifluoroacetic acid (TFA) solution to nano-pure water and also 0.1% TFA was added to the acetonitrile for organic phase. The analyses were performed by gradient elution starting with a primarily aqueous phase and the organic content was gradually increased.

Solvent system

A= HPLC grade water, 0.1% TFA

B= HPLC grade acetonitrile, 0.1% TFA

The following gradient time program was used for all the samples.

Time (min)	%B
0	20
10	60
15	80
15.1	20
20	20

Table 1: Gradient elution performed in HPLC to purify crude peptides.

3.2 Gold-Nanoparticles Synthesis

3.2.1 Surface Plasmon Resonance

Surface plasmon resonance (SPR) is a very sensitive technique to obtain the optical properties of metal nanoparticles.⁵⁵ SPR requires minimal use of equipment and can be performed with a standard ultraviolet-visible (UV-Vis) spectrometer. Many metallic nanoparticles such as gold and silver have a strong broad absorption band in the absorption spectra. The concept of surface plasmons originates in the plasma approach of Maxwell's theory. The free electrons of a metal are treated as an electron liquid of high density (plasma) and density fluctuations occurring on the surface of such a liquid are called plasmons or surface plasmons.

Surface Plasmon Resonance is the condition when the frequency of the incident light matches the natural frequency of the surface electrons and causes the collective oscillation of valence electrons. Being confined to the surface, this collective oscillation of the free electrons is sensitive to changes in the size of the particle. Hence, smaller size particles require higher energy to collectively excite motion of the surface plasmon electron.

The interaction of light by AuNPs is strongly dictated by their environment, size and physical dimensions. Small AuNPs absorb light in the blue-green portion of the spectrum while red light is reflected, yielding a rich red color. As the size of AuNPs increases, the wavelength of surface plasmon resonance absorption shifts to longer wavelengths yielding blue or purple color. When the size approaches the bulk limit, the surface plasmon resonance wavelengths move into the IR portion of the spectrum and most visible wavelengths are reflected yielding a clear color.

AuNPs with a diameter close to 10 nm absorb at visible wavelengths with a maximum absorbance near 520 nm. As the size of AuNPs increase the maximum absorbance wavelength increases from 520 nm to less than 100 nm. Figure 3.9 shows the variation in colors of different sizes AuNPs and their UV-vis absorbance. The smallest particle appears red wine color and absorbs light at 513 nm while the largest particle appears blue and absorbs light at 530 nm.

Figure 3.9: UV-vis absorbance of different size AuNPs. Resonances at 513, 515, 517, 525, and 530 nm are due to surface plasmons of gold colloids with increasing size from left to right.

3.2.2 Synthesis of gold nanoparticles

AuNPs was prepared by a method adapted from S. N. Sahu et al.⁵⁶ A solution of chloroauric acid was reduced with sodium borohydride (NaBH_4). The color of the solution turned purple, indicating the reduction of chloroaurate ions to form colloidal gold particles. Typically, 1mM HAuCl_4 solution in 100 mL water was reduced with 0.02g NaBH_4 . NaBH_4 solution was added

drop wise and the mixture was stirred vigorously to insure uniform particles. Upon addition of NaBH_4 , the solution color immediately changes from yellowish to ruby red, indicating the formation of gold nanoparticles. The solution was allowed to react for an hour. The chloroauric acid is very hygroscopic and must be stored in refrigerator.

3.2.3 Synthesis of Cys($\text{U-}^{13}\text{C},^{15}\text{N}$)AlaAla and AlaAlaCys($\text{U-}^{13}\text{C},^{15}\text{N}$) on gold nanoparticles

Both Cys($\text{U-}^{13}\text{C},^{15}\text{N}$)AlaAla and AlaAlaCys($\text{U-}^{13}\text{C},^{15}\text{N}$) was attached on the AuNPs using a well established protocol.⁵⁷ 1.38 mM Cys($\text{U-}^{13}\text{C},^{15}\text{N}$)AlaAla or AlaAlaCys($\text{U-}^{13}\text{C},^{15}\text{N}$) was added to 10 ml of 1.9 mM HEPES buffer (pH 7.2). This peptide solution was added to the AuNPs reduced with NaBH_4 and the solution was left undisturbed overnight under an argon atmosphere. The conjugation was made in the presence of excess peptide to ensure full coverage of AuNPs. Observation of a purple color after 12 hours confirms that aggregation of the AuNPs has not occurred. The peptide-AuNPs system were collected by centrifugation at 15,000 rpm for 30 minutes and washed three times with water to remove any unbound peptides. In order to check the absence of non-conjugated peptide, the supernatant after the third wash was checked by UV-vis absorbance spectrophotometer. The sample was lyophilized before characterization by with SSNMR spectroscopy.

3.3 Solid-state NMR Spectroscopy

MAS NMR spectra were obtained using a triple-channel custom-built spectrometer with a Tecmag libra pulse programmer and a 3.55 T magnet (proton frequency of 151.395 MHz). The triple-channel probe is a transmission-line design, and it incorporates a Chemagnetics 7.5 mm pencil rotor spinning assembly with a 14 mm long, 8.65 mm inner diameter, 6 turn coil made of 14 gauge wire. Radio-frequency field strengths were 114 kHz for ^1H decoupling, 49 kHz for ^1H - ^{13}C cross-polarization, 49 kHz for ^{13}C pulses, and 48 kHz for the ^{15}N pulses. The ^{13}C and ^{15}N

power amplifiers were under active control. All spectra were obtained with 1 s recycle delays and 1 ms matched ^1H - ^{13}C Hartmann-Hahn cross-polarization transfers. Sample were spun at 3125 Hz and controlled to $\pm 0.2\text{ Hz}$. ^{13}C spectra are referenced such that the ^{13}C resonance of the methyl carbon of L-alanine occurs at 20.0 ppm. The ^{15}N isotropic chemical shifts are referenced such that the ^{15}N isotropic shift for CH_3NO_2 is 0 ppm.

A Tecmag Redstone spectrometer with proton frequency of 300.161 MHz was used to obtain ^1H NMR spectra. A ^1H MAS probe designed around a Varian kel-F stator housing a 3.2 mm diameter pencil rotor was employed. The six-turn NMR coil was made of 22 AWG copper wire that was 6.4 mm in length with an inner diameter of 3.7 mm. All ^1H MAS NMR spectra were obtained at a spinning speed of 20 kHz.

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Chapter 4: Experiments Results and Discussion

4.1 Dynamic Light Scattering

The hydrodynamic diameters of the AuNPs after reducing with NaBH_4 were measured using dynamic light scattering (DLS). Measurements were conducted with 3 runs, each lasting for 2 minutes. The effective diameter of the AuNPs was found to be 20.5 nm in diameter. The hydrodynamic diameter measured by DLS accounts for the inorganic core along with any surfactant attached as well as the interaction with the solvent molecule. Hence, the average diameter obtained by DLS is always larger than the core size of the particles. Figure 4.1 shows an illustrative example of a sample of AuNPs reduced with NaBH_4 .

Figure 4.1: DLS plot of AuNPs. The effective diameter of the AuNPs was 20.5 nm.

4.2 Atomic Force Microscopy

The accurate size of the AuNPs was determined using Atomic Force Microscopy (AFM) by Nicole Shamitko from Dr. Legleiter group. AuNPs were deposited on mica and images with tapping mode AFM were obtained. A representative image and its histogram are shown in Figure 4.2. Several images were captured for each sample and analyzed so that large data sets of measured nanoparticle heights could be compiled into histograms. The average height of AuNPs was found to be 12.4 ± 2.7 nm.

Figure 4.2: AFM Image of AuNPs. The height of individual particles over several images was measured and used to construct size histograms of nanoparticles. The average height of AuNPs was 12.4 ± 2.7 nm.

4.3 UV-Vis Spectroscopy

UV-vis measurements were obtained using a Cary 50 Bio UV-Visible Spectrophotometer. The surface plasmon resonance (SPR) for naked AuNPs is shown in Figure 4.3. The data is presented in terms of absorbance (a.u.) versus wavelength (λ). An absorbance band at 518 nm is observed corresponding to the optical excitation of the SPR of the nanoparticles.

Figure 4.3: UV-Vis measurement of AuNPs. The peak at 518 nm is due to the Surface Plasmon resonance of AuNPs

4.4 SSNMR of Cysteine

REDOR spectrum on a U- ^{13}C cysteine sample is shown in Figure 4.4.¹⁹ The full spectrum at the bottom shows resonances for three carbons present in cysteine. The dipolar coupling between ^{15}N and $^{13}\text{C}_\alpha$ is recoupled by REDOR to produce the reduced spectrum. The intensity of the C_α is strongly reduced due to the short $^{13}\text{C}_\alpha$ - ^{15}N distance. Hence, the peak obtained in difference spectrum is the confirmation of the C_α carbon in close proximity to nitrogen. The full spectrum shows that the chemical shifts of C_α , C_β , and C_γ of the cysteine molecule are at 54 ppm, 28 ppm, and 173 ppm respectively.

Figure 4.4: a) ^{13}C REDOR spectra of $\text{U-}^{13}\text{C} \{^{15}\text{N}\}$ Cysteine, (b) $\text{U-}^{13}\text{C} \{^{15}\text{N}\}$ Cysteine REDOR reduced spectrum, and (c) $\text{U-}^{13}\text{C} \{^{15}\text{N}\}$ Cysteine REDOR difference spectra 10, 000 acquisitions were obtained for the full and reduced spectra. The insert shows the zwitterionic structure of cysteine.

4.5 SSNMR of Cysteine on AuNPs

Figure 4.5 is the REDOR spectrum of cysteine on AuNPs.¹⁹ The cysteine spectrum is shown at the bottom to compare the chemical shift information with cysteine on AuNPs. The full spectrum is due to the ^{13}C resonances of cysteine when attached on AuNPs. The REDOR full spectrum shows that the C_γ of cysteine-AuNPs did not shift but only got broader compared to cysteine. This indicates that cysteine does not interact on the gold surface through C_γ carbon. Multiple resonances were obtained for C_β and C_α instead of the two sharp peaks in cysteine. Four

peaks buried can be identified. Three peaks are readily apparent and the fourth is visualized after careful analysis of the upfield portion of the broad shoulder. The ^{13}C resonances for the C_α and C^*_α peaks make strong contributions to the REDOR difference spectrum indicating that these carbons are directly bonded to ^{15}N . This suggests that C_α carbons of cysteine interact on AuNPs by two different ways. The resonances of C_α and C^*_α are at 54 ppm and 66 ppm respectively. The C_α for cysteine-AuNPs resonance was similar to C_α resonance obtained from cysteine. While the C^*_α resonance of cysteine-AuNPs was shifted by 12 ppm compared to cysteine. C_β and C^*_β resonance for cysteine-AuNPs was obtained at 30 ppm and 43 ppm, respectively. The 30 ppm chemical shift was only shifted by 2 ppm compared to C_β of cysteine while C^*_β shifted by 15 ppm. The shifts of C^*_β and C^*_α relative to cysteine while on AuNPs indicates the chemisorption of cysteine on AuNPs through the thiol bond. The unshifted ^{13}C resonances from the C_β and C_α carbons of cysteine-AuNPs are associated with cysteine on AuNPs that are not chemisorbed. Since cysteine is chemisorbed on AuNPs through thiol-gold interactions, it should lose the thiol proton. If the chemisorbed cysteine is zwitterionic, then it can hydrogen bond to another cysteine molecule with amino and carboxylate group. The outer layer cysteine should have thiol proton present which can be detected by ^1H NMR spectroscopy. Hence, the presence of the thiol proton in ^1H NMR spectroscopy can provide an evidence of bilayer cysteine on AuNPs.

Figure 4.5: a) ^{13}C REDOR spectra of Cysteine b) ^{13}C $\{^{15}\text{N}\}$ REDOR full spectrum of Cysteine-AuNPs (713,100 acquisitions), (c) U- ^{13}C $\{^{15}\text{N}\}$ REDOR reduced spectrum of Cysteine-AuNPs (713,100 acquisitions), and (d) U- ^{13}C $\{^{15}\text{N}\}$ REDOR difference spectrum of Cysteine-AuNPs.

The top spectrum of Figure 4.6 shows the ^1H MAS NMR spectrum of cysteine.²¹ There are three broad resonances for cysteine molecule. The peak at 8.7 ppm is from the amino protons, the peak at 4.3 is from CH protons and the peak at 2.0 ppm is from CH_2 protons. The thiol proton appears around 2 ppm and is buried under CH_2 broad peak. The ^1H NMR spectrum of cysteine-

AuNPs (bottom) shows a broad feature at 7.6 ppm from amino protons and at 4.4 ppm from CH protons. The pair of intense sharp peaks at 2.1 ppm and 2.5 ppm is due to the CH₂ protons. The sharp peak at 1.9 ppm is from the thiol protons, indicating the presence of thiol proton on AuNPs. This thiol proton peak confirms that there is an outer layer of cysteine molecules on the surface that is not bound through thiol-gold interactions. This outer layer is hydrogen bonded to the first layer of cysteine that is chemisorbed on AuNPs. Hence, the chemical shifts obtained from ¹³C and ¹H NMR suggests the bilayer model of cysteine molecule on AuNPs as shown in Figure 4.7. The first layer of cysteine is made of cysteine forming a thiolate bond with the gold surface and with charged amino and carboxylate functional groups oriented away from the gold surface. The second layer of cysteine has amino and carboxylate functional groups oriented toward and interacting with the first layer and its thiol functional group oriented away from the gold surface.

Figure 4.6: ^1H MAS NMR spectrum of cysteine (top) and Cysteine-AuNPs (bottom). ^1H MAS NMR obtained on a 500 MHz Varian spectrometer at the Naval Research Laboratory (Washington, DC). 16 and 385 transients were obtained for Cysteine and Cysteine-AuNPs spectra at MAS speeds of 60 and 53 kHz, respectively.

Figure 4.7: Bilayer model of cysteine on AuNPs. The first layer is attached on AuNPs through thiolate-Au bond. The second layer of cysteine is hydrogen bonded to the first layer through amino and carboxylate functional groups. Taken from reference 2.

4.6 Solution NMR Spectroscopy

Solution NMR was obtained to characterize both tripeptides after purification. The crude peptide obtained after SPPS were purified using reverse-phase HPLC and lyophilized to obtain pure solid powder. Each peptide was dissolved in 700 μ L of D₂O to obtain ¹H and ¹³C solution NMR. Figure 4.8 is the ¹³C NMR spectrum of AAC after purification. All 3 carbons of cysteine residue of the peptide are uniformly ¹³C labeled. Resonances of -CH₂, -CH, and -COO carbons are at 26 ppm, 55 ppm and 173 ppm, respectively. These resonances are in accordance to the values obtained in literature for cysteine carbons. Figure 4.9 shows the ¹H resonances of AAC peptide. The doublets at 1.5 ppm are assigned to alanine methyl protons. The peak at 3.0 ppm is

assigned to $-\text{CH}_2$ protons and the peaks between 4 ppm to 4.5 ppm are from three $-\text{CH}$ protons.

Hence, from ^1H and ^{13}C NMR, the purity of AAC was confirmed.

Figure 4.8: ^{13}C solution NMR of AAC ($\text{U-}^{13}\text{C}$, ^{15}N (cysteine)). ^{13}C solution NMR spectrum was obtained on 600 MHz Varian Spectrometer.

Figure 4.9: ^1H solution NMR of AAC. ^1H solution NMR spectrum was obtained on 400 MHz Bruker NMR Spectrometer.

Figure 4.10 is the ^{13}C NMR spectrum of CAA after purification. All 3 carbons of cysteine residue of the peptide are uniformly ^{13}C labeled. Resonances of $-\text{CH}_2$, $-\text{CH}$, and $-\text{COO}$ carbons are at 25 ppm, 54 ppm and 168 ppm, respectively. Figure 4.11 shows the ^1H NMR of CAA. The ^1H NMR spectrum of CAA is similar to the ^1H NMR spectrum of AAC with slight chemical shift differences of each type of protons since the terminal cysteine is in different position.

Figure 4.10: ^{13}C solution NMR spectrum of CAA ($\text{U-}^{13}\text{C}$, ^{15}N (Cysteine)). ^{13}C solution NMR spectrum was obtained on 600 MHz Varian NMR Spectrometer.

Figure: 4.11: ^1H solution NMR spectrum of CAA. ^1H solution NMR spectrum was obtained on 400 MHz Bruker NMR Spectrometer.

4.7 CAA Interactions on AuNPs

CAA tripeptide was designed to attach to the surface of AuNPs. The basic design of the experiment was to create a small hydrophilic peptide that would readily chemisorb to AuNPs forming well-packed AuNPs that would have enhanced stability. AuNPs capped with tri-peptide CAA did not aggregate or cross link but resulted in highly stable CAA functionalized AuNPs. Cysteine was incorporated on N-terminus to induce an interaction on the gold surface through the thiol functional group and the presence of alanine promotes the self-assembly of the peptide due to its ability to self-assemble into a dense layer.

Figure 4.12: Tripeptide CAA zwitterionic structure. C_α , C_β , and C_γ represents uniformly ^{13}C labeled carbons.

Figure 4.12 is the structure of CAA zwitterionic form. The C_α , C_β , and C_γ of cysteine residue are uniformly ^{13}C labeled. The ^1H MAS NMR spectrum (Figure 4.13) shows a ^1H resonance for the hydroxide proton of CAA obtained at 13 ppm, showing that pure CAA is not in zwitterionic form. The ^1H NMR spectrum of CAA on AuNPs shows that CAA lost its carboxylate proton (i.e., no resonance peak at 13 ppm) when attached to AuNPs (Figure 4.14), suggesting that CAA on AuNPs is zwitterionic. The broad peak at 6 ppm is due to the $-\text{NH}$ and $-\text{CH}$ protons and the sharper peaks in the low frequency region are due to the $-\text{CH}_3$ and $-\text{CH}_2$ protons. The ^{13}C CPMAS of CAA is shown in Figure 4.15. The intense peak at 18 ppm is from the ^{13}C label placed on the methyl group of the second residue. The chemical shifts of C_α , C_β , and C_γ of pure CAA are at 52 ppm, 25 ppm, and 170 ppm, respectively. Figure 4.16 is the ^{13}C spectrum of CAA-AuNPs. There is a single set of resonances for the 3 carbons of the cysteine residue. The C_γ of CAA-AuNPs shifted to 184 ppm, suggesting that the carbonyl carbon of cysteine may be interacting with the gold. The chemical shifts of C_α and C_β shift to 71 ppm and 53 ppm, respectively. The large chemical shift change of 19 ppm in C_α and 28 ppm in C_β suggest that peptide CAA is strongly interacting to the surface of AuNPs through a thiolate-Au bond (recall the chemical shift changes

that occurred with cysteine on gold). The detailed ^{13}C chemical shift values of CAA and CAA-AuNPs is reported in Table 2. A single set of ^{13}C resonances for the C_α and C_β carbons indicates a monolayer of peptides on AuNPs. This is in contrast to the bilayer that cysteine forms on AuNPs.

Figure 4.13: ^1H MAS NMR spectrum of CAA. ^1H MAS NMR spectrum was obtained at 300 MHz spectrometer at spinning speed of 20 kHz. Sharp resonances in lower frequency region are from $-\text{CH}_3$ and $-\text{CH}_2$ protons and the broad peak at 12.8 ppm is assigned to $-\text{OH}$ protons.

Figure 4.14: ^1H MAS NMR spectrum of CAA-AuNPs. ^1H MAS NMR spectrum was obtained at 300 MHz Spectrometer at spinning speed of 20 kHz.

Figure 4.15: ^{13}C CPMAS NMR spectrum of CAA. All the carbons of cysteine and the methyl of middle alanine are ^{13}C labeled. ^{13}C CPMAS spectrum was obtained on 151.31 MHz Spectrometer with a spinning speed of 3125 Hz (508 acquisitions).

Figure 4.16: ^{13}C CPMAS of CAA-AuNPs. The cysteine residue was uniformly ^{13}C labeled. ^{13}C CPMAS was obtained on 151.31 MHz Spectrometer with a spinning speed of 3125 Hz (400,000 acquisitions).

4.8 AAC Interactions on AuNPs

^1H MAS NMR Spectrum of AAC is shown in Figure 4.18 and the resonance at 13 ppm shows the peptide is not a zwitterion. The absence of -OH peak in of AAC-AuNPs ^1H MAS NMR spectrum (Figure 4.19) confirms the zwitterionic structure of AAC when attached to the gold surface, similar to CAA on AuNPs. The ^{13}C spectrum of AAC (Figure 4.20) shows three peaks for C_α , C_β , and C_γ at 53 ppm, 26 ppm, and 171 ppm, respectively. The ^{13}C NMR spectrum of AAC on

AuNPs (Figure 4.21) has multiple resonances in a low frequency region. This low frequency region looks similar to the spectrum obtained from cysteine-AuNPs. The C_γ resonance shifted to 177 ppm while the broad region contains four peaks at 69 ppm, 50 ppm, 32 ppm, and 22 ppm. The peak at 69 ppm and 50 ppm are from two different C_α carbons while peaks at 32 ppm and 22 ppm are from C_β carbon. The two types of C_α and C_β suggest the multiple layers of AAC on AuNPs. The chemical shift change of 6 ppm of C_γ also suggests that AAC carboxylate functional group may interact with the gold surface. The resonances at 69 ppm and 50 ppm are the chemical shifts of two type of C_α . The 16 ppm chemical shift change is attributed to chemisorbed AAC on AuNPs and the 3 ppm shift from the outer layer of AAC that could be hydrogen bonded to the inner layer of AAC. Similarly, the 6 ppm downfield chemical shift perturbation is from chemisorbed C_β and 4 ppm upfield chemical shift is from the outer layer C_β . The ^{13}C chemical shift of AAC and AAC-AuNPs is also reported in Table 2 for comparison with cysteine and tri-peptide CAA. Unlike CAA on AuNPs, there are two populations of AAC on AuNPs.

Figure 4.17: Tri-peptide AAC Zwitterionic structure. C_α , C_β , and C_γ represents uniformly ^{13}C labeled carbons.

Figure 4.18: ^1H MAS NMR spectrum of AAC. ^1H MAS NMR spectrum was obtained at 300 MHz spectrometer at spinning speed of 20 kHz. Sharp resonances in lower frequency region are from $-\text{CH}_3$ and $-\text{CH}_2$ protons and the broad peak at 12.8 ppm is from $-\text{OH}$ protons.

Figure: 4.19: ^1H MAS NMR spectrum of AAC-AuNPs. ^1H MAS NMR spectrum was obtained at 300 MHz spectrometer at spinning speed of 20 kHz.

Figure 4.20: ^{13}C CPMAS NMR spectrum of AAC ($\text{U-}^{13}\text{C}$, Cysteine). ^{13}C CPMAS NMR spectrum was obtained on 151.31 MHz spectrometer with a spinning speed of 3125 Hz (8596 acquisitions).

Figure 4.21: ^{13}C CPMAS NMR spectrum of AAC on AuNPs ($\text{U-}^{13}\text{C}$, ^{15}N (Cysteine)) on AuNPs.

^{13}C CPMAS spectrum was obtained on 151.31 MHz spectrometer with a spinning speed of 3125 Hz (400,000 acquisitions).

Ligand	C _α (-CH)	C _β (-CH ₂)	C _γ (-COO or -CO)
Cysteine	54	28	173
Cysteine-AuNPs	54(0), 66 (12)	30 (2), 43 (15)	173 (0)
CAA	52	25	170
CAA-AuNPs	71 (19)	53 (28)	184 (14)
AAC	53	26	171
AAC-AuNPs	50 (-3), 69 (16)	22 (-4), 32 (6)	177 (6)

Table 2: ¹³C chemical shift (ppm) values of ¹³C resonances of cysteine residue. ¹³C isotropic chemical shifts are referenced such that the ¹³C isotropic shift for the methyl carbon of solid L-alanine is 20 ppm.

4.9 TGA Analysis

The number of peptide molecules bound to each gold nanoparticle was determined using TGA analysis (Figure 4.22). TGA was performed, on 1.75 mg AAC-AuNPs and 1.28 mg CAA-AuNPs under N₂ gas with a flow rate of 20 mL/min. The temperature was ramped from 35 to 900°C at a heating rate of 10°C/min. At about 220°C the weight loss was drastic. The resulting nanoparticle sample was only gold as a result of decomposition of surface peptides. From the measured weight loss, the number of peptide molecules on the surface of a nanoparticle was determined. The number of CAA per particle was found to be 2898 peptides/particle or 6 peptides/nm² and for AAC 16 peptides/nm² or and 7729 peptides/particle (calculations shown below). Uniform coverage of the surface is expected to be around 3 to 6 peptides/nm². Hence, it is proposed that the stability of AAC capped AuNPs and CAA capped AuNPs is due to the high peptide density at the surface of

AuNPs. The larger densities of AAC compared to CAA on AuNPs also suggest the bilayer AAC on gold surfaces.

TGA calculations

The initial mass used for TGA analysis contains the mass of peptide and the mass of AuNPs.

$$m_{initial} = m_{peptide} + m_{AuNPs}$$

After complete decomposition of the peptide, the final mass after the TGA analysis only contains AuNPs.

$$m_{final} = m_{AuNPs}$$

The difference of m_{final} and $m_{initial}$ is the $m_{peptide}$, i.e. $\Delta m = m_{peptide}$

$$\frac{\Delta m}{mass_{initial}} = \frac{m_{peptide}}{m_{peptide} + m_{AuNPs}} = f$$

f = mass of peptide which is equal to the % weight loss obtained from TGA analysis.

$$m_{peptide} = \left(\frac{f}{1-f}\right)m_{AuNPs} = \left(\frac{f}{1-f}\right)\rho_{AuNPs}V_{AuNPs}$$

$$\text{Since, } m_{AuNPs} = \rho_{AuNPs}V_{AuNPs}$$

$$\text{Also, } V_{AuNPs} = \frac{4}{3}\pi r^3$$

$$SA_{AuNPs} = 4\pi r^2$$

$$\frac{N_P}{SA_{AuNPs}} = \frac{1}{3} \left(\frac{N_A}{MW_{peptide}} \rho_{AuNPs} \right) (r_{AuNPs}) \left(\frac{f}{1-f} \right)$$

Where, m = mass, ρ_{AuNPs} = density of AuNPs, V_{AuNPs} = volume of AuNPs, r = radius of AuNPs,

SA_{AuNPs} = surface area of AuNPs, and f = % weight loss of peptide obtained from TGA

$$\rho_{AuNPs} = 19.8 * 10^{-21} \text{ g/nm}^3$$

$$r_{AuNPs} = 6.2 \text{ nm}$$

$$N_A = 6.022 * 10^{23} \text{ molecules/mol}$$

For CAA

Final peptide loss from TGA (f) = 6% = 0.06

Therefore,

$$\begin{aligned}\frac{N_{CAA}}{SA_{AuNps}} &= \frac{1}{3} \left(\frac{N_A}{MW_{Peptide}} \rho_{AuNPs} \right) (r_{AuNPs}) \left(\frac{f}{1-f} \right) \\ &= \frac{1}{3} \left(\frac{6.022 * 10^{23} \text{ molecules/mol}}{263.32 \text{ g/mol}} 19.8 * 10^{-21} \text{ g/nm}^3 \right) (6.2 \text{ nm}) \left(\frac{0.06}{1-0.06} \right) \\ &= 6 \text{ molecules/ nm}^2\end{aligned}$$

For AAC

Final peptide loss from TGA (f) = 15% = 0.15

$$\begin{aligned}\frac{N_{AAC}}{SA_{AuNps}} &= \frac{1}{3} \left(\frac{N_A}{MW_{Peptide}} \rho_{AuNPs} \right) (r_{AuNPs}) \left(\frac{f}{1-f} \right) \\ &= \frac{1}{3} \left(\frac{6.022 * 10^{23} \text{ molecules/mol}}{263.32 \text{ g/mol}} 19.8 * 10^{-21} \text{ g/nm}^3 \right) (6.2 \text{ nm}) \left(\frac{0.15}{1-0.15} \right) \\ &= 16 \text{ molecules/ nm}^2\end{aligned}$$

Figure 4.22: TGA data of CAA and AAC on AuNPs. Weight loss for CAA-AuNPs and AAC-AuNPs was calculated and found to be 6% and 15%, respectively. The weight loss after 600°C is the result of the instrumental drift and was observed during each analysis.

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Chapter 5: ^1H -Observe ^1H - ^2H Dipolar Recoupling by REDOR and Rotary Resonance Recoupling

5.1 Introduction

^1H magic-angle spinning NMR spectroscopy is useful in obtaining important structural information by providing a high-resolution spectra of a solid material.^{37a, 58} MAS reduces the line broadening factors by averaging the dipolar couplings and chemical shift anisotropies.⁴⁰ ^1H NMR spectroscopy has the potential advantage in studying organic and inorganic materials due to high sensitivity afforded by its high resonance frequency.⁵⁹ The inherent high sensitivity of ^1H could also be troublesome due to strong homonuclear dipolar interaction that broadens the proton signals of interest. Multiple pulse techniques combined with MAS and Lee-Goldburg homonuclear decoupling⁶⁰ yield substantial improvement in the resolution of ^1H spectra for solids.⁶¹ Recent work shows that very fast MAS significantly improves ^1H resolution.

REDOR provides information on heteronuclear dipolar coupling under magic angle spinning conditions.^{3d, 45, 62} Past REDOR experiments measured ^1H -X dipolar couplings by observing X nucleus. Unfortunately, this loses the sensitivity advantage of observing ^1H . Here, we explore measuring ^1H -X dipolar couplings with ^1H as the observe nucleus. Several experiments to measure dipolar couplings have been performed and potential experimental problems are identified and solved. All experiments are performed at a spinning speed of 20 kHz. Specifically, ^1H - ^2H REDOR^{45a, 45c} and ^1H - ^2H rotary resonance recoupling experiments⁶³ have been performed. Both experiments were successful in obtaining dipolar coupling information using ^1H as the observe nucleus. Potential limitations in both experiments were identified and the solutions to those limitations are discussed in detail.

5.2 Experimental Section

A Tecmag Redstone spectrometer with proton frequency of 300.161 MHz and deuterium frequency of 46.076 MHz was used for this work. The 300W high-power tuned rf amplifier for the proton channel is from Amplifier Systems, Inc. and built around a single Eimac 8874 tube. The 1 kW broadband American Microwave Technology, Inc. amplifier used for the deuterium channel was salvaged from a Chemagnetics II spectrometer. The custom-built ^1H - ^2H double-resonance magic-angle spinning probe is designed around a Varian kel-F stator housing a 3.2 mm diameter pencil rotor. The six-turn NMR coil is made of 22 AWG copper wire and is 6.4 mm in length and has an inner diameter of 3.7 mm. The proton rf field strength (measured as a nutation frequency) for the experiments was 39 kHz, and the deuterium rf field strength was varied from zero to 85 kHz. The spinning speed, ν_R , was set to 20 kHz for all of the experiments reported here.

Maier and Slater⁶⁴ described the method to measure the relative radiofrequency (rf) field strength inside the NMR coil. This method was recently utilized to measure the rf field strength in NMR coils by Paulson et al.⁶⁵ When a capacitor is placed parallel to the NMR coil a resonant circuit forms. In our work, the capacitor was chosen such that the circuit resonated at 142.850 MHz. A small annular piece of copper (0.5 mm outer diameter, 0.3 mm inner diameter, and 0.1 mm thickness) was attached to the end of a quartz fiber and positioned inside the coil. The quartz fiber had no effect on the circuit. The perturbation of the resonance frequency caused by the position of the copper ring was shown by Maier and Slater to obey $(\omega^2 - \omega_0^2)/\omega_0^2 = \alpha B_1^2$, where ω_0 is the unperturbed resonance frequency of the circuit, ω is the resonance frequency of the perturbed circuit, α is a geometric factor that depends on the shape of the copper ring, and B_1 is the rf magnetic field strength at the site of the copper ring for the unperturbed circuit.

Since circuit resonance frequencies are easily measured with great precision, this method gives a straightforward way to map the relative rf field strength in the coil. Assuming ω is never far from ω_0 , then $2(\omega - \omega_0)/\omega_0 = \alpha B_1^2$ is a reasonable approximation to the Maier and Slater formula. Let the maximum shift in the resonance frequency be $\Delta\omega_{\max} = (\omega_{\max} - \omega_0)$ and $B_{1,\max}$ be the corresponding B_1 value. The complicated geometric factor, α , can therefore be eliminated by using the ratio $(\omega - \omega_0)/(\omega_{\max} - \omega_0) = B_1^2/B_{1,\max}^2$. Hence, a plot of the ratio $\Delta\omega/\Delta\omega_{\max}$ as a function of coil position provides a map of $B_1^2/B_{1,\max}^2$ in the coil. Figure 5.7 shows $\Delta\omega/\Delta\omega_{\max}$ ratios of an NMR coil very similar to the one used in this work for frequencies 46.03 MHz, 142.85 MHz, and 303.20 MHz. The 46.03 MHz and 303.20 MHz values are near the resonance frequencies for ^2H and ^1H nuclei, and the 142.85 MHz is an intermediate frequency. Clearly, the rf field strength distributions are similar for all three frequencies. A sample centered in the middle one-third of this coil would experience a range of $B_1/B_{1,\max}$ from 1 to 0.96, and $B_1/B_{1,\max}$ would range from 1 to 0.83 for a sample centered about the middle two-thirds of the coil.

The inherent high sensitivity of ^1H nucleus often results in broad residual ^1H background signals from hydrogen containing materials used in stator and rotor parts. Also, the artifacts arising from pulse transients could appear in the ^1H spectra. Several methods were developed to eliminate background signals and pulse artifacts in SSNMR experiments.⁶⁶ A modified version of the blinking 180° pulse method described by Duncan et al.^{66a} was used to suppress the background ^1H signals and pulse artifacts. A block of back-to-back 180° pulses is applied prior to the application of the proton 90° pulse, converting longitudinal magnetization to transverse magnetization. The block is modified by alternating between $180^\circ_x 180^\circ_y 180^\circ_{-x}$ and $180^\circ_x 180^\circ_y$ with appropriate addition and subtraction of ^1H free induction decays. The 180°_{-x} pulse applied on alternate scans is represented by the dashed pulse in the pulse sequence diagrams. The phases of the two pre-

pulses were determined empirically for the ^1H - ^2H MAS NMR probe and proved to be very effective in producing flat baselines in the frequency domain and eliminating spurious ^1H signals.

The samples used were Ala(d3), CysAlaAla (d3), and polysiloxane. Ala(d3) (l-alanine-3,3,3-D3(99%)) is purchased from Cambridge Isotopes, Inc and re-crystallized from water.

The quadrupolar frequency $\nu_Q = 3e^2qQ/2h$ of the rapidly rotating methyl deuterons is approximately 80 kHz at room temperature.⁶⁷ Hence, the “horns” of the deuterium powder pattern are separated by ~ 40 kHz. CysAlaAla(d3) is a tri-peptide synthesized by solid-phase peptide synthesis using Fmoc-Cys(Trt)-OH, Fmoc-Ala-OH, and Fmoc-Ala-OH-3,3,3-D3 (99%). The methyl deuterons are on the C-terminal alanine. The protected amino acids were purchased from Sigma-Aldrich. A sheet of polysiloxane, referred to as PSO, was purchased from McMaster-Carr and was cut into small pieces for the NMR experiments.

5.3 results and Discussion

^1H - ^2H Rotational-Echo, Double-Resonance NMR Spectroscopy

Rotational-echo, double-resonance (REDOR) NMR is a powerful and versatile technique to measure heteronuclear dipolar interactions in SSNMR. REDOR measurements can be implemented to re-couple the dipolar coupling averaged by MAS, and hence precise internuclear distances between the heteronuclei can be obtained. REDOR experiments have been mostly performed on rare spin nuclei such as ^{13}C or ^{15}N . ^1H REDOR NMR has never been performed by directly observing ^1H signal. This work presents the ^1H - ^2H REDOR NMR with ^1H as the observe nuclei to measure the dipolar coupling interactions between ^1H and ^2H . The REDOR pulse sequence used for the work is shown in Figure 5.1 with xy-4 phasing. A block of ^1H 180° pulses prior to the REDOR experiment (as discussed in the Experimental Section) eliminated unwanted signals and transients. The pulse labeled “blink” is applied in an alternating fashion, and data

acquisition is appropriately phased to ensure addition of the ^1H signal from the sample. The 180° pulse is applied at the proton channel to refocus the isotropic chemical shifts. The xy-4 phase cycle scheme is applied to the 180° pulse to eliminate problems associated with pulse imperfections.^{62b}

Figure 5.1: ^1H - ^2H REDOR experiment. The block of three 180° pulses prior to 90° pulse are $180^\circ_x 180^\circ_y 180^\circ_{-x}$. The 180_{-x} pulse (illustrated with the labeled “blink”) is applied during alternate experiments. The 180° pulses on the deuterium channel are phased according to the xy-4 phasing scheme. REDOR is executed as a difference experiment. The ^1H signal acquired in the absence of the deuterium pulses produces the ^1H control signal, S . The ^1H signal acquired with the deuterium pulses applied during the dipolar evolution period produces the dipolar dephased signal, S_d . The ^1H signals from the two experiments are subtracted to yield a difference signal, $\Delta S = (S - S_d)/S$. The ratio $\Delta S/S$ is a measure of the dipolar dephasing of the ^1H signal caused by the ^2H nuclei.

^1H - ^2H REDOR experiments were performed on Ala(d3) sample. The REDOR curve obtained from ^1H - ^2H of Ala(d3) sample is shown in Figure 5.2. The CH and NH_3^+ ^1H resonances show strong dipolar dephasing as measured by $\Delta S/S$ as a function of dipolar evolution time. The CH REDOR data is best described (solid line) with a dipolar coupling of 920 Hz. The data point

at 0.8 ms is an outlier. The dipolar coupling of 920 Hz between the CH proton and methyl deuterons is 11% lower than the value obtained using x-ray determined distances for the alanine structure, but is consistent with fast small angle molecular motional averaging.

Figure 5.2: REDOR results for the Ala(d3) sample. Experimental results are the solid circles (CH) and open triangles (NH₃⁺). The result at 0.8 ms appears to be an anomaly. The solid line is an eye guide to REDOR calculations for the CH ¹H signal (plus symbols) using a 920 Hz ¹H-²H dipolar coupling.

We considered the methyl rotation while obtaining the coupling information but the fast motional averaging within the alanine molecule is not accounted for. One of the possible reasons for lower dipolar coupling value for alanine could be due to ignoring the molecular motion of

alanine. For example while obtaining the ^{13}C - ^2H dipolar coupling in zinc acetate between the carbonyl carbons ^{13}C spin label and deuterons in the methyl group, a dipolar coupling with 20% lower than the expected value was obtained by Schmidt et al.⁶⁸ The lower value compared to the x-ray crystallography was attributed to the additional molecular motion within the molecule since the methyl rotation was taken into account. Another possible reason could be due to the ^2H quadrupolar coupling. The power of the deuterium 180° pulses may not be sufficient enough to toggle the deuterium spin states perfectly. The effect of insufficient ^2H rf power was investigated by Sack et al. for ^{13}C - ^2H REDOR experiments.⁶⁹ It was found that the dipolar dephasing of the observe spin was weaker for a train of deuterium 180° pulses. This problem was solved by using the composite pulses to compensate for the insufficient deuterium rf power. In our experiment the deuterium rf field strength applied for the REDOR pulses was 78 kHz, which was close to the fast rotating methyl deuterons quadrupolar frequency of 80 kHz.⁶⁷ The deuterium rf field strength of 78 kHz used in our experiment might not be sufficient enough to toggle the deuterium spin states with quadrupolar frequency of 80 kHz. Applying composite pulses may result in obtaining better ^1H - ^2H dipolar couplings.

The transverse relaxation times for CH and NH_3^+ ^1H signals of Ala(d3) was found to be 560 ms and 360 ms at the spinning speed of 20 kHz. Use of short dipolar evolution times were sufficient to cause significant dipolar dephasing of the protons for the Ala(d3) sample. The use of longer evolution times would have been problematic since the ^1H magnetization decreases exponentially with longer dipolar evolution times. This decrease in ^1H magnetization during signal acquisition can be resolved by increasing the spinning speed. It has been reported that the ^1H spin-spin relaxation times get longer with the increase in the spinning speed.⁷⁰ Therefore, the longer

dipolar evolution times for ^1H -observe REDOR experiments can be used with faster spinning speed.

^1H - ^2H Rotary Resonance Recoupling and Bloch-Siegert Effects

The dipolar couplings between heteronuclear pairs of spin could also be obtained using rotary resonance recoupling (R^3) technique. A dipolar recoupling effect is observed when the strength of irradiation field is such as to bring about rotary resonance.⁷¹ R^3 of heteronuclear dipolar interactions can be obtained when continuous wave rf power on one of the channels is applied at an rf field strength, ν_1 , that is an integral multiple of the spinning speed, ν_R . The pulse sequence used in our work to demonstrate R^3 is shown in Figure 5.3. The sample used for this work is also Ala(d3). The applied pulse sequence consists of a 90° inspection pulse on ^1H channel and continuous wave power on the ^2H channel during ^1H acquisition. The pulse sequence consists of a block of 180° pulses at the beginning to eliminate the unwanted background signals and spectral artifacts. The deuterium field strength was increased from 0 to 85 kHz at the spinning speed of 20 kHz.

Figure 5.3: Rotary resonance recoupling pulse sequence. The block of three 180° pulses is the same as described in Figure 5.1.

Figure 5.4 shows the intensity of CH ^1H resonance with respect to the deuterium rf power. Rotary resonance recoupling is evident by the strong loss of ^1H signal at the deuterium rf field strengths of 20 kHz ($n=1$), 40 kHz ($n=2$), and 60 kHz ($n=3$).

Figure 5.4: Relative signal intensity of the CH ^1H resonance as a function of deuterium rf power.

While the R^3 was successfully obtained on Ala(d3) sample, we noticed that the positions of the ^1H resonances were also shifting to the higher frequency with the increase in deuterium power. The shifts obtained during R^3 were consistent with the Bloch-Siegert effect.^{33b, 72} The Bloch-Siegert shift is observed when applying an off-resonance rf field that causes a shift in the resonance frequency of the observed nucleus. In order to further characterize the effect of the deuterium rf field strength on the ^1H spectra, a sample of polysiloxane (PSO) was studied. PSO was chosen as it has many methyl groups rotating rapidly and is a rubbery polymer with large backbone motion, which yields a single sharp proton resonance.

The pulse sequence used for R^3 (Figure 5.3) was used to generate the ^1H spectra of the PSO sample. The deuterium rf field strength was varied from 0 to 85 kHz and the PSO sample was centered in the middle third of the NMR coil. The Bloch-Siegert shift was clearly observed with the increase in deuterium rf field strength. The Bloch-Siegert shift for a ^1H -observe experiment, in ppm, with applied deuterium rf power is given by $\Delta\delta = \left(\frac{\nu_H}{\nu_D}\right)^2 \left(\frac{\nu_{1,D}}{\nu_H^2 - \nu_D^2}\right)$, where $\nu_{1,D}$ is the deuterium rf field strength (in kHz) and ν_H and ν_D are the respective ^1H and ^2H Larmor frequencies (in MHz). The shift in ^1H resonance position for PSO was plotted as a function of the square of the deuterium rf field strength (Figure 5.5). The graph shows that $\Delta\delta$ is linearly dependent on $\nu_{1,D}^2$. The slope for the graph was obtained and found to be $5.0 \times 10^{-4} \text{ ppm/kHz}^2$. This value is comparable to $4.8 \times 10^{-4} \text{ ppm/kHz}^2$ predicted by the Bloch-Siegert formula.

Figure 5.5: The shift of the ^1H resonance for PSO as a function of the square of the deuterium rf field strength.

Figure 5.6 shows the ^1H spectra illustrating the effects of Bloch-Siegert shift on PSO ^1H spectra. The ^1H resonance of PSO also became skewed and broadened in shape when the deuterium rf power is applied. In order to understand the broadening of the ^1H resonance with deuterium power, the PSO sample was placed in middle third and two-thirds of the NMR coil. The ^1H peaks at the right side of the Figure 5.6(a) and 5.6(b) were obtained without applying the deuterium rf

power for PSO sample by placing at one-third and two-third, respectively. When the deuterium rf field strength was increased to 85 kHz, the ^1H resonances in both spectra shifted by more than 1 kHz and became broader (left peak at Figure 5.6 (a) and 5.6 (b)). The ^1H resonance of PSO centered in the middle two-thirds of the coil is significantly broader and skewed than the sample located in the middle one-third of the coil when ^2H rf power was applied.

Figure 5.6: Overlaid ^1H spectra of PSO. In the absence of deuterium rf power, the ^1H resonance is at 50 Hz for the two plots. The set of spectra in a) were taken with the sample centered in the middle one-third of the NMR coil. The set of spectra in b) were taken on a sample centered in the middle two-thirds of the NMR coil.

The rf field profile of the NMR coil used during the experiments is plotted in Figure 5.7. The sample placed inside the rf coil experiences different rf field distribution depending on the location within the coil. Therefore, the sample distributed about two-thirds of the coil resulted in broadened peak because of the large distribution of rf field strengths compared to the sample centered at one-third of the coil.

Figure 5.7: $B_1^2/B_{1,\max}^2$ distributions for the NMR coil as represented by the $\Delta\omega/\Delta\omega_{\max}$ ratios. Open circles, closed diamonds, and crosses are data taken at 46.03 MHz, 142.85 MHz, and 303.20 MHz, respectively. The solid line is a fit to the average of the three data sets.

The full-width half-maximum (fwhm) of the ^1H resonance for PSO sample with the increment of deuterium rf power was obtained with samples centered at one-third and two-thirds of the NMR coil (Figure 5.8). Without deuterium rf power, the fwhm of the ^1H spectrum is approximately 80 Hz for both samples. But as the deuterium rf field strength was increased, the fwhm of the ^1H spectra for both samples increased as well. The fwhm of the sample occupying larger volume of the NMR coil became broader than the sample centered in the coil. In order to obtain ^1H - ^2H rotary resonance recoupling experiments on high magnetic field, the deuterium rf field strengths with high rf power is required, which by nature will lead to greater shifts and broadening of resonance lines.

Figure 5.8: Full-width half-maxima (fwhm) for PSO. The diamonds and circles were obtained for samples occupying the middle one-third and two-thirds of the NMR coil, respectively.

The Bloch-Siegert shift will be a bigger issue for a sample that produces multiple ^1H resonances compared to the single ^1H resonance from PSO. The broadening of these peaks could eventually collapse on each other and cause them to be difficult to be resolved. Therefore, to understand the effect of Bloch-Siegert shifts, the ^1H spectra of CysAlaAla(d3) was obtained with the increase in deuterium rf power. Figure 5.9 shows the ^1H spectra of CysAlaAla(d3) at a spinning

speed of 20 kHz and a proton resonance frequency of 300.161 MHz. The proton spectrum without deuterium rf power is shown in the upper-left corner which is well-resolved. However, these resonances started to shift and became broad with the increase in deuterium rf power. As the deuterium rf field strength is increased, many of the sharp features began to broaden and eventually coalesce into one another. In order to verify the broadening effect, the CH₂ ¹H resonance from the cysteine residue was marked with an asterisk and observed by increasing the deuterium rf field. The CH₂ ¹H resonance shifted and became broader and eventually collapsed into other peaks and was no longer resolved.

Figure 5.9: ¹H spectra of CysAlaAla(d₃) taken in the presence of deuterium rf power. The applied deuterium rf field strength is indicated in each spectrum.

The Bloch-Siegert shifting and broadening of ^1H resonances can be avoided during rotary resonance recoupling by applying the pulse sequence shown in Figure 5.10. A ^1H spin-echo sequence was applied with deuterium rotary resonance recoupling before and after the ^1H 180° refocusing pulse. The broadening and shifting of ^1H resonances were not seen, since the deuterium rf irradiation was not applied during the detection of the ^1H free induction decay. Figure 5.10 shows two pulse sequences, one with and the other without a deuterium 180° pulse. Experiments performed with deuterium rf pulses set away from the rotary resonance condition had no effect on the proton signal intensity for these pulse sequence.

Figure 5.10: R^3 pulse sequences with rotary resonance recoupling pulses applied during the ^1H spin-echo sequence. The block of three 180° pulses is the same as described in Figure 5.1. Each R^3 pulse has the same duration (t).

Figure 5.11 shows results obtained on the Ala(d3) sample for the CH ^1H resonances with the $n=2$ rotary resonance recoupling condition. The relative signal intensity is plotted for different rotary resonance recoupling pulse lengths. The experiment without the deuterium 180° pulse

causes loss of the ^1H signal intensity for R^3 pulse lengths satisfying $\tau = (q + \frac{r}{8}) T_R$, where T_R is the rotor period, q is a whole number, and r is 1, 3, 5, or 7. For $\tau = (q + \frac{r}{8}) T_R$ and s equal to 0, 2, 4, 6, or 8, the experiment tends to refocus the proton magnetization. These features have also been observed in preliminary calculations using the SIMPSON package.⁷³ The experiment with the deuterium 180° pulse shows the opposite behavior, where signal loss occurs for $\tau = (q + \frac{r}{8}) T_R$ and refocusing behavior occurs for $\tau = (q + \frac{r}{8}) T_R$. The total time between the ^1H 90° pulse and the echo formation is 300ms for the data shown in Figure 5.11. The maximum value of $\Delta S/S$ is approximately 0.34 ($\Delta S/S = 1 - 0.66$ for the solid circle at $\tau \sim 2.76$). This R^3 $\Delta S/S$ is comparable to the value obtained with the same dipolar evolution time for the REDOR experiment (Figure 5.2).

Figure 5.11: Relative signal intensities for the two R^3 experiments illustrated in Figure 5.11. The data shown as circles were obtained with the experiment having a deuterium 180° pulse, and the data shown as triangles were obtained without a deuterium 180° pulse.

5.4 Conclusions

^1H -observe REDOR and R^3 experiments with MAS was successful in measuring ^1H - ^2H dipolar couplings. Dipolar couplings obtained for ^1H - ^2H REDOR experiments were slightly less than the values obtained using x-ray crystallography. This may be due to insufficient rf power to

toggle the deuterium quadrupolar spin states. Applying ^2H composite pulses for the ^1H - ^2H REDOR experiment may solve this issue. Transverse relaxation times of the ^1H spin system may limit the length of the dipolar evolution times, but going to faster spinning speeds than used in this work may alleviate that problem. A Bloch-Siegert shift was observed while performing rotary resonance recoupling experiments on ^1H - ^2H . Bloch-Siegert shifts can have a strong effect on the ^1H resonance positions and line widths in ^1H - ^2H R^3 experiments. The Bloch-Siegert effect can be eliminated by applying the R^3 rf pulses during the ^1H echo period. Also, the Bloch-Siegert effect can be minimized by going to high magnetic fields because of its $(\nu_H^2 - \nu_D^2)^{-1}$ dependence. For example, going from a 300 MHz system to a 600 MHz system reduces Bloch-Siegert shifts by a factor of four.

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Conclusions and Future Directions

The interactions of tri-peptide CAA and AAC on AuNPs were studied successfully using ^1H and ^{13}C SSNMR spectroscopy. SSNMR spectroscopy confirmed that both peptides were strongly attached on AuNPs resulting in a highly stable peptide capped AuNPs and prevented the AuNPs to cross link or aggregate on its own. ^1H and ^{13}C NMR experiments of free peptides and peptide bound on AuNPs were obtained. ^1H NMR suggested the zwitterion structure of both peptides when attached on AuNPs. ^{13}C NMR of CAA-AuNPs resulted in deshielding of all the ^{13}C resonances of cysteine residue. The deshielding of ^{13}C resonances confirmed the chemisorption of CAA on gold surfaces via thiol-gold interactions. Furthermore, these resonances also confirmed a single type of CAA on gold surfaces suggesting a monolayer of CAA on AuNPs. The anchoring point of CAA on AuNPs was through the thiol group present in cysteine molecule with the peptide lying close to the gold surface. The ^{13}C resonances of AAC-AuNPs were also deshielded but yielded two sets of resonances for the C_α and C_β carbons identical to cysteine-AuNPs. The anchoring point in AAC-AuNPs was through the thiol and carboxylate group of cysteine. As a result these peptides were prone to be in upright position and further interacted with other AAC molecule via hydrogen bond resulting in bilayer AAC on AuNPs.

The evidence of monolayer and bilayer was also supported by TGA analysis by calculating the number density of CAA and AAC on AuNPs. The number of CAA and AAC on AuNPs was found to be 6 and 16 peptides/nm² respectively. The larger densities of AAC compared to CAA on AuNPs suggested the multilayer AAC on gold surfaces. These peptide capped AuNPs were lyophilized and kept in the freezer that can be subsequently re-dissolved to yield stable AuNPs solution.

Polyalanine sequence is an important structural model of the crystalline region in spider dragline silk or wild silkworm.¹ The presence of polyaniline in proteins associated with human diseases provides urgency of solving its structure at atomic level.² Polyaniline is also known to form extremely stable parallel or anti-parallel β -sheets.³⁻⁵ Therefore, the future direction of this work is to synthesize polyaniline containing peptide with cysteine on either C or N terminus to anchor the peptide on gold surfaces. Polyaniline is very hydrophobic and difficult to work with which could be solved by anchoring the hydrophilic amino acid such as lysine to ensure solubility and stability of the peptide in water.

Gold clusters composed of few atoms exhibit quantized electronic states due to quantum confinement effect of electrons. These nanoparticles smaller than 2 nm have attracted wide research interest due to their interesting applications such as catalysis and biological labeling.⁶ Dodecane thiol stabilized Au₃₈ clusters have been synthesized in large scale with monodisperse Au₃₈(SC₁₂H₂₅)₂₄ nanoclusters in a two phase reaction system.⁷ Our future work also includes understanding the interaction of cysteine stabilized gold nanoclusters. With fewer atoms than nanoparticles the mode of interactions of cysteine on these nanoclusters could be completely different than 12.5 nm nanoparticles.

¹H-²H dipolar couplings with ¹H as the observe nuclei was successfully explored by REDOR and Rotary Resonance Recoupling experiments. Shorter relaxation times of ¹H limited the length of the dipolar evolution time, which could be alleviated by using faster spinning speeds. It was also found that ²H composite pulses may provide better performance for ¹H-²H REDOR experiments. The ¹H resonance positions and line widths in ¹H-²H R³ experiments could suffer from the Bloch-Siegert shift effect which could be minimized by using high magnetic field. These

problems are eliminated by placing the ^2H pulse during an evolution period and not during the detection period.

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