ABSTRACT

MARGITA, KALEIGH MARIE. Interactions and Conformational Changes of Double Stranded DNA with Cationic Ligand-Protected Au Nanoclusters by Spin-Labeling EPR and Ionizable EPR Probes (Under the direction of Dr. Alex I. Smirnov).

Integrations of stable and structurally tunable monolayer protected Au clusters (MPCs) with biological macromolecules and systems have been the focus of intense research efforts in the recent years. In applications to biomedicine, MPCs are actively researched as promising imaging agents and drug carriers that can be targeted to specific cells and/or tissues. For example, positively charged nanoparticles (NPs) are expected to readily interact with negatively charged DNA forming suitable delivery vehicles for penetrating cellular and nuclear membranes that typically exhibit negative electrostatic potential.\(^1\)–\(^3\) Despite some tremendous progress in the field, detailed atomic-level understanding of dynamic interactions between MPCs and DNA is still missing from the literature.\(^3\) Recently, a collaborating NCSU group of Prof. Yingling developed a series of atomistic molecular dynamic (MD) simulations to gain an insight into the interactions between DNA and cationic nanoparticles with a focus on conformational changes in the DNA structure such interactions could cause. The ultimate goal of such studies is to develop a toolbox to guide the synthesis of nanoparticles with properties optimal for gene delivery.\(^4\) Guided by these theoretical results a series of protonatable ligands and ionizable nitroxide ligands for modifying surfaces of Au NPs have been synthesized in the course of this Thesis project. Further, the surface electrostatics and local dynamics of the Au NP ligand layer were successfully examined by Electron Paramagnetic Resonance (EPR)\(^5\) before and after binding to DNA. To further understand the effects of solution ionic strength on pK\(a\) values and magnetic parameters of molecular pH-sensitive EPR probes, we have carried out studies of a model ionizable
nitroxide 2,2,3,4,5,5-hexamethylimidazolidin-1-oxyl. Next, a series of model oligonucleotides were spin-labeled with nitroxide probes in a site-directed manner and a 4-pulse Double Electron-Electron Resonance (DEER) experiment was conducted to examine the confirmations of the NP-bound DNA molecule.
Interactions and Conformational Changes of Double Stranded DNA with Cationic Ligand-Protected Au Nanoclusters by Spin-Labeling EPR and Ionizable EPR Probes

by
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A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Master of Science in Chemistry

Raleigh, North Carolina

2017

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DEDICATION

For those who taught me about passion, hard work, and dedication.
BIOGRAPHY

Kaleigh Margita was raised in Florence, South Carolina, where her love for science and learning was cultivated. She graduated from Newberry College in the spring of 2014 with a B.S. in Chemistry and a minor in Spanish. During her undergraduate career, she completed summer research projects in nanotechnology under the direction of Prof. Samuel Achilefu at Washington University in St. Louis and in bioelectronics under the direction of Prof. George Malliaras and Prof. Roisin Owens at the Centre Microélectronique de Provence of the Ecole Nationale Supérieure des Mines de Saint-Étienne. In the Fall of 2015, she joined the lab of Prof. Alex I. Smirnov at North Carolina State university to continue her graduate education in biophysical chemistry.
ACKNOWLEDGMENTS

I am incredibly grateful for the direction, unwavering support, and encouragement of my advisor Prof. Alex I. Smirnov. I would like to extend a special thanks to Dr. Maxim Voinov, who patiently answered my many questions. I also wish to acknowledge Dr. Sergey Milikisiyants and Ms. Melanie Chestnut for their help and many long nights of work on obtaining DEER Spectroscopy and SEM data. I would like to thank Prof. Alex Nevzorov and Prof. Gavin Williams for readily serving on my committee. To everyone in the Smirnov, Smirnova and Nevzorov labs, I would like to send my many heartfelt thanks for the encouragement and memories. Finally, I would like to thank my family and husband who have blessed me immeasurably. This work was supported by NSF CBET-1403871.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AIBN</td>
<td>Azobisisobutyronitrile</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N’-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric Acid</td>
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<tr>
<td>EPR</td>
<td>Electron Paramagnetic Resonance</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared spectroscopy</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscope</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
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**LIST OF COMPOUNDS SYNTHESIZED**

<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>1</td>
<td>11-(dimethylamino)undecane-1-thiol hydrochloride</td>
</tr>
<tr>
<td>2</td>
<td>10-(Acetylthio)decanoic acid</td>
</tr>
<tr>
<td>3</td>
<td>4-(2-aminoethylamino)-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazole</td>
</tr>
<tr>
<td>4</td>
<td>12-(Acetylthio)dodecanol</td>
</tr>
<tr>
<td>5</td>
<td>12-(acetylthio)dodecyl 1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylate</td>
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CHAPTER 1 INTRODUCTION

1.1 Effect of Ionic Strength on 2,2,3,4,5,5-hexamethylimidazolidin-1-oxyl (HMI) Nitroxide:
Over the years molecular probes based on stable free radicals (a.k.a. spin probes) and the associated Electron Paramagnetic Resonance (EPR) methods have been finding numerous bioanalytical applications from measuring oxygen concentrations in cells and tissues in vivo to obtaining unique data, on polarity of phase-separated liquids and microviscosity of cellular membranes. Most recently, proton transfer-related and surface electrostatic phenomena have been studied using pH-dependent EPR molecular probes. These molecular probes have a unique chemical functionality that is capable of a reversible ionization, such as protonation, in addition to possessing an EPR-active reporter nitroxide group (N-O•). In such molecular probes, the ionization event causes a formation of protonated and nonprotonated nitroxide species with different magnetic parameters ($A_{iso}$, $g_{iso}$) that are readily identified by EPR.

Solution ionic strength has a known effect on the chemical equilibrium of the charge species, and therefore is expected to affect the equilibrium constant ($pK_a$) of the reversible protonation of the nitroxide. However, until our work published earlier this year only some scarce data on effects of the solution ionic strength on the experimental $pK_a$ of ionizable nitroxide species can be found in literature. Specifically, we have carried out a series of EPR titration experiments using 2,2,3,4,5,5-hexamethylimidazolidin-1-oxyl (HMI) nitroxide and a wide range of electrolyte concentration (from 0 to 5M) in order to better the understanding this ionic strength effect. We choose this nitroxide because of an exceptionally large difference in isotropic nitrogen hyperfine coupling constant of the protonated and nonprotonated forms (see Figure 1). The Debye-Hückel formalism was used to analyze the subsequent data and the magnetic parameters of the ionizable nitroxides affected by the ionic strength are also discussed.
1.2 Site Directed Spin Labeling of Oligonucleotides:
At the center of most living organisms, the self-replicating material Deoxyribonucleic Acid (DNA) carries all the genetic information. DNA is comprised of four nucleotides, Adenine, Thymine, Guanine, and Cytosine, and its biological function arises from the structure and dynamics of the molecule. Much research has been devoted to understanding the biological interactions of DNA on the most basic level.\textsuperscript{9,10} Initially X-ray Crystallography was used to obtain the structure of DNA assemblies and to provide atom to atom measurements; however, as later it has been realized that the rigid crystal may not represent the definite structure as found under biological conditions in cells as well as model solutions.\textsuperscript{11} Nuclear Magnetic Resonance (NMR) has been used for biomolecules in solution to obtain information on the molecular dynamics and interactions of the nucleobases with an upper limit of approximately 50-100kDa molecules or 40-80 nucleotide strand of DNA (although molecular weight limits for solution NMR have been constantly pushed further towards heavier molecules).\textsuperscript{10,12} Additional biophysical methods have also been employed in DNA studies. For example, EPR and fluorescence spectroscopy are the two methods that can provide precise measurements of nanometer distances, structure, and orientation of larger biomolecules at biological conditions. Both methods involve an attachment of a molecular tag (a paramagnetic spin label for EPR or a fluorophore moiety for optical detection) to DNA. For biophysical studies spin-labeling EPR is viewed to be more suitable for DNA studies because of the relatively small perturbations to the native structure when a spin label is added in comparison to the attachment of a fluorophore.\textsuperscript{13}
Site-Directed Spin Labeling (SDSL) is an EPR technique that is based on covalent attachment of an EPR-active paramagnetic tag to a specific residue within the biological molecule. A spin label is a molecule with one lone electron, most commonly contained within a nitroxy group with a spin quantum number of $S=1/2$. The basic theory of SDSL involves the interaction between the electron dipole and the applied magnetic field, which is mathematically denoted the Equation 1 where $\mu_e$ is the dipole moment of the electron and the $B_o$ is the external magnetic field applied. The electron dipole also interacts with the nearby nuclei magnetic fields, $m_1$, or hyperfine interactions, $A$, which contribute to the overall energy difference as well. The electron dipole in the presence of a magnetic field has two spin states so the difference in energy of the spin states can be found using Equation 1.

$$\Delta E = h\nu = \mu_e B_o + m_1 A = g\beta_e B_o + m_1 A$$

*Equation 1: The energy between the two spin states, $\Delta E$, of the electron dipole, $\mu_e$, in the presence of a magnetic field, $B_o$. Where $g$ and $\beta_e$ are the electron spin $g$-factor and Bohr magnetron correspondingly. The additional hyperfine variables, due to interaction of the unpaired electron and nearby nuclei, is also included in terms of the nuclear spin state, $m_1$, and the hyperfine interaction $A$.*

Using continuous wave (CW) EPR methods, when the applied magnetic field is resonant with the $\Delta E$ between two energy levels, electrons absorb the energy and are excited to a higher energy state. This phenomenon is known as the Zeeman effect, with each excitation level giving rise to a peak in the first derivative EPR Spectrum as shown below in Figure 2. The quantity of spectrum peaks, due to the hyperfine splitting, is directly related to the dipolar interaction of the unpaired electron with the small magnetic fields of close nuclei. In a nitrooxide molecule, for example, the nearest nuclei is the Nitrogen molecule resulting in a three component spectra with splitting of the EPR signal into three spectral lines, as shown in Figure 2. The spectrum peaks are composed of both isotropic and anisotropic components, due to the elliptical dipole moment of the unpaired electron, meaning that the peaks resemble the averaged hyperfine interactions.
occurring as the dipole rotates. Spectral peak shape changes can then be directly correlated to the many rotational moments of the nitrooxide molecule as shown in Figure 3. Spectra linewidth broadening occurs when the nitrooxide rotational motion is hindered and narrows with an increase in rotational motion. This is particularly useful because the nitrooxide can be coupled to large biological molecules and provide information about the environment surrounding the labeling location.

Figure 2: Energy level transitions corresponding to the first derivative EPR spectrum (modified to include EPR spectrum from HMI Nitrooxide)

Figure 3: Rotational Dynamics present in R5 Nitrooxide and Theoretical Effect on EPR Spectra (modified illustration)
Electron-electron dipolar interactions, where the energy between the two electron dipoles, $E_{dip}$, is added to each electron energy level, can also be examined. Mathematically, with the case of two electron dipoles, the total energy is dependent on the distance between the two spins and the angle of the distance vector and the magnetic field, as illustrated in Figure 4.\textsuperscript{13}

![Figure 4: Electron-electron dipolar spin interaction where $B_0$ is the magnetic field and $\theta$ is the angle of the distance vector, $r_{AB}$ (modified\textsuperscript{13})](image)

The EPR spectra can be obtained of these pairwise interactions using the method double electron-electron resonance (DEER). The DEER method further explained in the subsequent chapter, uses a series of pulse sequences and measures the EPR signal in the time domain during the relaxation or echo period and is further discussed in the next section. The incorporation of two or more nitroxide molecules into a biological molecule and analysis with DEER can provide a wealth of information about not only the local environment but also provide accurate distances between portions of the molecule and the angle at which the nitroxides are oriented. In DNA, RNA, and Protein studies this data is crucial in understanding molecular conformations and association dynamics, which govern key biological functions.

There are numerous synthetic methods for incorporation of nitroxides into DNA strands from chemical modifications, enzymatic modifications, to the addition during solid phase synthesis of the nucleic acids. As shown in Figure 5, nitroxides can be covalently attached to DNA and categorized by the attachment location. Nitroxides can be incorporated at the 5’ and 3’ terminus, the phosphate, sugar, and base positions within the DNA strand, allowing for much
EPR data to be obtained about each site within the molecule. With each attachment location, it is important to note that each method for labeling is different with varying degrees of DNA perturbation.

![Figure 5: Locations for nitroxide labeling within DNA. 1. Internal Phosphate, 2. Sugar, 3. Nitrogenous Base (modified)](modified)

The most prevalent and highly efficient method involves assembling single nucleotides in the 3’ to 5’ direction and incorporating a functional group, for example, a thiol or amine which can be modified after a complete length of single stranded DNA is constructed. This allows the spin label to be added anywhere within the molecule. The replacement of a phosphate group with a phosphorothioate allows for the use of one method of SDSL proven particularly successful by the Qin group and shown in Figure 6 below. Phosphorothioate modified DNA assembled as described was commercially purchased for our studies. The phosphorothioate is further modified with a iodomethyl nitroxide (R5) moiety that attaches the nitroxide to the desired location with high efficiency. The attachment of the R5 nitroxide to the phosphorothioate group causes little to no perturbation of the DNA strand. The cost of the
phosphorothioate addition is also much less than developing modified oligonucleotides. However, while this method seems very flexible and advantageous some key aspects of the nitroxide must be discussed. The three single bonds which attach the nitroxide to the phosphorothioate group are rotatable, which gives rise to the limited perturbation of the DNA strand, but also introduces a degree of inconsistency in the labeling process. The solid-phase synthesis method introduces both diastereomers of phosphorothioate, resulting in the local environment of each nitroxide being slightly different as shown in Figure 7. The two forms have been successfully separated using HPLC methods, but the mixed diastereomers can still be used in EPR studies. With the attachment of the R5 nitroxide, one negative charge of the phosphate group is lost, which is a concern for obtaining electrostatic measurements and labeling at certain cites within DNA molecule. Overall, the phosphorothioate labeling scheme, is best suited for our experiments to obtain accurate distance measurements and local environment information for mapping of the DNA molecule using the R5 label.

Figure 6: A) Synthesis of iodomethyl derivative nitroxide R5 B) Phosphorothioate Labeling of DNA Scheme with R5 (redrawn
de)
Figure 7: Conformational diastereomers of R5 labeled phosphorothioate (redrawn\textsuperscript{15})

1.3 DEER Spectroscopy for Characterization of Nitroxide Labeled Oligonucleotides:
EPR fundamentally begins with the interaction of an electron in an external magnetic field. A simple energy-level diagram is shown below in Figure 2 for a particle, such as the R5 nitroxide, with a spin of $\frac{1}{2}$. The external magnetic field, $B$, is varied, changing the energy level separation until the frequency of the microwave radiation is resonant with the energy transition sending electrons from the lower to upper state. This relation can be explained by Equation 1, where the change in energy is directly described by the resonant frequency, $v$, and planks constant, $h$. Considering the spin angular momentum, in the presence of the magnetic field, the interaction of the $m_s=\pm1/2$ spin states can also be illustrated as a vector precession as shown in Figure 8. The frequency of the procession, known as the Larmor frequency, can be determined by the product of the gyromagnetic ratio of an electron and the magnetic field. Subsequently, the energy between the two states can also be given by Equation 2. This equation introduces contributions of the fundamental quantum of magnetic moment or Bohr Magneton and from the interactions of the electron with electromagnetic changes in the surroundings or the $g$-value. The
fingerprint like EPR spectrum arises from the splitting of the electrons individual resonance lines due to the interactions of the electrons with nuclei within the system. The hyperfine coupling constant, $a$, quantifies the degree of splitting from the center resonance line, $B$.\(^{17-19}\)

$$\Delta E = g_e \mu_B B_0$$

\textit{Equation 2: Energy equation for the precession of vectors dependent on the Bohr Magneton, $\mu_B$, the electronic spin state $m_l$, and the external magnetic field, $B_0$.}^{18,19}

\textit{Figure 8: Representation of angular momentum of an electron in an external magnetic field along the z-axis through vector precession with parallel and anti-parallel distribution.}^{18}

From examining electrons as vectors in precession, it is reasonable that a pulsed EPR method, reminiscent of a spin-echo double resonance NMR experiment which measures interactions throughout a time domain could have many advantages. Numerous EPR methods with varying pulse sequences have been developed. Each generally increasing the sensitivity and range of distances between paramagnetic centers that can be measured but each having niche applications.\(^{20}\) For our experiments a 4 pulse double electron electron resonance method was chosen.

Moving from the laboratory reference frame illustrated in Figure 8, where the magnetization is considered stationary (theoretical average of many electron spins), parallel to the z-axis, and aligned with $B_0$, into a rotating frame makes viewing linearly polarized
microwave pulses simpler. The linearly polarized microwaves can be represented as two circularly polarized components which are perpendicular to $B_0$, below in Figure 9. At resonance, one of the components of $B_1$ appears stationary and the second component or fast component which rotates with an angular velocity of $2\omega_1$ can be neglected because of effective fields. It can also be assumed that the magnetization, $B_0$, can also be neglected because it is not processing in the frame. This leaves a simple rotating frame and the components of $B_1$ and the net magnetization of many electron spins, $M_0$, to consider. $M_0$ can then be rotated to process around $B_1$ with a frequency termed the Rabi frequency, shown in equation 3 below. Pulses are labeled by the tip angle, $\alpha$, or the angle which $M_0$ must be rotated to process about $B_1$.\textsuperscript{17}

Typical EPR pulse sequences include characteristic pulses of $\pi/2$ which rotates $M_0$ 90° and $\pi$ which rotates $M_0$ 180°. The rotating frames of the four possible pulse phases are illustrated below where $B_1$ can be found on any of the ±x and ±y axes. In Figure 10, a $\pi/2$ pulse sequence is shown, where before the microwave pulse $M_0$ is aligned with $B_0$ until the microwave pulse and then $M_0$ is rotated to processes around $B_1$ until the pulse is completed and then spin-lattice relaxation occurs. Spin Lattice relaxation is the decay process of the magnetization back to alignment with the z-axis after the microwave pulse is removed. The phenomenon can be quantified by $T_1$, describing how quickly the magnetization returns to the z-axis or by the

\[ \omega_1 = -\gamma B_1 \]

Equation 4: Rabi frequency of precision of $M_0$ around $B_1$\textsuperscript{2,5}

\[ \alpha = -\gamma |B_1| t_p \]

Equation 3: tip angle equation where $t_p$ is the length of the pulse\textsuperscript{2,5}
transverse relaxation time, $T_2$, which is how quickly the magnetization disappears in the x-y plane.\textsuperscript{17,21} The Free Induction Decay (FID) spectrum can then be obtained and Fourier transformed into the EPR spectrum. To increase the signal, the FID is measured multiple times through a series of pulses and added together. However, there are several limitations in increasing the signal due to having to wait until the magnetization returns to the z-axis before the next pulse can be made and the inhomogeneous relaxation of electrons which cause decay. The decay of the FID signal is caused by the different frequencies at which the electrons relax, some are fast, others move slower based on their surroundings, and some even move out of the x-y frame.\textsuperscript{17,22}

Figure 10: A) four phases of $B_1$ in the rotational coordinate frame B) simple $\pi/2$ pulse diagram with subsequent behavior of magnetization vector (modified\textsuperscript{17})

Portions of the signal which have decayed away due to inhomogeneous broadening in the FID can be recovered using a Hahn echo. After the FID, a $\pi$ pulse ($180^\circ$) is applied to turn the magnetization into about the x-axis and while the magnetization rotates at the same speed and direction but towards the x-axis instead of away. The packets of electrons with lower frequency
of precession have time to “catch up” refocusing the magnetization, as shown below in Figure 11.\textsuperscript{17} The proceeding echo measures the FID of the refocusing and then the subsequent dephasing of the electrons.

![Figure 11: π pulse refocusing of magnetization before echo (modified\textsuperscript{17})](image)

From the three basic pulse EPR components, π/2 pulse, π pulse, and the echo, a pulse sequence can be constructed for obtaining DEER spectra and accurate distance measurements from 1.5nm-8nm. 4-pulse DEER has two pulse frequencies, observer frequency, $\omega_A$, and pump frequency, $\omega_B$, which correspond to the two electrons. The observer pulse refocuses the inhomogeneous line broadening of spin A, while the electrons, B, that are coupled to spin A are excited and subsequently inverted by the pump pulse frequency. As seen in Equation 6A, 4-pulse DEER begins with a π/2 pulse which begins the precession of the magnetization around $B_1$. Secondly a π pulse is used to refocus the spin A, which then transversely relaxes along with electrons that are coupled to A and excited by the same pulse and leads to the echo. Inhomogenities come from the hyperfine couplings, coupling of A spins to B spins which are not excited and g-value dispersions. The transverse relaxation of spin A and coupled spins leads to an echo attenuation described by the factor, shown in Equation 5. The decay rate constant, shown below in Equation 6 is directly dependent on $T_2$, and the instantaneous diffusion rate shown in Equation 7. These equations all show that as the concentration and inter-pulse delays increase, the signal will decrease. Electron spins B are then excited by a pump pulse frequency which inverts the B spins and that of the local field for the A spins, shown in Figure 12B. Due to the electron-electron
coupling of spin A with spin B, the inversion of spin B causes changes in the frequency of spin 
A, and can be related to changes in the echo amplitude through equation 8. The energy level 
diagram in Figure 12C, shows how the transition between the energy levels of spin A differ by 
the frequency of $\omega_{ee,i}$. During the time that B is flipped, A is allowed to evolve and then is also 
flipped by a $\pi$ observer pulse but it no longer aligns with the axis that initially flipped to after the 
first observer pulse. The difference in phase alignment corresponds to $\omega_{ee,i}$. It is then that the 
final echo FID oscillations are detected in the time domain and translated into the desired 
spectrum and shown in Figure 13.

\[
\exp[-2k(T_1 + T_2)]
\]

*Equation 5: Echo attenuation factor*\(^{23}\)

\[
k = \frac{1}{T_{2,A} + k_{ID}}
\]

*Equation 6: Decay rate constant of the transverse relaxation of spin A*\(^{23}\)

\[
K_{ID} = C_A K_A
\]

*Equation 7: Rate of instantaneous diffusion*\(^{23}\)

\[
\nu(t) = \prod_t \left[ 1 - \lambda_i [1 - \cos(\omega_{ee,i} t)] \right]
\]

*Equation 8: Inverse echo amplitude as a function of time*\(^{23}\)
Figure 12: A) 4-pulse DEER sequence B) effect of π pulse on B spins and local field of A spins C) energy level diagram for the exchange of energy between A and B spins (obtained from reference 23)

Figure 13: Theoretical DEER spectrum where the two spin packets have a difference in the frequency precession, ω_{ee} (obtained from reference 24)

The distance distribution between spins A and B can be determined by the following modification to Equation 8 shown in Equation 9. This equation makes the following key assumptions:

1) The external magnetic field is quantized and so are the spins.
2) Neglection of the electron spins exchange coupling can occur, simplifying the coupling to only dipole-dipole coupling.
3) The spin pairs are mostly isolated.
4) Within the fractional dimension there is a homogenous distribution of spins.
5) An average orientation of the spins is taken.
\[ \nu(t) = \left\{ 1 - \lambda \left[ 1 - \int_0^1 \cos \left( \frac{C_i}{r_i^3} (1 - \cos^2 \theta_i) t \right) \cos \theta \right] \right\} B(t) \]

*Equation 9: Distance distribution determination where \( C_i \) is the product of the g-values, \( \theta \) is the angle between the spin-spin vector, \( r \) is the distance between the two spins, \( \gamma \) is the fraction of spins and \( B(t) \) is the background function.\(^{25,26}\)*

The Fourier transform of \( \nu(t) \) gives the common dipolar pake pattern, in Figure 14, and using Equation 10 the frequency of the perpendicular spins can be used to determine the distance between two spins.\(^{10}\)

\[ r(\text{nm}) = \sqrt{\frac{52.04}{\nu(\text{MHz})}} \]

*Equation 10: Distance between spins calculation from perpendicular \( \nu \) of the pake pattern\(^{26}\)*

*Figure 14: Dipolar Pake pattern with the frequency of perpendicular spins (obtained from reference\(^{26}\)).*

More often, instead of performing a Fourier transform of the time domain computational programs are used assuming all orientations of the pake pattern are excited. Computation of distance distributions directly from the time trace or form factor has been thoroughly analyzed and it has been suggested that the Tikhonov regularization with an added nonnegativity constraint is the top transformation method.\(^{26}\) The resulting distribution includes information about, mean distance, width, and shape in increasing order of reliability. Several measurements often need to be observed to obtain accurate information limiting the length that can be measured. Overall the distance distribution can be incredibly useful in determining molecule conformation and understanding molecular interactions.\(^{23}\) For our experiments, a 4-pulse DEER
spectroscopy method will be used to determine the molecular conformation of DNA in the presence of cationic nanoparticles.

As in many spectroscopic methods, there are many experimental parameters to take into consideration which have potential to affect the system and decrease sensitivity of the measurements. Most importantly for DEER are the temperature and concentration parameters, the microwave frequency band, and the labeling efficiency of the sample. At low temperatures (40-60K), transverse relaxation is dominated by proton spin diffusion allowing for longer values for $T_{\text{max}}$, increasing sensitivity of the measurement and measuring longer distances between spins. The parameters of labeling efficiency and concentration work hand in hand. While it is important to have a sample with a high labeling efficiency so that there are many spin pairs to observe transitions between, it is just as important to maintain a homogenous distribution within the sample.$^{23,27}$ The microwave frequency band is also an important parameter to consider. Sensitive DEER measurements are typically done at X-band frequencies, but at Q-band frequencies and a high power set up can further enhance the measurements.

CHAPTER 2  RESULTS AND DISCUSSION
2.1 Potentiometric titration of aqueous solutions of 2,2,3,4,5,5-hexamethyllumidazolidin-1-oxyl (HMI) nitroxide: (The content of the following section was published by Springer in the fall of 2016.$^{28}$)

A series of titrations was completed in a thermostatic vessel with aqueous solutions of 2,2,3,4,5,5-hexamethyllumidazolidin-1-oxyl (HMI) nitroxide in the presence of a large range of electrolyte concentrations (0.05M to 5M). For each ionic strength salt solution, it was necessary to create a calibration curve, to graph the effect of pH vs potential (mV). The graphs were then
fit with the modified Nernst equation shown in Equation 11. The slope and $\varepsilon$ values for the calibration plots are shown in Table 1 below for each ionic strength solution.

$$E = \varepsilon - s \times pH$$

*Equation 11*: Modified Nernst equation where $\varepsilon$ is the total potential which remains constant for a given electrode and solution composition, $s$ is the slope of the plot, and $E$ is the experimentally measured potential (mV)

*Table 1*: Parameters (slopes and $\varepsilon$ values) for the calibration plots obtained from potentiometric titration of solutions with various ionic strength of NaCl

<table>
<thead>
<tr>
<th>[NaCl], M</th>
<th>$\varepsilon$ acidic</th>
<th>Slope acidic</th>
<th>$\varepsilon$ basic</th>
<th>Slope basic</th>
</tr>
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<tr>
<td>0.10</td>
<td>428.14</td>
<td>65.85</td>
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<td>0.30</td>
<td>431.41</td>
<td>65.92</td>
<td>749.00</td>
<td>80.05</td>
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<td>0.50</td>
<td>414.03</td>
<td>62.84</td>
<td>684.67</td>
<td>71.33</td>
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<td>1.00</td>
<td>416.14</td>
<td>63.06</td>
<td>808.41</td>
<td>91.49</td>
</tr>
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<td>2.00</td>
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<td>58.84</td>
<td>391.83</td>
<td>55.94</td>
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<tr>
<td>5.00</td>
<td>494.01</td>
<td>73.30</td>
<td>430.00</td>
<td>57.26</td>
</tr>
</tbody>
</table>

HMI solutions (0.2mM) in each ionic strength solution were titrated and the electric potentials recorded. From the measured electric potentials, $E$ (mV), and the slope, $s$, and the solution inherent $\varepsilon$ for the acidic or basic pH regions determined from the calibration plots were used with Equation 12 to calculate the pH of HMI solutions. This pH value is referred to as the calculated pH, while the reading from the electrode will be called the measured pH, illustrating the error that occurs in the pH measurements if the electrode was not calibrated using ionic strength-corrected buffer solutions.
Equation 12: \( pK_a = -\log_{10} K = pH - \log_{10} \left[ \frac{[R\cdot]}{[R\cdot H^+]} \right] \)

At each titration point, an X-band (9.5GHz) EPR spectrum was also taken, revealing two superimposed components and simulated assuming a two-center model as described in literature.\(^{29,30}\) A representative two component X-band EPR spectrum acquired at intermediate pH with a least squares decomposition of the experimental spectrum for components corresponding to the non-protonated and protonated forms of the nitroxide is shown below. The residual of the fit illustrates that experimental spectrum fits into the two component model rather well.

![Figure 15](image)

**Figure 15:** Least squares decomposition of the experimental X-band EPR spectrum acquired from HMI in 2 M NaCl solution for the spectral components corresponding to the protonated and non-protonated forms of the nitroxide.

Figure 16 shows the results of EPR titration of HMI in a 2 M NaCl aqueous solution. The pH values in the acidic and basic regions were calculated using the parameters from 2 M NaCl calibration plots and plotted against the weighed \( A_{iso} \) obtained from the fit of the two component EPR spectrum shown in Figure 15. The solid line represents the least squares fit of the
experimental data to the Henderson-Hasselbalch equation, shown in Equation 13, giving a \( pK_a \) of 5.36 ± 0.03. Table 2 below lists the experimentally determined \( pK_a \) values and weighed nitrogen hyperfine coupling constants, \( A_{iso} \), for each HMI solutions of varying ionic strength.

\[
A_{iso} = \frac{A_{iso} \left( R \cdot \right) \times 10^{(pH-pK_a)} + A_{iso} \left( R \cdot H^+ \right)}{1 + 10^{(pH-pK_a)}}
\]

*Equation 13: Modified Henderson-Hasselbalch equation used for fitting the experimental EPR titration data. \( A_{iso} \) represents the nitrogen hyperfine coupling constant averaged out proportionally to the weights of the individual spectral components (non-protonated and protonated forms of the nitroxide).*

![EPR Titration of HMI in 2.0M NaCl](image)

*Figure 16: Representative EPR Titration of HMI in 2.0M NaCl. Solid line represents the result of a least-squares fit to the modified Henderson-Hasselbalch equation*
Table 2: Experimentally determined pK<sub>a</sub> values and weighed nitrogen hyperfine coupling constants, A<sub>iso</sub>, for HMI solutions of varying ionic strength. A<sub>iso</sub> were determined from the least-squares fit of the experimental data to the modified Henderson-Hasselbalch equation

<table>
<thead>
<tr>
<th>[NaCl], M</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt; (measd)</th>
<th>A&lt;sub&gt;iso&lt;/sub&gt;, R&lt;sup&gt;+&lt;/sup&gt; G</th>
<th>A&lt;sub&gt;iso&lt;/sub&gt;, R&lt;sup&gt;+&lt;/sup&gt;H&lt;sup&gt;+&lt;/sup&gt;, G</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt; (calcd)</th>
<th>A&lt;sub&gt;iso&lt;/sub&gt;, R&lt;sup&gt;+&lt;/sup&gt;, G</th>
<th>A&lt;sub&gt;iso&lt;/sub&gt;, R&lt;sup&gt;+&lt;/sup&gt;H&lt;sup&gt;+&lt;/sup&gt;, G</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>4.63±0.01</td>
<td>15.95±0.01</td>
<td>14.64±0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.1</td>
<td>4.68±0.01</td>
<td>15.96±0.01</td>
<td>14.65±0.01</td>
<td>4.58±0.01</td>
<td>15.94±0.01</td>
<td>14.65±0.01</td>
</tr>
<tr>
<td>0.3</td>
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<td>15.98±0.01</td>
<td>14.67±0.02</td>
<td>4.66±0.03</td>
<td>15.97±0.01</td>
<td>14.62±0.01</td>
</tr>
<tr>
<td>0.5</td>
<td>5.05±0.06</td>
<td>15.97±0.02</td>
<td>14.67±0.03</td>
<td>4.78±0.06</td>
<td>15.97±0.02</td>
<td>14.66±0.03</td>
</tr>
<tr>
<td>1.0</td>
<td>5.42±0.09</td>
<td>16.01±0.04</td>
<td>14.66±0.04</td>
<td>5.12±0.09</td>
<td>16.02±0.05</td>
<td>14.64±0.05</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.36±0.02</td>
<td>15.99±0.01</td>
<td>14.66±0.01</td>
</tr>
<tr>
<td>5.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.35±0.02</td>
<td>16.01±0.01</td>
<td>14.71±0.01</td>
</tr>
</tbody>
</table>

As the ionic strength of the solution increases the pK<sub>a</sub> experiences an upward shift graphed in Figure 17. This trend can be explained by considering the electrostatic interactions of the Cl<sup>-</sup> with the protonated HMI; an increase in Cl<sup>-</sup> ions concentration results in a better stabilization of the protonated form of HMI and the equilibrium is shifted further towards the protonated HMI. There is also a consistent deviation of the pK<sub>a</sub> determined using the pH directly measured with the electrode and the pH calculated using the calibration plot, with a greater difference at higher salt concentrations. Figure 18 further examines the correlation between ionic strength and pK<sub>a</sub> by plotting the pK<sub>a</sub> against the ionic strength parameter (I<sup>0.5</sup>/1+I<sup>0.5</sup>) revealing the same trend.
Figure 17: $pK_a$'s of HMI plotted vs. NaCl concentration. Open circles – pH was calculated from ionic strength calibration plots; filled circles – pH was directly measured with a temperature-corrected 3-point calibrated pH electrode; red circle – a literature data$^{31}$ (0.01 M buffer, temperature was not specified) shown as a reference.

Figure 18: $pK_a$ from calculated pH values versus the ionic strength parameter

The EPR spectrum, in Figure 19, reveals two distinct components, that of the non-protonated form of the nitroxide with a larger nitrogen hyperfine coupling constant of approximately $A_{iso} = 15.95$ G and that of the protonated form with a smaller nitrogen hyperfine coupling constant of approximately $A_{iso} = 14.62$. As HMI is titrated the relative intensities of EPR spectral components change. At essentially the same pH, a series of EPR spectrums illustrates the trend seen in Figure...
15, where the higher ionic strengths cause an increase in the fraction of the protonated form of HMI.

![Figure 19: Representative X-band (9.5 GHz) EPR spectra of HMI acquired at essentially the same pH (pH is shown on the right) but at various NaCl concentrations.](image)

2.2 Chemical Synthesis: (Procedures adapted from a previous graduate student’s thesis)

2.2.1. Ligands-

**11-(dimethylamino)undecane-1-thiol hydrochloride (I)**

To study electrostatic properties of nanoparticles as a function of pH, 11-(dimethylamino)undecane-1-thiol hydrochloride was synthesized to serve as a ligand for attachment to gold nanoparticles. Thioacetic acid was combined with 11-bromo-1-undecene and AIBN to form thioacetic acid-S-(11-bromoundecyl) ester and characterized by $^1$H NMR and IR spectroscopy. Thioacetic acid-S-(11-dromoundecyl) ester was converted to 11-(dimethylamino)undecane-1-thiol using dimethylamine and then a solution of gaseous HCL in THF was used to make the hydrochloride form illustrated below in Scheme 1.

![Scheme 1: Structure of 11-(dimethylamino)undecane-1-thiol hydrochloride](image)
2.2.2 Spin Labeled Ligands-
Two thioacetyl ligands were successfully coupled to nitroxide radicals by DCC mediated reactions to form spin label ligands for the attachment to Au NPs and examination of the local environment of the Au NPs surface.

12-(Acetylthio)dodecanol-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylate (5) -
The substitution reaction of potassium thioacetate and 12-bromo-1-dodecanol was completed to form 12-(Acetylthio)dodecanol (4) and examined for impurities using TLC.\textsuperscript{34} The structure of the product is shown in Scheme 2.

![Scheme 2: Structure of 12-(Acetylthio)dodecanol](image)

The product was then coupled with the commercially available nitroxide 3-Carboxy-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl in the presence of DCC and DMAP to make 12-(Acetylthio)dodecanol-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylate shown below in Scheme 3.\textsuperscript{35} IR spectroscopy was used to characterize this ligand. Previous experiments have shown that while the compound is not inherently sensitive to the pH of the system, it is valuable as an EPR probe for the studying of ligand dynamics in self assembled monolayers.
Scheme 3: Synthesis of 12-(acetylthio)dodecyl-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrole-3-carboxylate EPR active Ligand

$S\text{-}10\text{-oxo}\text{-}10\text{-}(2\text{-}(1\text{-oxyl}\text{-}2,2,5,5\text{-tetramethyl}\text{-}2,5\text{-dihydro}\text{-}1H\text{-imidazol}\text{-}4\text{-ylamino})\text{ethylamino})\text{decel ethanethioate (6)}$

A nucleophilic substitution reaction between potassium thioacetate and 10-bromodecanoic acid produced 10-(acetylthio)decanoic acid (2) illustrated in Scheme 4 was completed.\textsuperscript{34} The ligand formation was confirmed by $^1H\text{-NMR.}$

Scheme 4: Structure of 10-(acetylthio)decanoic acid

The nitroxide 4-(2-Aminoethylamino)-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazole (3) was then synthesized for the coupling to 10-(acetylthio)decanoic acid.\textsuperscript{36} 2,2,5,5-Tetramethyl-2,5-dihydro-1H-imidazol-1-ol 3-oxide precursor was oxidized with MnO\textsubscript{2} to form aldonitrone 2,2,5,5-tetramethyl-3-imidazoline-1-oxyl. This intermediate was then reacted with 2-bromoethyl isocyanate to afford 1-(2(Bromoethyl)-6-oxyl-5,5,7,7-tetramethyltetrahydroimidazo[1,5-b][1,2,4]oxadiazol-2-one. Thirdly, 1-(2(Bromoethyl)-6-oxyl-5,5,7,7-tetramethyltetrahydroimidazo[1,5-b][1,2,4]oxadiazol-2-one was treated with with NaN\textsubscript{3} to give 4-[(2-Azidoethyl)amino]-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazole as an oily product. Finally, 4-(2-Aminoethylamino)-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazole was synthesized through the Staudinger procedure followed by the hydrolysis of the iminophosphoane formed.
The product shown below in Scheme 5 was characterized using IR spectroscopy and found to be identical with a previously reported compound.36

Scheme 5: Structure of 4-(2-Aminoethylamino)-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazole

The coupling of the nitroxide (3) to 10-(acetylthio)decanoic acid (2) shown in Scheme 6 was completed in the presence of DCC and DMAP.35 Due to the inoizable amidino group, S-10-oxo-10-(2-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-4-ylamino)ethylamino)decyl ethanethioate ligand has the potential to serve as a pH sensitive spin probe.

Scheme 6: Synthesis of pH sensitive ligand S-10-oxo-10-(2-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-4-ylamino)ethylamino)decyl ethanethioate

2.2.3 Gold Nanoparticles-

11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP (7)-

Using adapted one-pot synthesis literature procedures, stable 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP in small sizes were synthesized as illustrated in Scheme 7.37–39 11-(Dimethylamino)undecane-1-thiol hydrochloride (1) was added to tetrachloroaurate in a solution THF and acetic acid and stirred overnight at room temperature. The solution was then cooled to 0°C and ice cold sodium borohydride
solution was added to the mixture and stirred at 0°C for 24 hours. The gold nanoparticles were then characterized using Scanning Electron Microscopy, Dynamic Light Scattering, Transmission Electron Microscopy, and Thermal Gravimetric Analysis as subsequently shown below.

Scheme 7: Synthesis of 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NPs

Figure 20: SEM Images of Gold Nanoparticles approximately 10nm in size

Table 3: Number Statistics of Dynamic Light Scattering for size measurement of 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP
Figure 21: Dynamic Light Scattering analysis graphs generated by number% statistics in Table 3 of freshly prepared 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP

Figure 22: TEM Image of 11-dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP with 20nm scale bar (right) and corresponding histogram (left) of nanoparticle size and frequency that it appears in the image determined by ImageJ software.

Figure 23: TEM Image of 11-dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP with 5nm scale bar (right) and 2nm scale bar (left) with atomistic detail.
Table 4: Data from TGA analysis of 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Weight (mg)</th>
<th>Weight %</th>
</tr>
</thead>
<tbody>
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<td>200.2577</td>
<td>0.8516035</td>
<td>95.5784</td>
</tr>
<tr>
<td>297.2024</td>
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<td>297.5340</td>
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</tr>
<tr>
<td>500.3200</td>
<td>0.58662999</td>
<td>65.8395</td>
</tr>
<tr>
<td>Weight of Ligand (mg)</td>
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<td>0.265</td>
</tr>
<tr>
<td>Weight of Au (mg)</td>
<td>0.713</td>
<td>0.626</td>
</tr>
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</table>

SEM images showed that the nanoparticles had an average diameter of 10nm. However, TEM images reveal that the nanoparticles are polydisperse within a range of 1nm to 10nm in size. The largest populations of particles can be determined from the histogram above to be from 1-3nm. Using the equation below, the average size of Au NP can also be calculated using the statistical data from the DLS measurements. Mean Number % was used to avoid a small percent of large sized particles, attributed to dust, but in so small numbers that it was too low to contribute to the whole.
Using TGA, a mass loss which occurs from approximately 200°C to 500°C can be attributed to the loss of ligand from the gold core. From the information obtained through thermal gravimetric analysis, TEM, SEM and dynamic light scattering size measurements the potential compositions of 1nm, 2nm, 3nm, 6nm and 10nm size 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP was determined. The following representative calculations are completed using DLS data which reported 10nm particles. The same calculations were completed resulting in the table below for each of the potential sizes.

The volume of each nanocore can be determined as illustrated below.

\[ V_{NP} = \frac{4}{3} \pi r^3 = \frac{4}{3} \pi \left( \frac{100.0 \text{Å}}{2} \right)^3 = 523,599 \text{Å}^3 = 5.236 \times 10^{-25} \text{m}^3 = 5.236 \times 10^{-19} \text{cm}^3 \]

The volume of Au cubic unit cell is

\[ V_{Au \text{ Cubic Unit}} = t^3 = (4.0786 \text{ Å})^3 = 67.847 \text{ Å}^3 \]

Gold has a density of 19.3 g/cm³ so the mass of the gold nanocore = 1.011 x 10⁻¹⁷ g.

The number of gold atoms in the nanoparticle is found by

\[ \text{mass} \times \frac{197 \text{g/mol}}{197 \text{g/mol}} \times N_A = \frac{1.011 \times 10^{-17} \text{g}}{197 \text{g/mol}} \times 6.022 \times 10^{23} \frac{\text{particles}}{\text{mol}} = 30,891 \frac{\text{atoms}}{\text{NP}} \]

The surface area of the nanoparticle sphere is found by

\[ S = 4\pi r^2 = 4\pi \left( \frac{100.0 \text{Å}}{2} \right)^2 = 31,16\text{Å}^2 \]

The average length of an Au-Au bond on the surface of an Au NP, according to literature, is 2.93 Å.\textsuperscript{40} Using a lattice model, the number of Au atoms in each cell is then 1, and the number of cells on the surface can be determined by
Cells on Surface \( = \frac{s}{2.93^2} \approx 3659 \text{ cells} \)

If 3659 cells are on the surface then 27,232 cells are found in the core. TGA data showed from 200.26°C to 297.20°C that a sample contained 0.7128mg(3.637x10^{-6} \text{ mol of Au atoms}) of Au NPs or 1.177x10^{-10} \text{ mol of Au NPs}. The mass of ligand (0.178mg, 6.69x10^{-7} \text{ mols}) can then be used to determine the ligand:NP ratio of 5681.0 ligands: 1 NP. If we consider the second portion of the TGA curve, from 297.53°C to 500.32°C, the total mass Au (0.626mg, 3.19x10^{-6} \text{ mol of Au atoms, } 1.034x10^{-10} \text{ mols}) and the total mass of ligand (0.265mg, 9.94x10^{-7} \text{ mols}) making the ligand ratio that of 9613.0 ligands: 1NP. Overall the ligand to gold ratio for each size shown below is 1:3 respectively.

Table 5: Potential Nanoparticle Sizes from DLS, TEM, SEM and the composition determined from TGA data

<table>
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<th>Size</th>
<th>Composition</th>
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</tr>
<tr>
<td>6nm</td>
<td>( \text{Au}<em>{7197} \text{Lig}</em>{2267} )</td>
</tr>
<tr>
<td>3nm</td>
<td>( \text{Au}<em>{834} \text{Lig}</em>{260} )</td>
</tr>
<tr>
<td>2nm</td>
<td>( \text{Au}<em>{246} \text{Lig}</em>{77} )</td>
</tr>
<tr>
<td>1nm</td>
<td>( \text{Au}<em>{31} \text{Lig}</em>{10} )</td>
</tr>
</tbody>
</table>

To examine the stability of the nanoparticles, a pH titration of of 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP in 0.025M NaCl and 0.1M NaCl was completed with corresponding zeta potential measurements.
Figure 25: Titration of 11-(dimethylamino)undecane-1-thiol hydrochloride coated Au NP with 0.2M HCL/NaOH in 0.025M NaCl (dark circles) and 0.1M NaCl (open circles)

It was demonstrated that as the pH increases the zeta potential decreases until the amino groups are no longer positively charged and the nanoparticles are no longer stabilized by repulsion and aggregate. A different trend of salt concentration and pKₐ is also demonstrated in titrations of Au NP than in that of the titration of HMI nitroxide. Au NP titration in the presence of 0.025M NaCl has a pKₐ= 8.82, while those in 0.1M NaCl solution have a lower pKₐ=7.33. This affect can be contributed the screening of the nanoparticle by salt ions. In the top right of the graph, it can be seen that with an increase in salt concentration, more positive ligand cations are screened and therefore a reduction in the zeta potential is present. Fewer OH⁻ ions are needed to reach the half protention point in the high concentration of salt as well. Finally, on the left side of the graph we can see that the ligand charge is neutral and the gold core exhibits a negative charge, but slightly less negative in higher salt concentrations due to screening of the surface charges of the Au nanoparticle.

S-10-oxo-10-(2-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-4-ylamino)ethylamino)decyl ethanethioate modified Au NPs (8)-

S-10-oxo-10-((2-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-4-ylamino)ethylamino)decyl ethanethioate (6) was added to solution of Au NPs (7) as illustrated
below and ligand exchange was allowed to occur overnight. The modified Au NPs were purified to remove excess ligand and X-band CW-EPR was then taken, determining ligand attachment was successful.

Scheme 8: Attachment of S-10-oxo-10-(2-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-4-ylamino)ethylamino)decyl ethanethioate ligands to Au NPs through ligand exchange

Figure 26: X-Band EPR Spectrum of Au NP coated with S-10-oxo-10-(2-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-4-ylamino)ethylamino)decyl ethanethioate

12-(Acetylthio)dodecanol-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylate modified Au NPs (9)-

Theoretically, 12-(Acetylthio)dodecanol-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylate (5) modified Au NPs would be synthesized as shown in the scheme below and following previous methods of ligand exchange. However due to time constraints the ligand was stored for future attachment and use in Au NP experiments.
2.3 Site Directed Spin Labeling of Single Stranded Deoxyribonucleic Acid:

Three synthetic single stranded oligonucleotides, and their complementary strands, shown below, were custom-synthesized for site directed spin labeling. Each strand has two phosphorothioate modifications indicated by an asterisk (*) that replaces the phosphate group that links the two nucleosides before the asterisk. The average distance between the labeling sites can be calculated based on the approximate average length of a nucleotide of 0.34nm but this distance is usually an underestimation due to the length and flexibility of the linker tethering the nitrooxide to the oligonucleotide. Thus, the average distances calculations were completed using a NASNOX- a modeling program developed in the Qin lab which takes into consideration all the possible torsion angles and diastereomers of the R5 label which also contributes to the distance distribution.41
Several different labeling schemes were developed using two different nitrooxide molecules, 1-Oxyl-2,2,5,5-tetramethyl-3(methanesulfonyloxymethyl) pyrroline and 3-Iodomethyl-1-oxyl-2,2,5,5-tetramethylpyrroline, illustrated in Scheme 10.\textsuperscript{16}

Scheme 10: A) Structure of 1-Oxyl-2,2,5,5-tetramethyl-3(methanesulfonyloxymethyl) pyrroline B) Structure of 3-Iodomethyl-1-oxyl-2,2,5,5-tetramethylpyrroline

Initially, a modified literature procedure for labeling was attempted using 1-Oxyl-2,2,5,5-tetramethyl-3(methanesulfonyloxymethyl) pyrroline suggesting elimination of the conversion step into an iodo-derivative due to the resonance-stabilized methanesulfonyl group serving as an
equally good leaving group as iodine. NU_C_2_20 in tris buffer was combined with an excess 0.1M solution of 1-Oxyl-2,2,5,5-tetramethyl-3(methanesulfonyloxymethyl) pyrroline in ethyl acetate over a period of 48 hours at room temperature. To separate excess free label and the labeled DNA, HPLC was employed. A reverse phase HPLC method was developed using a C-4 column (Phenomenex Jupiter, 250x 4.60 mm) and triethylammonium acetate buffer solutions. Based on literature data, it was hypothesized that the free nitroxide spin label because of its hydrophilic nature would elute with a low retention time. This was confirmed by running free 1-Oxyl-2,2,5,5-tetramethyl-3(methanesulfonyloxymethyl) pyrroline label through the column. With the nitroxide serving as a reference, labeled single stranded NU_C_2_20 was injected into the column. The chromatogram in Figure 27 shows three HPLC peaks of free label, labeled single stranded NU_C_2_20 and unlabeled NU_C_2_20 with 8.2min, 9.94min, and 10.83min retention time, respectively, and the corresponding fractions were collected separately. However, upon examination, only the first peak, corresponding to free label was EPR active, suggesting that the second peak consisted only of unlabeled DNA and not a combination of labeled and unlabeled. It was concluded that the labeling method would also need further modifications.

![Figure 27: HPLC Chromatogram of purification of labeled NU_C_2_20 using Reverse Phase Chromatography](image)

A second HPLC method was developed using anion exchange column (DNAPac™ PA-100, Analytical, 4 x 250mm). A sample of DT18 was then used to approximate the elution time of
an unlabeled DNA strand, however the elution time was much earlier (Figure 28, 2.2min, 12.2%B) than expected (15-20 minutes, 83%B-100%). Labeled DNA, which has fewer charges, would theoretically appear between the free label and unlabeled DNA peaks, therefore separation with a shallower gradient of 2%B per minute from 25%B to 100%B was attempted before injection of the labeled single stranded NUCS_2_20 sample. With the new gradient conditions, as seen below, both DT18 (Figure 29, 17.85 min, 81.25%B) and free nitrooxide (Figure 30, 0.954min, 11.69%B) elute at reasonable times based on their charge. With a gradient method, twice shallower at 1%B per minute, the elution of unlabeled single stranded NUCS_2_20 can also be observed in Figure 31.

![Figure 28](image1.png)

*Figure 28: HPLC Chromatogram of purification of standard oligonucleotide DT18 using Anion Exchange Chromatography with gradient method 1*

![Figure 29](image2.png)

*Figure 29: HPLC Chromatogram of purification of standard oligonucleotide DT18 using Anion Exchange Chromatography with gradient method 2*
With the potential for successful purification using HPLC the labeling method was then examined. After careful consideration of the labeling method, it was determined that while the pH=8 of the labeling buffer protected the integrity of the nitroxide, it had potential to deprotonate the phosphate groups (pKₐ= 7.2) of the DNA backbone and therefore more than the phosphorothioate labeling sites (pKₐ= 5.4) had potential to be labeled, creating a molecule with significantly reduced negative charge, which would elute with the solvent front. The difference in the pKₐ is attributed to the larger size and polarizability of the sulfur atom in phosphorothioate which allows for a decrease in charge density in comparison to the oxygen molecule in phosphate.⁴⁴ A literature search found that a labeling buffer of 1M MES with pH=5.8 would be more effective.¹⁶ An iodo-derivative of the nitroxide label, illustrated previously, was also made.
by reacting 1-Oxyl-2,2,5,5-tetramethyl-3(methanesulfonyloxymethyl) pyrroline with NaI.\textsuperscript{16} 3-Iodomethyl-1-oxyl-2,2,5,5-tetramethylpyrroline (0.25M) was added to NUCS\_2\_20 and was allowed to react at room temperature for 48 hours. The attachment of this label to DNA should be highly effective due to I\textsuperscript{−} being a weak base, and therefore an excellent leaving group. With these modifications to the labeling procedure, single stranded labeled NUCS\_2\_20 was successfully constructed, and in the chromatogram, Figure 32, with elution of the free nitroxide as expected at the beginning of the spectra 1min, 1.6%B and elution of the single stranded labeled EPR active NUCS\_2\_20 at 15-18min, 25.0-29.8%B. The single stranded NUCS\_2\_20 sample portion is represented by the two overlapping peaks, the first which is labeled NUCS\_2\_20 and the second is unlabeled oligonucleotide. A steeper gradient condition of 2%B per minute within the elution window of NUCS\_2\_20 was developed to resolve the overlapping peaks, but proved unsuccessful. However, this optimization of the method allowed for a shorter run time of 12.98min, 25.96% with the same results. The unlabeled oligonucleotide was considered insignificant because it would not affect any of the future EPR experiments. Circular dichroism was then run on the single stranded labeled NUCS\_2\_20 resulting the in the spectrum, in Figure 34, with features between 225-295nm corresponding to the monomeric nucleotides correlating to literature data.\textsuperscript{12,45,46} The spectrum shows a right handed stacking formation, similar to B-DNA, with a maxima centered at approximately 275nm and a minima at approximately 240nm.\textsuperscript{47} This formation can be contributed to the hydrophobic interactions, and asymmetric sugar preferring a right-handed helicity. The absence of a peak beginning at the edge of our spectrum at 190nm suggests that there is no complexing between adenine and thymine residues which has been shown to indicated double strand formation.\textsuperscript{48}
Figure 32: Anion exchange HPLC chromatogram of labeled single stranded NUCS_2_20

Figure 33: Anion exchange HPLC chromatogram of labeled single stranded NUCS_2_20 with a 2% concentration gradient

Figure 34: Circular dichroism spectrum of single stranded labeled NUCS_2_20
The single stranded labeled NUCS_2_20 portion was then lyophilized and the complementary strand, NUCS_C, was combined and annealed following a standard literature procedure. The double stranded labeled NUCS_2_20 sample was then combined with 20 v/v% glycerol for a DEER spectroscopy sample. While the sample had strong signal when measured on X-Band (9.5Hz), there were only a few spin pairs detected, most likely because of low double labeling efficiency. A new sample of labeled NUCS_2_20 was prepared using an increased temperature of 37°C. The labeled NUCS_2_20 was then annealed to the complimentary strand before purification in attempts to increase the stability of the molecule. As show in Figure 35, double stranded labeled NUCS_2_20 elutes at a slightly later time of 17.8min, 35.6%B. The circular dichroism spectrum of double stranded labeled NUCS_2_20 shows a right-handed B-DNA formation and the coupling of all the bases with a positive band centered at approximately 275nm and a negative band centered at approximately 240nm. A large peak is visible in the 190nm range which suggests the complexing of adenine and thymine nucleotides. The decrease in intensity can be attributed to the bases being positioned further away from each other in a more defined structure, decreasing the electronic transitions. The disappearance of the peak around 210nm also suggests that a coil to helix transition has occurred.

Figure 35: Anion exchange HPLC chromatogram of nitroxide labeled double stranded NUCS_2_20
2.3.1 4-Pulse DEER Spectroscopy of Nitroxide Labeled Oligonucleotides -
Interspin distances in the spin-labeled double stranded DNA samples were determined
using DEER Spectroscopy. Each raw DEER data set was analyzed using two different
approaches, either fitting with Gaussian distribution, or using Tikhonov regularization method.
The results of the analysis are shown below. The average distance distribution between nitroxide
labels of NUCS_2_20 was found to be 5.8nm which correlates closely to the calculated distance
of 6.1nm. The labeling of NUCS_2_30 and NUCS_T_2_20 were also completed using the
optimized labeling and purification methods developed. NUCS_T_20 showed the best labeling
efficiency and an average distance between nitroxide labels of 6.2nm, which also correlates
closely to the calculated distance. Measuring the distance between the nitroxide labels of
NUCS_2_30 proved much more difficult due to the long distance, calculated as approximately
9.5nm between spins and low labeling efficiency (low spin pairs concentration). We speculate
that one of the reasons for low labeling efficiency could be the folding of longer oligonucleotides
upon themselves sterically inhibiting one of the labeling sites. For labeling of NUCS_2_30 the
temperature of labeling was increased to 45°C for a period of 72 hours. However, this did not
increase the labeling efficiency. The NUCS_2_30 sample was then annealed into double
stranded DNA before labeling to increase accessibility of the labeling site; however, this method was unsuccessful as well. It was anticipated that in the presence of positively charged nanoparticles, with a decreased distance between the nitroxides, the concentration of spin pairs would be sufficient for DEER detection.

Figure 37: NUCS_T_2_20 DEER echo modulation trace (left) and the distance distribution obtained from the raw data fitting (solid red line) using Tikhonov regularization method (right).

Figure 38: NUCS_T_2_20 DEER echo modulation trace (left) and the distance distribution obtained from the raw data fitting (solid red line) using Gaussian distribution method (right).
2.3.2 4-Pulse DEER spectroscopy of nitroxide labeled oligonucleotides in the presence of Au NP-

To elucidate the effect of positively-charged ligand-protected AuNPs on the conformational state of the double stranded DNA, the DNA samples containing AuNPs (see above) were prepared for DEER measurements. DEER measurement requires deep freezing of the sample, so the aqueous samples are typically doped with certain amount (~ 25-30 v/v%) of
glass-forming agents, e.g., glycerol. To make sure that glycerol does not cause nanoparticles’ aggregation, the X-band EPR spectra were measured from the DNA-AuNPs sample before and after the addition of glycerol. As shown below in the three spectra for the three DNA strands the high field component decreased illustrating that although the all spectra fall into the fast motion regime, with each addition of glycerol the rotation of the nitroxide label in DNA becomes more restricted because of the increased viscosity of the solution. Besides, the absence of the slow-motion or rigid-limit spectral components also reveals that the addition of glycerol does not cause aggregation of the nanoparticles. The CD spectra of double stranded DNA in the presence of Au NPs, while still exhibiting B-DNA conformation, has decreased intensity at 275nm and increase intensity at 295. From literature data, similarly to that of DNA in the presence of an amine terminated cationic ligand the ellipticity of the DNA is distorted and with a continued increase in concentration of Au NPs the peaks contributed to B-DNA would become negligible.\textsuperscript{50–52}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure41.png}
\caption{Effect of glycerol and Au NPs on the X-Band EPR spectrum of NUCS\textsubscript{2.20}}
\end{figure}
Figure 42: Effect of glycerol and Au NPs on the X-Band EPR spectrum of NUCS_T_2_20

Figure 43: Effect of glycerol and Au NPs on the X-Band EPR spectrum of NUCS_2_30
Table 8: Number Statistics of Dynamic Light Scattering for size measurement of 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP in the presence of 20% glycerol

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<th>Size (nm)</th>
<th>Mean Number %</th>
<th>Std Dev Number %</th>
<th>Size (nm)</th>
<th>Mean Number %</th>
<th>Std Dev Number %</th>
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<th>Mean Number %</th>
<th>Std Dev Number %</th>
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Figure 44: DLS analysis graphs generated by number% statistics in Table 5 of freshly prepared 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP in the presence of 20% glycerol

Figure 45: CD spectrum of labeled single stranded NUCS_2_20 (green), labeled double stranded NUCS_2_20 (red), labeled double stranded NUC_2_20 in the presence of 11-(dimethylamino)undecane-1-thiol hydrochloride coated water soluble Au NPs (blue)
An average interspin distance in the spin-labeled NUCS_2_30 in the presence of Au NP and 20 v/v% glycerol was measured to be 5.9nm, which is significantly smaller than the estimated distance of 9.5nm between the corresponding nucleotides (Table 7). Note that the interspin distance without nanoparticles was not even determined, most likely because it exceeded 9nm. We suppose that this detectable decrease in the distance in NUCS_2_30 indicates that the DNA was successfully bent around the positively-charged nanoparticle. The average distance Gaussian distribution is quite wide and using the Tikhonov regularization method several maxima are visible, suggesting that NUCS_2_30 is interacting with nanoparticles of several different sizes. When gold nanoparticles were added to the spin-labeled double stranded NUCS_2_20 only a slight decrease in the distance was observed from 5.8 to 5.6 nm, shown below in Figure 47 and Figure 48. A similar, only insignificant decrease in the interspin distance from 6.2nm to 6.0nm was observed for NUCS_T_2_20 in the presence of gold nanoparticles, shown in Figure 46. These two latter measurements most likely indicate that the lengths of the DNA fragments between the spin-labeled sites were too short to let DNA bend around the nanoparticle (or the nanoparticle was too large for such short DNA). However, the narrowing of the distance distribution suggests some ordered interaction of nanoparticles and DNA. The effect of solution ionic strength on the Au NPs-DNA interaction was also tested. From the molecular dynamics simulations, it was expected that at low ionic strengths and highly charged nanoparticles a severe kinked conformation of DNA, with deformations of the helix in few locations, would be observed. With the screening of charges in the presence of salt, DNA bending would be smooth, with subtle changing of the roll and tilt parameters. Based on the MD simulations, these two bending models were speculated to show a detectable difference in the distances between the terminal sites of the DNA. Thus, we expected that the DEER
technique would be sensitive enough to be able to distinguish between the smooth and kinked bending scenarios. For this reason, we chose truncated NUCS_T_2_20 for this experiment. However, the DEER measurements of Au NPs-NUCS_T_2_20 in the presence of 0.1 M NaCl did not show any change in the average interspin distance and the distance distribution widened. One of the reasons for this could be a flash freezing of the solution during the DEER sample preparation. This could cause a salt crystallization as a result of the solution vitrification. Another reason is that the electrostatic interaction between Au NPs and DNA could be so strong that it completely overwhelms the stabilizing effect of salt on the DNA double helix.\textsuperscript{53} And, finally, but not lastly, with this nanoparticle size, there was a chance that these two bending models did not cause any significant changes in the DNAs’ inter-terminus distance.

![Figure 46: NUCS_T_2_20 DEER echo modulation trace (left) and the distance distribution obtained from the raw data fitting of the DEER echo modulation using Tikhonov regularization method (right) compared to the sample without nanoparticles (red).](image)

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Figure 47: NUCS_2_20 DEER echo modulation trace (left) and the distance distribution obtained from the raw data fitting of the DEER echo modulation using Tikhonov regularization method (right) in comparison to the sample without nanoparticles (red).

Figure 48: NUCS_2_20 DEER echo modulation trace (left) and the distance distribution obtained from the raw data fitting (red line) of the DEER echo modulation using Tikhonov regularization method (left).
Figure 49: NUCS_T_2_20 DEER echo modulation trace (left) and the distance distribution obtained from the raw data fitting of the DEER echo modulation using Tikhonov regularization method (right) in the presence of 0.1M NaCl (black) compared to that without salt (red).

2.4 Conclusions:
1. The effect of a solution ionic strength on the pK\textsubscript{a} values of pH-sensitive nitroxide broadly employed in the biophysical EPR studies was elucidated. Within the 0.1 M to 2.0 M ionic strength range, the pK\textsubscript{a} of the nitroxide was found to change significantly from pK\textsubscript{a} ≈ 4.58 to pK\textsubscript{a} ≈ 5.34. The nitrogen hyperfine coupling constants, A\textsubscript{iso}, of the non-protonated and protonated forms of the nitroxide were found to be essentially the same no matter what was the ionic strength of a solution. Thus, it was concluded that the changes in pK\textsubscript{a} of the nitroxide were not the result of the change in the dielectric properties of a solution, but the result of the enhanced stabilization of the protonated form of the nitroxide. The ionic strength-correction was shown to be absolutely necessary for accurate determination of the pH in solutions using the pH electrode.

2. A series of ligands was synthesized for gold nanoparticle surface decoration. A cationic ligand (1) suitable for forming stable, small and water soluble Au NP (7) for investigating the dynamics and conformation of DNA was synthesized. Ligands (5) and (6) were synthesized through DCC mediated coupling and coated onto Au NP through ligand exchange reactions. Ligand (6) is a nitroxide modified thioacetate serving as excellent pH sensitive EPR probes. When attached to Au NP (8) it would be useful in continued studies into the electrostatic interactions within the ligand shell layer. Ligand (5), which is also a nitroxide modified thioacetate, is not intrinsically sensitive to the pH of solution but would be valuable in studying the dynamics of self-assembled monolayers. This ligand was not attached to Au NP, although attachment is theoretically illustrated forming (9), but due to time limitations was frozen for future studies.
3. Three oligonucleotide strands were site-directed spin labeled with 3-Iodomethyl-1-oxyl-2,2,5,5-tetramethylpyrroline, and annealed with the corresponding complimentary strand. To separate spin-labeled DNA from the unlabeled DNA and from excess label, a reproducible purification method was developed using anion exchange HPLC was developed. The successful labeling was confirmed by DEER spectroscopy measurements, which gave average distances of the linear double stranded DNA correlating to those calculated based on the distance of each nucleotide.

4. Spin labeled double stranded DNA was combined with 11-(dimethylamino)undecane-1-thiol hydrochloride coated water soluble Au NP and average interspin distances for each DNA strand were determined using DEER spectroscopy. The distance between spin labels in NUCS_2_30 was found to significantly decrease in the presence of Au NP, suggesting the bent conformation of DNA. For NUCS_2_20 and NUCS_T_2_20, only minor changes in the interspin distances were observed, suggesting that the lengths of the DNA fragments between the spin-labeled sites were too short to let DNA bend around that nanoparticle.

CHAPTER 3: EXPERIMENTAL
3.1 Materials and Instruments:

- Sodium Chloride, Hexanes, Sodium Hydroxide, Sodium Bicarbonate, DMSO,
  Dichloromethane, Potassium Thioacetate (98%), Sodium Borohydride, Sodium Iodide,
  Tris Base, Sodium Perchlorate, Acetonitrile, Tetrahydrofuran, Silica Gel, and
  Tetrachlorauric(III) Acid Trihydrate were purchased from Fisher Scientific.
- Chloroform, Methanol, Acetic Acid, Magnesium Sulfate (Anhydrous), Ethyl Acetate, Sodium Sulfate (Anhydrous) were purchased from EMD.
- Dimethylamine and Manganese Oxide were purchased from Alfa Aesar.
- Deuterated Methanol, Deuterated Chloroform, 12-bromo-1-dodecanol (98%), 10-Bromodecanoic acid (95%), DCC (99%), 4-(Dimethylamino)pyridine (99%), 11-bromo-1-undecene (95%), Thioacetic Acid (96%), and Azobisisobutyronitrile were purchased from Sigma-Aldrich.
- Deuterated Glycerol was purchased from CDN Isotopes.
- Deuterium Oxide was purchased from Cambridge Isotopes Laboratories.
- Standard pH Solutions and DMF were purchased from VWR.
- Phosphorothioate Modified Oligonucleotides were purchased from Biosynthesis.
- pH-sensitive nitroxide radical HMI was synthesized at the Institute of Organic Chemistry, Siberian Branch of the Russian Academy.
- pH and electrochemical potential measurements were completed with a Mettler Toledo InLab® Micro Combination pH Electrode model 51343160 using Symphony SB70P pH meter (VWR International).
- Distilled deionized water was obtained from Milli-Q, Millipore Synergy® UV Water Purification System, Merck Millipore, Billerica, MA.
- All conventional titration experiments were conducted in a double-walled glass vessel thermostated at 21.0 ± 0.5 °C using a circulating bath Model 9710 with a digital temperature controller (PolyScience, Niles, IL).
- All X-band (9.5 GHz) CW EPR spectra were recorded with a Varian (Palo Alto, CA) Century Series E-109 spectrometer interfaced to a PC. For pH titration experiments, the
temperature in the EPR cavity was maintained with stability better than ±0.02 °C and a
gradient below 0.07 °C/cm over the sample region by a second digital temperature
controller. Polytetrafluoroethylene (PTFE) capillaries (0.81 × 1.12 mm, NewAge
Industries, Inc., Southampton, PA), were used to hold aqueous solutions and the capillary
was folded and inserted into 3 × 4 mm clear fused quartz EPR tube open from both ends
(VWR International). Typical spectrometer settings were as follows: modulation
amplitude, 0.625 G; time constant, 64 ms; incident microwave power, 2 mW; sweep time,
30 s; scan width, 100 G. Typically, from 5 to 50 individual scans were acquired, digitized
to 2048 data points, and averaged out.\textsuperscript{28} For experiments with nitroxide labeled
oligonucleotides and gold nanoparticles, the EPR cavity was maintained at room
temperature. Samples were deposited into 1.6mm O.D. sealed quartz tube, which was
then inserted into a 3 x 4 mm clear fused quartz EPR tube that is open at one end and
plugged with cotton in the other. Spectrometer settings remained consistent except for an
increase of modulation amplitude to 1.0G and an increase to acquiring 200 individual
scans.

- All DEER Spectroscopy measurements were completed on a custom-built Bruker
  ELEXSYS E580 spectrometer equipped with SuperQ-FT bridge and SuperQ-FT Solid
  State 10 W Amplifier (all from Bruker Biospin, Billerica, MA, USA) operating at Q-band
  frequencies of 33.8GHz. The temperature of the EPR cavity remained constant at 76K
  using a Bruker ER 4118CF flow cryostat using liquid nitrogen as coolant.

- Circular Dichroism Spectra were obtained using a Jasco J8115 CD Spectrometer with a
  nitrogen flow of 9.4 L/min and temperature control by a Peltier unit set to 21°C. The CD
spectra of NUCS_2_20 in 500μL of sterile water in a 1.00mm glass cell was recorded from 195nm-230nm with a total of 50 scans.

- DLS measurements were carried out suing a Malvern Zetasizer (Malvern Instruments Inc., Westborough, MA).
- HPLC purification was performed on a Shimadzu LC10AS Liquid Chromatography system coupled with a SPP-10AV UV-vis detector using an anion exchange column (DNAPac™ PA-100, Analytical, 4 x 250mm).

3.2 Nitroxide pH EPR probe 2,2,3,4,5,5-hexamethylimidazolidin-1-oxyl Ionic Strength Studies:

CO₂- Free water was prepared by boiling Milli-Q ultra-pure water for 30 minutes and then flushing water with N₂ for 30 minutes. Solutions of 0.05 M, 0.1 M, 0.3 M, 0.5 M, 1.0 M, 2.0 M, and 5.0 M NaCl and standardized 1M HCl and 2M NaOH solutions were prepared with CO₂ free water. The various ionic strength salt solutions were pH-titrated in a thermostatically controlled vessel, 21±0.5 °C, and the electric potentials (mV) were measured. The acidic and basic regions were titrated separately, plotted and fit with the Nerset equation to serve as calibration curves. Then NaCl solutions of various concentrations, containing 0.2 mM of HMI, were prepared and titrated in the same way as the ionic strength calibration solutions. Both the electric potential and pH were measured for each titration point, and a corresponding EPR spectrum was obtained. The cavity of the EPR spectrometer was also temperature-equilibrated at 21±0.5 °C. The changes in the hyperfine splitting were graphed as a function of the pH and fit using the least squares fit of the Henderson-Hasselbach equation as further discussed in the results section.
3.3 Ligand Synthesis:

3.3.1 Synthesis of 11-(dimethylamino)undecane-1-thiol hydrochloride (1):

In a round bottom flask AIBN (713.0mg, 3.06mmol), thioacetic acid (3mL, 41.52mmol), 11-bromo-1-undecene (2.12mL, 9.67mmol) were combined in 100mL of dry toluene. The reaction was flushed with argon gas for 20 minutes and then refluxed for 4 hours with vigorous stirring. After cooling, the organic layer was then washed three times with 500mL of a saturated NaHCO₃ solution and once with brine solution. The organic layer was then dried over MgSO₄ overnight. The drying agent was filtered off and the solvent was removed under reduced pressure, resulting in a reddish-orange oil. This reaction was later scaled up to make more thioacetic acid-S-(11-bromoundecyl) ester with AIBN (3.55g, 21.63mmol), thioacetic acid (15.5mL, 241.5mmol), 11-bromo-1-undecene (9.41mL, 42.9mmol). The combined products were then purified by chromatography on silica gel using a hexane/CH₂Cl₂ mixture as an eluent of a gradient from 4:1-2:1 v/v. The combination of the two reactions resulted in (12.22g, 75.29%). Due to a small impurity still present a second purification by chromatography on aluminum oxide with a hexane/CH₂Cl₂ eluent mixture (2:1) was required. This yielded (11g, 40.6mmol, 67.8%) of yellow product. IR (KBR, cm⁻¹) 2927, 2852 (CH Stretching), 1464
(CH₂ Bending), 1638 ((C=O)-S). \(^1\)H NMR (400 MHz, CDCl₃, δ): 3.40 (t, 2H, CH₂Br), 2.86 (t, 2H, CH₂S), 2.31 (s, 3H, CH₃CO), 1.85 (m, 2H, BrCH₂CH₂ and SCH₂CH₂ and possible contribution of minor product), 1.56 (m, 2H, SCH₂CH₂CH₂), 1.27(m, 12H)

![Figure 51: Synthesis Scheme of thioacetic acid-S-(11-bromoundcyl) ester](image1)

![Figure 52: IR Spectrum of thioacetic acid-S-(11-bromoundcyl) ester](image2)

![Figure 53: \(^1\)H NMR of thioacetic acid-S-(11-bromoundcyl) ester](image3)
The thioacetic acid-S-(11-bromoundecyl) ester in 40mL of THF was added dropwise to dimethylamine (530mmol) in 247.6mL of THF over a period of 6 hours at a temperature of 0°C as illustrated below. After complete addition, the reaction was stirred for 12 hours at room temperature. The reaction mixture was filtered to remove dimethylamine bromohydrate salt precipitates and the solvent was removed under reduced pressure. To remove impurities from the product, chromatography was run on silica gel using a CH₂Cl₂:MeOH:AcOH mixture (89:10:1 v/v) as an eluent. The solvent from the product fraction was removed under reduced pressure. NMR measurements revealed that some of the impurities had been removed but not all.

Ethyl acetate was combined with the product fraction and the solution was washed with NaHCO₃ to remove residual CH₃COOH. The organic layer was separated and dried over MgSO₄ overnight. A second chromatography was run on silica gel with a MeOH:CH₂Cl₃ mixture in a gradient fashion from 1:10-2:10 v/v. as an eluent. The solvent from the product fractions was removed under pressure.

![Figure 54: Synthesis Scheme of 11-(dimethylamino)undecane-1-thiol](image)

The pure product was then converted to 11-(dimethylamino)undecane-1-thiol hydrochloride following the scheme below. The pure product was taken up in a solution of gaseous HCL in THF (18.2 wt.%) and allowed to react for one hour. The solvent was removed under reduced pressure and then treated again with HCL/THF solution. The solvent was removed under reduced pressure and a small portion of diethyl ether was added to induce crystallization. The white crystals were recrystallized and then the product was dried overnight in the vacuum desiccator.
This reaction afforded 11-(dimethylamino)undecane-1-thiol hydrochloride as a white powder (63.64 mg, 1.07%). $^1$H NMR (400 MHz, CD$_3$OD, δ): 3.31 (s, 2H, HS+NH), 3.11 (m, 2H, CH$_2$NH(Me)$_2$), 2.87 (s, 6H, NH(Me)$_2$), 2.67 (t, 2H, HSCH$_2$), 1.67 (m, 2H, CH$_2$CH$_2$NH), 1.57 (q, 2H, HSCH$_2$CH$_2$), 1.38-1.33 (m, 14H).

Figure 55: Synthesis scheme of 11-(dimethylamino)undecane-1-thiol hydrochloride

3.3.2 Synthesis of 10-(Acetylthio)decanoic acid (2)-

10-bromodecanoic acid (978.15 mg, 3.88 mmol) was added to 16 mL of dry DMF and cooled to 0°C in ice. Potassium thioacetate (634.0 mg, 5.58 mmol) was then added and the reaction was stirred overnight at room temperature. The reaction mixture was then diluted in 16 mL of DMSO and 32 mL of ethyl acetate and then washed with brine (3x25 mL) to remove DMF. The organic layer was then dried over Na$_2$SO$_4$ overnight. The drying agent was then filtered off and the solvent was reduced under pressure affording orange brown crystals. The crystals were dissolved in 3 mL of hexanes at 90°C and gravity filtered while hot to remove
inorganic impurities. The solution was put in the fridge at 4°C to recrystallize and then filtered through sintered glass and dried. This reaction resulted in pure 10-(Acetylthio)decanoic acid (211.091mg, 22.1%). This reaction was later repeated and resulted in an increased yield of 10-(Acetylthio)decanoic acid (577.8mg, 59.3%). $^1$H NMR (400 MHz, CDCl$_3$, δ): 2.87 (t, 2H, CH$_2$S), 2.33 (m, 5H, CH$_3$+CH2COOH), 1.59, (m, 4H, CH$_2$CH$_2$COOH+CH$_2$CH$_2$S), 1.30 (m, 10H, CH$_2$)

![Figure 57: Synthesis Scheme of 10-(Acetylthio)decanoic acid](image)

3.3.3 Synthesis of 4-(2-aminoethylamino)-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazole (3)-

2,2,5,5-Tetramethyl-2,5-dihydro-1H-imidazol-1-ol 3-oxide (7.49g, 47mmol) was combined with MnO$_2$ (9.97g, 247mmols) in 170mL of CHCl$_3$. The solution was stirred for three hours and then gravity filtered to remove the MnO$_2$. The solvent was then removed under reduced pressure. Chromatography was run on silica gel column with MeOH:CHCl$_3$ eluent (1:100). The solvent from the product fractions was evaporated under reduced pressure and pure
aldonitrone 2,2,5,5-tetramethyl-3-imidazoline-1-oxyl (4.49g, 28mmol) resulted. Aldonitrone 2,2,5,5-tetramethyl-3-imidazoline-1-oxyl was then added to 2-bromoethyl isocyanate (5.04g, 33.6mmol, 3.036mL) in 50mL of dry chloroform. TLC was run on the product and some starting material remained so an extra 300μL of 2-bromoethyl isocyanate to push the reaction towards the products. When 1-(2(Bromoethyl)-6-oxyl-5,5,7,7-tetramethyltetrahydroimidazo[1,5-b][1,2,4] oxadiazol-2-one was obtained it was purified by chromatography on silica gel with ethyl acetate:hexane mixture (1:1) eluent. Some impurities remained so chromatography was run a second time on silica gel of a smaller particle size. The solvent was removed from the product fractions under reduced pressure. The 1-(2(Bromoethyl)-6-oxyl-5,5,7,7-tetramethyltetrahydroimidazo[1,5-b][1,2,4] oxadiazol-2-one (2.868g, 9.4mmol) was combined with NaN₃ (1.22g, 18.8mmol) in 5.8mL of water and 60mL of dry DMF. The mixture was then heated in an oil bath at 100°C. The reaction was monitored with chromatography TLC plates on silica gel with CHCl₃:MeOH (15:1) and after six hours still showed some reactants. The solution was then allowed to react at room temperature overnight. The solution was then diluted in 20mL of H₂O and washed with chloroform (5x10mL). The organic layer was then washed with brine solution (4x10mL) and the organic layer was dried over MgSO₄ for three hours. The product solution was concentrated under reduced pressure and tested with chromatography as described above for impurities. Chromatography on silica gel was run with ethyl acetate eluent to remove the impurities found. The solvent was then removed under reduced pressure and 4-[(2-Azidoethyl)amino]-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazole resulted as an oily residue (1.103g, 4.96mmol, 53.0%). PBu₃ (2.12mL, 7.72mmol) was added to the 4-[(2-Azidoethyl)amino]-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazole in 10mL of dry THF. The reaction mixture stirred under argon for 1.5 hours and then 20mL of EtOH/H₂O mixture
(1:1) was added. The reaction was then stirred overnight to remove one nitrogen. The solvent was then removed under reduced pressure. The remaining aqueous layer was then acidified by the addition of 3% HCL to a pH of 4. The solution was then washed with CHCl₃ (5x10mL) and the aqueous layer was neutralized to pH of 7 with NaHCO₃. The solution was then washed a second time with CHCl₃ (5x10mL). The aqueous layer was then basified with NaOH to pH of 12 and washed a third time with CHCl₃ (7x10mL). After this step the organic layer contains the product and was dried with NaSO₄. The drying agent was then filtered off and the solvent was removed under reduced pressure. The remaining product had negligible yield so the process was restarted and pure yellow powder 4-[(2-Azidoethyl)amino]-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazole (2.9249g, 13.15mmol, 70.4%) resulted. IR (KBR, cm⁻¹) 3341, 1563 (N-H), 2102 (N₃), 1630 (C=N). A small portion was converted to 4-(2-Aminoethylamino)-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazole for use at a future date to make S-10-oxo-10-(2(1-oxyl, 2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-4-ylamion)ethy lamion)decyI ethanethioate in combination with 10-(Acetylthio)decanoic acid. IR (KBR, cm⁻¹) 3295 (NH₂), 1617 (C=N), 1563 (NH).

Figure 59: Synthesis Scheme of 4-(2-Aminoethylamino)-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazole
3.3.4 Synthesis of 12-(Acetylthio)dodecanol (4)-
12-bromo-1-dodecanol (135.6mg, 0.51mmol) was added to a flask with 8mL of dry DMF and cooled to 0°C. Potassium thioacetate (84.7mg, 0.74mmol) was added to the solution and the solution was stirred overnight at room temperature. The solution was then diluted with 8mL of DMSO and 16mL of ethyl acetate and washed with brine solution (3x25mL). The organic layer was separated and dried overnight with Na₂SO₄. The drying agent was then removed and solvent removed under reduced pressure. Some solvent still remained so the washing procedure was
repeated again. After a solid remained it was recrystallized in hexanes affording a few white crystals (34.48mg, 29.0%). The reaction was repeated with approximately 6 times the amount of reactants resulting in pure 12-(Acetylthio)dodecanol (486.0mg, 60.7%).

![Figure 62: Synthesis Scheme of 12-(Acetylthio)dodecanol](image)

3.3.5 Synthesis of 12-(acetylthio)dodecyl 1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylate (5) -

12-(Acetylthio)dodecanol (4) (51.0mg, 1.962x10⁻⁴ mols) is added to DMAP (1.4mg, 1.14X10⁻⁵ mols) and DCC (55mg, 2.667x10⁻⁴ mols) in 2mL of dry chloroform. The 3-Carboxy-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl (48.7mg, 2.63X10⁻⁴ mols) was added to 2mL of dry chloroform and then dropwise added to the mixture of Ligand 4, DMAP, and DCC. The mixture was stirred at room temperature overnight. The solution was then concentrated under reduced pressure and impurities were removed using PTLC on silica gel with a CHCl₃ eluent. The pure product was dried in the vacuum desiccator and 478.29mg of 12-(acetylthio)dodecyl 1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylate resulted. This reaction was performed again producing 74mg of product. IR (KBR, cm⁻¹) 1716 (C=O of conjugated ester group), 1688 (C=O of thioacetyl group).

![Figure 63: Synthesis Scheme of 12-(acetylthio)dodecyl 1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylate](image)
3.4 Gold Nanoparticle Synthesis:

3.4.1 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP (7) - 11-(dimethylamino)undecane-1-thiol hydrochloride (1) (79.5mg, 0.4935mmol) was added to tetrachloroaurate (44.8mg, 1.16mmol) in 5mL of THF and 800μL of glacial acetic acid. The milky white solution stirred overnight at room temperature. The solution was then cooled to 0°C for 30 minutes and then sodium borohydride (73.6mg, 1.95mmol) in 1.5mL of ice cold ultra-pure water. The solution changed to dark purple in color immediately and was then allowed to stir for 24 hours at 0°C. The solvent was then evaporated under reduced pressure; the residue was rinsed from the round bottom with 6mL of ultra-pure water and then filtered through sintered glass. The Au NP solution is then dialyzed against ultra-pure water (4x1L) for 6 hours. DLS was used to determine the average size of the nanoparticles. The temperature during Au NP growth, ligand to gold ratio, the reducing agent, amount of reducing agent, and speed of addition of the reducing agent to solution are key parameters that contribute to size and both were varied to synthesize the smallest possible size of nanoparticles summarized in the table below. The 4nm size of 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP (12.02mg, 16.09% over 3 reactions) were used with Nitroxide labeled DNA and underwent ligand exchange to make Au NP with Nitroxide ligands.
Table 9: Summarization of 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP synthetizes and conditions

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<th>Trial</th>
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<td>Slow</td>
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</tr>
<tr>
<td>2</td>
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<td>3:1</td>
<td>1.98mmol NaBH₄</td>
<td>Slow</td>
<td>100nm</td>
</tr>
<tr>
<td>3</td>
<td>0°C</td>
<td>3:1</td>
<td>1.02mmol NaBH₄</td>
<td>Fast</td>
<td>4-10nm</td>
</tr>
<tr>
<td>4</td>
<td>0°C</td>
<td>2:1</td>
<td>0.175mmol NaBH₄</td>
<td>Fast</td>
<td>10-100nm</td>
</tr>
<tr>
<td>5</td>
<td>0°C</td>
<td>2:1</td>
<td>0.125mmol NaBH₃CN</td>
<td>Fast</td>
<td>10-100nm</td>
</tr>
<tr>
<td>6</td>
<td>0°C</td>
<td>3:1</td>
<td>0.125mmol NaBH₄</td>
<td>Fast</td>
<td>4-10nm</td>
</tr>
</tbody>
</table>

Figure 65: Synthesis Scheme of 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP

3.4.2 S-10-oxo-10-(2(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-4-ylamino)ethylamino)decyl ethanethioate modified Au NP (8)

Trial 1 Au NP were used for a ligand exchange reaction shown below. S-10-oxo-10-(2(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-4-ylamino)ethylamino)decyl ethanethioate (6) (0.85mg, 2x10⁻⁶ mol) was combined in 50μL of MeOH with 11-(dimethylamino)undecane-1-thiol hydrochloride-coated water soluble Au NP (7) (2.2mg, 5x10⁻⁸mol). The solution was flushed with argon gas and ligand exchange occurred overnight at 50°C. The solvent was then evaporated using nitrogen gas and dissolved in 500μL of ultra-pure water. The aqueous solution was then extracted with dichloromethane (5x2mL) and then the product layer was dialyzed for 24 hours (3x1L). X-band EPR was used to determine that the nanoparticles had nitrooxide ligands attached.
3.5 Site Directed Spin Labeling of Oligonucleotides:

3.5.1 Single-strand DNA labeling in a two-phase system:

Site directed spin labeling of single-strand DNA was attempted through adaptations of methods developed by the Qin lab. An iodo-derivative of the nitroxide label was made, as shown in the scheme below, by reacting 1-Oxyl-2,2,5,5-tetramethyl-3(methanesfonyloxymethyl) pyrroline (1.75mg, 9.5x10^-6 mols) with NaI (0.77mg, 5.1x10^-6 mols) in 500μL of dry acetone for 60min at 37°C. Na₂OSO₂CH₃ formed as a cloudy white substance and when centrifuged a pellet was formed at the bottom of the tube. The supernatant was pulled off and the pellet was washed with dry acetone (3x1mL) and combined. The acetone was then evaporated by nitrogen gas and 3-Iodomethyl-1-oxyl-2,2,5,5-tetramethylpyrroline (0.0157g, 5.71x10^-5 mols). A 1.14M solution of the nitroxide was made and then a dilution was made making a 0.25M solution.

3.5.2 Synthesis of double stranded labeled DNA:

A standard synthesis procedure was developed combining 3-Iodomethyl-1-oxyl-2,2,5,5-tetramethylpyrroline, (0.25M in 20μL of acetonitrile) with the DNA strand of choice (5.667nmol), 10μL of 1.0M MES at a pH=5.8 and 60.3μL of sterile water. The solution was spun overnight at 37°C for 48 hours. The complimentary strand (5.667nmol, 22μL) was then added and the tube was heated to 95°C in a water bath for 3 minutes, cooled to room temperature.

Figure 66: Attachment of S-10-oxo-10-(2(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-4-ylamino)ethylamino)decyl ethanethioate EPR Sensitive Ligand to Gold Nanoparticles
for 20 minutes, and repeated. 200μL of a solution containing 5mM NaCl and 2.5mM Tris Base at a pH of 7.5 was added and the solution was spun for 6 hours.

3.5.3 Purification of labeled double stranded DNA strands by Anion Exchange HPLC-
An anion exchange HPLC method using the linear gradient shown below was used with UV detection at 260nm for purification of labeled double stranded DNA strands. Two HPLC peaks with retention time 1.0min and 10-20 min, respectively, are observed on a chromatogram corresponding to free label and labeled DNA fractions that are collected separately. Free nitroxide label elutes within 1.5min, 3.0%B and double stranded labeled NUCS_2_20 eluted at 17.8min,35.6%B as a single peak as shown in Figure 67.

HPLC Method 6-

Anion Exchange HPLC Buffer A: 1mM NaClO₄, 20mM tris-HCL, 20% vol/vol ACN, pH=6.8
Anion Exchange HPLC Buffer B: 400mM NaClO₄, 20mM tris-HCL, 20% vol/vol ACN, pH=6.8
Flow Rate: 2.0mL/min
Pressure: 4000psi

<table>
<thead>
<tr>
<th>Time</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.050</td>
</tr>
<tr>
<td>10.00</td>
<td>20.00</td>
</tr>
<tr>
<td>25.00</td>
<td>50.00</td>
</tr>
<tr>
<td>30.00</td>
<td>0.05</td>
</tr>
<tr>
<td>31.00</td>
<td>0.05</td>
</tr>
</tbody>
</table>
The fractions collected containing labeled double stranded DNA are then desalted using a sephadex column, and lyophilized overnight. Circular Dichroism (CD) was then used to further characterize the double stranded labeled fractions of NUCS_2_20.

3.5.4 Preparation of DEER Spectroscopy Samples -
The solid double stranded labeled DNA sample was then diluted in 50μL of D₂O. 1nmol of DNA was then taken and brought to a total volume of 20μL with D₂O and combined with 20% glycerol was then immediately frozen in liquid nitrogen and DEER spectroscopy was run.

To 1nmol of DNA in the presence of 20% glycerol, 2nmol of Au NP were added. X-Band CW-EPR spectra with each of the additions (glycerol, NP) to DNA were taken. DEER measurements
were completed using a dead time free version of the DEER pulse sequence. The length of the pump pulse was 26ns. For all DEER experiments, length of the observer \(\pi/2\) and \(\pi\) -pulses was 12 and 20ns respectively. The delay between the first pulse and the second observer pulses was 240ns. Initial separation between the second observer and the pump pulse was 140ns. Position of the third observer pulse was adjusted according the total length of the measured DEER trace such that time separation between the pump pulse at its extreme position and the third observer pulse was 140ns. To obtain a DEER trace, echo intensity was integrated with 4ns time constant over 54ns gate and plotted versus the position of the pump pulse which was incremented with 12ns step. All spectra were analyzed using DeerAnalysis software package.
REFERENCES


(21) Likhtenshtein, G. In Electron Spin Interactions in Chemistry and Biology; Biological and Medical Physics, Biomedical Engineering; Springer International Publishing, 2016; pp 93–122.


(27) Fajer, P. G.; Brown, L.; Song, L. In ESR Spectroscopy in Membrane Biophysics; Biological Magnetic Resonance; Springer US, 2007; pp 95–128.


(41) Frey, P. A.; Sammons, R. D. Science 1985, 228 (4699), 541–545.


(49) Circular Dichroism Spectroscopy of Nucleic Acids


