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Development of An Spr Method To Monitor the Chemical Interaction of Dna With A Small Reactive Molecule: Peroxynitrite As An Example

Divya Sharma
Cleveland State University

DEVELOPMENT OF AN SPR METHOD TO MONITOR THE CHEMICAL INTERACTION OF DNA WITH A SMALL REACTIVE MOLECULE: PEROXYNITRITE AS AN EXAMPLE

DIVYA SHARMA

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We hereby approve this thesis for

DIVYA SHARMA

Candidate for the Master of Science in Chemistry degree for the

Department of Chemistry

and the CLEVELAND STATE UNIVERSITY

College of Graduate Studies

Thesis Chairperson, Dr. Mekki Bayachou

Department & Date

Thesis Committee Member, Dr. Valentin Gogonea

Department & Date

Thesis Committee Member, Dr. Christopher W. Boyd

Department & Date

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DEVELOPMENT OF AN SPR METHOD TO MONITOR THE CHEMICAL INTERACTION OF DNA WITH A SMALL REACTIVE MOLECULE: PEROXYNITRITE AS AN EXAMPLE

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ABSTRACT

Peroxynitrite is an anion with the formula $ONOO^-$. It is an unstable structural isomer of nitrate, NO_3^-. Although its conjugate acid (ONOOH) is highly reactive, it is quite relatively stable in basic solutions. Generation of peroxynitrite in-vivo occurs through the diffusion-controlled reaction between superoxide anion and nitric oxide free radical. It is a strong oxidizing and nitrating agent, and its formation has been correlated with many pathological conditions. Because of its oxidizing properties, peroxynitrite can damage a wide array of molecules in cells, including DNA and proteins. Reactions of peroxynitrite with DNA, proteins, and lipids, trigger a wide array of cellular responses ranging from subtle modifications of cell signaling to oxidative injury committing cells to necrosis and apoptosis. Peroxynitrite-induced DNA modifications include formation of 8-nitroguanine and 8-oxoguanine as well as DNA single strand breakage and base modifications when exposed to different concentrations of peroxynitrite. However, the accurate measurement of peroxynitrite concentration has been a challenge since this analyte reacts with many cellular targets. Development of analytical techniques capable of rapid and sensitive detection of this fast reacting and damaging agent is an important research goal not only to monitor its dynamic concentration but also to correlate its amount with observed chemical damage that this oxidant generates.

Surface Plasmon Resonance (SPR) is an analytical platform that can detect interactions of immobilized ligand with running analytes. The change detected is related to a change in the refractive index as a result of analyte binding to immobilized ligands. Peroxynitrite reaction with DNA is expected to result in significant structural changes in the tethered DNA double helix of attached probes. We hypothesize that although peroxynitrite is a small molecule and is not expected to bind or cause significant changes in refractive index on SPR chips, the expected effect of its reactivity with DNA bases will result in collective structural configuration of immobilized DNA probes with can be monitored using SPR chips. To address this hypothesis, we propose the following 4 specific aims:

Specific Aim1: Optimization of methods of immobilization of oligonucleotides on nanogold SPR interfaces**.**

Specific Aim2: Characterization of the DNA-functionalized SPR gold chip**.**

Specific Aim3: Study of the interaction of PON with immobilized DNA structures on the SPR chips**.**

Specific Aim 4: Compare and contrast SPR results with electrochemical methods using the same DNA sequences immobilized on electrode surfaces.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

Scheme Page

CHAPTER I

GENERAL BACKGROUND

1.1 Introduction

DNA determines genetic characteristics in living organisms. Analysis of genetic material has gained prominence in the diagnosis of a variety of diseases. Most of the techniques rely on the unique structure of DNA which is made up of two complementary antiparallel strands held together by highly specific interactions.[1] Single stranded DNA (ss DNA) anneals with complementary specific sequences through spontaneous hybridization. The DNA sequence can be affected by physical factors like high temperature, effects of radiation as well as by chemical factors like reaction highly reactive chemicals such as reactive oxygen species (ROS) or mutagenic chemicals. Peroxynitrite (PON, $ONOO^-$) is one such chemical that has been shown to induce single-strand breaks and base chemical damage in DNA.

Peroxynitrite is a powerful oxidizing and nitrating agent. It is formed in biological systems when nitric oxide and superoxide are produced in proximity at physiological sites. The two species readily react at diffusion-controlled rates to produce the highly reactive PON.[2] $ONOO^-$ causes strand breaks in plasmid and eukaryotic cell DNA.[3] It also causes nitration and nitrosation leading to the formation of products like 8-nitroguanine, 4-

hydroxy-5-nitrosooxy-guanine and various oxidation products like oxazolone, 8-hydroxyadeine and 8-hydroxyguanine in isolated DNA or nucleosides.[4] DNA damages cause changes in the structure of the genetic material and prevent the replication mechanism from functioning and performing properly.

Optical methods are among the oldest and best-established techniques for sensing chemical changes in biomolecules. The advantages these techniques have are non-destructive in character and usually come with high sensitivity. The Surface Plasmon Resonance (SPR) based methods are one kind of such optical methods that can be used in real-time biosensing. Its working principle is based on the ability to sense subtle changes in the refractive index as a result of binding or structural change of a sample near the SPR sensing surface (more details about the method will be given later).

The focus of this work is to quantify and detect peroxynitrite-induced DNA damage on gold sensor surface and gold electrodes.

1.2 Peroxynitrite – Formation and Reactivity

Peroxynitrite is the primary product of the reaction of superoxide ion ($O_2^{\bullet -}$) and nitric oxide ion ($NO^{\bullet}$).[5]The reaction is shown in the equation below.

$$\mathbf{O_2^{\bullet -} + NO^{\bullet} \rightarrow ONO_2^-}$$

Scheme 1.1. Diffusion-controlled reaction between superoxide anion and nitric oxide radical

Nitric oxide and superoxide anion react very fast in a diffusion-controlled reaction to produce peroxynitrite. The reactivity of $ONOO^-$ is affected by an intermediate which could

be peroxynitrous acid (ONOOH) or its activated isomer (ONOOH*).[6] Peroxynitrite is present in two conformational isomers: the cis-isomer and the trans-isomer.

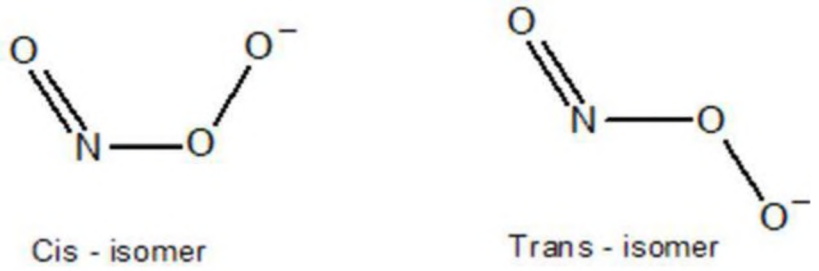


Figure 1.1. Two conformational isomers of peroxynitrite

Vibrational spectroscopy shows that cis isomer is more stable than trans isomer.[7] Peroxynitrite chemistry strongly depends on pH. It behaves as anion at pH 7.4 whereas at lower pH forms peroxynitrous acid i.e. ONOOH (its pK_a= 6.8).[8] The anionic form is very reactive and reacts with many targets such as CO_2 to form nitrosoperoxycarbonate adduct ($ONOOCO_2$). The homolytic cleavage of O-O bond in both ONOOH and $ONOOCO_2^-$ results in generation of harmful radicals ($^{\bullet}OH$), ($^{\bullet}NO_2$), ($CO_3^{\bullet-}$). These radicals cause disruption of membrane lipids, nucleobase oxidation/nitration and DNA strand breaks which are all irreversible impacts.[6, 9] Peroxynitrite in both its anionic form ($ONOO^-$) as well as its protonated form (ONOOH) participates in oxidation reactions (Fig. 1) with a variety of macromolecules and cellular targets.

1.3 Peroxynitrite Induced DNA Damage

Peroxynitrite mostly brings about oxidative change in DNA. Nitric oxide also reacts with DNA however, the damage caused by peroxynitrite is more deleterious. In fact, chemical damage that once was assigned to nitric oxide, turned out to be the result of peroxynitrite when it was later established that the latter is produced at the same sites where

superoxide anions are formed with nitric oxide as a result of nitric oxide synthase decoupling or other inflammatory reactions. Ischiropoulos and co-workers detected significant concentration of peroxynitrite (up to 0.11 nmol/10^6 cell per min) as a marker of peroxynitrite activity.[10]

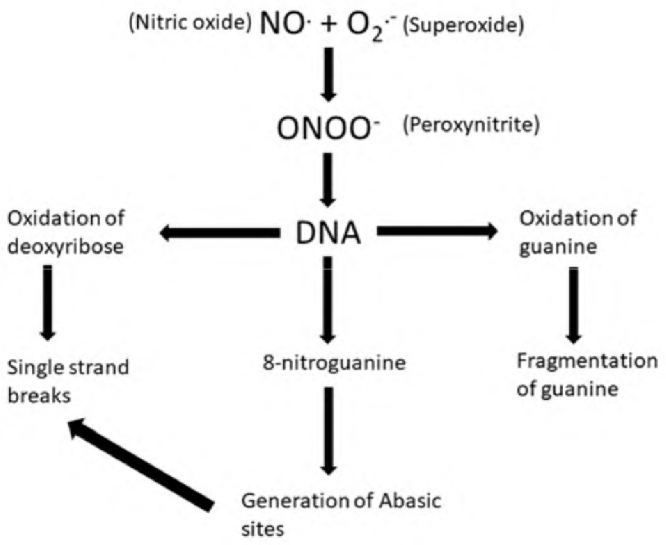


Figure 1.2. The superoxide and nitric oxide reacts to form peroxynitrite which reacts with DNA.[2]

1.3.1 DNA base modifications. Reactions of peroxynitrite with nucleosides and nucleobases showed that it affects purine bases (i.e. adenine and guanine) more than the pyrimidine bases. Further analysis by techniques like high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) revealed the products formed are 8-nitroguanine and 8-oxo-guanine, where the former is the major product. 8-nitorguanine was formed at pH 8 and increased with the increase in the concentration of peroxynitrite.[11] Peroxynitrite is also considered as the biomarker for the DNA damage with the formation of 8-nitroguanine.

Analysis of reaction products by HPLC and TLC showed that the reaction of peroxynitrite with nucleobases formed several new products as outlined below in Scheme 1.3 .[11] Reactions below shows reaction between peroxynitrite and guanine residue to form 8-nitroguanine (A), 5-guanidino-4-nitroimidazole (B), 8-oxoguanine (C) and 2,5-diamino-4H-imidazol-4-one (D) and it undergoes hydrolysis to form 2,2,4-triamino-5(2H)-oxazolone (E) at physiologic pH.[12] However, formation of 8-oxoguanine takes place in the presence of other species such as hydrogen peroxide and several metal ions.

Scheme 1.2. Reaction of peroxynitrite with guanine residues

1.3.2 DNA single strand breakage. Over the past years single strand breakage in DNA upon exposure to peroxynitrite or superoxide or NO has been the focus of a number or research groups. Peroxynitrite has been reported to induce single strand breakage in plasmid DNA with concentration as low as 1uM whereas higher concentrations (1mM) were needed to induce double strand breakage.[13] DNA cleavage caused by peroxynitrite was seen at almost every nucleotide with little more at guanine residues. Since peroxynitrite-induced DNA damage occurs at acidic pH rather than neutral or alkaline pH, it was hypothesized that hydroxyl radical-like intermediate(s) or peroxynitrous acid (ONOOH) are responsible for the single strand breakage.[14]

The mechanism for DNA strand breakage upon exposure to peroxynitrite has not been studied well and has been focus of intense research.

1.3.3 Peroxynitrite induced apoptosis. Once the level of cellular damage caused by peroxynitrite exceeds the threshold of viable cellular repair, the cell eventually dies. Peroxynitrite and NO have been shown to cause cell death in two ways: either acute cell death (necrosis) or delayed cell death (apoptosis) depending on a cell type. Low levels of exposure to NO or peroxynitrite leads to apoptosis whereas sudden exposure to high concentrations causes necrosis.[15, 16]

The defective cells are incapable of repairing single strand breakage in DNA, these unrepaired single strands give rise to double strand breaks, which eventually leads to cell death.[17] Hence, it is quite clear that peroxynitrite-induced DNA damage determines the fate of the cell in many ways.

1.4 Biosensors as Sensing Platforms for Detection of DNA Damage Induced by Peroxynitrite

Biosensors are used for the real-time and label-free study of biochemical reactions between various ligands and analytes. The rapid development in the biosensor technology has made it possible to study the nucleic acid interactions and related kinetics with various reactive analytes. Detection of point mutations[18], simultaneous screening of nucleic acid samples on high density DNA arrays[19] and DNA base modifications[2] are few potential applications of biosensing platforms in the field of nucleic acid analysis. DNA detection in any biosensing setup usually happens in two steps: PCR amplification that makes DNA assay very sensitive as few copies of DNA could be detected during amplification process, and then reaction with a transducer follows which converts the recognition event into a measurable signal.[20] Optical, piezoelectric or electrochemical instruments are used as transducers in DNA biosensing.[21]

Optical techniques are one of the oldest and most reliable techniques for sensing biomolecules. The advantages offered are their non-destructive character and the relative high sensitivity. Although some optical methods based assays exhibit sensitivities that allow to observe the interactions between the biomolecules without the need to label.[22, 23] most methods rely on the introduction of an optical chromophore for signal transduction. The potential and efficient use of surface-based biosensors depends on the highly programmable positioning of biomolecules on surfaces. DNA chips are important tools in this regard and have been in steady development on multiple fronts. Yet, the success of DNA chips is still influenced by number of factors like (i) good accessibility and functionality of the surface-bound probes, (ii) density of attachment and (iii) attachment chemistry.[24, 25]

Immobilization is the preliminary step towards developing of a whole range of microarray methods. It can be defined as the attachment of molecules to a surface resulting in the possibility to offer localized chemical reactivity that can be monitored *in-situ*.[25] The mechanism by which immobilization takes place determines the property of the sensing surface. The DNA probes can be either made base-by-base or pre-synthesized to be immobilized on the surface.

Over the past years, various immobilization techniques have been developed and used. However, they mainly fall into three important categories: (1) physical adsorption, (2) covalent bonding and (3) affinity-based immobilization (such as biotin-avidin type immobilization).

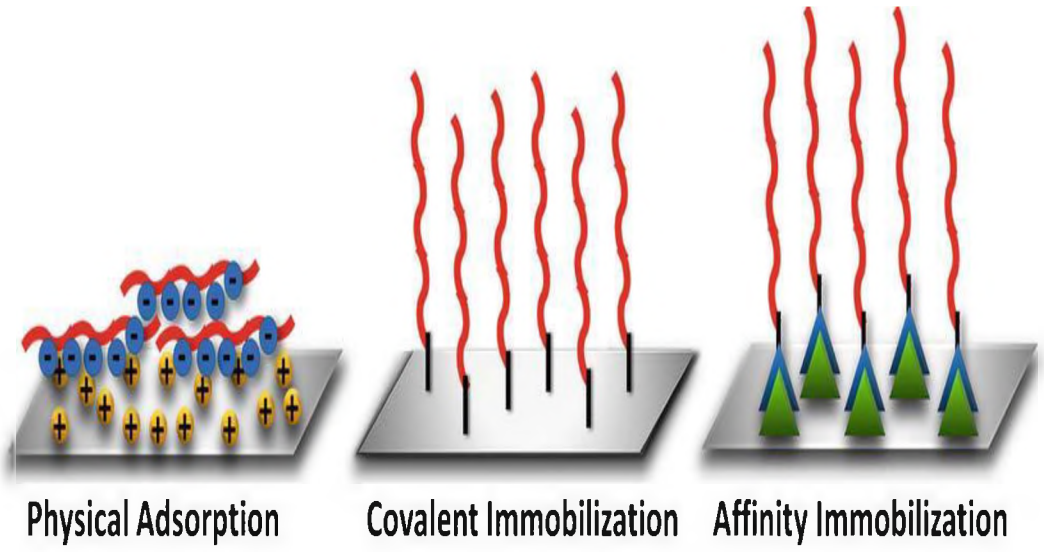


Figure 1.3. Immobilization techniques for fabrication DNA microarray[25]

Figure 1.3 illustrates different mechanisms by which immobilization process takes place. However, these immobilization techniques also lead to non-specific binding. It is very important to minimize non-specific binding in order to ensure high reactivity, proper

orientation and stability of the surface-bound molecules. The table given below lists the advantages and disadvantages of the different immobilization methods.

Table I. Tabulation of advantages and disadvantages of different immobilization techniques

Immobilization Method	**Interaction**	**Advantages**	**Disadvantages**
Physical[26] Adsorption	Charge-charge interaction	-Simple -Fast -Direct method -Appropriate for DNA, RNA and PNA	-Random orientation -Poor reproducibility -Crowding effect
Covalent[27, 28] Bonding	Chemical bonding	-Good stability -High binding strength -Use during long term	-Use of linker molecules -Slow, irreversible -Crowding effect -Island formation
Streptavidin-Biotin Interactions[29]	Specific Streptavidin-Biotin Interactions	-Improved orientation -High specificity & functionality -Well-controlled -Reversible	-Expensive, slow -Crowding effect -Poor reproducibility

1.4.1 Charge-driven DNA surface immobilization. This immobilization method is based on ionic interactions between the negatively charged phosphate backbone of the DNA probes and positively charged functionalized surfaces. Charge-driven immobilization is the simplest method used as it does not require any DNA modifications or any linkers to attach the DNA probes to the surface. However, the resulting modified surface with DNA or oligonucleotides with this mode of immobilization are likely to be heterogeneous in nature with random orientations on the surface.[30] The molecules are

randomly oriented since each molecule can form many contacts in different directions to minimize the repulsive interactions with already present DNA probes.

Random orientation is not the only limiting factor that makes this immobilization method less reliable. Leaching is also a potential problem since electrostatic interactions are not very strong and this may lead to DNA molecules gradual removal from the surface when the surface comes in contact with salts and detergents during experiment.[30] Moreover, problems like mass transport effect, high background signals and non-specific binding can result in false calculations.

1.4.2 Covalent attachment. Covalent coupling of target species to surfaces gives rise to highly stable microarrays with strong binding strength. Covalent bonding coupling methods are many and are commonly used for chemical immobilization.[30, 31] This type of attachment method is applicable to the different kinds of couplings. Some of them are listed below:

Amine Coupling. This chemistry is the mostly used for the attachment of proteins and other biomolecules covalently on the sensor surface. The carboxyl groups on the sensor surface are activated with EDC/NHS to give reactive succinimide esters. These ester groups then react with the primary amine groups or other nucleophilic groups in the ligand.

Thiol Coupling. In this method, thiol group is attached on the ligand and the sulfur atom of the thiol group reacts with the metals like Au.

Aldehyde Coupling. Ligands containing aldehyde group are immobilized by activating the surface with hydrazine or carbohydrazide. Aldehyde coupling provides an alternative approach for immobilizing glycoproteins and other glycoconjugates. In this work, we focus on thiol-metal coupling.

1.4.2.1 Thiol-gold linkages. Self-assembled monolayer films of alkanethiols or disulfide alkanes on gold surfaces have been studied extensively, [32] including factors that affect their formation and stability.[33]Chemical modification of metal surfaces is the primary step to enhance the interfacial reactivity of a metal. [34] Factors like intermolecular interactions, adsorbate-substrate binding are key to form a stable surface-films.[32, 35] Organosulfur molecules are known to bind strongly to metals like Fe, Au, Ag and Cu. This is attributed to the capability of sulfur atom to form strong linkage with the metals. Thiol groups are used as one way to tether biomolecules to gold surfaces because of their strong affinity towards this metal. Raman spectroscopy provides experimental evidence of the loss of hydrogen and formation of S-Au bond on adsorption of organo-thiol molecules on Au gold atoms.[36]This method has been widely used to immobilize thiol-modified oligonucleotides on surface functionalized with gold nanoparticles by self-assembly of thiolated DNA probes.[37]

Self-assembled monolayers (SAM) play a vital role in minimizing the non-specific binding, and therefore presents a simple and effective means to control the density and availability of the surface targets. Non-specific binding (NSB) takes place when the analyte binds non-specifically to the surface rather than the target ligand. SAMs are obtained by thiolated DNAs mixed with alkanethiols or disulfide alkanes which use sulfide bonds to anchor DNA probes on the gold surface. The secondary thiol displaces the non-specifically adsorbed DNA molecules, leaving the tightly bound molecules in an upright position.[38] Another important feature of this adsorption chemistry is the stability of the biomolecule monolayer attached on the surface.

As shown in figure below, thiol-metal interactions are one example of covalent binding of thiolated biomolecules on gold surfaces.

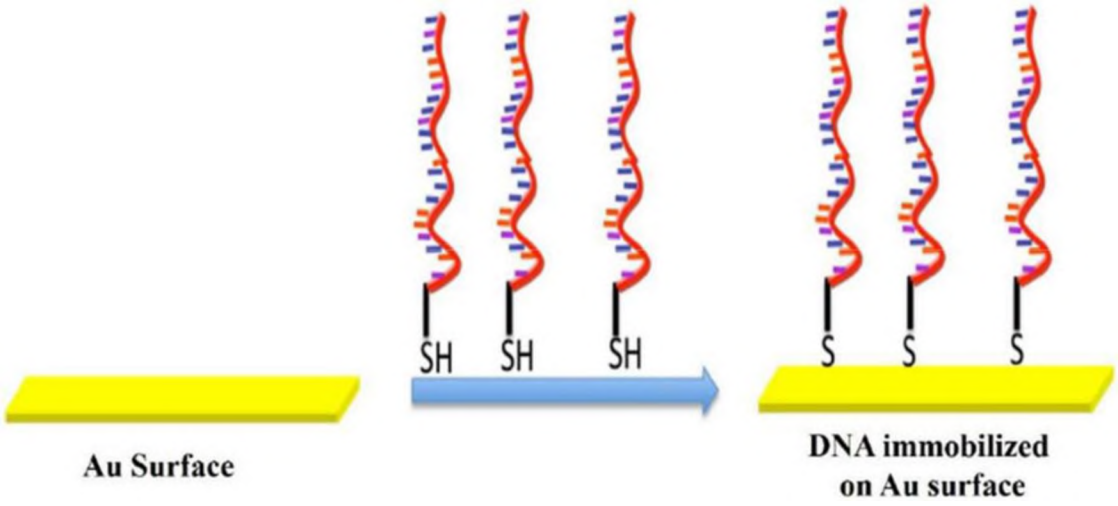


Figure 1.4. DNA immobilization on Au (Gold) surface[25]

Covalent coupling chemistry requires however the prior chemical modification of the DNA molecule with the right functionalization of the surface. However, there are many factors that influence the preparation of DNA-modified surfaces to construct microarrays. Some of these factors are immobilization chemistry, buffer, DNA concentration. The test surfaces should be developed in such a manner that all the molecules should be evenly spaced to allow high specificity and avoid crowding and non-specific binding. Also, DNA probes that are closely packed have limited exposure for hybridization or reactivity with other analytes.

Another method that takes advantage of gold-thiol chemistry and introduces other chemical functionalities that can be used to attach DNA or other molecules is the use of self-assembled monolayers of cystamine and cysteamine on gold. This method has been used for the preparation of biosensors and modified electrodes.[39] Self-assembled monolayers of cystamine or cysteamine form building blocks, where the sulfur atoms of

the molecules bind to the gold surface and the amino atoms are used for the attachment of other groups on the self-assembled thiol layer. The formation of cystamine monolayers is considerably slow when compared with the formation of cysteamine on gold surface. This is due to the slow cleavage of sulfur-sulfur bond of the disulfide in cystamine molecule.[39]

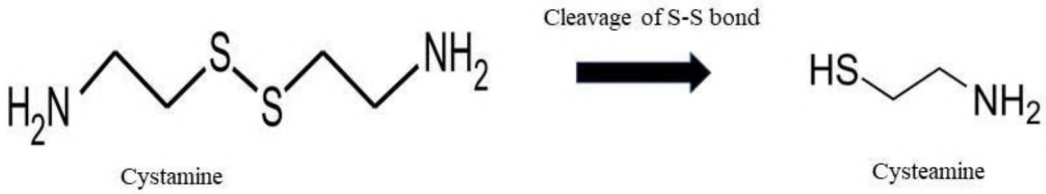


Figure 1.5. Reduction of cystamine to cysteamine[40]

1.4.2.2 Thermal stability of DNA functionalized gold particles. DNA functionalized gold nanoparticles (DNA-AuNPs) have shown great potential as biosensors to study induced DNA damage for disease diagnostics and treatment. It is important to maintain a stable conjugation between DNA oligonucleotides and gold nanoparticles under thermally stress conditions. There are few factors affecting the thermal stability of DNA-AuNPs like organo-sulfur anchor groups and packing densities. Fluorescence assay was used to determine the thermal stability of DNA bound to gold surface using different anchor groups. The Au-S bond formed with acyclic disulfide was thermally more stable than the bond formed using cyclic disulfide.[41]

DNA packing density on gold surface also played a role in determining the thermal stability of the DNA-gold nanoparticles. The effect was maximum at temperature as low as 37^{o}C and was minimized as temperature increased to 85^{o}C.

1.4.3 Streptavidin-biotin interactions. Streptavidin homo-tetramers have high affinity for biotin. The binding of biotin to streptavidin is one the strongest non-covalent

interactions known.[25] Therefore, the highly specific bonding between streptavidin and biotin can also be used to immobilize DNA on the surfaces. This interaction usually takes place in two steps (i) derivatization of the biomolecule with biotin using a crosslinker reagent (ii) and a second step to attach the biotinylated molecule to the streptavidin surface.[42]

Streptavidin and avidin are tetramers having four binding sites available for biotin.

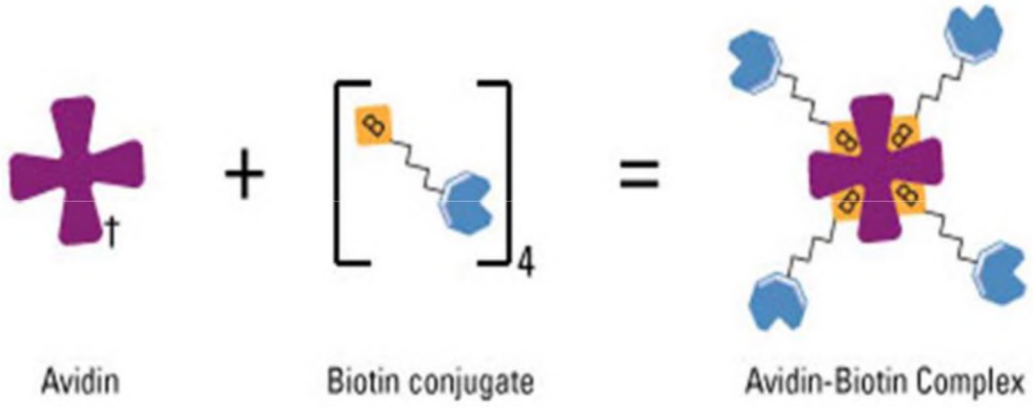


Figure 1.6. Biotin-avidin interactions[43]

Streptavidin is preferably used over avidin to avoid non-specific interactions. Although, streptavidin-biotin interactions are very strong, the binding capacity decreases over time. Moreover, this immobilization process is complex involving multiple steps like fabrication of the surface, modification of the biomolecule and then blocking. All these steps increase the non-specific interactions, instability of the immobilized molecule, production time, and cost.[44]

CHAPTER II

METHODS AND INSTRUMENTS

2.1 Introduction

We studied DNA-peroxynitrite interactions using *OpenSPR benchtop* instrument from Nicoya LIFESCIENCES, which is based on the Surface Plasmon Resonance phenomenon. We also conducted a parallel comparative study using Cyclic Voltammetry.

2.2 Surface Plasmon Resonance

2.2.1 Theory. Surface Plasmon Resonance (SPR) is an optical phenomenon that is used to study the biomolecular interactions at functionalized surfaces in real-time and without the use of labels. SPR instruments are used to measure the binding between partners such as proteins, or DNA and a protein, among other ligand-analyte interactions. It studies the binding kinetics and binding affinity of the ligand and analyte. The biosensing technique gives the "ON" (association) and "OFF" (dissociation) rates of real-time binding. The data obtained from these studies helps to determine how fast molecules interact, and the molecular mechanisms followed in their interaction.

Figure 2.1 gives the typical binding curve consisting of an association phase, during which the analyte binds to the ligand, followed by dissociation phase where only buffer is flowing through the sensor surface.

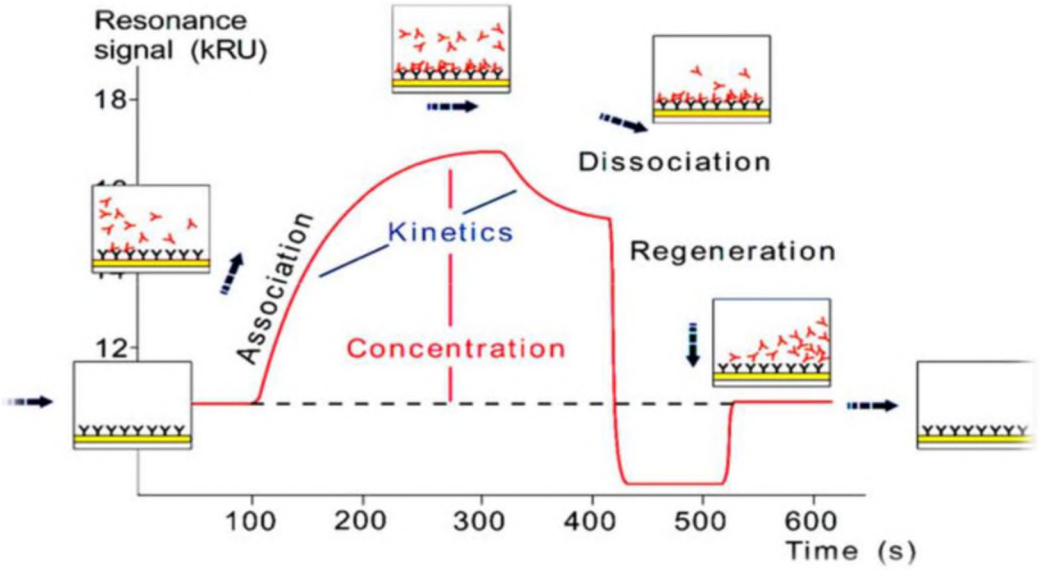


Figure 2.1. Binding curve generated from an SPR experiment[45]

The final phase is the regeneration phase where regeneration solution is used to remove the analyte from the ligand. SPR instruments can typically measure concentrations ranging from pM to mM.

This technique offers number of advantages:

Label-free technique (less expensive and convenient)

Requires small sample volumes

High sensitivity

Real-time binding

Quantitative

The method relies on optical measurement to determine very sensitive changes in the refractive index that occurs at the surface of the sensor chip upon binding or removal of molecular partners at the functionalized surface. The sensor chip is a glass substrate with gold coated film that is chemically modified to accommodate the immobilization process.

One of the binding partners is immobilized on the sensor chip and is called *ligand*. Whereas, the other molecule that passes through the sensor chip is called *analyte*.

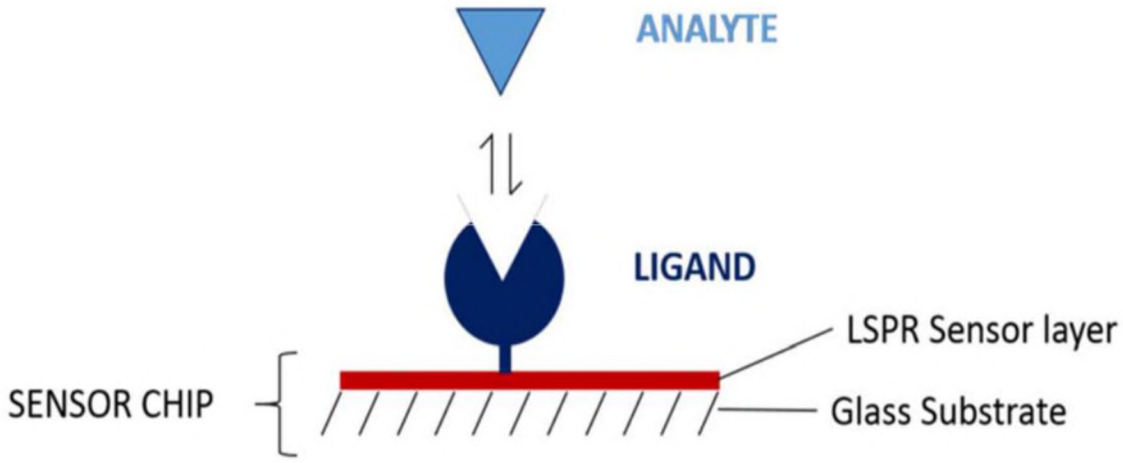


Figure 2.2. Cartoon symbolizing the SPR sensor chip surface functionalized with the ligand as the specific target for the added analyte[46]

The sensor chip is inserted in the fluidic system within a small flow cell. This helps the user to inject analyte at different concentrations with intermittent washing steps. The fluidic system regulates the buffer flowing through the flow cell and across the sensor chip in a controlled manner. The analyte is injected through the sensor surface for a specific amount of time (interaction time) as adjusted by the flow rate.

The optical system consists of a light source, which illuminates the gold film and a detector, which is used to measure the unique optical spectrum produced by SPR. When a molecule binds to the surface of the sensor chip, the refractive index of the space in which plasmonic wave is propagating through changes, which causes the shift in reflectance detected optically. The amount of shift depends on the mass of material bound to the surface, and hence the shift is a direct measure of real-time interaction of the analyte with

the modified surface or, for that matter, of any change in structure of immobilized species at the surface.

2.2.2 Workflow. The typical SPR experiment starts with a first step of immobilizing the target ligand on the surface of the sensor chip. This could be done in many ways taking advantage of chemical functionalization as described in the previous chapter. After immobilizing the ligand at a certain surface density, the next step is to block the remaining binding sites to avoid non-specific binding. Meanwhile, the running buffer is continuously flowing through the sensor chip surface. Once the surface is stable, one can proceed with the different analyte injections. During the analyte injection, the flow rate is set according to the interaction time needed for the specific ligand-analyte. Binding of the analyte will increase the signal until it reaches the equilibrium. As soon we switch to plain running buffer solution, bound analyte molecules come off the surface, which will cause the signal to decrease. The resulting curve will give the binding between the ligand and analyte.

2.2.3 Sensor chips and binding curves. The preliminary step to obtain good binding kinetics is the correct choice of sensor chips for the experiment. There are number of ways in which a sensor chip can be functionalized for different types of applications. Plain gold sensor chips are used to immobilize thiolated ligands due to strong Sulfur-Gold linkage. Biotinylated ligands can be immobilized on streptavidin coated chips. Carboxylated chips are used for the immobilization of ligand having amine groups using the standard EDC coupling. Similarly, other derivatized glass chips are available to immobilize chemically modified ligands with appropriate functional groups.

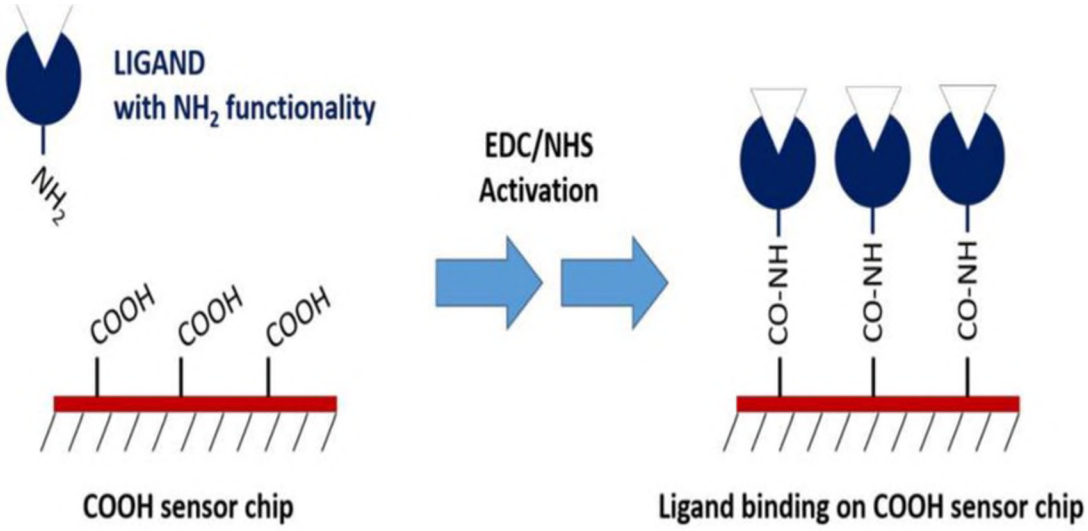


Figure 2.3. Covalent coupling of ligands with amine (-NH_2) functional group on to carboxylic acid modified sensor chips[46]

Avoiding non-specific binding is also important in order to determine the right kinetics. Non-specific binding happens because of the interactive forces between the analyte and the surface. To prevent this process, it is important to block the surface of the sensor chip with a suitable blocker to ensure the response is due to the binding between the ligand and analyte. The figure below shows the non-specific binding of the analyte with the surface vs. specific binding of the analyte to the ligand.

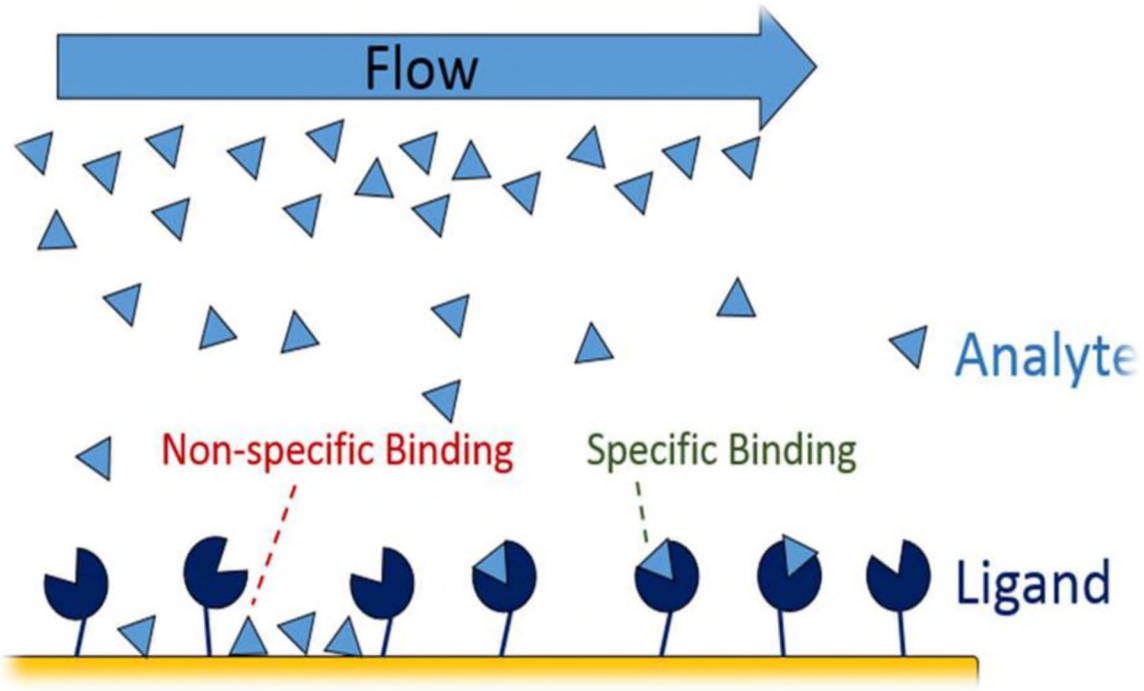


Figure 2.4. Non-specific binding vs. specific binding[46]

In order to get accurate binding curves, one must repeat the experiment with different analyte concentrations. We typically perform the first step with lowest concentration and then increase accordingly. If the level of NSB is minimal, then the user may proceed with the experiment under the same conditions. However, there are other factors as well which affect the binding curves and one of them is mass transport effect. Mass transport effects will show up in kinetic data as binding curves that have very little curvature. Reducing ligand density is very important in order to get a typical SPR response.

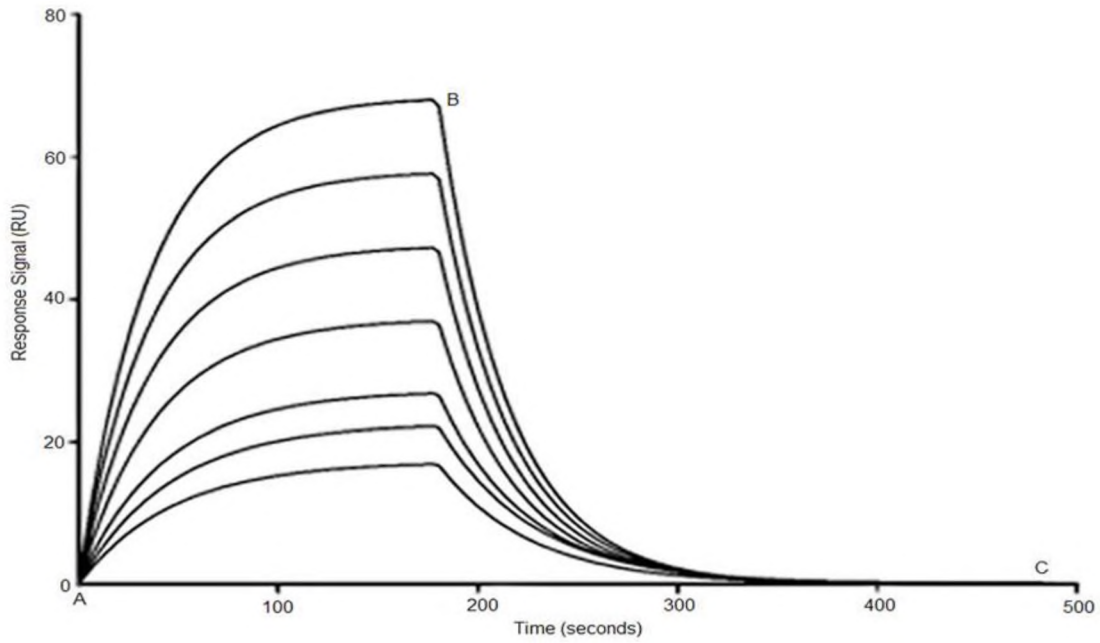


Figure 2.5. Example response graph of a kinetic titration experiment, with increasing concentrations of analyte injected over the sensor surface

2.2.4 Regeneration. Regeneration is used to wash the analyte from the sensor surface so that the higher analyte concentration can be injected without the interference of previous analyte injection. However, it is only required if analyte-ligand bond is very strong and analyte is not washed away with the washing buffer. In such cases, an effective regeneration buffer is specific to the types of molecules being used and their affinity for each other. To regenerate the binding surface, an acidic, basic, salt or surfactant solution is injected to break the ligand-analyte bond.[47] The regeneration buffer to be used is determined according to the binding pair used in the experiment. Regeneration should be started with least harsh conditions and subsequently harsh conditions.

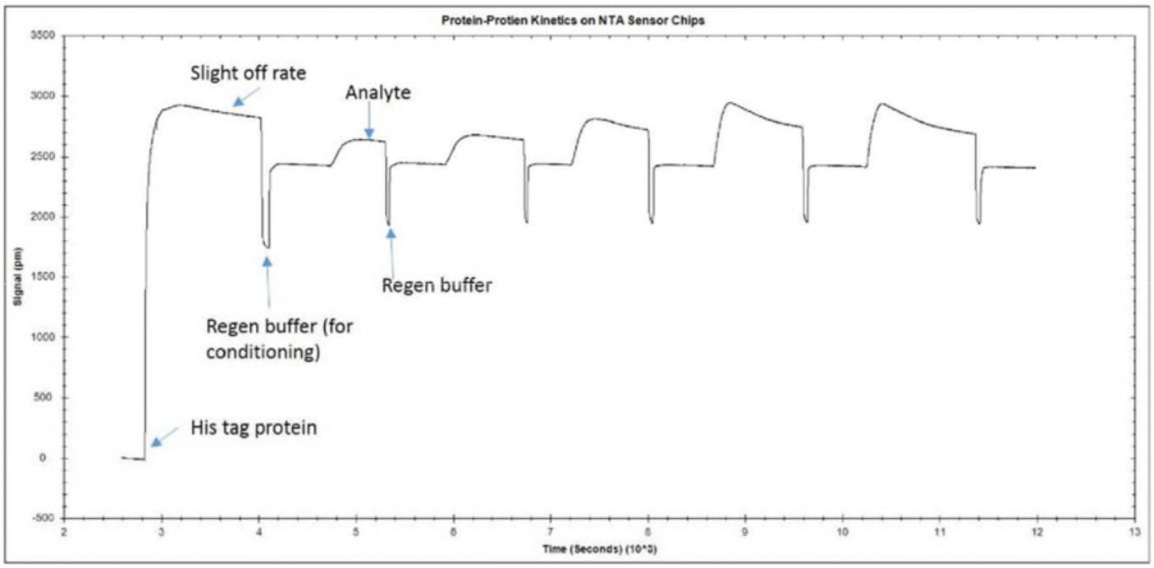


Figure 2.6. Example response graph with regeneration of surface between subsequent analyte injections[47]

2.3 Electrochemistry

2.3.1 Introduction. In this section, we address electrochemical techniques that we used in parallel with SPR to study the chemical reactivity of peroxynitrite on DNA chips. Electrochemical methods fall into two major branched (1) bulk techniques, which effect overall change of the solution in the electrochemical cell, and (2) analytical methods which only affect analytes at the electrode interface and determine concentrations of species without changing the properties of the original solution.[48] All the methods that we use in this work are analytical in nature.

2.3.2 Electrochemical method used: cyclic voltammetry. Cyclic Voltammetry is an analytical technique used to study the kinetics and thermodynamics of the oxidation and reduction processes of the target molecular species. It can also be used for analytical purposes to monitor the concentration of target species. In this method, the potential of the electrode is varied linearly with time and the resulting current is monitored.[49] The potential

is then switched at a pre-set value and is scanned back to monitor the current of the reverse reaction. The resulting graph of current as a function of the potential is called cyclic voltammogram.[50] Cyclic voltammetry can be conducted at various scan rates, which gives, in certain cases, the possibility to study the kinetics of electron transfers or related chemical reactions at the electrode interface.[51] The figure given below shows a typical cyclic voltammogram of a reversible system.

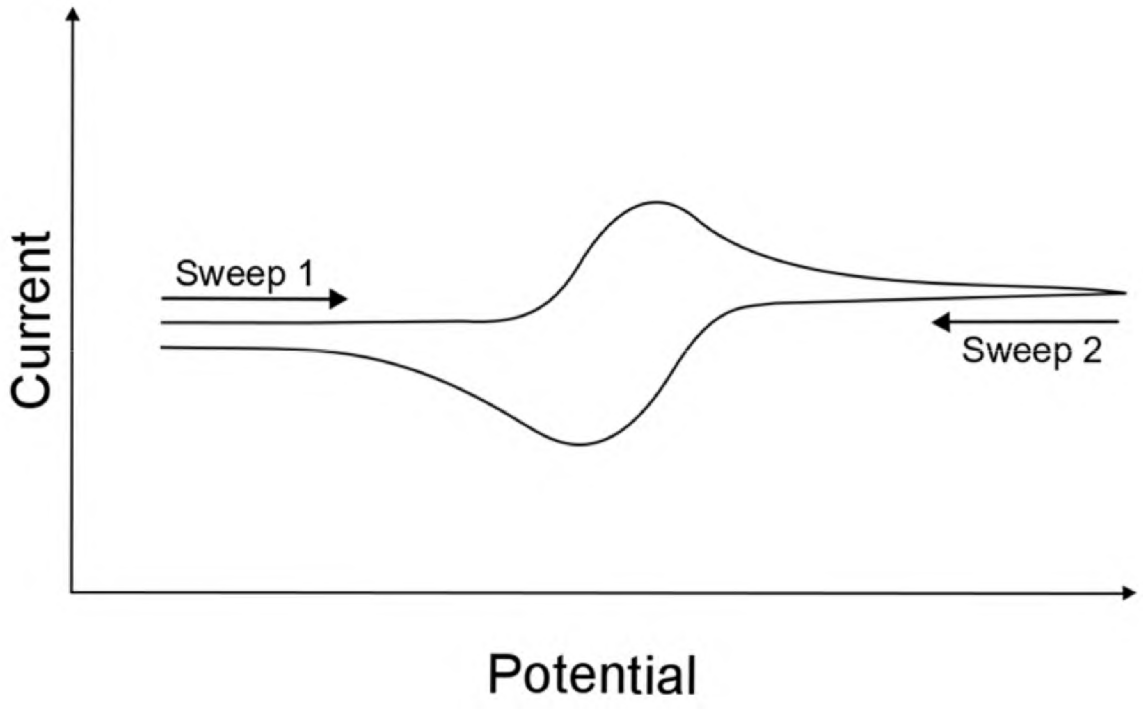


Figure 2.7. Typical cyclic voltammogram of a reversible electrochemical system. The potential is scanned linearly with time and the resulting current is plotted on the y-axis.[49]

For the purposes of this work, we first scan the potential negatively (potential is lowered moving to the right of the graph) which result in a reductive current. The potential is then switched a pre-set value and is scanned back positively (moving left on the graph) which results in an oxidative current.[50]

2.3.3 Electrode modification and immobilization method used. The gold electrode is first sonicated and polished using standard procedures. A drop of the solution of thiolated DNA sequence is then cast on the surface of the electrode and allowed to react with under controlled conditions that prevent solvent evaporation. This results in the formation of self-assembled monolayer of the thiolated molecules on the substrate.

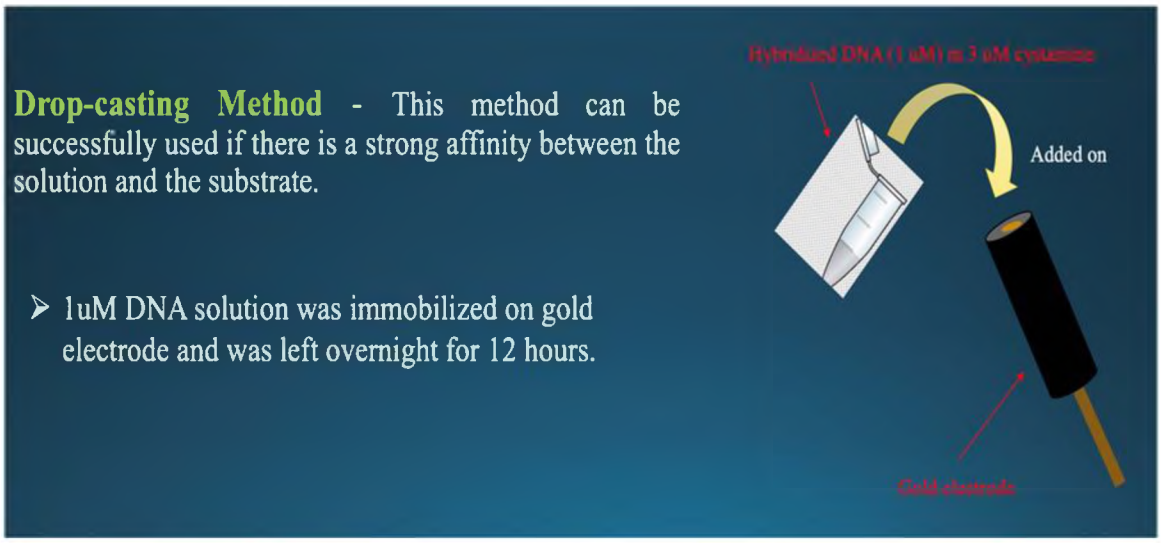


Figure 2.8. Drop casting method used to immobilize DNA on gold electrode

This drop-cast method can be used whenever there is a strong affinity between the target molecules and the electrode substrate. This is the case for thiolated oligonucleotide on the gold electrode.[52] The synthesized DNA probe is linked with the group of thiols (SH) to bind covalently to the metal surface.[53] .

CHAPTER III

EXPERIMENTAL

3.1 Introduction

This chapter outlines the general experimental methods and materials used throughout this work and addresses the specific aims outline in the summary page.

3.2 Synthesis and General Reagents

Phosphate buffer. Phosphate-buffered saline solution (PBS) of 5mM and 50mM sodium chloride (NaCl) was prepared by dissolving 3.28 g of sodium di-hydrogen phosphate (NaH_2PO_4), 4.12 g of disodium hydrogen phosphate (Na_2HPO_4) and 1.46 g of NaCl in deionized water (DI water) and fill the volume up to 500 ml. The pH of the resulting buffer is adjusted with a few drops of concentrated acid or base to pH 7.4.

Cystamine solution. Cystamine was prepared by adding 6.7 mg of cystamine in 10 ml of PBS solution. 10 ul of resulting cystamine solution (3 mM) is then diluted in 10 ml of PBS solution.

Preparation of double-stranded oligonucleotides probes. Single stranded thiolated oligonucleotides having 15 base pairs were purchased from Biosynthesis, Inc. The thiol groups are tethered to the oligonucleotides using a 6-mercapto-hexyl group attached to the 5'-phosphate of the oligonucleotide. The sequences are as follows:

1. [Thiol C6]-AG TAC AGT CAT CGC G,

2. CGC GAT GAC TGT ACT,

3. [Thiol C6]-GG GGG GGG GGG GGG G,

4. CCC CCC CCC CCC CCC)

All oligos were suspended in deionized water to prepare stock solution of about 2ug/ul concentration. Equal volumes of each ssDNA and the corresponding matching sequence (1+2 and 3+4) were hybridized in PBS (5mM phosphate, 50mM NaCl, pH 7.4) by heating the mixture for 5-10 minutes at 95^0C in a heat block. The heated solution was then allowed to cool down slowly to room temperature over 3-4 hours. We used absorption spectroscopy to estimate the final DNA concentration using the known DNA extinction coefficient at 260 nm.

Synthesis of peroxynitrite. Chemicals- 40ml of NaOH (5N) was prepared, 27 ml Isoamyl nitrite (0.2 M), 23 ml of Hydrogen Peroxide (H_2O_2) (30%), 5 ml of Diethylenetriamine pentaacetate (DTPA) was prepared in 0.05 N sodium hydroxide (NaOH).

Procedure- 23 ml of H_2O_2 was mixed with 40 ml of NaOH (5 N) in a round bottom flask kept in chilled ice bath. The solution could equilibrate for 15-20 minutes on an ice bath. Then, 5 mL of 0.04 M DTPA (prepared in 0.05 N NaOH) was gently mixed with the solution. The total volume of buffered H_2O_2 was then increased by adding deionized water to 100 mL with a 0.2 M concentration. Finally, this solution was mixed with equimolar concentration of isoamyl nitrite (0.2 M, 27 mL). This solution was stirred vigorously overnight at room temperature. Deep yellow color marks the formation of peroxynitrite. The peroxynitrite remained in the aqueous phase and isoamyl alcohol remained in the

organic phase, where organic phase corresponds to isoamyl nitrite and hydroperoxide anion is the aqueous phase.

The peroxynitrite synthesis follows the following chemical reaction where the hydroperoxide ion from hydrogen peroxide under alkaline conditions reacts with isoamyl nitrite.

$$\mathbf{RONO + HOO^- \rightarrow HOONO + RO^- \rightarrow ONOO^- + ROH}$$

Scheme 3.1. General reaction between alkyl nitrites (isoamyl nitrite in this case) and hydroperoxide ion to form peroxynitrite

Purification- Isoamyl alcohol and traces of isoamyl nitrite are removed by washing the solution with dichloromethane and chloroform. The aqueous phase where peroxynitrite accumulates is separated from the organic layer. The solution is then passed through a column filled with granular MnO_2 to remove any unreacted H_2O_2. Pure peroxynitrite is then collected and stored in aliquots in a freezer at -80oC. The needed amounts are thawed and kept on ice prior to experiments.

Preparation of solutions of redox active probes. A 2 mM stock solution of potassium ferricyanide K_3 [Fe $(CN)_6$] is prepared in pH 7.0 PBS. When needed, the ferricyanide solution of mixed with methylene blue at a final concentration of 2μM methylene blue in the same pH 7.0 PBS buffer.

3.3 SPR Method

Instrument and Software. All SPR experiments were performed using OpenSPR™ instrument purchased from Nicoya LIFESCIENCES. The OpenSPR™ operates using a UL certified Class 2 power supply with an input of 100-240 V, ~50-60 Hz and 0.31A MAX.

The software works with Windows 7 or higher. The data was analyzed using TraceDrawer 1.8.1 by Ridgeview Instruments AB.

Specific Aim 1: Optimization of Methods of Immobilization of Oligonucleotides on Nanogold Interfaces

Single-stranded oligonucleotides with the tethered thio-hexyl group is first hybridized to its complementary sequence in PBS buffer (5 mM PBS with 50 mM NaCl, pH 7.4) using a heating block. The mixture is heated 5-10 minutes at 95^0C and then allowed to cool down slowly to room temperature over 3-4 hours.

DNA immobilization on the sensor chips. It important to mention first that the Nicoya sensor chips use gold nanoparticles on the glass chip rather than a gold film. The gold nanoparticles result rather in localized surface plasmon resonance (LSPR), which gives number of advantages over regular gold film SPR, including robust angle-independent responses, as well as lower variability due to bulk sample effects.

The gold nanoparticles on the sensor chip can modified the same way any gold surface is modified with thiol-derivatized probes. In our case, 10 ul drop of hybridized DNA was cast on the Nicoya sensor chip. The covered chip was kept in chamber with controlled moisture overnight allowing enough time for the oligonucleotide to form self-assembled monolayers on the gold nanoparticles. Each functionalized gold chip was prepared using this procedure for each experiment. In addition, in order to make sure that the exposed surfaces of all nanoparticles are covered and to ensure an appropriate density of oligos on the nanoparticles, we used cystamine as a blocking agent in a 1:3 (DNA: Cystamine) ratio. Figure 3.1 illustrates this nano-gold modification process.

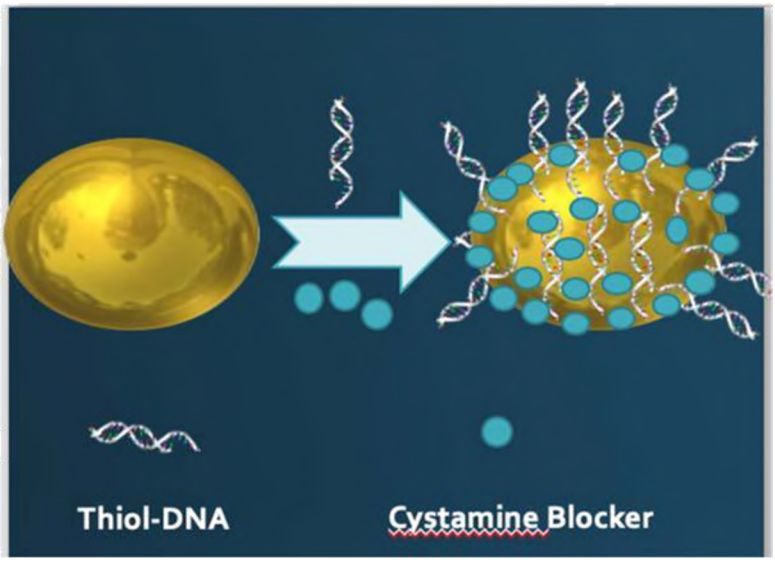


Figure 3.1. Modification of nanogold on the Nicoya sensor chip with thiolated hybridized DNA in the presence of cystamine blocker

Figure 3.2 shows the process of hybridization of thiolated an oligo and its complementary sequence, as well as the immobilization of the resulting hybridized probe on the SPR chip.

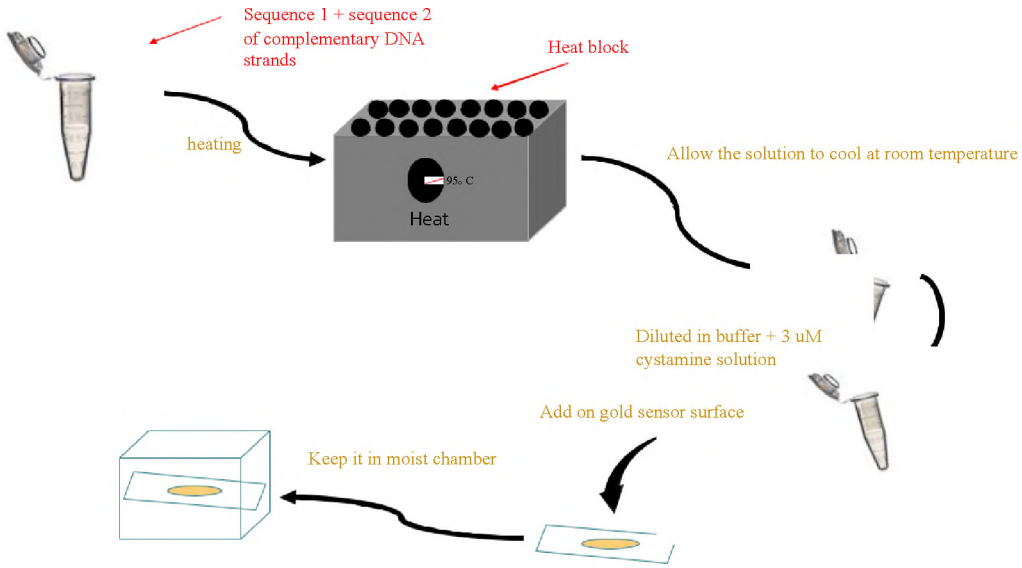


Figure 3.2. Process of hybridization of a thiolated oligonucleotide and its complementary sequence followed by the immobilization of the resulting probe on the Nicoya SPR chip

In effort to optimize the immobilization conditions of thiolated DNA on our SPR chips, we have tried various exposure times under an environment with controlled moisture to ensure that the drop of the solution with the target thiolated DNA does not evaporate during the immobilization process.

We have first followed established procedures in our lab calling for about 6 hours of immobilization time. However, the characterization of resulting chips showed that the DNA immobilization was not complete. We have therefore prolonged the chip exposure time to 12 hours overnight. Subsequent characterization of these chips showed appropriate DNA densities on the chips and reliable SPR signals. We therefore selected these immobilization conditions for the remainder of the work.

Specific Aim 2: Characterization of the DNA-Functionalized SPR Gold Chip

Before the use of the SPR chips modified with thiolated DNA, we wanted to ensure that the thiolated probes are in fact tethered to the gold nanoparticles on the SPR chip. For this reason, we used scanning electron microscopy with elemental analysis using energy dispersive spectroscopy.

The rationale for this method is the fact that in the presence of immobilized oligonucleotide probes not only the morphology of the nanoparticles on the chips is expected to change, but also the characteristic signature peaks of phosphorus (phosphate backbone) and nitrogen (bases) are expected to signal the presence of the tethered oligonucleotides. Although EDS can be used for semi-quantitative determination of elements on the analyzed surfaces, we used here purely qualitatively to indicate the presence of elements that are intrinsic to the added DNA probes.

Figure 3.3 shows the comparative EDS spectra of representative areas of the SPR chip before and after immobilization of oligonucleotides. The insets are typical SEM images of the chips before and after DNA immobilization. The EDS spectrum of the bare nanogold SPR chip shows the presence of Na and Si elements that are attributed to the glass substrate of the SPR chip. We also observe the Au element signal, which represents the attached nanogold particles on the glass SPR chip. The EDS spectrum of the DNA-modified SPR chip shows the appearance of surface N and P elements, which are attributed to the oligonucleotides attached to the gold nanoparticles using thiol-Au bonding.

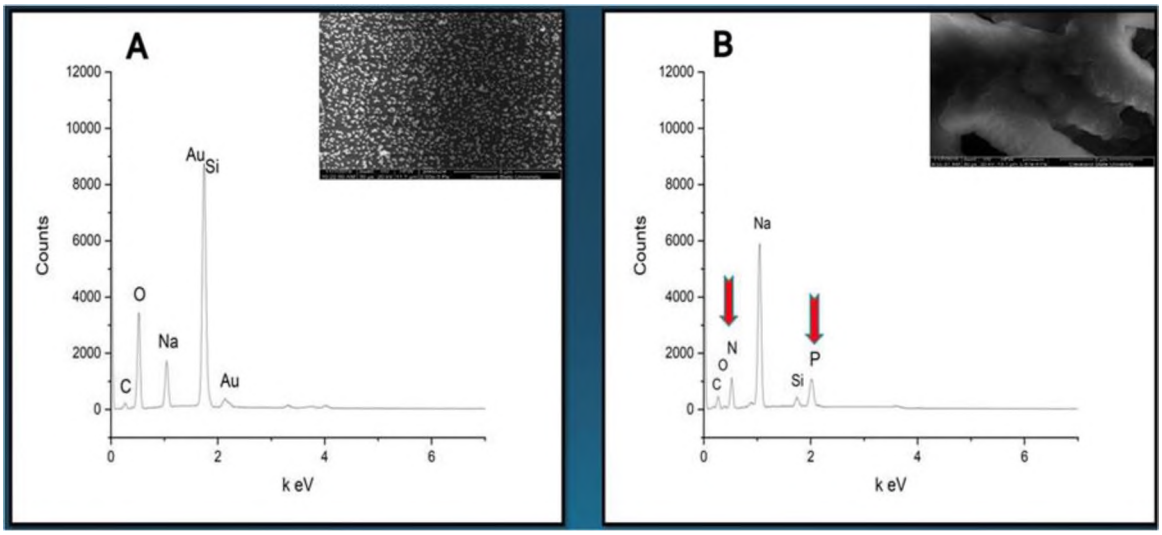


Figure 3.3. EDS spectra of nanogold SPR chip before (A) and after (B) immobilization of the thiolated oligonucleotide probes. Red arrows in (B) point to the peaks of N and P elements of the attached DNA. Insets are typical scanning electron microscopic images of the SPR chip before (A) and after (B) DNA probe immobilization.

Conclusion. The coupled SEM-EDS surface characterization shows that the procedure and conditions we used for the immobilization of thiolated DNA on the gold nanoparticles on the SPR chips results in modified chips with the target DNA probes.

Specific Aim 3: Study of the Interaction of PON with Immobilized DNA Structures on the SPR Chips

Figure 3.4 shows a typical sensor chip. The colored spot in the middle is the attached nanogold that is used to generate the LSPR phenomenon. The same gold nanoparticles are subject to DNA modification as described above. The glass chip is then inserted in a microfluidics system that direct analytes flow on the nanogold surface to monitor resulting SPR changes.

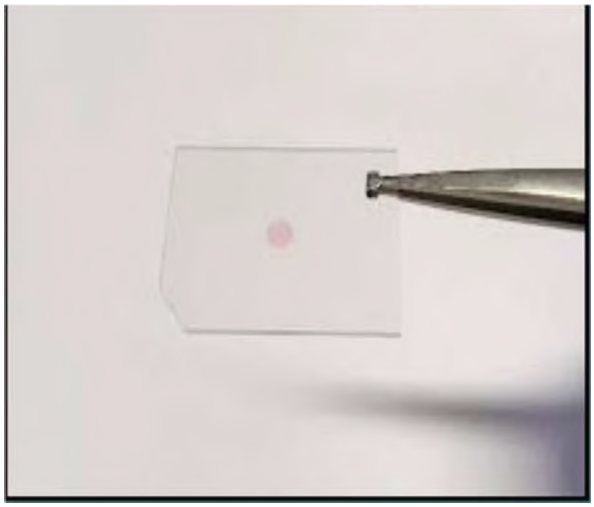


Figure 3.4: SPR glass chip: The colored spot in the middle is the active nanogold surface of interest and that generates the localized surface plasmon resonance signal.

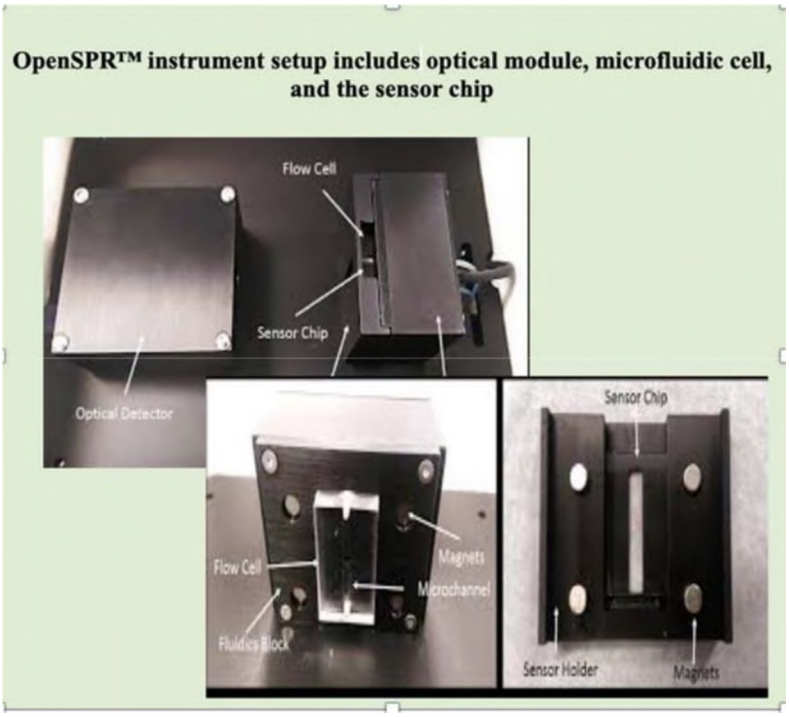


Figure 3.5. OpenSPR instrument used in this work. The top portion shows the placement of the flow cell and the optical detector. The lower portion show two views of the SPR chip housing within the flow cell module.

Instrument is first primed using the buffer solution (5 mM PBS, 50 mM NaCl, pH 7.4). This is done at flow rate of 150 ul/min. New reference spectrum is taken after removing the sensor chip and the sensor holder. Once the reference is taken, the functionalized sensor chip is loaded into the flow cell module. The reference absorbance plasmon peak of the sensor is found and recorded and is used to track any binding interactions on the sensor surface, which result in a change in the position of the absorbance peak. Collection of real-time data then begins. Once a stable baseline is obtained the instrument and the inserted sensor chip are ready for use.

All SPR test are performed at a flow rate of 20 uL/min and injection are allowed 5 minutes to complete (the volume of the loading loop is 100 uL).

In order to test if the surface of DNA-modified gold nanoparticles on the SPR chips is saturated with cystamine as the blocking agent, we performed injections of 3 uM cystamine solution over the DNA-modified SPR chip. As shown in figure 3.6, injection of cystamine solution results in an increase in the SPR signal indicating the blocker is depositing on the DNA modified sensor surface. This indicates that the original modification with the 3:1 (cystamine: DNA) ratio under our conditions does not result in DNA-nanoparticles fully saturated with cystamine blocker. One injection was enough to block all bare spots on the DNA-modified nanoparticles as indicated by a stable baseline.

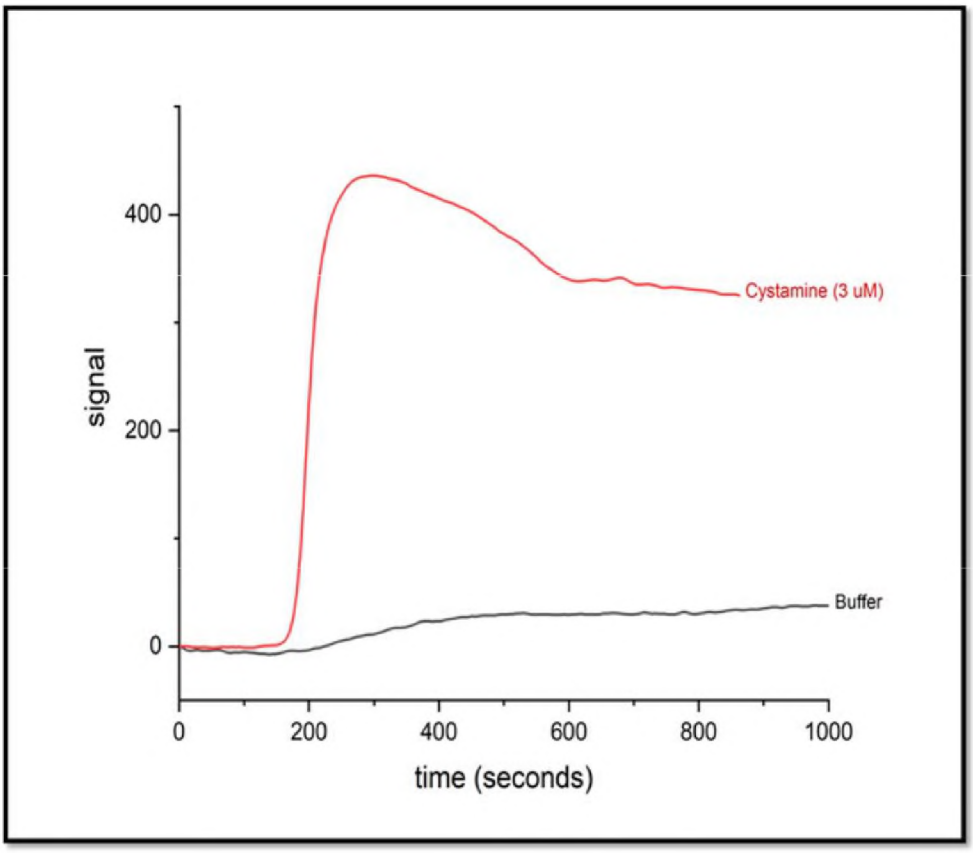


Figure 3.6. Typical SPR response of DNA-modified SPR chip to injections of running buffer and of 100 uL of 3.0 uM cystamine solution at a flow rate of 20 uL/min. The change with buffer injection is minimal. The increase in the SPR signal upon cystamine injection indicates that the DNA-modified nanoparticles on the SPR chips have bare spots that are further blocked with added cystamine.

After we confirmed that the surface of DNA-modified gold nanoparticles is fully blocked, and that the SPR signal is stable, we proceeded with peroxynitrite injections at different concentrations. Again, each injection is performed at 20ul/min for five minutes. Figure 3.7 shows that the addition of peroxynitrite (as aliquots stored in NaOH at pH 12) results in a decrease of the SPR signal. The decrease does not seem to be proportional to the increase in peroxynitrite concentration. Rather, repeated additions of PON in NaOH, no matter what the concentration used, resulted in gradual decrease in the SPR signal. We therefore suspected that the degradation of the SPR signal may be simply due to the chemical effect of the highly alkaline pH in which PON is stored.

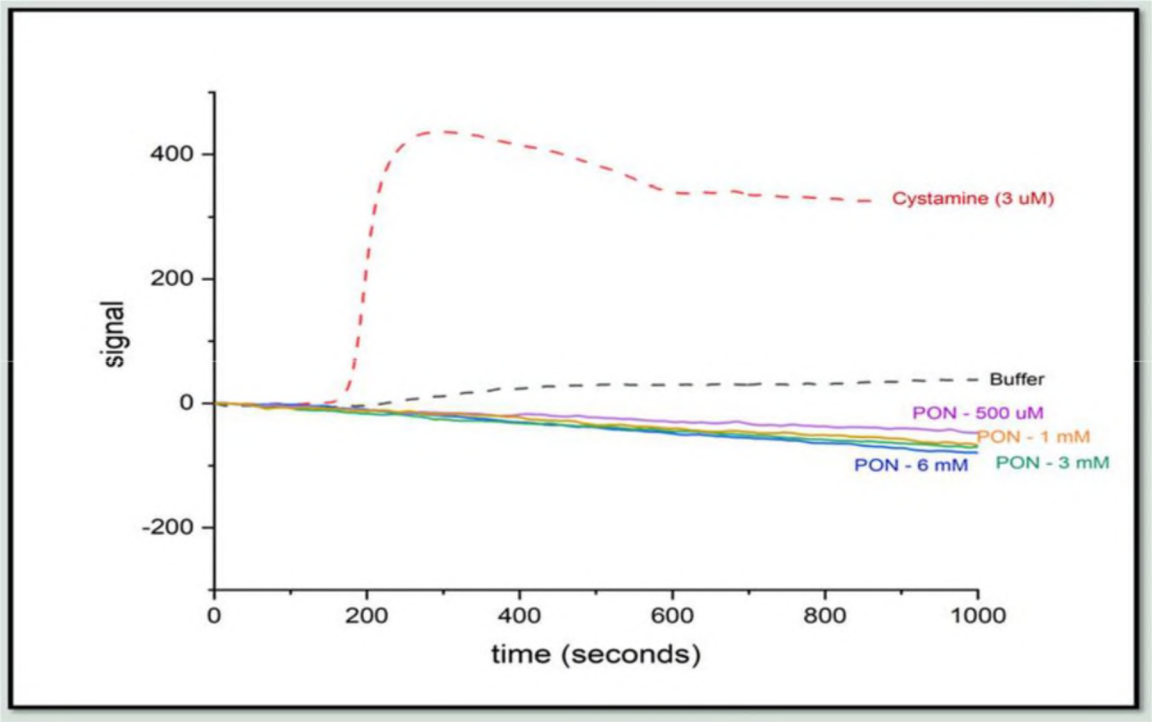


Figure 3.7. Typical SPR response of DNA-modified SPR chip to injections of running buffer and of 100 uL of 3.0 uM cystamine solution at a flow rate of 20 uL/min. The change with buffer injection is minimal. The increase in the SPR signal upon cystamine injection indicates that the DNA-modified nanoparticles on the SPR chips have bare spots that are further blocked with added cystamine.

In order to confirm if the mere high concentration hydroxide solution is behind the indiscriminate decrease of the SPR signal, we examined the effect of injections of NaOH at the same pH=12 but in absence of PON. Figure 3.8 shows that the addition of plain NaOH at pH=12 results in a significant decrease of the SPR signal. The decrease of the SPR signal may simply be the result of the degradation of the SPR chip with the possible removal of the immobilized DNA. In fact, high pH is known to favor thiol dissociation from gold nanoparticles.[54]

While we do not know the mechanism of adhesion of the gold nanoparticles to the glass substrate (Nicoya's proprietary information), it seems that the high pH seems to also contribute in washing away the nanoparticles off the glass surface as supported by visual examination of the chips before and after injections of NaOH at pH=12 (Figure 3.9).

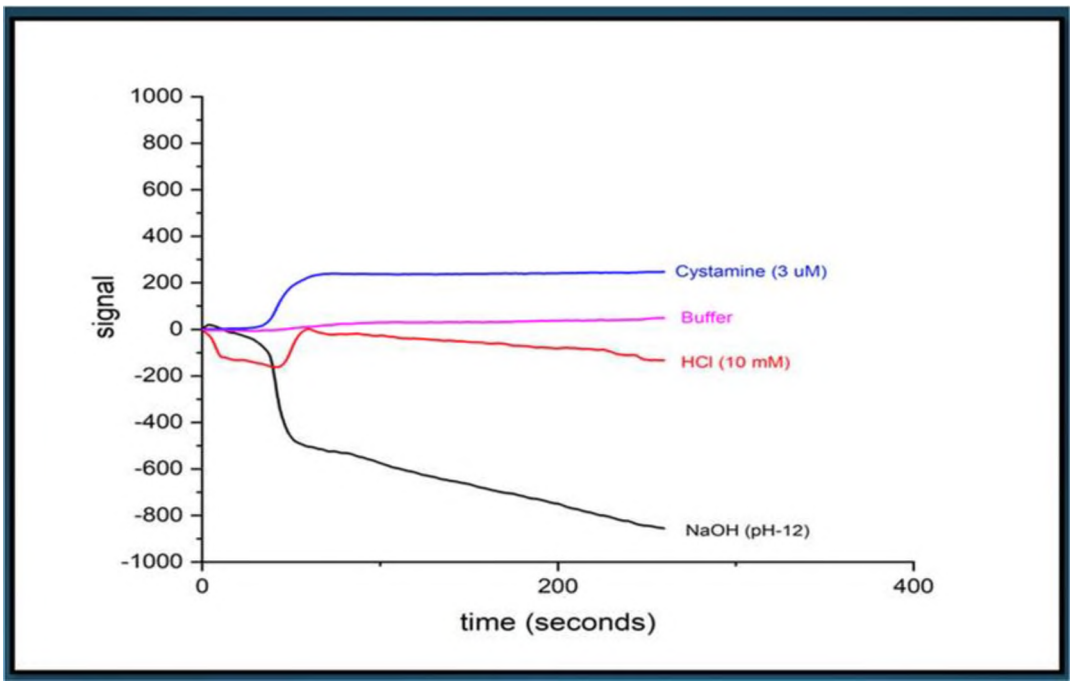


Figure 3.8. Typical SPR response of DNA-modified SPR chip to injections of running buffer and of 100 uL of 3.0 uM cystamine solution at a flow rate of 20 uL/min. The change with buffer injection is minimal. The increase in the SPR signal upon cystamine injection indicates that the DNA-modified nanoparticles on the SPR chips have bare spots that are further blocked with added cystamine.

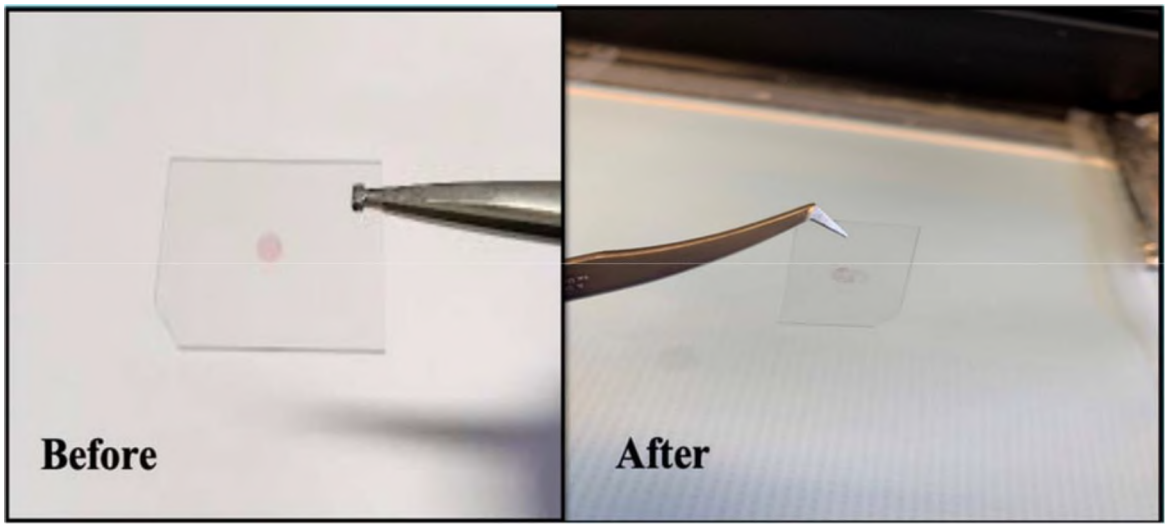


Figure 3.9. Exposure of the SPR chip to repeated injections of NaOH at pH 12 results in the degradation of the chip with clear visual decrease of nanogold on the glass surface.

Given the intrinsic degradation problems that high pH aqueous NaOH storing medium brings, we wanted to know if we can work with PON at relatively neutral pH. We examined if we used peroxynitrite stored at pH 7.4. Peroxynitrite is relatively unstable at low pH and this is one reason why we store it at high pH. However, using absorbance spectroscopy, we monitored the degree of degradation of PON at pH 7.4 (5 mM PBS, 50 mM NaCl). We monitored the absorbance of peroxynitrite absorbance band at 302 nm over time. It turns out that after 50 minutes in this medium, only 7.9% of the original PON degraded. The typical SPR experiment takes much less time. We therefore decided to work with peroxynitrite at pH 7.4 to monitor the interaction of PON with the immobilized thiolated DNA on the gold nanoparticles on the SPR chips. To this end, a small volume of PON from the original concentrated stock solution at pH 12 and is withdrawn and then diluted into a larger volume of PBS buffer at pH 7.4. the solution is kept on ice for a few minutes at most before injection (Figure 3.10)

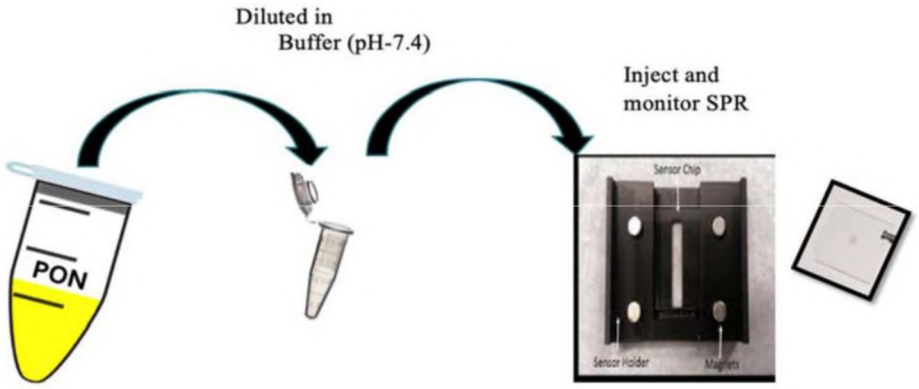


Figure 3.10. Transfer of PON form high pH to physiologic pH at 7.4 prior to injection into the microfluidic cells with the DNA-modified SPR chips.

Figure 3.11 shows the sensor response of DNA-modified gold nanoparticles SPR chip to peroxynitrite injections at pH 7.4 at increasing peroxynitrite concentrations.

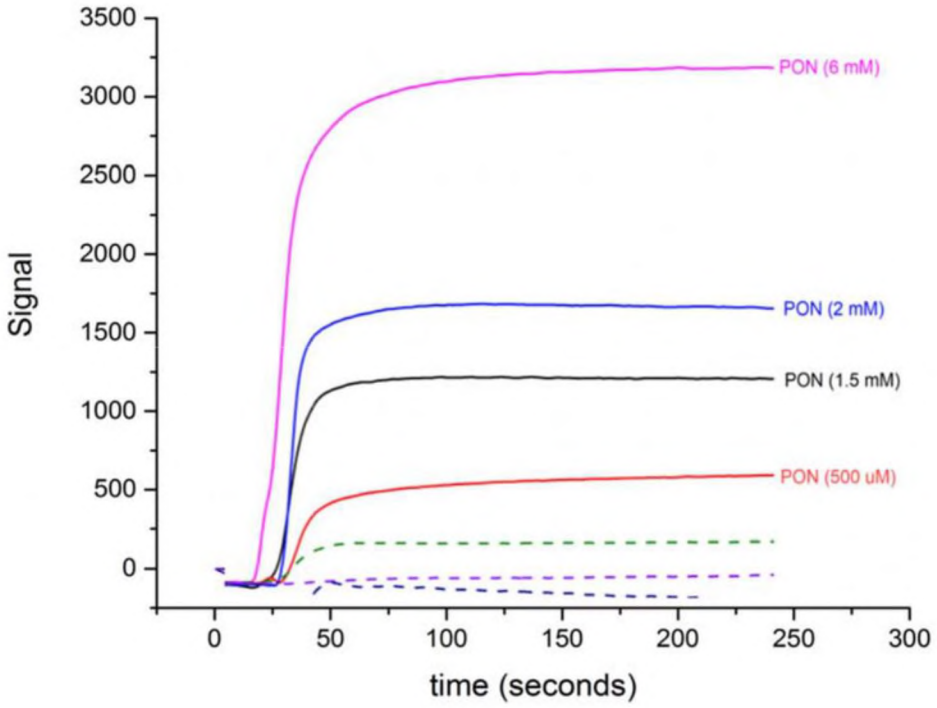


Figure 3.11. Typical SPR response of DNA-modified SPR chip to injections of peroxynitrite at pH 7.4 with increasing concentrations from 500 uM to 6 mM (solid traces). The dotted curves are the base line responses to running buffer and 3.0 uM cystamine solution at a flow rate of 20 uL/min.

It is interesting to note that the SPR response increases in a relatively proportional way with the concentration of peroxynitrite over the range of 0.5 mM to 6.0 mM.

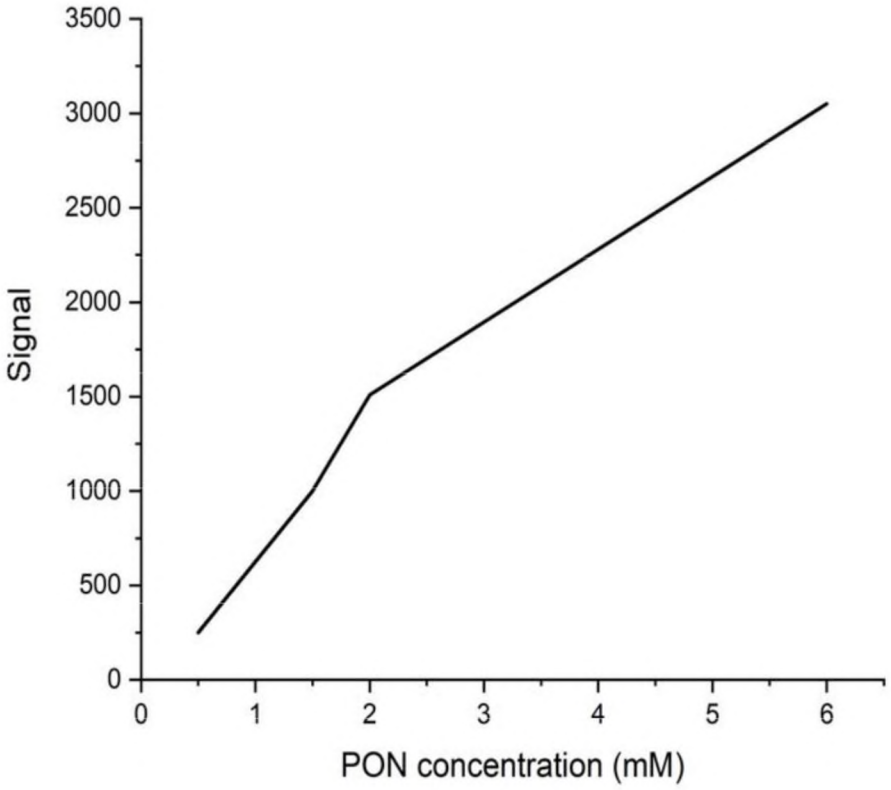


Figure 3.12. Typical SPR response with increasing concentrations of peroxynitrite

The reactive nature of peroxynitrite with DNA bases and backbone is known to result in significant chemical damage and consequently an expected unwinding of the hybridized oligonucleotide probes attached to the gold nanoparticles. This structural "swelling" of the DNA probes attached to gold nanoparticles upon peroxynitrite additions, combined with the efficient detection of subtle conformational changes using the localized

surface plasmon resonance (LSPR) are attributed to the observed changes in the SPR signal.

Conclusion. DNA-modified gold nanoparticles on SPR chips can be used to monitor the reactivity of peroxynitrite with the DNA double helix probes. The observed change in the SPR signal as a function of peroxynitrite concentration is due to structural changes of DNA probes induced by chemical damage combined with the efficient detection of conformational changes on gold nanoparticles taking advantage of efficient field confinement in localized surface plasmon resonance.

Specific Aim 4: Compare and Contrast SPR Results with Electrochemical Methods using the same DNA Sequences Immobilized on Electrode Surface.

Apparatus. All electrochemical measurements were performed using a CHI-440 electrochemical workstation interfaced with a PC system.

Electrodes. Gold electrodes (CHI Inc.) were cleaned and polished before surface modification. Electrodes were hand-polished with 0.3- and 0.05-micron alumina slurries on a Buehler micro-cloth, followed by sonication for 15 min to ensure the elimination of the alumina particles from the surface before surface modification procedures. This procedure was repeated for each set of experiments.

Cell Setup. Standard glass multi-armed electrochemical cells were used. The cells were thoroughly rinsed with de-ionized water before use. The three electrodes consisted of the gold electrode as the working electrode, a platinum wire as an auxiliary electrode, and Ag/AgCl (3.0 M KCl) was used as the reference electrode.

Electrochemical study of the interaction of peroxynitrite with DNA probes on electrodes. We next examined the interaction of peroxynitrite with immobilized DNA on gold electrodes using electrochemical means.

Surface modification procedures. DNA probes were immobilized on polished gold electrodes by casting a 10-ul drop of thiolated hybridized DNA on the gold electrode surface. The electrode is then covered and kept in a chamber with controlled moisture overnight to prevent evaporation and to allow enough time for the oligonucleotide to interact with the gold surface. After immobilization, the electrode is washed gently and stored until the time of electrochemical analysis.

Electrochemical investigation of the interaction of peroxynitrite with DNA probes immobilized on gold electrodes. The presence of immobilized DNA and the interaction of peroxynitrite with immobilized DNA are studied using electrochemically active species.

First, we take advantage of ferricyanide as a negatively charged reversible redox couple to monitor DNA immobilization on gold electrodes. The presence of negatively charged DNA probes on the electrode surface should repel ferricyanide from the electrode surface resulting in small or no reduction current of this redox probe. This is exactly what we observe in Figure 3.13. This figure shows the ferricyanide redox couple with a standard potential as expected at 0.2 V/Ag-AgCl[55] on a bare gold electrode. The reduction and reverse oxidation currents are the result of direct electron exchange with the electrode surface. When thiolated DNA is immobilized on the same gold electrode, the redox couple disappears because the negatively charged DNA film prevents ferricyanide from reaching the electrode surface and undergoing direct electrochemical reduction.

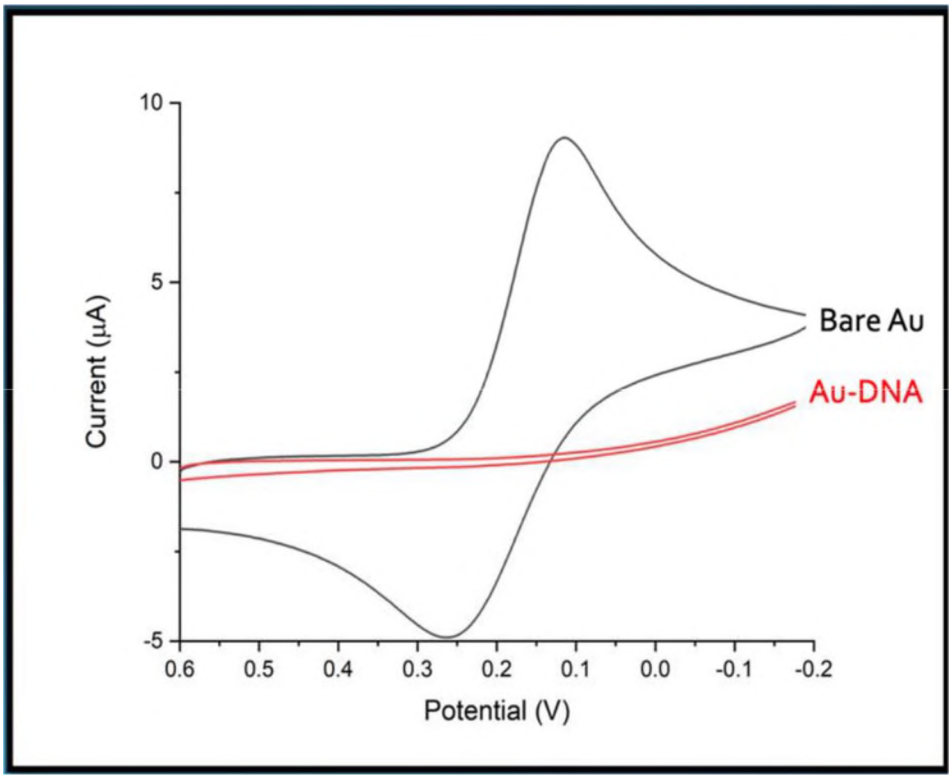


Figure 3.13. Cyclic voltammograms of 2.0 mM $K_3[Fe(CN)_6]$ at a bare gold electrode (black trace) and at the same electrode after immobilization of thiolated DNA (oligo# 1 hybridized to its complementary sequence) (red trace) in phosphate buffer pH 7.4

In order to investigate the interaction of peroxynitrite with the selected DNA probes we examined the response of DNA-modified electrode in the presence of ferricyanide solution after exposure to various concentrations of peroxynitrite. To this end, we incubate the DNA-modified gold electrode in peroxynitrite at a given concentration for 20 minutes. The electrode is then washed with deionized water and buffer before it is immersed back into the test solution in the presence of ferricyanide.

Figure 3.14 shows the typical response of DNA immobilized gold electrode after exposure to peroxynitrite solution with increasing concentration. We observe that bathing the DNA-modified electrode in peroxynitrite solution with increasing concentration results in gradual recovery of the current of the ferricyanide redox probe. This behavior is not unexpected. In fact, the interaction of peroxynitrite with the immobilized DNA is expected

to result in chemical damage and unwinding of the DNA double helices within the DNA film immobilized on the electrode surface.

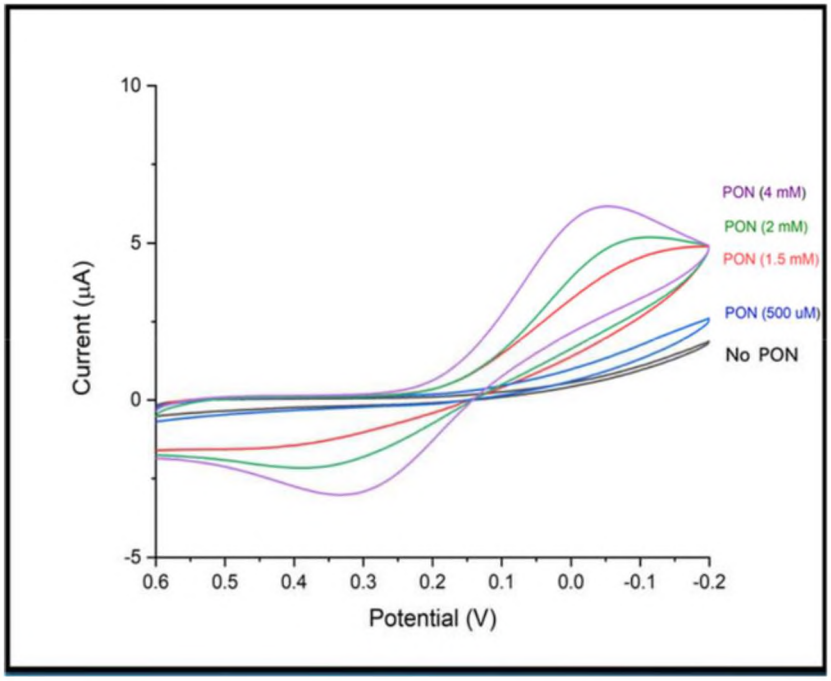
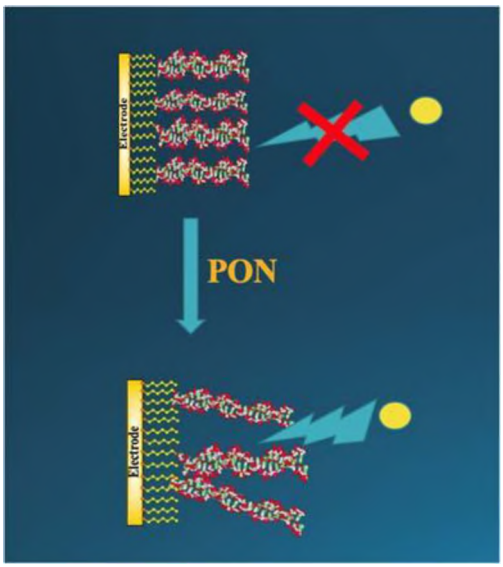


Figure 3.14. Cyclic voltammograms of 2.0 mM $K_3[Fe(CN)_6]$ at a DNA-modified electrode (oligo# 1 hybridized to its complementary sequence) in phosphate buffer pH 7.4 after exposure to increasing peroxynitrite concentrations

The putative chemical damage induced by peroxynitrite opens the DNA film and introduces structural defects (Scheme 3.2). As a result, this allows the ferricyanide redox probe in solution to reach the electrode surface and undergo direct electrochemical reduction, which explains the gradual increase of the redox current.

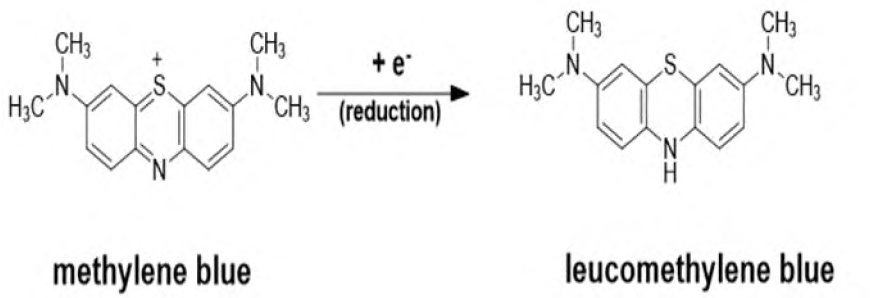


Scheme 3.2. Representations of DNA-modified electrodes before and after reaction with peroxynitrite (PON): (Top) Before incubation in PON solution. Ferricyanide redox probes (represented by yellow ovals) are repelled and not permitted to exchange electrons with the electrode surface. (Bottom) After exposure and incubation in PON solution. In this case ferricyanide can undergo direct electrochemical reduction with the electrode through film defects induced by chemical damage.

Analysis of ferricyanide's reduction current increase as a function of the increase in PON concentration shows a linear relationship from 0.5 mM until 2 mM. This behavior is smilar to what we observe in SPR although the slope of the linear relationship of signal increase as function of PON concentration is higher for the SPR method.

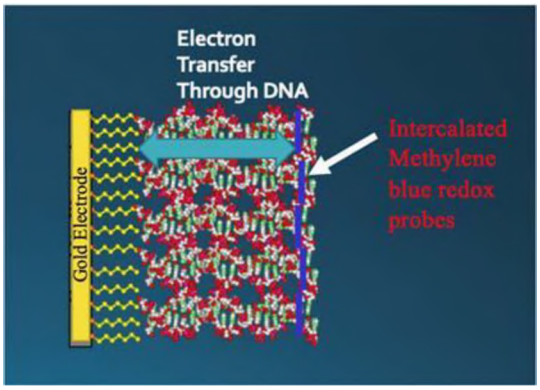
We also noticed that this electrochemical method is not very sensitive for the low concentration of PON (500 uM or lower). We therefore explored another electrochemical method that rather relies on electrocatalysis at DNA-modified electrodes. We wanted to examine if the fact that this method is electrocatalytic in nature results in a method that is potentially more sensitive for lower concentrations of PON (lower that 500 uM).

To this end, we called upon an aromatic redox probe, methylene blue, that is known to undergo reversible reduction in solution with a bare electrode according to the the equation in Scheme 3.3.



Scheme 3.3. Electrochemical reduction of methylene blue to its reduced form. The reduction is electrochemically reversible.

Methylene blue is also well-known to intercalate between DNA bases. If hybridized oligonucleotides are immobilized on an electrode, the intercalated methylene blue redox probes are capable of exchanging electrons with the electrode surface through the attached DNA double helix (-way), Scheme 3.4.



Scheme 3.4. Representation of a DNA-modified electrode with intercalated methylene blue (blue bars) at the top of the DNA film. This representation depicts the possible electron transfer through the aromatic bases of hybridized DNA

However this electrochemical current is expected to be very small since the amount of redox probes (methylene blue) intercalated at the outside of the compact DNA film on the electrode is very small.

Figure 3.15 shows the relative responses of the redox current of methylene blue in solution on a bare gold electrode (Red trace; redox couple at -0.2 V vs. Ag/Ag/Cl[56]). This is to be compared to the response of the limited amount of methylene blue intercalated at the outside of the compact DNA immobilized on the gold electrode.

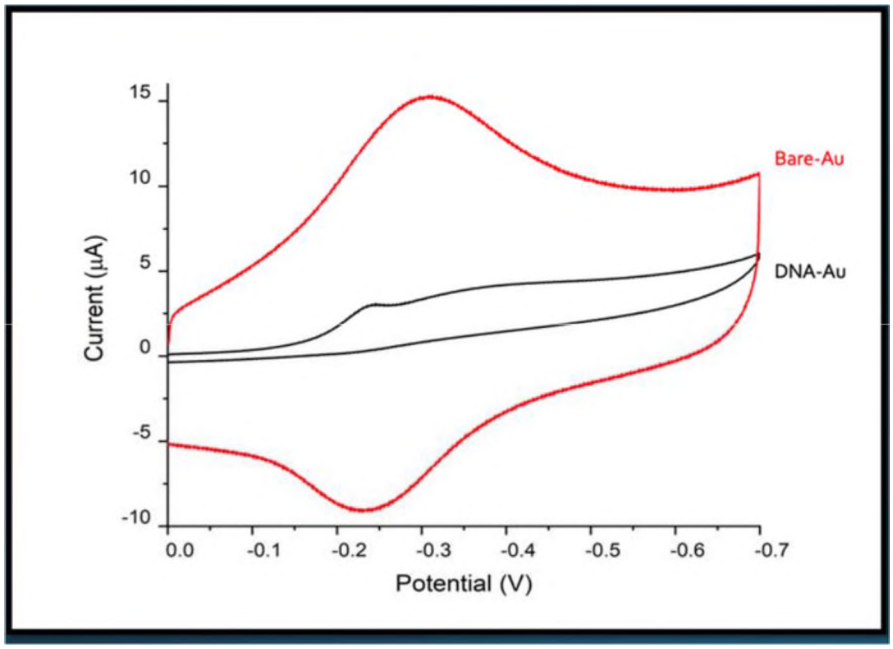
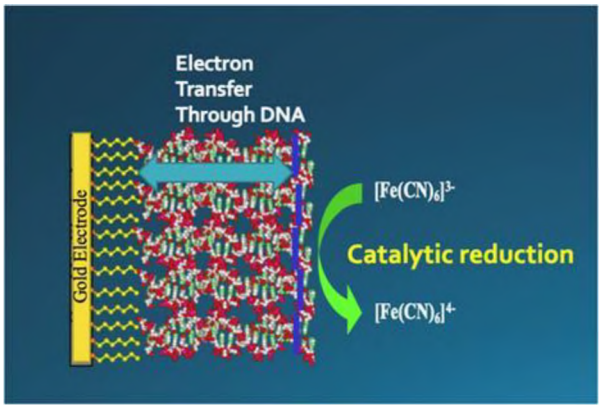


Figure 3.15. Cyclic voltammograms of methylene blue in solution at a bare gold electrode (red trace) and at a DNA-modified electrode (DNA -modified electrode: oligo #1 hybridized to its complementary sequence) in phosphate buffer pH 7.4 after exposure to increasing peroxynitrite concentrations

When reduced, the intercalated methylene blue at the outside of the compact DNA layer is capable of transfering electrons to a proper redox active molecule in solution if that species is easier to reduce (i.e. has a more positive redox potential). Ferricyanide fits very well this requirement and has been used for this purpose in similar situations.[57] In fact, as we showed earlier, ferricyanide's redox potential is at +0.2 V vs. Ag/AgCl, which is about 400 mV more positive than methylene blue. This situation makes the electron transfer from

reduced methylene blue intercalated at the outside of the compact DNA film to the ferricyanide in solution thermodynamically feasible. Figure 3.16 shows a significant increase of the methylene blue reduction curent when we add 2 mM ferricyanide to the solution. The current increase is the result of an electrocalalytic process where the reduction of methylene blue through immobilized DNA layer is coupled to the reduction of ferricyanide as a freely diffusing redox probe, [58] as illustrated in Scheme 3.5.



Scheme 3.5. Representation of a DNA- modified electrode with intercalated methylene blue (blue bars) at the outside of the DNA film. The reduction of ferricyanide by intercalated reduced methylene blue drives an electrocatalytic process that result in a significant increase of current.

It is important to note that the negatively charged ferricyanide cannot enter the negatively charged DNA film and interacts with methylene blue only at the outside of the DNA layer. The electrocatalytic process (reduction of freely diffusing ferricyanide mediated by DNA-intercalated methylene blue) is manifested through a significant increase of the reduction current upon addition of ferricyanide.

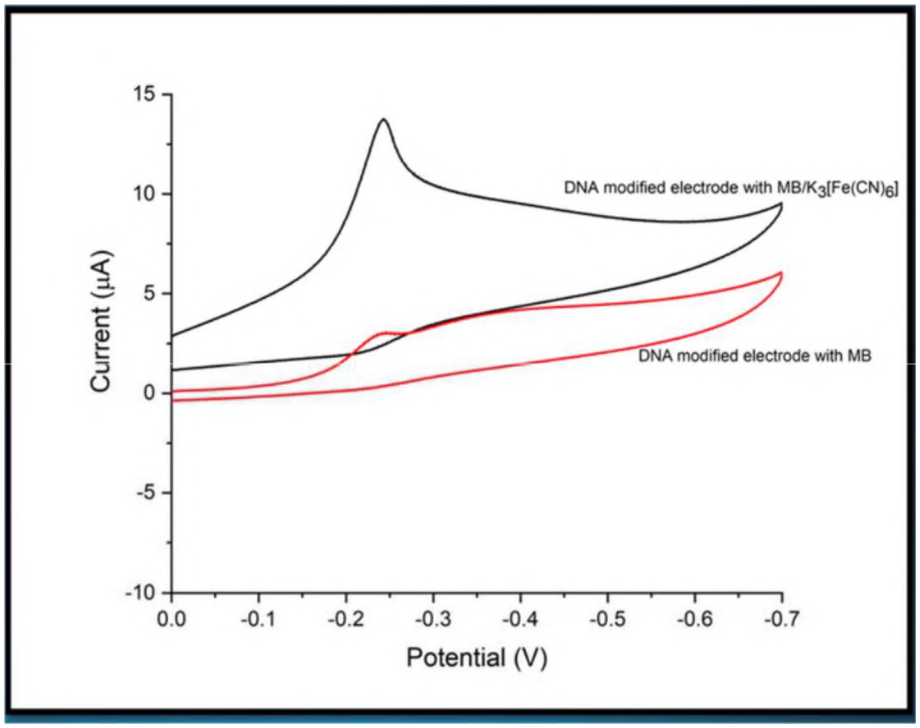


Figure 3.16. Cyclic voltammograms of methylene blue intercalated in immobilized DNA (DNA-modified electrode: oligo #1 hybridized to its complementary sequence) in phosphate buffer pH 7.4 (red trace). Response of the same electrode after addition of 2.0 mM ferricyanide to the solution (black trace)

This electrocatalytic process with significant increase in current through intercalated methylene blue is only efficient if the immobilized DNA film maintains its structural integrity that makes electron transfer through its stacked aromatic bases possible. We therefore examined if we can use this system to monitor DNA damage induced by peroxynitrite.

Figure 3.17 shows that exposure to a peroxynitrite concentration as small as 500 uM, results in almost complete collapse of the electrocatalytic current. This indicates that the catalytic efficiency driven by ferricyanide reduction in solution is tightly dependent on the strucural integrity of the immobilized DNA film where the mediator methylene blue is intercalated.

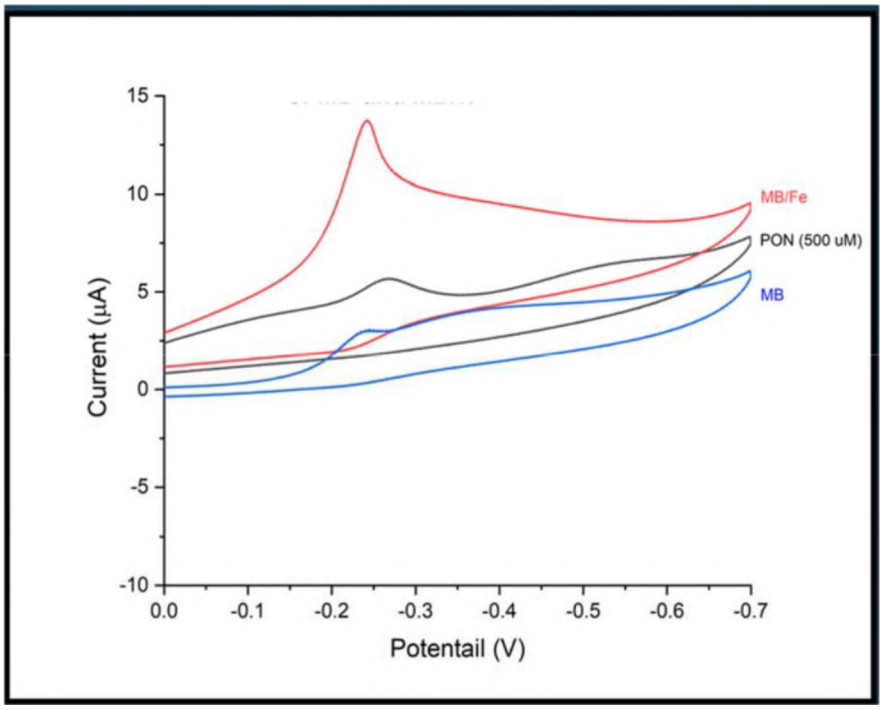


Figure 3.17. Cyclic voltammograms of methylene blue intercalated in immobilized DNA (DNA-modified electrode: oligo #1 hybridized to its complementary sequence) in phosphate buffer pH 7.4 (blue trace). Response of the same electrode after addition of 2.0 mM ferricyanide to the solution (red trace). Response of the same electrode after incubation in peroxynitrite solution with a concentration of 500 uM (black trace).

CHAPTER IV

CONCLUSIONS AND FUTURE PROSPECTS

The fact that the "ferricyanide/methylene blue/DNA" electrocatalytic system is significanlty sensitive to a concentraton of peroxynitrite as small as 500 uM, gives us a system to monitor DNA damage induced by very small concentrations of peroxynitrite. This is important if we want to study the very subtle structural changes in the DNA double helix after transient exposure to very small peroxynitrite concentration. On the other hand, the non catalytic simple ferricyanide system on immobilized DNA gives the possibility to monitor the effect of peroxynitrite-induced DNA damage at moderate concentrations above 500uM. The simple ferricyanide system exhibits a linear relationship between the peroxynitrite concentration used and the observed current increases as expected. This is similar to our observation using the change of SPR signal on the surface of gold nanoparticles in localized SPR (LSPR) mode.

In the future, we will explore the linear range of peroxynitrite concentration versus DNA damage at moderate (millimolar) concentrations for both SPR and the ferricyanide electrochemical system.

We will also examine the effect of a range of lower concentrations (nanomolar to low micromolar) using the ferricyanide/methylene blue/DNA electrocatalytic system and compare it to the SPR method for the same range.

Finally, we plan to vary the nature of DNA sequences used (i.e. various G content) to make the immobilized DNA more suscepltible to oxidative reactivity with peroxynitrite. To this end, we will monitor how SPR and electrochemical methods compare in predicting the extent of DNA chemical damage induced by peroxynitrite.

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