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Bio-Nano Interfaces: Enzyme Immobilization for Biomimetic Energy Harvesting

Rachel Hjelm

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Bio-Nano Interfaces: Enzyme Immobilization for Biomimetic Energy Harvesting

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Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Engineering, Nanoscience and Microsystems
The University of New Mexico, Albuquerque, New Mexico
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Abstract

In the face of today’s rapidly growing energy demands accompanied by limited, non-renewable supplies, development of novel energy alternatives that are both renewable and inexpensive has become more important than ever. Development of 3D structures exploring the properties of nano-materials and biological molecules has been shown through the years as an effective path forward for the design of advanced bio-nano architectures for enzymatic fuel cells (EFCs). Despite advantages over conventional fuel cells, EFCs still suffer from several problems including low efficiency and stability. Overcoming these limitations in order to make them more viable for real world application is an ongoing challenge for researchers.

Functionalized carbon nanotubes (CNTs) were covalently bonded to diazonium salt modified gold surfaces through carbodiimide chemistry creating a brush-type nanotube alignment. Having CNTs ordered in this nature developed a highly ordered structure with markedly high surface area that allowed for the attachment of protein/DNA assembly. The specificity of the enzyme immobilization was controlled by small protein structural motifs,
called zinc fingers (ZnF) that bind to specific dsDNA sequences and may be genetically bound to small laccase (SLAC-3ZnF) or other redox enzymes. Utilizing scaffolds with gold nanoclusters (AuNC) for mediated electron transfer (MET), we test the capabilities for oxygen reduction reactions (ORR) by SLAC-3ZnF. Direct absorption on SWNT results in poor ORR while using DNA results in slowed reaction kinetics. With the addition of AuNC, ORR and electron transfer are improved. Analytical techniques such as x-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), and enzymatic activity analysis, allowed characterization at each stage of development.

Additionally, two methods for co-immobilization on consecutive tricarboxylic acid cycle (TCA) enzymes were investigated. Encapsulation using deacetylated chitosan (chit) and tethering through amide bond linkage with 1-pyrenebutanoic acid, succinimidyl ester (PBSE) were used to immobilize porcine heart malate dehydrogenase (MDH) and citrate synthase (CS) on multi-walled carbon nanotubes (MWNTs). The effects of each method on the oxidation of L-malate (M) by MDH in the presence of CS are reported. Poly-(methylenegreen)-modified MWNT “buckypaper” (PMG-BP) was used to reduce cell overpotential through regeneration of oxidized nicotinamide adenine dinucleotide (NAD\(^{\prime}\)) from reduced form (NADH).
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Chapter 1: Biomimetic Energy Harvesting

Biomimetic energy harvesting is a process that utilizes artificially built systems of biological molecules such as enzymes for the purpose of mimicking natural energy processes that occur in cells. The use of biological element for catalysis in energy harvesting has its advantages over traditional methods [1]. Reductive or oxidative enzymes may be used for this purpose. Enzymatic fuel cells (EFCs) for example are similar to conventional fuel cells, however they differ in the type of catalyst being used. Conventional fuel cells use inorganic catalysts such metal and metal oxides, which are generally expensive, non-renewable and can suffer from fuel cross-over and poisoning. Since enzymes are derived from living cells, they are easy to mass produce and can be specifically designed to process organic compounds such as sugars or alcohols. These biofuels used can be manufactured cheaply and on a mass scale.

1.1 Fuel Cell Concepts

A fuel cell is an electrochemical device used to produce electricity from chemical energy or visa versa and can either produce energy from chemicals within the cell or through channels from outside of the cell [2]. The schematic in Figure 1 shows the design, reactions and electron flow of a general fuel cell which consists of an anode and cathode. Unlike batteries where chemicals present react with each other to produce electricity; fuel cells use a continuous source of fuel to operate. Oxidation occurs at the anode driven forward by catalysts. In a conventional fuel cell, these catalysts are generally metals such as platinum.
An example of a conventional technology is a proton exchange membrane (PEM) fuel cell. PEM cells are low temperature/pressure systems that produce electrical energy from the reaction between hydrogen and oxygen:

**Anode:** \[ H_2 \rightarrow 2H^+ + 2e^- \] \hspace{1cm} (Eq. 1)

**Cathode:** \[ \frac{1}{2} O_2 + 2H^+ + 2e^- \rightarrow H_2O \] \hspace{1cm} (Eq. 2)

**Overall:** \[ H_2 + \frac{1}{2} O_2 \rightarrow H_2O \] \hspace{1cm} (Eq. 3)
These cells function by transporting protons through a membrane that is located between electrodes. At the anode, \( \text{H}_2 \) is split using catalysts like platinum (Eq. 1), while at the cathode an oxygen reduction reaction (ORR) occurs from reaction of \( \text{O}_2 \) with the proton transported through the membrane and electrons from an external circuit (Eq. 2). Platinum is a common catalyst used in PEM and considered the most effective. Eq. 3 shows the overall cell reaction that takes place.

Fuel cell performance can be directly attributed to the types of fuel and catalyst that are used in the devices. Looking at fuel cells from a thermodynamic perspective can give a general idea of how a cell should perform given absolutely ideal conditions and one hundred percent maximum output without energy loss. The theoretical values of chemical energy conversion to electricity can be described through thermodynamic calculation. For instance, the theoretical maximum amount of chemical energy that a system can convert to useful electricity is given by the change in free energy (\( \Delta G \)) (Eq. 4). This states that the energy of a system at given conditions is dependent on the absolute temperature (\( T_{\text{ref}} \)) and pressure (\( P_{\text{ref}} \)) of the surrounding systems; and the chemical energy (\( U \)), entropy (\( S \)), and volume (\( V \)) of the cell itself. The increase seen in chemical energy inside the cell according to the classical law of conversion (1st law of thermodynamics) can be put in terms of external energy added to the system as seen in Eq. 5, where \( \Delta U \) is the change in energy of the system; \( H \) is the enthalpy and \( W \) is the mechanical or electric work done by the surrounding on the system.

\[
\Delta G = \Delta U - T_{\text{ref}} \Delta S + P_{\text{ref}} \Delta V \quad \text{(Eq. 4)}
\]
\[ \Delta U = \int dH + \int dW \]  
(Eq. 5)

The amount of work (W) done by an electrochemical device on the surrounding can also be described in the same way. When the volume of a device is held constant, the last term which describes the behavior with change of volume is zero. The electric work \( \Delta W_{\text{elec}} \), the work done by a device attached to an external circuit can also be expressed as the difference in potential between the cathode and the anode (Eq. 6). In devices where volume is held constant the energy produced will correspond to the loss of free energy from the cell (Eq. 7) [2].

\[ -\Delta W = -\Delta W_{\text{elec}} + \int PdV \]  
(Eq. 6)

\[ -W_{\text{elec}} = -\Delta G \]  
(Eq. 7)

The change in standard Gibbs free energy (\( \Delta G^o \)) can be related to the ideal cell potential by the equilibrium constant (\( K_{eq} \)) and by the Faraday constant (Eq. 8) where \( R \) is the ideal gas constant (8.314 J/(mol K)). The concentration of reduced products over oxidized reactants (\( Q = [\text{red}]/[\text{ox}] \)) when the system is at equilibrium such that the cell potential (E) expressed in volts is zero, is equal to \( K_{eq} \). The ideal potential of an electrochemical cell (\( E_{\text{cell}} \)) can be described by the Nernst equation which relates cell potential to the concentration of reactants and products (Q) in the cell and the standard cell potential (\( E_{\text{cell}}^o \)) for either the full (Eq. 9) or half reaction.
\[
\Delta G^\circ = -RT \ln K_{eq} = -nF E^\circ \quad \text{(Eq. 8)}
\]

\[
E_{cell} = E_{cell}^\circ - \frac{RT}{nF} \ln Q \quad \text{(Eq. 9)}
\]

It is unfortunately not possible for fuel cells to match the ideal thermodynamic maximum energies and cell performance. Ideal performance relies on no loss in electric energy when current is drawn. The ideal cell is contrary to what is actually observed due to irreversible losses. Cell potential losses called polarization or overpotential (\(\eta\)) are caused by a variety of factors but result in less than ideal cell behavior.

1.2 Enzyme-Based Fuel Cells

There are many features of EFCs that make them a favorable choice over batteries and conventional fuel cells, such as selectivity and specificity of enzymatic substrate binding. Hypothetically, this reduces chances of fuel cross overs and simplifies the fuel cell design reducing the need for compartmentalization of electrodes. The fuel is also generally safer and less expensive to mass-produce. EFCs can also operate under mild pH and temperatures (pH=7, T=25-37°C) and are therefore explored as biosensors. Problems that arise from using enzymes for electrocatalysis are low power densities and short lifetimes due to the fragility of enzymes outside of their natural biological cell environment. One of the reasons for this problem that researchers are trying to overcome is the proper immobilization of enzymes on the electrode in order to increase electron flow to or from the surface as well as increase stability/lifetime [4, 5]. Another problem is that
most EFCs generally only utilize one type of enzyme for fuel oxidation. This means that the fuel may not be fully oxidized and full power potentials not reached. [3, 6, 7].

There are two mechanism utilized in EFCs for electron transfer. The first is mediated electron transfer (MET) and the second is direct electron transfer (DET) (Figure 2). In MET, an additional reactant or cofactor is present to aid in shuttling electrons to or from an electrode surface. In the case of anodic cells that use dehydrogenases that are dependent on nicotinamide adenine dinucleotide (NAD+/NADH), which is used to transport the electrons either being free in buffer solution or immobilized on the electrode surface. Additional means may be used, as with films that may oxidize the reduced form (NADH) regenerating oxidized form (NAD\(^+\)). Mediators may often help to reduce cell overpotential, enhance reaction kinetics and increase electron density. However, a mediator may also increase the distance from the electrode when immobilized on the surface, in effect increasing electron tunneling time and reducing cell performance. Ideally, not having to rely on a mediator would diminish this affect such as with DET, however the design of electrodes is highly dependent on enzymatic structural and catalytic properties.
Figure 2: Representation of a) mediated electron transfer for oxidation of glucose to gluconolactone by glucose oxidase with ferrocene and b) direct electron transfer of electron to laccase for oxygen reduction to water [3].

DET is most commonly used in reduction at the cathode, particularly with oxygen reduction reactions (ORR), where O$_2$ is reduced to H$_2$O. Multi-copper oxidases (MCOs), such as laccase or bilirubin oxidase are the most commonly used enzymes for catalyzing enzymatic ORR.

Solving the issues with using enzymes for biocatalysis is still a problem for researchers and design of systems that overcome these challenges are being extensively researched. The designs that have been implemented utilize immobilization as a method for increasing electron flux, increasing stability and improving overall cell performance. These techniques include various immobilization methods [8-10], MET [11, 12], DET [13, 14], and use of nanomaterials [9, 15-18] for improved catalyst stability and higher power outputs.

1.1. Electron Transfer Between Enzymes and Electrodes

Oxidoreductase enzymes normally interact with a biological cofactor to transfer electrons between the active site and a substrate. Some of these natural cofactors (FAD, NAD, NADP), although effective in their native biological settings, have poor electrochemical properties at common electrode materials, such as carbon, gold, and platinum, and can be expensive. Therefore, to successfully transport electrons between enzymes and electrode surfaces, natural cofactors are frequently replaced, altered, or removed completely, depending on the enzyme and the type of biofuel cell. Biofuel cells can be categorized by the type of electron transfer used, and two methods have become predominant: mediated electron transfer (MET) and direct electron transfer (DET) (Figure 3a and b, respectively). In MET, molecules with multiple redox states are used to accept or donate electrons from the enzyme active site and to assist in transporting charge to or from the electrode.
Preferentially, EFCs may one day be used as an implantable power source since they may use the glucose in the blood as a fuel and can operate in physiological conditions. However, concerns of foreign body rejection by the body must be considered for their design. A more realistic application at the moment would be to use them for battery replacement. Current density from 1-25 mA/cm$^2$ [19-22] has been achieved which is enough to power a small electronic device. Most small batteries only produce about 100-200 mA/cm$^2$.

1.3 Multi-Enzyme Cascades

It has long been a hypothesis that enzymes involved in cellular reaction pathways, including the tricarboxylic acid cycle (TCA; also known as the Krebs cycle), spontaneously and physically interact with one another forming multi-enzyme complexes (MECs) called metabolons [23, 24]. The formation of MECs is suggested and in some cases observed to be the reason for the high efficiency of energy producing pathways due to sufficient transport of substrates between active sites [23-25]. This observation has made using artificial MECs an attractive concept in the development of bio-mimetic devices such as bio-sensors and enzyme-based biofuel cells (EFCs) [1, 6, 26]. In recent years, the importance of having multiple enzymes in a redox system has been demonstrated through the development of electrodes for bioelectrocatalysis [27-30]. The goal is to create artificial pathways that mimic the natural metabolic cycles for deep or complete oxidation of fuels such as ethanol, methanol and pyruvate [11, 29-31] Figure 3 shows the general concept of MEC’s and the mass transport of substrates/electrons through the complex.
Evidence for this concept was first presented by the complete fuel oxidation of methanol using alcohol dehydrogenase, aldehyde dehydrogenase and formate dehydrogenase [33]. In 2009, Arechderra et al. demonstrated the complete oxidation of glycerol using a three-enzyme system finding that fuel oxidation was at only 70% without a third enzyme present [29, 34]. The same year, Sokic-Lazic et al., showed the importance of using MECs utilizing natural metabolic pathways where complete oxidation occurred only in the presence of all TCA cycle enzymes do to product inhibition (Figure 4). The power representation of five separate pyruvate/air bioanodes is shown here demonstrating the effect of multi-enzyme assemblies on cell power density [31].
The effect of each additional enzyme on the biofuel cell performance. (b) enhance substrate channeling in multienzyme cascades. Krebs cycle enzymes are immobilized on an electrode surface to achieve the complete oxidation of pyruvate, and a metabolon is used to carry for highly efficient and stable EFCs [3, 7, 27, 35]. Using MECs that oxidize fuel is one of the many problems associated with EFCs is the low power density that results from single enzyme utilization, thus novel methods for multi-enzyme cascades (MECs) must be developed and implemented. Natural metabolic processes observed in nature carry out complex fuel oxidation and reduction reactions through the use of MECs. The capability to reproduce these processes would help to ensure high-level fuel processing and allow for highly efficient and stable EFCs [3, 7, 27, 35]. Using MECs that oxidize fuel is one of the many problems associated with EFCs is the low power density that results from single enzyme utilization, thus novel methods for multi-enzyme cascades (MECs) must be developed and implemented. Natural metabolic processes observed in nature carry out complex fuel oxidation and reduction reactions through the use of MECs. The capability to reproduce these processes would help to ensure high-level fuel processing and allow for highly efficient and stable EFCs [3, 7, 27, 35].
an attempt to mimic the natural metabolic pathways, such as TCA is a current focus for EFC design. It has been demonstrated that TCA enzymes likely interact physically with each other forming a pathway for substrate channeling and electron transfer using NAD [25, 36-40]. The cycle, which takes place in mitochondrion, serves two major functions:

1. Supply electrons for respiration using cofactors such as NAD for electron transport;
2. Provide starting materials for biosynthesis.

The cycle is essentially a hub for multiple metabolic pathways including the breakdown of carbohydrates, fats and amino acids; and an important resource for energy supply producing three NADH molecules per cycle. It involves a series of reactions that cause the complete oxidation of acetyl coenzymes A (acetyl coA) to CO₂ and H₂O; and release of electrons. Acetyl coA is introduced into the cycle upon oxidation of pyruvate. The first enzyme that starts the cycle is citrate synthase (CS), which binds acetyl coA and oxaloacetate for the production of citrate and coA. Acetyl coA is completely oxidized after a sequence of eight reactions that end with regeneration of oxaloacetate from malate oxidation by malate dehydrogenase (MDH) and release of two CO₂ molecules (Figure 5).
Figure 5: Representation of tricarboxylic acid cycle.

The importance of a complete TCA cycle for pyruvate oxidation in biofuel cells was demonstrated by Sokic-Lazic et al.. In their study, bioanodes consisting of pyruvate dehydrogenase (PDH) with the addition of one or more successive TCA enzymes immobilized on poly-(methylen green)-modified carbon paper were tested. Although increasing oxidation was observed with additional dehydrogenases (DHs), the linear progression that was expected was not seen. Additionally, complete oxidation was not achieved until all enzymes were present where a 31.2-fold increase in power density was observed between the addition of fumerase and malate dehydrogenase (MDH) (Figure 4) [31]. Thus it was concluded that product inhibition plays a large role in EFC efficiency when complete oxidation is not achieved and the CO₂ is not released.
The research reported in Chapter 6 focuses on the use of mitochondrial MDH and CS from porcine heart for anodic cells. MDH is the final dehydrogenase in the TCA cycle. It is an oxidoreductase which catalyzes the oxidation of malate to oxaloacetate with the help of NAD$^+$ (Figure 6a) that in metabolism carries electrons to the electron transport chain (ETC) as NADH. In biocatalysis, it has been studied for its instability in aqueous forums and dependence on NAD. NAD has shown effectiveness in MET for NAD dependent catalysis using DHs such as MDH [33, 35, 41, 42].

Figure 6: a) Oxidation of L-malate with NAD$^+$ to oxaloacetate and NADH and b) reaction of acetyl-S-coA with oxaloacetate forming citrate and coA-SH.

The interactions between MDH and CS including the channeling of oxaloacetate through MDH/CS complexes have been studied thoroughly [23, 25, 36, 37, 40, 43]. Citrate formed from reaction between oxaloacetate and acetyl coA (Figure 6b), has been observed
to participate in MDH regulation by possible allosteric binding. Activation of MDH L-
malate oxidation is seen at relatively high concentrations of NAD$^+$ ($[\text{NAD}^+] \geq 5\text{mM}$) regardless of L-malate concentration while inhibition of oxaloacetate binding is observed regardless of NADH concentration [44, 45]. This is significant because the formation of oxaloacetate is highly unfavorable using both CS and MDH meaning that the equilibrium in the presence of citrate is shifted in the direction of oxaloacetate production, making it a useful mechanism for increasing reaction rates in MDH-based cells.

The challenge faced by researchers for MECs-systems is the development of immobilization methods that are stable, allow multiple enzymes to align in the spatial orientation necessary for functional and efficient catalytic pathways, facilitate electron transfer (ET) to and from electrode surfaces, and result in complete fuel oxidation for optimized power output.

1.3.2 Protein-Protein Interaction and Substrate Channeling

The formation of an MDH/CS complex and channeling of oxaloacetate through these complexes has been shown through mathematical and experimental means [36-38, 40, 46] however, structural evidence was not discovered until recently. Wu et al. have demonstrated, through chemical cross-linking and mass spectroscopy studies, the existence of TCA cycle MECs formation and physical interaction between MDH and CS [43]. They were able to identify several cross-links between lysine residues and found that multiple residues were within 20 Å in one subunit of each MDH and CS that suggested participation of one C-terminal and one N-terminal in forming a binding interface. Electrostatic potential data obtained from models of the resulting complex confirmed that a positively charged
zone forms at association along the protein-protein interface which would facilitate negatively charged substrate transport from one active site to another. **Figure 7** illustrated two possible models for MECs of MDH, CS and aconitase.

![Figure 7: Illustration of malate dehydrogenase (green), citrate synthase (gold) and aconitase (purple) in two proposed complex structures [43]](image-url)

**Figure 7: Illustration of malate dehydrogenase (green), citrate synthase (gold) and aconitase (purple) in two proposed complex structures [43]**
1.4 Enzyme Immobilization

A key component in the design of EFCs, photo-bio energy harvesting devices, nano-biosensors, bio-actuators and other bio-nano-interfacial architectures is the effectiveness, specificity and stability of the enzyme immobilization [47, 48]. Immobilization of enzymes is believed to be advantageous in EFCs when compared to free enzymes due to observed reduced electron loss; higher recovery rates and often times improved stability [49, 50]. Over the years, researchers have developed methods for integration of enzymes and nanomaterials. Some of these methods include direct protein adsorption [51, 52], enzyme tethering [8, 53, 54], polymers [14], cross-linking [55, 56], chemical bonding [48, 57, 58], biomolecules [59] protein engineering to form hydrogels [60-62] or fusion of proteins to create novel bioactive materials [63].

1.4.1 Carbon Nanotubes

Nanostructured materials, carbon nanotubes (CNTs) in particular, are an ideal material for enzyme immobilization due of their high surface to volume ratio, comparable size to enzymes and other biomolecules, intrinsic electronic properties, and apparent positive effect on enzyme stability [64-68]. CNTs get their name from their long hollow structure. Two forms of CNTs exist: single walled carbon nanotubes (SWNTs) which consist of a single roll of graphene, and multi-walled carbon nanotubes (MWNTs) (Figure 8). The electronic properties of CNTs are highly dependent on their unique wrapping represented by the indices (n,m), having characteristics from semi-conductive to metallic behavior and a band gap range anywhere from 0 to 2 eV.
Enzymes that have been properly immobilized on nanomaterials have exhibited higher levels of stability and higher power outputs when placed in non-physiological environments compared to free enzymes [9, 50, 67, 70]. Enzymes have a high affinity for direct physical absorption onto CNTs due to hydrophobic interactions and π-π stacking between aromatic rings. The small radius of CNTs is also thought to allow them to get in close proximity to active centers thereby shortening electron tunneling distance to or from electrodes. Carbon paper and CNTs aggregate paper called “Buckypaper” (BP) have also been used as electrodes and for enzyme and/or cofactor immobilization. [71, 72]. The paper can be made up of both SWNT and MWNT and can be modified with films for enhanced electron transfer.
Unfortunately, in some instances the hydrophobic property of pure CNTs has been shown to change enzymatic structure and function resulting in lowered catalytic activity and shortened lifetimes more probably due to altered enzyme tertiary structure and denaturing. This is likely due to locations of hydrophobic regions within the enzyme folded structure. Using surfactants, DNA and polymer modified CNTs are methods that have been developed to overcome this problem. These methods are non-covalent and thereby do not directly interfere with the intrinsic properties and structure of either the enzymes or the CNTs [67].

1.4.2 Targeting Through Recognition: Zinc Fingers and DNA

Immobilization of artificially produced MECs require methods that direct enzyme orientation in such a way that reduces diffusion losses by creating efficient catalytic pathways, much like in nature. This cannot be achieved using nanotubes alone where orientation is not controlled and is highly dependent on enzymatic properties. One possible method, deoxyribose nucleic acid (DNA) directed self-assembly, has shown to not only alleviate many stability issues but also allow for regulation of enzyme orientation and position from one enzyme to the next [27]. Single-stranded DNAs phosphate-sugar backbone is believed to interact with CNTs through parallel π-stacking. π-stacking is an attractive but non-covalent interaction that occurs between aromatic rings. DNA’s phosphate-sugar backbone interacts with the aromatic carbon rings of CNTs [73-76]. However, at high buffer concentrations the wrapping of the DNA/CNTs is not as strong. This is because at higher ion concentrations, it has been observed that DNA does not favor a helical conformation around CNTs that may be due to electrostatic interaction of the
negatively charged sugar phosphate backbone and ions over interaction with the CNTs. For this reason, lower salt concentrations are necessary to ensure that DNA is wrapped around CNTs and does not easily dissociated.

An example of an advanced DNA/CNTs interfacial architecture has been demonstrated by Rawson et al., who vertically aligned SWNTs (VASWNTs) on an indium tin oxide (ITO) surface and explored them for intracellular electrochemical sensing in eukaryotic cells [77]. To achieve uptake of the CNTs into the cells, the VASWNTs were wrapped with single stranded DNA (ssDNA) (Figure 9). The resulting ssDNA/VASWNTs assemblies were taken up naturally by a mouse macrophage cells and used to electrochemically investigate the intracellular environment and activity.

![Figure 9: Schematic of surface modification, SWNT alignment and DNA wrapping [77].](image-url)
Transcription factors called zinc fingers (ZnFs) have been commonly used for controlled DNA-binding of biomolecules because of their highly selective DNA binding capability to specific DNA sequences and the customization of the DNA-binding domain making them ideal for use in controlled enzyme immobilization [78-82]. ZnFs are largely abundant protein structural motifs that get their name from their coordination of one or more zinc ions and from their multiple finger-like protrusions. Engineering of sequence specific ZnFs has been largely studied and the development of ZnFs that bind to guanine-rich sequences are most common due to the strong interactions between proteins and guanine over other bases [81, 83]. Znf268 belongs to the Cys2His2-like fold group and contain a DNA binding amino acid motif [80]. With a very specific DNA binding sequence, 5’-GCGTGGGCG-3’, structural stability and its ability to be genetically fused to enzymes such as small laccase (SLAC), it can easily and stably attach to the DNA/SWNT scaffold for enzyme immobilization.

The effectiveness of Znf268 for binding DNA for immobilization was demonstrated using trimeric SLAC genetically fused to three ZnFs. The study found that the presence of the DNA binding domain showed a high specificity to binding to the specific DNA sequence. Even while bound to DNA, electrochemical activity was maintained [84].

1.4.3 Chitosan: Biopolymer Entrapment

Polymer entrapment of enzymes is one of the most common methods for constraining enzymes on support materials [14, 48, 85-89]. Unfortunately, enzyme distribution throughout the polymer matrix can be heterogeneous [48]. On the other hand, protein engineering strategies has enabled proteins to self-assemble while either retaining or
enhancing their catalytic performance [60]. Chitosan (chit) is an example of biopolymer that has been used for entrapment to modify nanomaterials [90-94].

Chit is a linear polysaccharide derived from of chitin [95], a structural element of the exoskeleton of crustaceans. It is composed of randomly ordered \( \beta(1/4) \) linked D-glucosamine and N-acetyl-D-glucosamine (Figure 10). It has been studied for use in bioelectrocatalysis because it is biocompatible, non-toxic, biodegradable, chemically inert and has high mechanical strength. In addition, it displays polyelectrolyte behavior due to the protonated amino group present along the polymer backbone [96]. In recent years it has been used for entrapment immobilization of enzymes through its formation as a porous scaffold [90-93, 97-103].

The solubility of Chit in aqueous solution can be increased by further removal (up to 95%) of the acetylamine group on its backbone through a process called deacylation. This is largely due to the increased number of reactive amines available through removal of the acetyl functional group. Hydrophobic modification of Chit has been shown to improve enzyme immobilization because of increased formation of micelles that are more

![Figure 10: Deacylated chitosan (Wikipedia)](image-url)
stable than surfactant micelles [104, 105]. Increased degrees of deacylation of hydrophobically modified Chit has shown to provide a better scaffold for protein immobilization through improved enzyme activity [101]. Chit has been used to form films on CNTs using electrostatic interaction [106] and can act as a cationic emulsifier for non-covalent nanotube dispersion given acidic conditions [107]. These properties make it very useful for EFCs applications.

1.4.4 Tethering: 1-Pyrenebutanoic Acid, Succinimidyl Ester

Tethering enzymes to carbon nanotubes using linking molecules can reduce effects from direct nanotube interaction and result in higher maintained activity than when physically adsorbed [8, 64, 97, 108, 109]. 1-Pyrenebutanoic acid, succinimidyl ester (PBSE) is one example of this. PBSE first interacts with nanotubes by means of $\pi-\pi$ stacking of the aromatic rings [8]. It also forms an amide bond between the ester group on PBSE with available primary amines on the protein structure [110].
PBSE has been used to tether both multi-copper oxidases and dehydrogenases to CNTs and has been observed through improved cell performance to be a candidate for stabilization of these systems [8, 56, 110].

1.5 Dissertation Objectives

The objective of this research is to develop new 3D structures that optimize the efficiency of EFCs by enhanced electron transfer kinetics and stabilization of enzymes for longer fuel cell lifetimes. Using biomolecules, electrode surface modification and nanomaterials, redox enzymes are immobilized and characterized using X-ray photoelectron spectroscopy (XPS), linear sweep (LSV) and cyclic voltammetry (CV), chronoamperometry (CA), and open circuit voltage (OCV).
The initial focus here is a proof of concept for ability to immobilize enzymes using DNA/SWNT scaffolds with various characterization methods to show correct interactions and the positive effect of these assemblies for electrochemical activity and further transfer of the concept in the design of multi-enzyme anodes. Consecutive TCA cycle enzymes are also used to test immobilization using chitosan and PBSE for co-localization EFCs design for the purpose of assessing the utility of these extensively studied methods for multiple enzyme systems. The overall objectives are as follows:

**Objective 1:** Development of DNA and DNA-binding proteins as a scaffold for nano-scale templating and arrangement of individual proteins.

**Objective 2:** Electrochemical study of SLAC-3ZnF on DNA/CNTs scaffold with gold nanoclusters to show capabilities in oxygen reduction reactions (ORR) on cathodes.

**Objective 3:** Electrochemical study of the co-localization of consecutive TCA cycle enzyme on different modified-CNT scaffold to show utility for metabolon immobilization.

This research is approached from several different aspects for single and multiple enzyme immobilization. The first is the development of a single enzyme scaffold architecture using SLAC-3ZnF docked on SWNT wrapped with DNA that contains zinc finger recognition sequences. Vertically aligned SWNTs on an electrochemically modified
gold electrode are developed in a bottom up approach starting with gold modification, followed by SWNTs alignment and finally wrapping with DNA. DNA/SWNTs is also made in suspension for testing normal catalytic behavior and electrochemical characteristics of SLAC-3ZnF. **Figure 12** shows the general outline for SLAC-3ZnF experimental design. Electrode development involves a bottom up, step-wise fabrication of each layer of the scaffold matrices and is followed by subsequent enzyme immobilization.

![Figure 12: General experimental schematic for SLAC-3ZnF scaffold development and testing.](image)

The second approach is testing systems containing one or more enzymes, including sequential TCA cycle enzymes, MDH and CS. Scaffolds made up of chitosan or PBSE on
MWNT are used for co-immobilization of MDH and CS. Each enzyme/scaffold composite is tested on buckypaper electrodes modified with a poly(thiazine) film. This is to determine: 1) the affects of CS presence on MDH oxidation potential using immobilization method tested for MDH alone and 2) the affects of tethering versus encapsulation on the same systems to take a step forward in optimizing scaffold design for MECs development.
Chapter 2: Electrode Surface Modification for Scaffold Design and Enhanced Electron Transfer

2.1 Diazonium Salt reduction on Au Electrodes

The electrochemical grafting of gold surfaces with diazonium salts functionalizes surfaces for further modifications such as the controlled attachment of carboxyl-modified nanotubes. This modification allows the development of aligned in a brush form CNTs that create a “CNT forest”. CNTs aligned on the electrode surface provide high surface area of nanomaterials available for enzyme immobilization and electron transfer.

Preparation of diazonium salt is done through “diazotation” of an aromatic amine such as p-phenylenediamine (PPD) with nitrous acid in presence of sodium nitrite (NaNO₂) and hydrochloric acid (HCl). The produced diazonium salt is highly unstable; therefore, the reaction with the gold surface is carried out immediately following synthesis. Figure 13 illustrates the process of forming the diazonium salt.

![Formation of diazonium salt from in situ reaction of hydrochloric acid, sodium nitrite, and p-phenylenediamine.](image)

Figure 13: Formation of diazonium salt from in situ reaction of hydrochloric acid, sodium nitrite, and p-phenylenediamine.
Electrochemical reduction at the electrode surface is performed by removal of N₂. As a result, a radical is formed that bonds covalently with the surface. This process can form both a monolayer and multilayers. Multilayers are formed from the interaction of the radical with the already present monolayer. The radical tends to attach at the ortho position to NH₂ on the aromatic ring, which creates a disordered multilayered film. Factors that affect the surface modification are the reduction potential, deposition time and concentration of the diazonium salt. More negative potentials have shown to produce greater reduction peaks and are more likely to form multilayers [111]. By controlling each of these factors, the thickness of the film can be controlled. Ensuring that the surface has an ordered monolayer will provide the least amount of interference when used for energy harvesting purposes. Figure 14 shows the process of the monolayer formation and subsequent layers, respectively [112, 113].

![Figure 14: Mechanism for gold surface modification with PPD to form monolayer and multilayer at ortho position.](image)

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2.2 Nanotube Forrest

Nanotubes have been found to be useful in energy harvesting due to their high surface area, chemical stability, similar size scale to enzymes and high conductivity. Functionalized CNTs may be attached to NH\textsubscript{2}-modified surface where the carboxyl groups (f-CNT) are located at the ends of the nanotubes resulting in a brush formation after attachment. The use of N,N’-dicyclohexylcarbodiimide (DCC) allows covalent bonding of f-CNTs to the arylamine modified surfaces through carbodiimide chemistry. The DCC reacts with the carboxyl group present at the ends of the nanotubes and the arylamine layer on the modified surface creating amide bond that results in the f-CNTs attachment to the surface. **Figure 15** shows the amide formation excluding all byproducts, which are removed after attachment through washing with polar solvents.
Figure 15: Mechanism for single wall nanotube attachment via carbodiimide chemistry using DCC excluding bi-products.

The formation of the ester-CNT product using this method results in main side reactions. The COOH-SWNT further reacts with the intermediate o-acylisourea, which contains an activated leaving group to give the desired amide product through a replacement reaction. [114]

2.3 Methylene Green Polymerization

Modification of electrode surfaces for lowering overpotential and facilitating electron transfer is an important area of research. At carbon electrode surfaces, the
overpotential for NADH oxidation is significant, going as high as 1.0 V, leading to possible side reaction with undesirable byproducts and energy inefficiencies [115, 116]. The use of poly(thiazine) such as poly(methylene green) (PMG) has been reported for oxidation of NADH and FADH molecules on anodes [41, 42, 115-118]. Methylene green is a heterocyclic aromatic chemical compound similar to methylene blue that is often used as a dye (Figure 16). In electrochemical cells, PMG has been used for the regeneration of NAD\(^+\) from NADH as a polymerized film on carbon surfaces including glassy carbon electrodes and BP.

![Methylene green structure](image)

**Figure 16: Methylene green structure.**

\[
PMG_{ox} + NADH \rightarrow PMG_{red} + NAD^+ + H + 2e^- \\
\text{(Eq. 10)}
\]

There is still debate on PMG film structure and why it is able to oxidize NADH so readily, however PMG-modified electrodes have consistently demonstrated improved cell kinetics because of this. Eq. 10 shows the possible reaction for NADH oxidation by PMG.
Chapter 3: Physical and Electrochemical Analytical Techniques

3.1 X-Ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) is a useful characterization technique for sample composition. XPS obtains surface information of samples through irradiation of the material, measuring the chemical and electrons states, composition (ppm) and empirical formula. Obtained spectrums give counts of electrons at the ordinate as a function of the binding energy at the abscissa. The observed peaks correspond to the electrons within the 1s, 2s, 2p orbitals and so on, giving valuable information about atoms and the types of interactions present in a sample.

3.2 Atomic Force Microscopy

Physical characteristics of a given sample can be analyzed in a variety of ways. Atomic force microscopy (AFM) gives a physical interpretation of a samples surface. AFM is a type of scanning probe microscopy in which a mechanical probe using a piezoelectric element moves along the surface producing images with resolution on the scale of nanometers. The typical AFM setup consists of a tip fixed to the end of a cantilever, cantilever support, piezoelectric element to oscillate the cantilever at its Eigen frequency, a detector, sample stage and an X,Y,Z drive to move the stage. The sharpness of the tip is chosen based upon the size of the features under investigation. In no contact modes, the AFM tip does not physically come in contact with the surface. Instead, when the tip
approaches the sample surface, forces between them cause the tip to deflect. The resulting image give an idea of the physical surface characteristics including topography from 3D image manipulation. The resolution is dependent on experimental protocols including scan rates and force applied (Hz) It may be used to study a range of surface characteristics including topography.

3.3 Linear sweep and cyclic voltammetry

Voltammetry measurement is an electrochemical analytical method used to measure the current as a function of changing potential of an analyte in a half reaction. Linear sweep (LSV) and cyclic voltammetry (CV) are just two kinds of a variety of voltammetry measurements both using a general three electrode setup measuring the potentials of a working electrode against a known reference and counter electrode. LSV measures from an initial set potential (V) to a final potential giving information about either the reductive or oxidative properties of a system. CV does the same, except does a return scan back to the initial potential or a new set potential and can be repeated consecutively numerous times. When a reaction is only occurring in one direction, CV does not provide any more information than LSV.

3.4 Rotating Disk Electrode

Enzyme and scaffold efficiency can be determined using rotating disk electrodes (RDE) in combination with LSV and CV. An RDE is comprised of a conductive disk, like glass carbon. The disk is encased by an insulating material (Figure 17). RDE experiments test current against a changing potential range while spinning the electrode at different
rotations per minute (rpm) $\omega$. Samples are placed on the disk and the RDE is submerged in a solution containing an analyte for measurement of the current.

![Figure 17: Rotating Disk Electrode with rotating rate $\omega$](image)

If a limiting current is obtained, that is the point in which system reaches a maximum current at an applied potential value, the information obtained allows for the calculation of the diffusion coefficient and the mass transfer rate of electrons to or from the electrode using the Levich and Koutechy-Levich plots. The mass transfer rate ($J$) according to Fick’s first law may be obtained from the diffusion coefficient of the transferred ion, the concentration of the ion on the surface ($c_s$), the concentration in the bulk solution ($c_o$) and the thickness of the diffusion layer (Eq. 11). The diffusion layer is found from the diffusion coefficient, kinematic velocity ($v$) of the electrolyte and rotation rate ($\omega$) of the RDE (Eq. 12).
\[ J = \frac{D(c_a - c_o)}{\delta} \quad \text{(Eq. 11)} \]

\[ \delta = 1.61Dv\omega \quad \text{(Eq. 12)} \]

The RDE measures current with respect to applied potential where the measured current \((i_l)\) is related to the charge transferred across the diffusion layer \((i_d)\) and across the interface \((i_k)\) seen in Eq. 13 and expressed in amperes (A). If the reaction is limited by mass transfer meaning it is limited by the rate in which the system can react, the limiting current can be related to diffusion coefficient and mass transfer rate using the Levich equation (Eq. 14).

\[
\frac{1}{i_l} = \frac{1}{i_k} + \frac{1}{i_d} \quad \text{(Eq. 13)}
\]

\[
i_{\text{lim}} = 0.62zFAD^{\frac{2}{3}}\omega^{-\frac{1}{2}}c_0 \quad \text{(Eq. 14)}
\]

The Koutecký-Levich equation describes the behavior in a system not governed by the mass-transfer rate (Eq. 14). The equation is obtained by substituting the Levich equation \((i_{\text{lim}})\) into Eq. 13.
3.5 Michaelis-Menten Kinetics

The kinetic behavior of an enzyme can be done using a substrate concentration study that measured the current obtained as a function of the concentration of substrate present in the cell. The simplest model describing this behavior is based on single substrate binding without cooperative or uncooperative binding. The model developed by biochemists Leonor Michaelis and Maude L Menten used the simple two-step scheme of substrate binding to enzymes and product release (Eq. 15) to determine the initial rate at its maximum value ($V_{max}$) of the reaction when the substrate is saturated such that all enzymes are bound to substrate (ES).

$$\frac{k_2}{k_1} E + S \overset{k_2}{\underset{k_1}{\rightleftharpoons}} ES \rightarrow P + E \quad \text{(Eq. 15)}$$

$$V_{max} = k_2 [E_0] \quad \text{(Eq. 16)}$$

Incorporating steady state kinetics into the conditions determined by Michaelis and Menten assuming that at [ES] reaches a constant value, the rate of reaction can be described in terms of the Michaelis-Menten constant ($K_M$), substrate concentration ([S]) and the maximum current reached at saturation [S] (Eq. 17). $K_M$ describes the concentration of a system when it is at half of $V_{max}$.

$$v_0 = \frac{V_{max} [S]}{K_M + [S]} \quad \text{(Eq. 17)}$$
**Figure 18** is a graphical representation of the initial rate as a function of concentration. The value for $V_{\text{max}}$ is found at the plateau and $K_M$ at $\frac{1}{2} V_{\text{max}}$. $K_M$ can be used to describe the binding affinity of a substrate. Smaller values of $K_M$ suggest a higher affinity for substrate binding than with large $K_M$.

![Graphical representation of plot initial rate ($v_0$) and substrate concentration ([S]).](image)

Although Michaelis-Menten kinetics can be used to determine parameters of many enzyme systems including multi-substrate binding and inhibition, it cannot be used to determine properties where allosteric and non-reversible inhibition occur. For such instances, other kinetic models must be employed such as the Hill equation.
Chapter 4: Functional Interfaces for Biomimetic Energy Harvesting: CNTs-DNA Matrix for Enzyme Assembly

4.1.1 Introduction

In this study we demonstrate a scaffold design utilizing carbon nanotubes (CNTs), DNA and ZnFs that allows for directed immobilization of a single enzyme. The specificity of ZnF-DNA interactions was explored for controlled enzyme immobilization. As proof of concept, ZnF268 was genetically fused to the small laccase (SLAC) from *Streptomyces coelicolor* (e. coli) and attached to a three-dimensional DNA/CNT assembly (Figure 19). In a previous studies it was demonstrated that a multifunctional SLAC-3ZnF fusion protein could be engineered, expressed and successfully immobilized on dsDNA [84]. Here this finding was expanded towards the incorporation of the SLAC-3ZnF protein into a more sophisticated design of the supporting assembly. For the creation of the supporting scaffold, CNTs aligned in a brush-type formation were wrapped with DNA engineered to be recognized by the ZnF, acting as a docking place for its attachment. Analytical techniques such as X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), and enzymatic activity analysis, allowed characterization at each stage of development of the bio-nano architecture.
Figure 19: Schematic representation of the approach and architecture developed in this study where I) modification of the gold surface, II) arylamine modified gold surface, III) attached SWNTs to the support, IV) dsDNA wrapped around the aligned SWNTs, V) SLAC-3ZnF attached to the DNA scaffold on the SWNTs “forest”.

4.2 Reagents and Materials

Dibasic potassium phosphate (K₂HPO₄, Lot # 2014091787), monobasic potassium phosphate (KH₂PO₄, Lot #2012022368), dimethyl sulfoxide (DMSO, Lot # SHBC2756V), 1N hydrochloric acid (HCl, Lot # 49279) and HPLC Omnisolv water (Lot #57184) were purchased from EMD Millipore (Billerica, MA). Single walled carbon nanotubes 99 were purchased from cheaptubes.com. Carboxylic acid functionalized single walled carbon nanotubes (f-SWNT, Lot # 03619LD), 2,6-dimethoxyphenol (DMP, Prod. No. D135550-
25G), p-phenylenediamine (PPD, Low # MKBJ5024V), N,N-dicyclohexylcarbodiimide (DCC, Lot # SHBC2756V) and sodium nitrite 97+%(NaNO₂, Lot # 08316DJ) were purchased from Sigma Aldrich. Acetone (Lot # 050713E) was provided by BHD chemicals. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Gold Biotechnology (St. Louis, MO). HALT protease inhibitor, precast NuPAGE SDS-PAGE gels, NuPAGE SDS MES running buffer and Novex Sharp Pre-Stained Protein Standard were from Thermo Fisher Scientific (Waltham, MA). HisTrap HP columns and the ÄKTA FPLC system were purchased from GE Healthcare (Piscataway, NJ). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity unless otherwise specified.

### 4.3 Bottom-up fabrication of DNA/SWNT-Au scaffold

Electrodes were made in a bottom up procedure starting with Au deposition on glass, modification of resulting gold with an arylamine film, covalent attachment of SWNTs and finally DNA wrapping of SWNTs “forest”. The following sections outlines scaffold fabrication procedures.

#### 4.3.1 Gold modification

Gold deposition on glass support was carried out through spray evaporation. Glass covered slides were cleaned using ozone prior to deposition. A 3nm layer of chromium and 100nm layer of gold were deposited subsequently. The gold surface was further modified through electrochemical grafting. Three-electrode set-up with a gold covered slide used as working electrode; Ag/AgCl as reference and Pt-wire as counter electrodes, respectively
was used for the electrochemical grafting. 10ml solution of 7mM p-phenyleneamine diazonium salt was used as electrolyte and modifying solution. The arylamine diazonium salt was synthesized when 7mM p-phenylenediamine (PPD) interacted with 1mM NaNO₂ in 0.5M HCl for 5min under ice [111]. Cyclic voltammetry (CV) was utilized as electrochemical technique for the grafting procedure. CVs at 100mV/s in three potential windows starting from 0.4V to -0.6, -0.4 and -0.2V vs. Ag/AgCl, respectively was carried out to form an arylamine layer on the gold surface [111, 119].

4.3.2 Nanotube attachment

The next step was covalent attachment of carbon nanotubes onto the modified gold slides. Carboxyl-functionalized single walled carbon nanotubes (f-SWNT, 3 to 5µm) were explored due to the presence of carboxyl functional groups at the mouth of the nanotubes necessary for the chemical bonding with the amine groups of the arylamine layer deposited onto the support surface. 0.4mg/mL f-SWNTs were suspended in dimethyl sulfoxide (DMSO), sonicated for 15min and then combined with a solution of 0.4mg/mL N,N'-dicyclohexylcarbodiimide (DCC) in DMSO. The modified gold samples were submerged in the solution and sonicated for 15min. The submerged samples were then transferred into an oven and heated in a closed cell for 24hrs at 55°C. Samples were then washed with a series of polar solvents starting with 2-min sonication in acetone followed by 10 seconds sonication in isopropyl alcohol and rinsed with HPLC grade water.
4.3.3 DNA scaffold

The ssDNA sequence: 5’-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCGCCCACGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3’ and ssDNA of complimentary ZnF specific binding base sequence 5’-GCGTGGGCG-3’ were ordered as custom oligomers from Sigma Aldrich. The complementary DNA (5’-GCGTGGGCG-3’) was marked with thiol-SS-C3, a disulfide-containing oligomer modifier, at the 5’ terminus and with thiol-SS-C6 at the 3’ terminus. DNA hybridization took place in solution. dsDNA was allowed to hybridize through 15-minute sonication prior to submerging SWNT-Aryl-Au samples or f-SWNTs (1:1 molar ratio), depending on the experiment. Samples underwent sonication for 15 minutes and were left in solution for 24hrs before being removed, rinsed with HPLC water and dried with N₂.

4.4 SLAC-3ZnF expression and immobilization

4.4.1 SLAC-3ZNF complex expression and purification

Expression and purification of SLAC-3ZnF with the plasmid pSLAC-3ZnF was performed as described in Szilvay et al. 2011 (Supplementary Methods) [84] with minor modifications. Cells were grown in 2xYT media at 37°C until the OD₆₀₀ reached approximately 1.5. Protein expression was induced with 0.4mM IPTG, and protein expression continued for 20 hours at 25°C before cells were collected by centrifugation and stored at -20°C. Pellets corresponding to 1L of culture were thawed and suspended in 50mL of binding buffer (20mM Na Pi pH 7.3, 50mM NaCl, 40mM imidazole) supplemented with HALT EDTA-free protease inhibitor. Cells were sonicated on ice for 6 minutes with a microtip probe, and lysates were clarified by centrifugation. Lysates were
then purified using a HisTrap HP column equilibrated with binding buffer. A gradient of elution buffer (20mM NaPi pH 7.3, 500mM NaCl, 500mM imidazole) was applied, and fractions were collected and analyzed with SDS-PAGE. Fractions containing SLAC-3ZnF-His shown at 50kDa were pooled together. The protein was then dialyzed against binding buffer containing 1mM CuSO₄ and 1mM ZnSO₄ at 4°C overnight. The sample was then dialyzed against ammonium bicarbonate buffer and concentrated by ultrafiltration. They were then frozen overnight in micro-centrifuge tubes at -80°C, followed by a minimum of 24hrs of lyophilization.

4.4.2 Enzyme Immobilization

A solution of 1 mg/ml SLAC-3ZnF in HPLC water was prepared. dsDNA/SWNT complexes aligned on the gold support were submerged in the enzyme solution and left for 24hrs at 4°C for enzyme immobilization. The sample was removed from the SLAC-3ZnF solution, washed three times and dried under N₂.

4.5 Analytical Methods

4.5.1 Atomic Force Microscopy

Surface imaging was done using an Asylum MFP-3D-BIOTM Atomic Force Microscope and NanoWorld Innovative Technologies SuperSharpSilicon™-Silicon SPM-Sensor non-contact mode (SSS-NCH-10, #76807F4L969). 10x10µm and 5x5µm images were taken of gold deposited on glass substrate, arylamine modified gold layers for the arylamine modifications and the aligned SWNT-arylamine-gold sample. 5x5µm scans were taken at a scan rate of 2.5µm/s and 10x10µm scans were taken at a scan rate of 5µm/s.
4.5.2 X-ray Photoelectron Spectroscopy

XPS spectra were acquired using a Kratos AXIS Ultra DLD photoelectron spectrometer with a monochromatic Al Kα source operating at 150W. Charge compensation was accomplished using low energy electrons. Standard operating conditions for good charge compensation were –3.1V bias voltage, -1.0V filament voltage and filament current of 2.1A. The following take-off angles (TOA) are selected for angle resolved studies: 90°, 30° and 15°. All spectra processing was done in CasaXPS. A linear background was used for all high-resolution spectra. Atomic % were calculated from areas under the peak in electron count per second using sensitivity factors provided by the manufacturer. All the spectra were charge referenced to the aliphatic carbon at 284.8eV or Au 4f at 84eV for samples where Au was used as substrate. A 70% Gaussian /30% Lorentzian (GL (30)) line shape was used for the curve-fits. Full width at half maximums (FWHMs) used for curve fits of C 1s spectra were constrained to 1.0±0.2eV for all spectra acquired by monochromatic Al Kα source.

The thickness of each individual layer was calculated by using substrate/overlayer model in Arctick. Areas under the peaks in counts of electrons were used for Arctick calculations.

4.5.3 Activity Assay

Two samples containing SLAC-3ZnF were prepared in 500µl of 0.01 M phosphate buffer (pH 7.5):

1. 1 mg/ml SLAC-3ZnF adsorbed on 1 mg/ml f-SWNT in suspension.

Solution containing f-SWNT was prepared first by sonication of a
250µl suspension of 2mg/ml f-SWNT and then diluting to final volume of 500µl with a suspension of 2mg/ml SLAC-3ZnF. This gave a final weight concentration of 1 mg/ml for f-SWNT and SLAC-3ZnF. The sample was left to incubate at 4°C for 24hrs. After incubation, it was centrifuged for 30 min to remove supernatant and then washed twice with 0.01M phosphate buffer to remove any unattached enzyme. Sample was suspended to 500µl with phosphate buffer for final testing.

2. 1 mg/ml SLAC-3ZnF attached to 2mg/ml dsDNA, wrapped onto 1mg/ml SWNTs in suspension. SLAC-3ZnF sample with dsDNA was prepared by 5-min sonication of a 250µl suspension of 2mg/ml f-SWNT with 4mg/ml dsDNA followed by addition of 250µl 2mg/ml SLAC-3ZnF. The sample was left to incubate at 4°C for 24hrs. After incubation, it was centrifuged for 30min to remove supernatant and then washed twice with 0.01M phosphate buffer to remove any unattached enzyme. Sample was suspended to 500µl with phosphate buffer for final testing.

3. A 1mg/ml suspension of f-SWNT and a 2mg/ml dsDNA/SWNT assembly were prepared as controls for UV-Vis spectroscopic background subtraction.

The samples were prepared and an activity assay was carried out. Then the samples were stored at 4°C for 24hrs when activity assay was carried out again. Prior to acquiring the
activity assay the samples were centrifuged for 30 min to remove supernatant and then washed twice with 0.01 M phosphate buffer to discard the unattached enzyme and the enzyme that has been detached during storage. Samples were then suspended to 500 µl with phosphate buffer and 4 µl of the suspension were transferred in a 96 well plate. Aliquots of 196 µl of DMP were then added to the samples (to a final DMP concentration of 100 mM). The reaction rate of DMP oxidation from the enzyme was examined through UV-Vis spectroscopy at 468 nm for 5 min. The amount of enzyme active units was calculated based on the change in DMP absorbance over 5 min and extinction coefficient of DMP equal to 14,800 M⁻¹ cm⁻¹. All measurements were taken using SoftMax® Pro5 software and the SpectraMax® M5 Multimode Microplate Reader from Molecular Devices, LLC at 23°C and 0.8 atm.

4.6 Results and Discussion

4.6.1 Development of CNTs “forest”

The utilization of diazonium salts for surface modification of metal and carbon materials has been heavily explored in the recent years. As a result, their surface can be populated with different chemical groups (-NH₂, -COOH, -OH, etc.). The aryl diazonium salts are very unstable at temperatures above 0°C and usually are produced right before their use. The process of forming diazonium compounds is called "diazotation" and is a chemical reaction of aromatic amines with nitrous acid, generated in situ from sodium nitrite and hydrochloric acid at 0°C. Subsequently, the aryl group (Ar = -C₆H₅-R, where R could be –NH₂, -COOH, -OH, etc.) is covalently attached to the electrode surface by electrochemical reduction, releasing N₂. During the electrochemical grafting, the
diazonium salt reacts with the surface where the diazonium group is lost (Eq. 18) and the formed aryl radical reacts with the surface (Eq. 19) [119]. The electrochemical grafting is carried out by cyclic voltammetry (CV), where the layer coverage depends on the potential range of the CV and the number of cycles.

\[
\text{ArN}_2^+ + 1\text{e}^- \rightarrow \text{Ar}^\cdot + \text{N}_2 \quad \text{(Eq. 18)}
\]

\[
\text{Surface} + \text{Ar}^\cdot \rightarrow \text{Surface-Ar} \quad \text{(Eq. 19)}
\]

\[
\text{Ar}^\cdot + 1\text{e}^- \rightarrow \text{Ar}^- \quad \text{(Eq. 20)}
\]

In this study, an electrochemical modification with p-phenyleneamine diazonium salt (NH₂-C₆H₅-N₂) was carried out to create an Ar layer on the gold surface used as a support material (Figure 19 (I and II)). The phenyleneamine diazonium salt was produced in situ by chemical interaction of p-phenylenediamine (C₆H₄(NH₂)₂, PPD) with sodium nitrite in presence of 0.5M HCl at 0°C. Factors that affect the surface modification are the reduction potential, deposition time and concentration of the diazonium salt. It is known that expanding the potential toward more negative values and increasing the number of cycles leads to the formation of multilayers with increasing thickness [111]. Therefore, to determine the optimal procedure for the Ar monolayer deposition we prepared three samples. CVs with different potential ranges were used: 1) from 0.4 to -0.6V vs. Ag/AgCl; 2) from 0.4 to -0.4V vs. Ag/AgCl; and 3) from 0.4 to -0.2V vs. Ag/AgCl. Only one cycle of CV was carried out for each sample (Figure 20a). Two reduction peaks at 0.245V and 0.020V vs. Ag/AgCl were observed when the potential window was expanded toward more negative potentials and only one peak at 0.245 V vs. Ag/AgCl was recorded swiping the
potential between 0.4 and -0.2V vs. Ag/AgCl. The first peak at 0.245V vs. Ag/AgCl is associated with the formation of aryl radical and the second peak at 0.020V vs. Ag/AgCl most likely is referred to the reduction of this radical to aryl anion (Eq. 20) and subsequent development of multilayers, which inhibit further the electron transfer [113, 120]. The formation of Ar layer can be easily detected (Figure 20b) through the disappearance of the reduction wave of the Ar salt when more then one cycle is performed.

Figure 20: Electrochemical grafting of arylamine layer on gold support a) using three different potential windows for the carried out cyclic voltammetry, and b) two subsequent cycles in the potential window 0.4 to -0.2V. Scan rate 100 mV/s.

The modified gold surfaces were then examined through Atomic Force Microscopy (AFM) (Figure 21b, c and d). For comparison, a control sample of bare gold was also studied (Figure 21a). The AFM images revealed noticeable changes in surface topography after the modification step. The features characteristic for the bare gold surface (Figure 21a) could not be seen after the electrochemical grafting (Figure 21b, c and d), revealing successful coverage of the gold presumably from an Ar layer. Differences in the
topography of the Ar layer were also observed depending on the potential window used for the modification step. The most uniform and smoothest surface coverage was achieved with the shortest potential window used most likely due to the development of a single layer of Ar.

![AFM images of gold samples modified via electrochemical grafting of p-phenylenediamine diazonium salt through cyclic voltammetry carried out at three potential windows: a) unmodified gold, b) 0.4 to -0.6V, c) 0.4 to -0.4V and d) 0.4 to -0.2V vs. Ag/AgCl. Size: 10x10µm, Rate: 5µm/s for 5µm scan, Frequency: 0.2Hz](image)

The thickness of the Ar layer strongly influences the conductivity through the layer. A monolayer provides the necessary surface coverage without introducing significant constraints for electron transfer while the deposition of multilayers of Ar will decrease the conductivity of the material and dramatically hinder the electron transfer rate. Therefore, further analyses were carried out to study the layer composition and thickness. XPS analysis was explored for resolving the surface content and angle resolved XPS was used to determine the thickness of the deposited layer (Figure 22). The angle resolved analysis showed that thicker layers were deposited in potential ranges 0.4 to -0.4V and 0.4 to -0.6V vs. Ag/AgCl, confirming the effect of the potential window on the thickness of the deposited Ar layer. The thinnest Ar layer (0.33nm), close to a monolayer, was created when
the CV’s potential range was 0.4 to -0.2V vs. Ag/AgCl. Thus for the subsequent experiments, this electrochemical modification leading to monolayer deposition was applied.

**Figure 22:** XPS spectra of modified gold surface using one cycle of CV with potential window 0.4 to -0.2 V vs. Ag/AgCl a) 90° TOA, b) 30° TOA. High resolution C 1s, O 1s, N 1s and Au 4f spectral lines are shown. Areas under the peaks of N 1s and Au 4f were used for calculating thickness of arylamine. Elemental composition for 90° TOA is shown in Table 1.

XPS spectra at 90° and 30° TOAs of the monolayer modified gold surface demonstrated the presence of O 1s, C 1s and N 1s along with 4f Au (Figure 22). The appearance of C and mainly N-atoms on the sample surface confirms the success of the electrochemical grafting. The oxygen content for was determined to be 13 % (atomic), the carbon content was 56 % and the N-atoms composed 4.8 % of the surface coverage (Table 1).

The next step was covalent attachment of f-SWNTs on the modified gold surface in a brush-like formation (Figure 19 (III)). Carbodiimide chemistry was used for this purpose as it has been successfully utilized for nanotube-surface covalent bonding [77].
This approach uses an organic compound containing carbodiimide group to catalyze the formation of amide bond from carboxylic and amine groups [114]. The carbodiimide activates the carboxylic group creating a highly reactive intermediate carboxylic ester, which further reacts with the amine group and creates an amide bond. In order to explore this chemistry and create a covalent bond between CNTs and the solid support, carboxyl-functionalized SWNT were used. The -COOH groups of the SWNTs were attached to the amide groups of the grafted arylamine layer via the utilization of N,N'-dicyclohexylcarbodiimide (DCC) as a homogenous catalyst. Since the carboxyl groups of the modified SWNTs are located at the mouth of the SWNTs, the designed SWNTs-Au assembly had brush-like formation of the CNTs on the support surface.

XPS and AFM analyses were carried out after the attachment of the CNTs to confirm the nanotubes presence and to study the changes in the sample topography. XPS spectra of N 1s, C 1s, O 1s and Au 4f revealed an increase in carbon and oxygen content as a result of the presence of f-SWNTs on the gold surface (Figure 23 and Table 1). The greater increase in oxygen (~13%) than carbon (~5%) is likely due to the location of carboxyl groups at the mouths of the aligned nanotubes. The O-atoms present on the sample surface prior to the SWNTs attachment is most likely due to the atmospheric O2. In addition, the percentages of N 1s and Au 4f reduction additionally demonstrate an expanded coverage of f-SWNTs over the sample surface.
Figure 23: XPS spectra of f-SWNTs on the gold. High resolution C 1s, O 1s, N 1s and Au 4f spectral lines are shown. Elemental composition is shown in Table 1.

Table 1: XPS atomic composition of modified Au support before and after the covalent attachment of f-SWNTs. Data at 90° TOA

<table>
<thead>
<tr>
<th>Sample</th>
<th>% O 1s</th>
<th>% N 1s</th>
<th>% C 1s</th>
<th>% Au 4f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au-Ar</td>
<td>13.02</td>
<td>4.80</td>
<td>56.06</td>
<td>26.12</td>
</tr>
<tr>
<td>Au-SWNTs</td>
<td>26.28</td>
<td>1.67</td>
<td>61.38</td>
<td>10.67</td>
</tr>
</tbody>
</table>

Further surface analyses using AFM demonstrated changes in the surface topography of the sample. The SWNT-Au surface (Figure 24) showed a much rougher structure in comparison to arylamine-modified gold surface (Figure 21d). The three-dimensional view of the AFM image (Figure 24b) revealed a brush-like construct on the surface with attached CNTs having a relatively short length (< 300 nm).
4.6.2 Design of DNA Scaffold and Enzyme Immobilization

The family of ZnFs includes a broad group of proteins having extraordinarily diverse functions including DNA recognition, RNA packing, transcriptional activation, protein bonding and assembly, and lipid binding [121, 122]. A common feature of these proteins is the presence of one or more zinc atoms gripped in between four amino acids (cysteine or histidine), arranged as a tetrahedron around the zinc [122]. ZnFs curl around DNA and binds to the grooves with positioned amino acids inward to read the DNA bases [78, 121]. ZnF268 plays an essential role in eukaryotic gene expression. It belongs to the Cys₂His₂-like fold group and contains a DNA binding motif, which recognizes and binds to the DNA sequence 5’-GCGTGGGCG-3’ with high accuracy and specificity [80]. It has been shown that ZnF268 interacts with its amino acid residues from the N-terminal portion of the α-helix with three base pair of the DNA scaffold mainly through the guanine residues [123]. The specificity of the ZnF-DNA recognition is provided by side chain-base interactions (Figure 25) [78, 123, 124]. ZnF268 has been genetically fused to small laccase (SLAC)
creating SLAC-3ZnF assembly and since SLAC is a trimer the final assembly has three ZnFs (SLAC-3ZnF). Using surface plasma resonance and magnetic bead capture assay it was demonstrated that the engineered SLAC-3ZNF attaches specifically to target DNA in a catalytically active manner as compared to the enzyme without the ZnF (SLAC-His) [84].

Single-stranded DNA with the following sequence 5’-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3’ was used to create a ssDNA/CNT scaffold. The twenty thymine bases on either side of the ZnF specific sequence were utilized to wrap around the CNTs. The middle sequence of the ssDNA was further hybridized with 5’-GCGTGGGCG-3’, which served as a docking and binding site for the ZnF module from the fused SLAC-3ZNF.

![Figure 25: Overview of the 3ZnF-DNA complex (PDB code 1A1F) [124]. Jmol was used for the visualization: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/](image)

It has been shown through molecular dynamics simulations that all nucleotides can bind to SWNTs in a similar fashion via π-π stacking of the DNA backbone and the SWNTs sidewalls [125]. In our study the success of the DNA hybridization when attached to the
CNTs will depend on the strength of the thymine-SWNTs $\pi-\pi$ interactions. If the interactions are not strong enough the DNA hybridization will strip the ssDNA from the SWNTs, destroying the DNA scaffold. Therefore, the DNA hybridization was performed before its attachment to the CNTs. The complementary DNA (5’-GCGTGGGCG-3’) was marked with thiol-SS-C3 at the 5’ terminus and with thiol-SS-C6 at the 3’ terminus (Scheme 2). The presence of S-atoms, and more specifically the presence of S-S bond, in the complementary DNA sequence allowed the utilization of XPS analysis to determine the success of the DNA hybridization step. The ssDNA was hybridized with its complementary DNA, marked with thiol-SS-C3 and thiol-SS-C6, in solution and then wrapped to f-SWNTs suspended in DI water (dsDNA/SWNTs). A suspension of f-SWNTs wrapped with ssDNA (ssDNA/SWNT) was tested as a control. The samples were washed three times with DI water prior XPS analysis. The results from the XPS study are shown in Table 2.

![Figure 26: Composition of thiol-SS-C3 and thiol-SS-C6 marked complementary DNA](image)

### Table 2: XPS analysis of SWNTs in suspension, wrapped with single stranded (ssDNA) or double stranded (dsDNA) DNA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% O 1s</th>
<th>% N 1s</th>
<th>% C 1s</th>
<th>% S 2p</th>
<th>% P 2p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssDNA/SWNT</td>
<td>25.77</td>
<td>11.63</td>
<td>58.69</td>
<td>0.00</td>
<td>3.91</td>
</tr>
<tr>
<td>dsDNA/SWNT</td>
<td>38.14</td>
<td>8.43</td>
<td>50.95</td>
<td>0.44</td>
<td>2.05</td>
</tr>
</tbody>
</table>
The presence of phosphorus in the ssDNA/SWNT and dsDNA/SWNT XPS spectra reveals the attachment of DNA to the CNTs. At the same time the appearance of S 2p peaks in the dsDNA/SWNT spectrum is indication for the successful DNA hybridization since S-atoms are present only in the sequence of the complementary DNA.

Once the DNA hybridization and subsequent immobilization on CNTs in suspension was proven, the same approach was explored for the attachment of dsDNA on the aligned CNTs (Figure 19 (IV)). Two samples were composed, one with aligned CNTs on gold support (SWNT-Au) and another with dsDNA wrapped around the aligned SWNTs, which were covalently attached to the gold substrate (dsDNA/SWNT-Au). The samples were washed with DI water to remove the excess of unattached DNA and subjected to XPS analysis (Table 3).

### Table 3: Results from XPS analysis of aligned SWNTs on Au support with and without dsDNA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% O 1s</th>
<th>% N 1s</th>
<th>% C 1s</th>
<th>% P 2p</th>
<th>% S 2p</th>
<th>% S-S</th>
<th>% S-C</th>
<th>% S-Ox</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWNT-Au</td>
<td>13.78</td>
<td>1.57</td>
<td>82.62</td>
<td>0.00</td>
<td>31.8</td>
<td>10.3</td>
<td>57.8</td>
<td></td>
</tr>
<tr>
<td>dsDNA/SWNT-Au</td>
<td>17.88</td>
<td>3.82</td>
<td>75.11</td>
<td>1.19</td>
<td>61.8</td>
<td>24.8</td>
<td>13.4</td>
<td></td>
</tr>
</tbody>
</table>

A detailed examination of S 2p XPS spectra shows the presence of three types of S-bonds: S-S, S-C and S-O_x (Figure 27 and Table 3). The S-C and S-O_x can be due to impurities in the material or to residuals from the solvent (DMSO) used during sample preparation, but the dramatic increase in the amount of S-S bonds can be ascribed to the presence of the thiol-SS-marker in the dsDNA sequence. Accordingly, along with the
appearance of phosphorus in the XPS spectra of the dsDNA/SWNTs-Au sample, we can conclude that dsDNA was successfully attached to the CNTs matrix.

Figure 27: XPS S 2p spectra of a sample composed of aligned SWNTs on midwifed gold support a) without dsDNA and b) with dsDNA wrapped around the CNTs.

After wrapping dsDNA around the SWNT-Au, SLAC-3ZNF was introduced into the system creating SLAC-3ZNF/dsDNA/SWNT-Au-assemblies (Figure 19 (V)). As mentioned before, the genetically modified SLAC-3ZnF has three ZnF motifs, which allow the enzyme to attach to more than one SWNT or to attach to one and the same SWNT using two of the fused ZnFs. The latter would provide more stable enzyme immobilization and/or better interactions with CNTs.

Since the capability of SLAC–3ZnF to bind to dsDNA have been already demonstrated [84], in this study we only confirmed the presence of SLAC-3ZnF onto the developed assembly using XPS analysis. XPS spectra of the SLAC-3ZnF/dsDNA/SWNT-Au sample showed the presence of phosphorus and copper atoms (0.43 % and 0.1 %, respectively) (Figure 28). The recorded phosphorus can be due to the presence of DNA
and the observed copper can be associated only with the presence of the enzyme. SLAC belongs to the family of multi-copper oxidases named as such based on the incorporation of four and more copper atoms in their active centers. Therefore, the presence of Cu 2p spectrum unambiguously demonstrates the success of the enzyme immobilization.

Figure 28: C 1s, Au 4f, O 1s, N 1s, P 2p and Cu 2p XPS spectra of attached SLAC-3ZNF complex onto aligned f-SWNT/arylamine-modified gold surface through dsDNA scaffold.

4.6.3 Enzyme Activity and Stability

It has already been demonstrated that the SLAC-3ZnF enzyme can oxidize DMP and ABTS [84]. To further study the influence of the immobilization of SLAC-3ZnF onto the designed dsDNA/SWNT matrix, we monitored the amount of active enzyme attached to the DNA/SWNT scaffold. For that purpose an activity assay was carried out. f-SWNT were wrapped with dsDNA in solution of 0.01M phosphate buffer. Low buffer concentration was used to minimize electrostatic interactions between buffer cations and the phosphodiester backbone of DNA and ensure helical wrapping of DNA around the f-
SWNTs. SLAC-3ZnF was then added to the dsDNA/f-SWNT suspension and allowed to immobilize (Figure 29). The unimmobilized SLAC-3ZnF was removed from the sample by washing and resuspension in phosphate buffer three times. A control containing f-SWNTs with adsorbed SLAC-3ZnF (Figure 29) was used to compare the enzyme activity after direct enzyme adsorption onto the CNTs and the amount of active enzyme present onto the sample surface when the DNA scaffold was used for enzyme immobilization. The unattached SLAC-3ZnF was also removed from the sample by washing and resuspending it in phosphate buffer three times. 24hrs after preparation, the two samples were washed three times again to remove enzyme that did not attach during storage. The samples were resuspended again in phosphate buffer and aliquots were taken for carrying out the activity assay. 100 mM 2,6-dimethoxyphenol (DMP) was used as a substrate for the activity assay. The amount of DMP oxidized by the enzyme was monitored by measuring the absorbance of the DMP with UV-Vis spectrometer at 468 nm for five minutes and the amount of enzyme active units (U=1μmol/min of DMP) was calculated based on extinction coefficient of DMP equal to 14,800 M⁻¹ cm⁻¹.
Figure 29: Amount of enzyme active units present for SLAC-3ZnF physically adsorbed onto SWNTs (SLAC-3ZnF on SWNTs) along with SLAC-3ZnF attached to a dsDNA, adsorbed on SWNTs (SLAC-3ZnF to DNA/SWNT). The samples were prepared in 0.01M phosphate buffer tested after their preparation and 24 hours after being stored at 4°C. Prior to each measurement the samples were washed three times to remove the unattached and detached during storage protein.

The amount of active enzyme immobilized on the SWNTs either through physical adsorption or through the designed DNA scaffold was initially similar (Figure 29). However, 24 hours after the samples were stored at 4°C, the amount of enzyme active units decreased by approximately 40% for physically adsorbed SLAC-3ZnF and remained unaltered for SLAC-3ZnF attached to dsDNA/SWNT. Thus the active enzyme immobilized through the DNA scaffold after 24 hours of storage was significantly higher (P<0.002) than the physically adsorbed SLAC-3ZnF. This indicated that enzyme immobilization through dsDNA provides more stable enzyme attachment or leads to preserved enzyme activity.
4.7 Conclusions

In this study, the design of a three-dimensional DNA/CNTs scaffold for specific immobilization of a single enzyme was demonstrated. This design included the alignment of SWNT to form a brush-type structure on an Ar modified gold electrode surface where the SWNTs were then wrapped with partially hybridized strands of DNA. The dsDNA was used for the docking of protein engineered SLAC-3ZnF complex. The enzyme immobilization relied on the selectivity of dsDNA-ZnF interactions. Each step of the development of this assembly was discussed in detail and supported through AFM and/or XPS analyses. Finally, an activity assay of the attached through the DNA scaffold SLAC-3ZnF was carried out showing more stable enzyme attachment and preserved enzyme activity.

The engineered SLAC-3ZnF/DNA/CNTs assembly demonstrated here is an essential step in the development of complex three-dimensional bio-nano architectures and a step forward in the design of spatially oriented single- and multi-enzyme assemblies. The incorporation of two or more variants of zinc fingers that bind to different DNA sequences will allow for the precise placement of multiple proteins within nanometer distances, which will be the goal of a future study.
Chapter 5: Electrochemical Enhancement of Small Laccase DNA complex with DNA-Templated Gold Nanoclusters

5.1 Introduction

SLAC was chosen for proof of concept for single enzyme immobilization due to its high thermal and pH stability (up to pH 9.4), and high catalytic activity [26, 126, 127]. The genetic engineering of trimeric SLAC fused with the three zinc finger domains (SLAC-3ZnF), specifically ZnF268 has allowed for controlled docking of the enzyme conjugate with DNA [84, 128]. The position of the SLAC active centers in the protein molecule and the presence of ZnF for the immobilization do not allow rapid electron transfer from the electrode surface to the T1 center of the enzyme and thus limits the electrochemical efficiency of the system. Therefore, to decrease the electron transfer tunneling distance and improve the electron transfer rate in this study we explored the addition of metal nanoparticles to the SLAC-ZnF conjugate. The metal particles are bound to sequence specific DNA constructs to be recognized by SLAC-bound ZnF domains.

In this study, we have incorporated the use of gold nanoclusters (NCs) as means for enhanced electron transfer and overall improved EFC performance with ZnF directed immobilization of SLAC on DNA/CNTs architecture (Figure 30). Although bulk gold is inert, gold nanoparticles (AuNPs) have been shown to act as catalysts in several reductive reactions including ORR [129-135]. It was recently reported that a DNA-templated NC
(D3NC) on CNT facilitated the electron transfer during bilirubin oxidase catalyzed reduction of $O_2$ to $H_2O$ [136]. The goal of this research was to develop a stable architecture for SLAC-3ZnF binding in the presence of DNA-template NCs and demonstrate improved electron transfer and therefore higher ORR rate. The NCs were synthesized using the DNA template (D3). A ZnF recognition sequence was hybridized at the DNA sequence (D3D$_2$H-), and poly T tails at both ends were used for CNT wrapping. The resulting product (D3D$_2$-H-NC) was immobilized on SWNTs and used for SLAC-3ZnF attachment.

![Figure 30: Oxygen reduction to water by SLAC-3ZnF via gold nanocluster mediated electron transfer.](image)

We believe enzyme immobilization in this manner will lead to future MECs development that would allow for very controlled and customizable systems for two or
more enzymes. The latter will allow the design of systems with maximized power densities and lower overpotential for electrocatalysis. Herein we demonstrate that the D3NCs, when co-assembled with SLAC-3ZnF and SWNTs, improve both the ORR reaction kinetics and electron transfer from the electrode to the active site of SLAC.

5.2 Materials and Reagents

HDPlas™ single-wall/double-wall carbon nanotubes 99 wt% (diameter = 1-2 nm, length = 3-30µm) were purchased from cheaptubes.com. Custom oligomer sequences were ordered from Integrated DNA Technologies, Inc. Tetrabutylammonium bromide (TBAB-Nafion) was provided by Professor Shelley Minteer (University of Utah, Salt Lake City, UT). Lyophilized SLAC-3ZNF was expressed and purified as previously reported [128].

5.3 Synthesis and Purification of DNA-Templated AuNC Containing Zinc Finger Recognition Sequence

We designed a DNA sequence bearing the NC forming sequence, ZnF recognition sequence, and poly-T tails to enhance wrapping of the DNA with CNTs. The full-length sequence thus designed is represented as D3Dz:

5’-
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCCGAACCTGGGCTACCACCCTTACCCCCGCACCCGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3’
where the nanocluster forming sequence, as reported previously, [136] is shown in red, and the zinc finger recognition sequence in blue. We employed two approaches to synthesize the NC. In one approach we first synthesized the NC with D3DZ followed by subsequent hybridization of D3DZNC with the complimentary zinc finger recognition sequence H: 5’-GCGGGTGCG-3’ (Tm = 43.8°C). The hybridized sequence containing the cluster is represented here as D3DZNCH, implying that first the NC is synthesized with D3DZ, followed by hybridization. In the second approach, we first hybridized the zinc finger sequence followed by NC synthesis. The final cluster sample thus prepared is represented as D3DZHNC.

5.3.1 Preparation of D3DZNCH

For AuNC synthesis, 15 µM D3DZ was equilibrated with 225 µM HAuCl4·3H2O (Sigma-Aldrich, ≥ 99% trace metal basis) for ~18 h in 20 mM phosphate buffer pH 7 and 1 mM Mg(OAc)2 (Fisher Scientific) solution at 22°C. Upon equilibration, the solution color changed from colorless to light yellow. Cluster formation was initiated by addition of 2.25 mM dimethylamine borane (Sigma-Aldrich) as reducing agent followed by equilibration for additional 18 h at 22°C. At the end a light purple color solution was formed which was purified by spin filtration through 50 kDa MWCO membranes (Millipore). The light purple solution was retained in the membrane indicating the presence of a small amount of plasmonic Au particles. Pure NC was obtained as a light yellow color solution in the filtrate. An equimolar solution containing 91 µM D3DZNC and H (concentrations determined based on ε260 = 838.1 mM⁻¹cm⁻¹ of D3DZ and 83.9 mM⁻¹cm⁻¹ for H as provided by IDT) was hybridized by heating the mixture at 80°C for 5 min in
hybridization buffer (30 mM HEPES pH 7.5, 100 mM KOAc) followed by slow cooling to 4°C. The final hybridized solution was then buffer exchanged to 20 mM phosphate buffer pH 7 with 1 mM Mg(OAc)$_2$ using 10 kDa MWCO membranes and stored at 4°C until further use.

5.3.2 Preparation of D3D$_2$HNC

The hybridization of D3D$_2$ and H was performed as described above. After hybridization, the sample was buffer exchanged to 20 mM phosphate buffer pH 7 with 1 mM Mg(OAc)$_2$. Synthesis and purification of NC was performed using similar conditions as described above except that this time the temperature was maintained at 4°C to ensure that the hybridization is maintained at all times during cluster synthesis.

5.4 Development of SLAC-3ZnF Control Composites

Two control composites without NC were made in order to observe the effect of the NC on enhancing the electro-catalytic activity of SLAC-3ZnF represented by SZ$_3$ (Table 5) with and without the ZnF recognition sequence.

5.4.1 SWNTs Mixture

All samples were made with a mixture of 1.5% SWNT and 0.1% TBAB-Nafion in a 4:1 water to EtOH. The SWNT suspension was sonicated for 30 minutes to disperse the tubes. SLAC-3ZnF was suspended at an initial 67mg/ml in 0.01M potassium phosphate buffer (pH 7.5) for use in all samples.
5.4.2 Preparation of SLAC-3ZnF on SWNT-TBAB Scaffold

SLAC-3ZnF immobilized through direct adsorption on SWNT was prepared with 10µl SLAC-3ZnF and 40µl SWNT mixture described above. Samples were allowed to incubate for 24 h at 4°C prior to testing.

5.4.3 Preparation of SLAC-3ZnF with Zinc Finger DNA Recognition Sequence

For control samples with DNA but no NC, we designed DNA with the zinc finger recognition sequence and poly T tails for efficient wrapping of CNTs. The sequence is represented by DZ:

5’-TTTTTTTTTTTTTTTTTTTCGCCACGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3’

where zinc finger sequence is in blue. The sequence was hybridized with the complimentary H 5’-GCGTGGGCG-3’. The resulting hybrid is represented by DZH. DNA was stored at 4°C until ready for use. 5µl of DZH (10 mg/ml) was then suspended with 40µl of SWNT mixture, gently mixed and allowed to equilibrate for 12 hours at 4°C. 10µl SLAC-3ZnF was added to the suspension and left to incubate for 24 hours at 4°C. The final molar concentrations of DNA and SLAC-3ZnF were approximately 51.45 and 243.99 µM, respectively.
5.5 Development of SLAC-3ZnF Composites with NC

Samples containing NC with and without zinc finger recognition sequence were prepared to compare the effect of the recognition sequence on directing the SLAC-3ZnF to the DNA-NC and the resulting electrocatalytic performance of the composite. All samples were prepared with 1.5% SWNT 0.1% TBAB-Nafion mixture (initial concentration) and 67mg/ml (initial concentration) SLAC-3ZnF suspensions prepared as the control samples.

5.5.1 Preparation of SLAC-3ZnF with D3NC

We first combined 5 µl D3NC with a 40 µl SWNT mixture and left to allow for D3NC to attach to the nanotubes for 12 hours at 4°C before adding 10 µl SLAC-3ZnF suspension. Samples were left to incubate overnight for 24 hours at 4°C.

5.5.2 Preparation of SLAC-3ZnF with D3DzNCH

Samples containing zinc finger recognition sequences hybridized prior to NC synthesis were made by first mixing 5µl D3DzNCH with 40µl SWNT mixture and left for 12 hours at 4°C. 10µl SLAC-3ZnF was then added to each samples and allowed to incubate for 24 hours at 4°C.

5.5.3 Preparation of SLAC-3ZnF with D3DzHNC

Samples containing zinc finger recognition sequences hybridized after NC synthesis were made by first mixing either 5µl D3DzHNC with 40µl SWNT mixture and left for 12 hours at 4°C. 10µl SLAC-3ZnF was added to each samples and allowed to incubate for 24 hours at 4°C.
5.6 Analytical Methods

5.6.1 MALDI-MS

MALDI-MS data were collected on ABSciex 4800 Plus TOF/TOF MALDI mass spectrometer using sinapinic acid (Sigma-Aldrich) as the matrix. Molecular weight of D3D\(_2\)NC sample was determined to be ~32221 Da indicating the formation of ~10 atom gold cluster (MW of D3D\(_2\) = 30264 Da).

5.6.2 Electrochemical Measurement

Open circuit potentials (OCP) were run for 30 minutes or until stable.

Linear sweep voltammetry (LSV) was performed on all SZ\(_3\)/SWNT composites using a Princeton Instruments rotating disk electrode (RDE) with 0.2465cm\(^2\) glassy carbon electrode and Versa Stat MC equipped with Pine Company Instruments rotating motor and control box. The RDE was polished prior to each use with 0.05 mm alumina and rinsed with deionized water. 20 \(\mu\)l of each SZ\(_3\)/SWNT composite was dropped on the RDE and allowed to air dry at 4\(^\circ\)C. Reduction currents were measured at rotation rates 1600, 1200, 800, 400 and 0 rotations per minute (rpm) from applied potential of 0.8 to -0.6 V with a scan rate of 100 mV/s with RDE submerged in 0.1 KPB (pH 7.5). Testing was done under dissolved, saturated or depleted O\(_2\) environment. Depleted and saturated O\(_2\) was achieved by purging the buffer with compressed N\(_2\) or O\(_2\), respectively, for a minimum of 20 minutes prior to testing. Ag/AgCl and platinum wire electrodes were used as reference and counter electrodes, respectively. The swept potential was represented both versus Ag/AgCl and reversible hydrogen electrode (RHE).
Potentiostatic polarization curves were carried out by chronoamperometric (CA) measurements starting from OCP to -0.2V vs. Ag/AgCl with a step of 50mV. The samples tested were placed on a small glassy carbon cap electrode (GCCE). GCCE was polished before each use with 0.05 mm alumina and rinsed with DI water. 5µl of the samples were dropped on 0.085 cm² multi-walled carbon nanotubes buckypaper (BP) electrodes, placed on GCCE and allowed to air dry at 4°C. The GCCE was submerged in 0.1 M KPB (pH 7.5) and tested using Ag/AgCl reference and platinum wire counter electrodes. Potentials were held for 300 seconds each from OCP to -0.25 V at 0.05V increments in saturated O₂ electrolyte. Conversion to RHE for both LVS and CA were done using $E_{RHE} = E_{Ag/AgCl} + 0.059pH + E^0_{Ag/AgCl}$ with $E^0_{Ag/AgCl} = 0.197$ V.

5.7 Table of DNA Templates and Enzyme Composites

The following table show the description of each component and abbreviations used for the purpose of simplification:
Table 4: Description of DNA sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Nucleotide Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3</td>
<td>NC Template</td>
<td>ACCCGAACCTGGGTACCAACCCTTAATCCCC</td>
</tr>
<tr>
<td>D3DZ</td>
<td>ZnF Recognition with NC Template</td>
<td>ACCCGAACCTGGGTACCAACCCTTAATCCC CGCACCCGC</td>
</tr>
<tr>
<td>D3H</td>
<td>ZnF Recognition Sequence</td>
<td>-CGCCCACGC-Poly T</td>
</tr>
<tr>
<td>Poly-</td>
<td>Poly Thymine Tail</td>
<td>-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT</td>
</tr>
</tbody>
</table>

Table 5: Description of SLAC-3ZnF composites

<table>
<thead>
<tr>
<th>Composite</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWNT</td>
<td>SWNT</td>
<td>Single-walled carbon nanotubes</td>
</tr>
<tr>
<td>D3H/SWNT</td>
<td>D3H</td>
<td>ZnF hybrid recognition sequence wrapped with no AuNC</td>
</tr>
<tr>
<td>SZ3/SWNT</td>
<td>SZ31</td>
<td>SLAC-3ZnF directly adsorbed on SWNT</td>
</tr>
<tr>
<td>SZ3/D3H/SWNT</td>
<td>SZ32</td>
<td>SLAC-3ZnF bound by ZnF recognition sequence on SWNT</td>
</tr>
<tr>
<td>SZ3/D3NC/SWNT</td>
<td>SZ3NC</td>
<td>AuNC without ZnF recognition sequence</td>
</tr>
<tr>
<td>SZ3/D3D2NCH/SWNT</td>
<td>SZ3NC1</td>
<td>ZnF hybrid recognition hybridization after to AuNC synthesis</td>
</tr>
<tr>
<td>SZ3/D3D2HNC/SWNT</td>
<td>SZ3NC2</td>
<td>ZnF hybrid recognition hybridization prior AuNC synthesis</td>
</tr>
</tbody>
</table>
5.8 Results and Discussion

5.8.1 XPS Characterization of D3D₂NCH and D3D₂HNC

We used x-ray photoelectron spectroscopy (XPS) to probe the oxidation state of Au in the NC samples. Both the D3D₂NCH and D3D₂HNC samples display intense peaks at 84.9 eV, 88.6 eV (Figure 31a: D3D₂NCH), and 84.7 eV, 88.4 eV (Figure 31b: D3D₂HNC), corresponding to the Au 4f\textsubscript{7/2} and Au 4f\textsubscript{5/2} components, respectively (Figure 31: blue lines). Deconvolution of the spectral envelope yielded single Au(I) species (Figure 31: green lines for Au 4f\textsubscript{7/2} and gray dotted lines for Au 4f\textsubscript{5/2} components) corresponding to 86% and 92% of total in D3D₂NCH and D3D₂HNC, respectively. The remaining Au is present as some residual unreduced Au(III) species (Figure 31: dotted magenta lines for Au 4f\textsubscript{7/2} at 87 eV and gray dotted lines for Au 4f\textsubscript{5/2} at 90.6 eV).

Figure 31: The NCs consist of primarily Au(I) oxidation state in both D3D₂NCH (a) and D3D₂HNC (b) samples. Experimental data: blue curves, simulated data: red curves, Au 4f\textsubscript{7/2} for Au(I) is shown as green lines, residual Au(III) species is shown as dotted magenta lines. The corresponding Au 4f\textsubscript{5/2} components are shown as gray dotted lines.
5.9 Electrochemical Analysis of SLAC-3ZnF Composites

Recently we reported the design of a directed immobilization of SLAC-3ZnF using the high specificity of the ZnF binding to a specific DNA sequence. Although the catalytic activity in terms of DMP reduction was shown for both SZ31 and SZ32 [128], it was discovered that electrochemical activity towards ORR for of these composites is poor. It was posited that the impeded electron transfer was in part due to increased distance of the enzyme active centers from the SWNT scaffolds resulting in poor electrocatalytic performance by SLAC. Encouraged by a recent study where a D3NC was shown to improve the electrocatalytic performance of bilirubin oxidase (BOD) in catalyzing ORR while not affecting the 4e\(^-\) reduction of O\(_2\) to H\(_2\)O [9], we hypothesized that by using gold nanoclusters the ORR reaction kinetics and electron transfer could be improved in the SLAC-3ZnF system.

5.9.1 Linear Sweep Voltammetry Characterization

In order to study the effectiveness of NCs on improving the electron transfer we performed linear sweep voltammetry (LSV) experiments on five different SLAC composites employing different immobilization methods. We tested each composite in 0.1 M KPB under dissolved (~6.91 mg/L), saturated (8.66 mg/L), and depleted (~0.66 mg/L) O\(_2\) environments at 1600 rpm, respectively. Under depleted O\(_2\) conditions only modest currents were observed.

Figure 32 and Table 6 show the results from each sample tested under saturated O\(_2\) conditions. The control composites SZ31 (Figure 32, black line) demonstrated modest
catalytic activity even in the presence of saturated O\textsubscript{2} with SZ\textsubscript{3}2 (Figure 32, red line) displaying lower onset potential E\textsubscript{onset} = 0.130V (Table 6) in comparison to all other samples. Obviously the presence of the NC caused significant changes in the ORR profile displayed by the SZ\textsubscript{3}NC/SWNT composites (Figure 32, red, blue and green lines). More positive onset potential of ORR was observed for SZ\textsubscript{3}NC1 (Figure 32, pink line), SZ\textsubscript{3}NC (Figure 32, green line) and SZ\textsubscript{3}NC2 (Figure 32, blue line) in comparison to both SLAC-3ZnF samples with no D\textsubscript{2}H present (See Table 6). The higher onset potential for those composite materials showed enhanced thermodynamics when compared to composites without NCs. In addition, the electrocatalytic current density was increased to ~1.125 \textmu A/cm\textsuperscript{2} at -0.1V vs. Ag/AgCl, an increase of ~880 \textmu A/cm\textsuperscript{2} compared to SZ\textsubscript{3}2 composite and ~925 \textmu A/cm\textsuperscript{2} compared to SZ\textsubscript{3}1. These results, therefore, suggest that the presence of NC enhances the ORR activity of the SLAC by lowering the overpotential and increasing the kinetics of the reaction.
Figure 32: LSV of SZ31 (black); SZ32 (red); SZ3NC (green); SZ3NC1 (pink); and SZ3NC2 (blue) composites in O₂ saturated 0.1 M potassium phosphate buffer (pH 7.5). Three individually prepared samples of each composite were tested and averaged. Standard deviation represented by the shaded areas. Potential sweep vs. Ag/AgCl was performed from 0.8 to -0.6V, scan rate=0.01V/s; and rotation rate (ω)= 1600 rpm. The potential was also converted to RHE using $E_{RHE} = E_{Ag/AgCl} + 0.059pH + E^\circ_{Ag/AgCl}$ with $E^\circ_{Ag/AgCl} = 0.179$ V.
Table 6: LSV Electrochemical Results of SLAC-3ZnF/SWNT Composites in O₂-Saturated Buffer.

<table>
<thead>
<tr>
<th>Composite</th>
<th>$E_{onset}/V$ vs Ag/AgCl (V vs RHE)</th>
<th>$\Delta j/\mu A/cm^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SZ₃₁</td>
<td>0.130 (0.752)</td>
<td>141</td>
</tr>
<tr>
<td>SZ₂₂</td>
<td>0.113 (0.735)</td>
<td>222</td>
</tr>
<tr>
<td>SZ₃_NC</td>
<td>0.225 (0.847)</td>
<td>1050</td>
</tr>
<tr>
<td>SZ₃_NC₁</td>
<td>0.252 (0.874)</td>
<td>1137</td>
</tr>
<tr>
<td>SZ₃_NC₂</td>
<td>0.240 (0.877)</td>
<td>1114</td>
</tr>
</tbody>
</table>

Potentials vs. RHE are in parenthesis. $\Delta j$ is the differential current density between the current at the onset potential and the current at -0.1 V.

5.9.2 Polarization Curve for SLAC-3ZnF Immobilized on ZnF Recognition DNA

Potentiostatic polarization curves of the SZ₃/NCs-DNA composites have been performed to evaluate their applicability as cathodes for ORR. Slightly different cathode design has been explored this time. Composite materials of SZ₂₂, SZ₃_NC₁ and SZ₃_NC₂ were dropped on circular disk of multi-walled carbon nanotubes “Buckypaper”. The BP electrodes were then placed on glassy carbon cap electrode and allowed to air dry at 4°C.
The polarization curves were carried out under saturated O₂ conditions to minimize the mass transfer limitations. The resulting polarization curves show an increase in current density from 100.04 +/- 2.9 µA/cm² for SZ₃2 (red, Figure 33) to 210.22 +/- 35.8 µA/cm² for SZ₃NC2 (blue, Figure 33) and 298.59 +/- 15.7 µA/cm² for SZ₃NC1 (pink, Figure 33). The latter supports the data collected with the LSV, where improved current density was seen in presence of NC. It can also be seen that when the NCs were synthesized prior to DNA hybridization a higher ORR rate results and thus higher current densities were recorded.

Figure 33: Polarization curves for SZ₃2 (red), SZ₃NC1 (pink), and SZ₃NC2 (blue).
As we mentioned previously the proposed mechanism by which the NC enhances the ORR activity of the SLAC/SWNTs composites is by facilitating the electron uptake of the enzyme from the poised electrode surface. This is possible due to the electrochemical activity of the NCs, their mediation role and the decreased electron traveling distance.

5.10 Conclusion

In this communication, we report the effects of the synthesis of DNA-templated gold nanoclusters with addition of ZnF recognition DNA. NC synthesis was confirmed by a combination of MALDI-MS and XPS analysis. We showed dramatically increased current densities when the electron transfer was facilitated by the presence of the NC. It was also observed that the SLAC-3ZnF/NCs-DNA composite with synthesized NCs prior DNA hybridization are having higher performance in terms of ORR and generated current densities.
Chapter 6: Malate Dehydrogenase and Citrate Synthase on Modified Multi-Walled Carbon Nanotubes for Mediated L-Malate Oxidation

6.1 Introduction

The TCA cycle is an important part of the natural metabolic mechanism responsible for producing energy, adenosine triphosphate (ATP) regulation and certain biosynthesis building blocks. In eukaryotes, the TCA cycle begins within the mitochondria as pyruvate is converted to oxaloacetate and acetyl coenzyme A (acetyl coA) by pyruvate carboxylase (PCB) and pyruvate dehydrogenase (PDH), respectively. Oxaloacetate and acetyl-CoA then bind to citrate synthase (CS) forming citrate. The cycle ends when oxaloacetate is regenerated by the oxidation of L-malate by malate dehydrogenase (MDH) and the oxidized form of nicotinamide adenine dinucleotide (NAD\(^+\)). Resulting NADH molecules are then transported into the oxidative phosphorylation cycle. Several NAD-dependent redox enzymes are responsible for the oxidation of intermediate products throughout the cycle.

The enzyme that completes the TCA cycle, MDH, has been one enzyme of interest due its electrochemical properties, general instability in aqueous solution and NAD dependence. MDH catalyzes the reversible oxidation of L-malate to oxaloacetate in the presence of NAD\(^+\). Oxaloacetate is then channeled through citrate synthase and used with acetyl-CoA to produce citrate. The formation of an MDH/CS complex and channeling of
oxaloacetate through it has been shown through mathematical and experimentally means [39, 45]. Given this evidence along with evidence of MDH/CS complex formation, it would logical to conclude that additional means for enzyme orientation would not be necessary when using sequential enzymes and would simplify methods used for optimizing reaction pathways due to lowered risk to structural integrity. However, because naturally occurring MECs are unstable and dissociate quickly, finding methods for increased stabilization are a necessary step in producing EFCs that can compete against the current energy technology.

Immobilization on nanomaterials is one area that has been extensively researched to address stability issues. Nanomaterials are ideal for this purpose due to their comparable to enzymes size, nanomaterials structural and electronic properties, and ability to interact with biological molecules[17, 18, 67, 74, 75, 89, 93, 94, 137-144]. For MECs, numerous co-localization techniques have been explored for EFCs including entrapment, covalent bonding, direct-physical adsorption and cross-linking using biomolecules, polymers and surfactant [27]. However, choosing the best method is highly dependent on enzyme properties. Direct adsorption onto some nanomaterials can lead to denaturing of the enzyme resulting in loss of function. PBSE and biomolecules like chitosan (chit) have also been used for immobilization of enzyme on nanomaterials in sensors and EFCs [41, 91-93, 100, 101, 103].

For the purposes of this study, we focused on the incorporation of nanomaterials, specifically multi-walled carbon nanotubes (MWNTs) for stabilization and increased electron transfer to electrode surfaces of PMG-modified MWNT Buckypaper (PMG-BP). Our goal was to identify methods in which the native structure and therefor oxidative
function of the MDH with CS are not negatively impacted. Two methods that we address here for MDH/CS co-localization include entrapment via deacylated chit or tethering with PBSE. These two methods differ significantly in enzyme interaction but both have been shown to produce stable scaffolds for immobilization on nanotubes. We report in this chapter the effects of concentration and applied potential on each immobilization method in the presence of CS.

6.2 Reagents

Malic acid (No. M1000), aconitase (porcine heart, lyophilized powder, No. A5384), citrate synthase (porcine heart, (NH₄)₂SO₄ suspension, No. C3269), β-nicotinamide adenine dinucleotide hydrate (No N7004), chitosan (No. 03312A7) and 1-pyrenebutanoic acid succinimidyl ester (PBSE, No. MKBQ6358V) were purchased from Sigma Aldrich (St. Louis, MO). Citrate synthase (CS) was purified using centrifugation to remove from (NH₄)₂SO₄ immediately prior to use. Malate dehydrogenase (porcine heart, lyophilized powder, No. 18670) was obtained from Affymetrix USB products (Cleveland, OH) and used as received. Multi-walled carbon nanotubes (MWNT) were obtained from www.cheaptubes.com (Cambridgeport, VT). Tetrabutylammonium bromide (TBAB-Nafion) was provided by Professor Shelley Minteer (University of Utah, Salt Lake City, UT)

6.3 Materials

A Reference 600™ potentiostat/galvanostat/ZRA from Gamry Instruments (Warminster, PA) was used for all electrochemical methods. Cells for electrode testing
were constructed from a proprietary polypropylene cell with graphite plate current collector
provided by CFD Research Corporation (CRDRC, Huntsville, AL), 3M KCl Ag/AgCl reference and platinum wire counter electrodes. The Buckypaper (BP) for electrodes (20 gsm, M and C-grade mixture) was purchased from Nanotech Labs Composites (Yadkinville, NC).

6.4 Electrode Preparation Protocol

6.4.1 Polymerization of Methylene Green Film on BP

Electropolymerization was used for depositing a PMG film onto the carbon nanotube paper. To accomplish this, first, a buffer solution of 0.6mM methylene green (MG) in 0.1M potassium phosphate buffer (KPB, pH 7) and 0.1M potassium nitrate (KNO₃) was prepared and purged with compressed nitrogen (N₂) for 15 minutes to remove oxygen. Working electrodes were made from high conductivity, c/m-grade blend MWNT Buckypaper (BP) cut to approximately 1.77 cm² each. Reduced hydrophobicity was achieved through brief treatment with isopropyl alcohol (IPA) and then rinsed with DI water immediately prior to film deposition. A standard three-electrode cell using Ag/AgCl reference electrode, stainless steel mesh counter electrode and BP electrode was set up with 15 ml MG solution with N₂ purging. Polymerization was carried out through 10 cyclic voltammetry cycles with an applied potential range of -0.5 to 1.3V at a scan rate of 50 mV/s resulting in the deposition of a PMG layer on the BP surface (PMG-BP). Each PMG-BP electrode was thoroughly washed with DI water and air-dried.
6.4.2 Chitosan/MWNT Composite Preparation

All enzymes immobilized on MWNT using entrapment with chitosan were done in a 95% chitosan to 5% MWNT mixture (Chit/MWNT). Deacylation of medium weight chitosan to 95% was carried out through autoclaving for 20 minutes at 121°C and 15 psi in 45% NaOH solution and dry vacuumed for 24 hours [99, 101]. A 1% deacylated chitosan in 0.25 acetic acid (Chit, pH 6) suspension was prepared and stored at 4°C. From this suspension, a mixture of MWNT and chitosan with a 2.5 wt.% MWNT was made (Chit/MWNT). The mixture was stored at 4°C and stirred immediately prior to use to ensure uniform distribution and prevent polymerization while at room temperature.

6.4.3 Malate Dehydrogenase Entrapment with Chitosan/MWNT

1.5 mg of MDH were combined with 150µl of Chit/MWNT mixture and left to incubate at 4°C for 30 minutes. Samples were then transferred and distributed on prepared PMG-BP electrodes and left to air dry overnight at 4°C.

6.4.4 Malate Dehydrogenase and Citrate Synthase Entrapment with Chitosan/MWNT

138 µl of CS suspension in ammonium sulfate (9 mg/ml) was centrifuged at 1000xg for 10 minutes to remove CS from solution. The resulting pellet (~1.28 mg CS) was combined with 1.5 mg MDH and then immediately and carefully mixed with 150 µl Chit/MWNT mixture (MCChit). The mixture was allowed to incubate for 30 min at 4°C before being transferred and distributed on the prepared PMG-BP electrodes.
6.4.5 PBSE /MWNT Composite Preparation

For all PBSE/MWNT samples, 2.5%wt MWNT with 0.1 % TBAB-Nafion, were suspended in 0.1M KPB (pH 7.5), placed under water sonication for 30 minutes and stored at room temperature. 4 mg/ml PBSE in ethanol (EtOH) suspension was prepared using water sonication. Suspensions of PBSE and 2.5%wt MWNT (20% PBSE, 80% MWNT) were combined, briefly mixed using a vortex mixer and left for 1 hour at 23° C to combine.

6.4.6 Malate Dehydrogenase Cross-Linkage with PBSE on MWNT

1.5 mg of MDH were combined with 200 µl of MWNT-PBSE suspension and left to incubate at 4°C for 18 hours (MP). Samples were then transferred and distributed on prepared PMG-BP electrodes and left to air dry at 4°C before testing.

6.4.7 Malate Dehydrogenase and Citrate Synthase Cross-Linkage on MWNT with PBSE

As with chit/MWNT samples, 138µl of CS suspension in ammonium sulfate (9 mg/ml) was centrifuged at 1000xg for 10 minutes to remove CS from solution. The resulting pellet was combined with 1.5mg MDH and then carefully mixed with 200µl of the PBSE-MWNT mixture (MCP). The mixture was allowed to incubate for 24 hours at 4°C before being transferred and dried on the prepared PMG-BP electrodes (Figure 34).
Electrochemical Measurement

Cells were assembled using prepared electrodes placed on graphite plate electrodes and secured against a polypropylene cell for all composites. All potentials were tested against Ag/AgCl reference with a platinum wire counter electrode. 1.75M L-malate solution was prepared from L-malic acid in DI water and adjusted to pH 7.4 with concentrated NaOH for use with all measurements. For cells with MDH only, approximately 2.6ml of a solution containing 0.1 KPB, 0.1 KCl and 5 mM NAD⁺ (pH 7.4) solution was transferred into the poly-propylene cells. For cells with MDH and CS, a solution of 0.1M KPB, 0.1 M KCl, 5 mM NAD⁺ and 1 mM Acetyl Co-A (pH 7.4) was transferred into the poly-propylene cells. Cells were checked for air bubbles in the solution and on the electrode surface prior to running any measurements. An open circuit potentials (OCP) measurement was run for 1 hour or until voltage was stable for each electrode.
6.5.1 Kinetic-Concentration Measurement

After OCP measurements were complete, chronoamperometry (CA) was used to measure current at increasing concentrations of L-malate. Prior to L-malate addition, CA was left to stabilize to where a steady-state current was observed. Aliquots of L-malate were then added at 300 second intervals until a concentration of 0.1M was reached. Results were plotted as current ($i$) versus L-malate concentration ($[M]$).

Michaelis-Menten equation (Eq. 21) was used to determine parameters for MP, MCP and MChit electrodes and bi-hill equation was used to determine MCChit parameters (Eq. 22).

\[
i = \frac{i_{\text{max}} [M]}{K_M + [M]} \quad \text{(Eq. 21)}
\]

\[
i = P_m \frac{1+(K_a [M])^{H_a}}{1+(K_i [M])^{H_i}} \quad \text{(Eq. 22)}
\]

Where $i$ is the reaction rate or current ($\mu$A) at $[M]$; $i_{\text{max}}$ is the maximum current reached by the system, $[M]$ is the concentration of L-malate; $K_M$ is the concentration at $\frac{1}{2} i_{\text{max}}$; $P_m$ is the peak current observed; $K_a$ is the L-malate concentration at $\frac{1}{2} P_m$ during activation; $K_i$ is the L-malate concentration at $\frac{1}{2} P_m$ during inhibition; and $H_a$ and $H_i$ are the hill coefficients for activation and inhibition, respectively.
6.5.2 Polarization Measurement of Electrodes

150µl of L-malate solution (final concentration of approximately 0.1M) was added to cell prior to start of the polarization measurements. Each sample was measured for 300 seconds at a constant potential from -0.35 to 0.35V at 0.05V increments. Resulting current density (j) were plotted against the applied potential (E).

6.6 Results and Discussion

6.6.1 Electropolymerization of PMG on MWNT Type “Buckypaper”

The use of PMG for NAD$^+$ regeneration has been extensively studied [115, 145, 146] and been implemented for use in NAD dependent DH-based electrodes due to its effectiveness in reducing overpotential [12, 17, 41, 42, 72, 116]. Previous studies have demonstrated the usefulness of various immobilization techniques in conjunction with mediators such as PMG for the regeneration of NAD$^+$ using MDH in anodic electrodes [41, 42, 99]. These studies addressed the issue of NAD$^+$ regeneration for reducing overpotential and improving the overall statistics of the electrode. We have chosen PMG for the modification of MWNT type BP for the purpose of NAD$^+$ regeneration in MDH and MDH/CS electrodes immobilized through PBSE/MWNT and Chit/MWNT scaffolds. The electrodes were modified prior to enzyme/scaffold addition using cyclic voltammetry as described earlier. The number of cycles and range used was determined based on prior SWNT PB modification with PMG film [42].
Figure 35: Representative cyclic voltammogram results for poly-methylene green deposition on MWNT type BP. 10 cycles were carried out consecutively from an applied potential range of -0.5 to 1.3 V with a 50 mV/s scan rate. Cycle 1 and cycle 2 are indicated here and are evidence of altered surface composition.

Ten CV cycles from -0.5 to 1.3 V were run to form the PMG film. The voltammetry results were typical for PMG deposition observed in previous studies (Figure 35) [41, 42, 117] and is evidence of MG polymerization on the surface of BP. The peak observed at approximately -0.2V, which represents the un-polymerized MG reduces with each cycle applied until no longer present at cycle 10; while the polymerization peak at approximate 0.1V shifts and becomes increasingly present.
6.6.2 L-Malate Oxidation by MDH-Based PMG-PB Electrodes

In order to study the affects of CS interaction with MDH and immobilization techniques for co-localization on fuel cell oxidation potentials, two methods were utilized. The first was to immobilize MDH alone and in the presence of CS using entrapment via chit-modified MWNT. The second was to use covalent crosslinking to MWNT via PBSE, for a direct comparison. Coenzyme/KBP solutions, PBSE, chit, MWNT and enzyme concentrations were held constant throughout experiments and were the same for all composites. Deacylated chit was chosen as one method because it has been shown to be an optimal structure for enzyme immobilization [90, 101, 102] and ability to solubilize carbon nanotubes [98, 147]. PBSE was chosen because it forms an amide bond with proteins using primary amines and pi-stacks on nanotubes structures.

For composites containing both MDH and CS, we added each enzyme simultaneously to allow for equal interaction time between enzymes and scaffolds. The goal was to see how L-malate oxidation would be affected when MDH was in complex with or in possible competition for immobilization with CS depending on the method used. The resulting composites were deposited on the prepared PMG-BP and measured by applying a constant potential of 0.3V with increasing concentrations of L-malate (Figure 36). Comparing the two MDH immobilization methods, PBSE or chitosan, we saw that the maximum current \( i_{\text{max}} \) reached with PBSE/MWNT \( i_{\text{max}}=11.1 \pm 0.2 \mu\text{A} \) was twice higher than Chit/MWNT \( i_{\text{max}}=5.7 \pm 0.2 \mu\text{A} \) and the corresponding apparent Michaelis-Menten constant, \( K_{\text{Mapp}} \), for MChit was 1.2 times higher in comparison to the PBSE tethering technique (Figure 36, Table 7). The higher \( K_M \) value for MChit suggests lower binding affinity of L-malate to MDH although it may be due to mass transport inhibition of L-malate through chitosan.
electrodes behavior deviates from the Michaelis-Menten model used to fit all other electrodes resembling inhibition-like behavior at [M]>20mM. A bi-hill equation fit was used to determine $P_m$ and $K_a$ and estimate the binding affinity for L-malate (Figure 36). While there is a two-fold increase in $i_{\text{max}}$ of MCP in relation to MP, at $P_m$ ($P_m=35.0\mu\text{A}$) of MCChit, at approximately [M]=20mM was two times higher than observed at the same malate concentration for MCP ($i_{20\text{mM}}=17.5\mu\text{A}$) (Table 7) indicating higher conversion efficiency for the MCChit electrodes. The $i_{\text{max}}$ for MCChit is also 12 times higher than observed with MChit electrodes. At [M] between 20 and 40 mM, product inhibition behavior is seen where current decreases from $35.0\mu\text{A}$ to $24.9\mu\text{A}$ before reaching apparent saturation. This inhibition effect is most likely a result of the binding of citrate to an allosteric site of MDH as proposed previously. The latter is possible due to the presence of
CS in the artificially created MDH/CS metabolon. $K_a$ of MCChit was compared to $K_M$ of MP, MCP and MChit. For MDH only electrodes, $K_M$ for MP and MChit were approximately 14.5mM and 17.3mM, respectively. The binding affinity increased with addition of CS to the MDH on PBSE and chitosan where $K_M$ reduced to 4.9mM and $K_a = 3.7mM$ (Table 7).

There are several factors that may account for this behavior seen in entrapment versus covalent crosslinking:

1. Since there is evidence for MDH and CS complex formation through residue cross-linking and the likely involvement of C- and N- termini in surface interfaces [43], and PBSE forms amide bonds from reaction between primary amine in the proteins and its ester group; the close proximity of the N- and C-termini may be interfered with due to reaction with the ester and primary amine for cross-linking to carbon nanotubes leading to fewer MDH/CS complex present at MWNT surfaces.

2. CS and MDH may be competing for binding to PBSE.

3. The deacylation of chitosan where acyl groups (–COCH$_3$) are removed increases amine groups and makes it less reactive to nucleophilic attack from primary amines on proteins. The reaction with the resulting amine with carboxyl groups at the C-terminus is non-spontaneous and would require heat in order to create a bond. Therefore, it is unlikely that the protein complex would be disrupted by chitosan and more likely to have higher amounts of MDH/CS interaction than with amide bonding.
4. Although, lower activity of enzymes immobilized in chitosan versus free enzymes has been reported and thought to be a result of the lowered mass transport of substrates through the porous structure [101]; at low [M], mass transport is likely not as hindered. In addition, citrate may bind to an allosteric binding site in the MDH, shifting equilibrium in favor of oxaloacetate/NADH production resulting in increased reaction rate [39, 45] therefor a higher current detected.

5. Saturation of CS with citrate inhibits oxaloacetate binding to the enzyme, resulting in a higher concentration of free oxaloacetate [Ox] in the buffer solution. High [Ox] inhibits L-malate oxidation by MDH [148].

The results from this study show that in the presence of CS, the oxidation of L-malate through MDH is enhanced. The data suggests that MDH/CS complex formation may not be as favored with PBSE as with chitosan scaffolds. This would suggest that chitosan is a more appropriate method for MDH/CS immobilization than covalent tethering at low substrate concentrations. The increase in recorded current of MDH/CS using PBSE may however still be evidence of MDH/CS complex formation.
Table 7: Summary of concentration study parameters obtained for chronoamperometry measurements of PMG-BP electrodes.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Composition</th>
<th>$K_M$ (mM)</th>
<th>$i_{max}$ (µA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP</td>
<td>MDH immobilized on PBSE/MWNT</td>
<td>14.5</td>
<td>11.1 ± 0.2</td>
</tr>
<tr>
<td>MCP</td>
<td>MDH and CS immobilized on PBSE/MWNT</td>
<td>4.90</td>
<td>22.7 ± 0.4</td>
</tr>
<tr>
<td>MChit</td>
<td>MDH immobilized in a Chit/MWNT</td>
<td>17.2</td>
<td>5.66 ± 0.2</td>
</tr>
<tr>
<td>MCChit</td>
<td>MDH and CS immobilized in a chit/MWNT</td>
<td>$K_a=3.7$</td>
<td>$P_m=35.01$</td>
</tr>
</tbody>
</table>

6.6.3 Polarization Curves of MDH-Based PMG-BP Electrodes

A potentiostatic polarization curves were also carried out to support the results of the concentration experiment. A series of chronoamperometry measurements were performed for 300 seconds each to study the behavior of the system at incremental potential increase. The resulting current density ($j$) for the anodes was plotted versus the applied potential (Figure 37). The results from the polarization curves supported the concentration data with CS containing electrodes out-performing the MDH electrodes by 2-fold increase in current density. Both single enzyme and two-enzyme electrodes showed similar current densities. At 0.3 V, $j$ is approximately $17.4 \pm 1.5 \, \mu A/cm^2$ for MP, $14.6 \pm 1.2 \, \mu A/cm^2$ for MChit, $28 \pm 1.4 \, \mu A/cm^2$ for MCChit and $29 \pm 0.6 \, \mu A/cm^2$ for MCP.
Figure 37: Anodic polarization curve of MDH (black) and MDH/CS (red) enzymes on a) PBSE/SWNT and b) Chit/MWNT scaffolds in, respectively. Potentials were applied from -0.35 to 0.35V at 0.05V increments.

6.7 Conclusion

In this study, the affects of CS presence on oxidation of L-malate by MDH using two methods of immobilization, tethering with PBSE and entrapment in deacylated chitosan on MWNT were tested using chronoamperometry. BP electrode surfaces were first prepared using poly-(methylene green) modification. Prepared MP, MCP, MChit, MCChit were deposited on the PMG-BP and tested for oxidation potential of L-malate by MDH. It was observed that in the presence of CS, the MDH-based electrodes performance was significantly improved showing a 2-fold increase in current density. This may support the hypothesis that citrate plays a roll in MDH regulation.
Chapter 7: Conclusion: Biocathode and Bioanode

Design for Enzyme Immobilization

In the preceding chapters the use of CNT with immobilization methods for redox enzyme immobilization was demonstrated. The objectives of this research were to show the usefulness of these techniques at the anodes and cathodes for enzyme-based biofuel cells. The resulting systems were not combined into a fully functional cell and were therefore treated individually for experimental design.

7.1 SLAC-3ZnF Immobilization on DNA/SWNT Scaffold

7.1.1 Fabrication of DNA/SWNT Scaffolds

A Novel 3D scaffold for stable immobilization of one or more enzymes was designed and fabricated in a step-wise bottom up fashion. Gold on glass electrodes were electrochemically modified with an arylamine film and f-SWNT. DNA was then wrapped around the resulting SWNT “forest” like structure. Characterization was done at each step of the process. AFM was carried out on bare gold, arylamine modified gold and after the SWNT had been aligned. The resulting images showed a significant change in surface topography after the modification of Au with arylamine as well as with f-SWNT confirming successful development of the SWNT “forest”.

XPS was used as well to confirm changes in the surface composition. Analysis confirmed CNTs presence on the Ar-gold surface through % increases in C 1s, O 1s; and decreases in Au 4f and N1s compared to just Ar-gold. After thiol-DNA wrapping, XPS confirmed presence of ssDNA and hybridize DNA by the presence of phosphorous from
the DNA backbone and S-S from that could only be from the thiol marker. This showed that the scaffold had been successfully fabricated as it would be unlikely for the DNA to remain on the surface after washing without having wrapped the SWNT.

SLAC-3ZnF was immobilized after conformation of DNA/SWNT on Ar-gold electrodes. Because SLAC is in the class of multi-copper oxidases, XPS was used to identify the presence of Cu. After the sample had been submerged, it was washed and dried. XPS spectrum revealed Cu 2p and P 2p peaks showing that both DNA and SLAC-3ZnF were present confirming SLAC-3ZnF immobilization in the presence of the DNA.

Improved stability over time of SLAC-3ZnF on DNA/CNTs over direct adsorption was demonstrated using UV-Vis spectroscopy determination of DMP reduction over 5 minutes. SLAC-3ZnF/DNA/SWNT was made in solution by first wrapping SWNT with DNA and then immobilizing the SLAC-3ZnF. The samples were washed to remove any free enzymes from the suspension. It was tested against directly absorbed SLAC-3ZnF on SWNT at 24 and 48 hours of incubation. While the SLAC-3ZnF on DNA/SWNT system maintained its activity over this time, the absorbed SLAC-3ZnF lost activity. This showed that immobilization in this method improved stability thereby increasing the lifetime of the enzyme making this method an excellent candidate for immobilization.

7.1.2 SLAC-3ZnF Electrochemical Activity with AuNC Enhancement

Despite successfully showing improved stability and activity for DMP reduction, SLAC-3ZnF showed low electrocatalytic activity likely caused by increased distance from active centers and the electrode surface. Use of Au nanoclusters bound to DNA consisting of NC synthesis template and ZnF recognition sequences were implemented to improve
electron transfer rates and improve reaction kinetics of oxygen reduction reaction carried out by SLAC-3ZnF. Linear sweep voltammetry and chronoamperometry along with rotating disk electrodes and glassy carbon cap electrodes were used to determine the onset potentials of ORR and limiting current densities of the system. Results showed marked improvement in charge transfer rates in the presence of the AuNC supporting previous reports of improved kinetics. Polarization analysis showed a 2 and 3-fold increase between DNA and DNA-AuNC samples.

7.2 Multi-Enzyme Immobilization on Cathode Surfaces

TCA cycle enzymes were used to test the applicability of PBSE and chitosan for multi-enzyme co-localization. Malate dehydrogenase and citrate synthase were immobilized together on each scaffold and tested for charge transfer kinetics as functions of L-malate concentration or applied potential. It was hypothesized that CS would complex with MDH and improve reaction kinetics through substrate channeling and subsequent allosteric binding by the citrate produced. Michaelis-Menten and Bi-Hill analysis of the resulting voltammograms showed significant increases in $i_{\text{max}}$ and $K_M$ when both MDH and CS were on the electrode versus just MDH. Polarization analysis further supported the hypothesis showing the same trend with increased current densities compared to MDH only electrode. Interestingly, although systems with MDH immobilized using chitosan had lower $i_{\text{max}}$ that with PBSE, there was no difference when both MDH and CS were immobilized at saturated L-malate.
7.3 Significance of Research Moving Forward

Development of stable and efficient biomimetic devices is essential for developing alternative energy technology to the current conventions like batteries and metal-based fuel cells. The research presented in the previous chapters shows the effectiveness of several different enzyme immobilization methods. The demonstration of these stable and kinetically enhanced electrodes for EFCs application is a step forward for biomimetic design. Moving forward, researchers can expand on this research towards more energy efficient devices that can outlast and outperform batteries for small device powering using multiple-enzymes cascades for complete fuel oxidation.
References


