Chemically specific protein immobilization strategies on surfaces were explored. The goal of this work is to capture and bind proteins to the surface without losing their physiological properties. This approach permits to express functions of proteins after immobilization process. Using nitrilotriacetic acid (NTA) linkers and polyhistidine-tagged proteins, several synthetic strategies for presenting these linkers to the surface are illustrated. Gold was used as a primary surface. Data was also obtained for other potentially useful surfaces, including Indium-Tin Oxide (ITO) and germanium. Variable Angle Reflectance FTIR, Polarization-Modulation-IR Reflection Absorption Spectroscopy and Atomic Force Microscopy were used to characterize assembly process and structures on the surfaces. In-situ system assembly optimization was attempted by using a Ferrocene-based electroactive probe. Extensive study of specific and non-specific binding was conducted for a wide range of proteins.
CHEMICALLY SPECIFIC PROTEIN IMMOBILIZATION
STRATEGIES ON ANALYTICAL SURFACES

by

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirement for the Degree of
Master of Science

CHEMISTRY

Raleigh

2004

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For my parents, friends and above all Aneta
Pracę tą dedukuję rodzicom, przyjaciołom, a szczególnie Anetce
BIOGRAPHY

Szymon Dembowy was born on March 11, 1976 in Sulechów, Poland to Zenon and Marianna Dembowy. He has one brother, Dawid. He spent his childhood in his hometown of Babimost where he attended Elementary School. The last 2 years in elementary school were decisive about his future as he met Dr. Marian Mielniczak and started chemical education under his guidance. Szymon continued his education in Sulechów High School, graduating in 1995. The same year he was accepted into Chemistry program at Adam Mickiewicz University in Poznan, where he also met his future wife Aneta. Szymon graduated in June of 2000. In September 2000 he started his graduate education at North Carolina State under the guidance of Dr. Christopher B. Gorman and Dr. Stefan Franzen. He received his Master’s Degree in August 2004.
ACKNOWLEDGEMENTS

I would like to thank Dr. Christopher B. Gorman and Dr. Stefan Franzen. Without their continuing support and guidance it would not be possible for me to accomplish goals of this project. I also want to mention the invaluable help on personal level received from Dr. Franzen before and after I was admitted to the program. In addition I would like to thank my coworkers in 508, especially Young-Rae Hong and Chris Monceaux. Young-Rae’s synthetic expertise came to my help many times throughout my stay here. Chris Monceaux shared my interest in military aviation and photography and we spent some time in interesting discussions. Great thanks go to Scott Brewer and Dr. Simon Lappi for their help with IR instrument and theory. The entire STM/AFM crew was also extremely supportive in answering my question about Self-Assembling Monolayers. Dr. Tyson Chasse along with Drew Wassel helped me whenever I was having problems with my electrochemical experiments. This work would also not be complete without input from Chris Cameron. His help involved solving issues with synthesis, spectroscopy and protein experiments. It often evolved from heated discussion during group meetings. Those discussions were helpful by themselves teaching me to defend and present my data in a clear but forceful manner.

I met a lot of good friends during various stages of my life. Most of them are long-time companions from high school and college. Unfortunately there is a significant distance barrier and I do not get to see them as often as I would want to, but all of them hold a special place in my heart. Opportunity to work at NC State and in the US allowed me to meet many more interesting people. Some of them became my very close friends. I would like to thank them for letting me relieve stresses and hardships of being a graduate student during numerous social gatherings and meetings.
I thank my wife and best friend Aneta for her love and support. She always has stood by my side and respected my decision whether agreeing with them or not. She instinctively knew when I was down and always cheered me up. I cannot stress enough how much I love her.

At last but definitely not least, I would like to thank my great parents, Zenon and Marianna. Without them I would never become the person I am. They always guided me to become better and achieve more. No matter the situation they always were there for me. Unfortunately, we are separated by 5000 miles but I feel their touch on my life in whatever I do. They are by far the greatest parents in the world.
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1.1. Biosurfaces

1.1.1. Self-Assembling Monolayers

The field of biosurfaces emerged from the study of organic matter and the chemical and physical principles behind self-assembly. Biological and material sciences are two major disciplines whose fusion allowed defining this field. In order to achieve highly controllable biomolecular surface in-depth studies of more basic, precursor assemblies were needed. That included mono- and multilayer structures prepared either by Langmuir-Blodgett or self-assembly techniques. The properties of thin film materials have significant impetus not only on biosurfaces but on other fields as well, including adhesion, lubrication, microelectronics, photochemistry and electrochemistry. One of the earliest works to characterize self-assembled monolayers was performed by Nuzzo et al. in 1983 [1]. This research showed stable, oriented and polyfunctional organic monolayers prepared by the spontaneous organization of organic disulfides on polycrystalline gold substrates. Very high coverage that approached the equivalent of the bulk-phase densities of the absorbate materials was shown. It was concluded that bonding to the surface was highly specific, since chemisorption of the disulfide is preferred over other moieties. This approach also allowed preparing a broad variety of organic surfaces with well-defined microscopic and macroscopic properties. Several different examples of prepared films were given and a wide pool of characterization tools were used in order to study them. The techniques included infrared and photoelectron spectroscopies. The methods to modify the surface of gold by formation of self-assembled monolayers of alkanethiols were also developed by Whitesides and Gorman [2]. In this case authors describe and characterize SAMs of alkanethiols. There is also a discussion and
review of possible procedures of patterning SAMs using contact printing, microwriting, micromachining, photolithography and electron-beam writing presented by these authors.

1.2. Coupling Techniques

1.2.1. Maleimide Coupling

An interesting approach to bind proteins and cells to analytical surfaces was shown by a Mrksich et al. [3] (Figure 1.1). This approach is based on monolayers presenting maleimide and penta(ethylene glycol) groups. Maleimide group reacts efficiently with thiol-terminated ligands, while PEG-terminated groups prevent the surface from non-specific absorption of the protein to the substrate. Cyclic voltammetry was used to characterize the rate and selectivity of the immobilization of ferrocene-thiol. Examples of the biochips prepared using this approach are also presented in the paper. The first example presents the study of four carbohydrate-thiol conjugates immobilized on the surface and their lectin-binding properties are investigated by fluorescence and Surface Plasmon Resonance (SPR). The second biochip was used to study the enzymatic phosphorylation of the immobilized peptide IYGEFKKKC by the tyrosine kinase c-src. Monolayers presenting this peptide were then used to study the inhibition of the enzyme in the array format. The final class of substrates, which presented tripeptide Arg-Gly-Asp, were used for studies of integrin-mediated cell adhesion. This immobilization approach is useful for preparing substrates for a wide range of applications in basic science and biotechnology by combining the structural order and inert properties of SAMs with the efficient reaction between soluble thiol and surface-bound maleimide groups. This research group also showed a surface cell immobilization technique which enabled the researchers to turn on and off cell migration
using electric current. All of these achievements are useful steps leading to better understanding of solid-liquid or solid-air interface and are making it possible to construct nanochips with possible uses as biosensors or molecular machines.

1.2.2. Ni-NTA coupling

Another approach to specifically bind proteins is to use Ni-NTA-functionalized surfaces. It was studied previously and presented in a paper by Sigal et al. published in 1996 [4] that describes the generation of a self-assembled monolayer made of 2 components: NTA-terminated thiol to specifically bind His-tagged proteins and PEG-terminated thiol to protect the surface from non-specific binding. Using SPR techniques it was shown that NTA-functionalized surface showed high specificity to bind His-tagged proteins while the rest of the surface remained inert due to its protection by PEG-terminated SAM. To test for
resistance against non-specific binding, NTA surface was exposed to a solution containing a highly “sticky” protein, fibronogen. SPR measurements showed no protein after washing the surface with buffer solution which proved the suitability of this approach. This work was later copied by Vogel et al. in 1995 [5]. Their paper describes binding of anti-lysozyme F\textsubscript{ab} fragment modified by 6His extension at C-termini to NTA-functionalized surface. The binding was monitored by the SPR and apparent dissociation constant was also determined using this technique. The retention of secondary protein structure was demonstrated by infra-red spectroscopy. Similar work was published in 1999 by the same group [6]. Process of system assembly developed by Whitesides is shown below in Figure 1.2

![Figure 1.2 Ni-NTA Coupling Scheme](image-url)
1.3. Proteins on analytical surfaces – potential applications

1.3.1 Myosin-Actin Molecular Machine

1.3.1.1. Introduction

Myosin has long been known to be a crucial component of muscle contraction (Figure 1.3). It forms bipolar thick filaments (Figure 1.4) that interdigitate with thinner actin filaments (Figure 1.5) in the sarcomere, forming the basic contractile unit of muscle (Figure 1.3). This type of myosin exists in virtually all eucaryotic cells. Its abundance was therefore a deciding factor to use in a system. To distinguish it from other motors of myosin superfamily, it’s called Myosin II.

1.3.1.2. Myosin-Actin Working Cycle

The basis for actin-myosin motor cycle is transduction of the chemical energy derived from ATP hydrolysis. The mechanism is still not fully understood, but it can be explained...
based on the hypothesis that myosin exists in two states while tightly bound to actin: a state before the power stroke of the globular head domain of myosin (upper left panel of Figure 1.6) and a post-stroke state (upper right panel). Because myosin is anchored in a thick filament by its α-helical tail (a small portion of which is shown in Figure 1.4), the transition from the pre-stroke to the post-stroke state gives rise to about 10 nm displacement of the actin filament relative to the myosin. Step 1 in Figure 1.6 involves the binding of ATP to the globular head domain (head shown in Figure 1.4) of the myosin molecule, which results in sufficient conformational change to cause rapid dissociation of the myosin from the actin. In step 2, the ATP is rapidly hydrolyzed to ADP and Pᵢ, which stays tightly bound to the myosin. This myosin•P•ADP is in rapid equilibrium with the actin•myosin•P•ADP.
complex, but this is a low-affinity interaction. This step is followed by a slow transition (step 3) to an activated, high-affinity form of the actin•myosin complex. Literature states this actin•myosin•P•ADP complex presumably comprises a conformationally strained form of the myosin now in its pre-stroke state. The total cycle time ($t_c$) is limited to step 3 and is determined by how fast the low-affinity actin•myosin•P•ADP is converted to the high-

Figure 1.6 Actin-activated Myosin-II ATPase cycle
affinity actin••myosin••P;•ADP. This conversion triggers the release of P$_i$ (step 4) [7, 8] which in turn triggers a putative large conformational change (step 5) that gives rise to a step in motion of about 10 nm. This conformational change allows ADP to dissociate (step 6) [9]. The combination of steps 4 and 5 is referred to as the ‘strongly bound-state time’ (ts), as the myosin is strongly bound to the actin throughout these steps. In the case of skeletal muscle myosin, ts is about 2 ms [10]. Dissociation of ADP allows rapid binding of ATP and the crossbridge cycle repeats. Some variations for the above mechanism have been proposed in the literature. Described mechanism of myosin-actin interaction in presence of ATP should occur upon immobilization of myosin onto a surface. This hypothesis is a basis for future experiments as presented in the next section.

1.3.1.3. Potential Experiment

One of the possible goals of this project is the assembly of a working molecular machine. In order to do that His-tagged myosin which as described in the introduction as a component of muscles can be used. This approach allows binding myosin specifically with further advantage of obtaining correct orientation of the protein once on the surface. Such a prepared surface can be exposed to actin filament solution and spontaneous assembly of actin on top of myosin is predicted to occur at this point. Use of labeled actin allows studying its movement on the surface after exposing the entire system to ATP and ions needed to effectuate the movement (Figure 1.7).
1.3.2. Reaction Center-Cytochrome C System

1.3.2.1. Potential Experiment

The goal of this experiment is to characterize the interaction of the Reaction Center (RC) with cytochrome (cyt) c2 using Doubly Resonant Sum Frequency Generation (DR-SFG) as represented in Figure 1.8. To accomplish this task, the transmembrane 6x His-tagged Reactive Center (RC) will be anchored to a Ni-NTA SAM. The step-by-step chemistry necessary for building the layers of the SAM has been shown here. DR-SFG will be used to probe the electronic resonance of the RC to determine its structural properties during electron transport with cyt c2. In addition, the orientation of cyt c2 will be measured to determine if the preferred binding of the molecule differs from that observed in the absence of the RC. The electron transport process is readily initiated by light from the visible and/or UV lasers used to generate electronic resonance. Of particular interest in this study is the orientation and alignment of Tyr L162 of the RC which is thought to be the...
reactive site responsible for binding cyt c2. Finally, the complete reaction that drives ATP photosynthesis will be characterized using DR-SFG. Ni-NTA SAMs will be used to bind 6X His-tagged RCs and 6X His-tagged cyt bc1 to Au surface. The CV profile will provide insight into the redox potential which also creates a unique opportunity to acquire DR-SFG pump/probe measurements as a function of the events which are occurring in the redox cycle. The DR-SFG setup can be configured to perform femtosecond pump/probe experiments which are well-suited to repeat earlier studies that report picosecond dynamics for the structural changes that occur upon binding of cyt c2 with the RC [11]. The confirmation of results from previous studies of *Rb. sphaeroides* and cyt c will validate the usefulness of DR-SFG as a low-resolution technique for the structural characterization of membrane proteins with unknown properties.
1.4. Proteins and cells - Non-specific binding

After achieving primary goals of controlling the assembly process an explosive trend of immobilizing biological species to analytical surfaces started. After the initial euphoria of being able to do that, another problem emerged. After exposure of the surface to protein solution there was always a substantial amount of non-specific binding. The mechanisms that describe that behavior were extensively studied and are very complex. There are still many aspects of the mechanism of non-specific binding that remain speculative.

The model for non-specific binding assumes that many processes leading to binding initially involve the hydrophobic interaction between the surface of the material and those areas on the protein surface that are also hydrophobic. Initial adsorption that is probably weak and reversible is subsequently followed by denaturation and unfolding of the protein to expose more of its hydrophobic areas to the surface. This unfolding ultimately leads to irreversible attachment to the surface. In order to overcome that problem multiple different termination groups were studied. This work performed by the group of George Whitesides ended in the conclusion that monolayers terminated with poly(ethylene glycol) are the best suited to protect the surface from non-specific binding. The discussion of the length of the PEG unit concluded with the determination that three ethylene glycols are the minimum, and six the optimum to prevent unwanted protein from denaturing over the studied surface. Structure-property relationship was also established for the surfaces that resist non-specific absorption. Certain criteria for these groups were proposed including hydrophilic, hydrogen-bond acceptors but not donors and electrically neutral. Multiple research groups did similar
studies using different surfaces, monolayers and proteins [15-19]. To date it seems that PEG-terminated surface is the best choice for analytical surface in terms of passivation and protection against non-specific binding. Another interesting example of research involving the protein absorption was published by the Whitesides group in 1998 [20]. It describes a Surface Plasmon Resonance (SPR) study of surface wettability and its influence on the non-specific protein and detergent adsorption to self-assembled monolayers. It proved that adsorption of both proteins and detergents depended on the wettability with the trend of proteins binding to the least wettable surfaces. Among the proteins studied, the largest of them adsorbed to some extent to all the surfaces with the exception of the one protected by the PEG-terminated SAM.

1.5. References


CHAPTER II – IR INVESTIGATION OF Ni-NTA SYSTEM
2.1. Introduction

The general idea for this project is to use Ni\textsuperscript{2+}-NTA – His-tagged protein interaction. It has been used in metal-affinity chromatography to purify proteins [1] for over 25 years and is based on observation that proteins with exposed histidine and cysteine side chains have an affinity for certain metals, such as Ni\textsuperscript{2+}, Co\textsuperscript{2+}, Zn\textsuperscript{2+}, Cu\textsuperscript{2+} [2], Fe\textsuperscript{3+} [3] and Mn\textsuperscript{2+} [4]. Ni\textsuperscript{2+} and the tetradeutate nitritoltriacetic acid (NTA) were found to be an optimal design [2], Ni\textsuperscript{2+} having six coordination sites for binding and NTA with three carboxylic groups and one nitrogen to coordinate Ni\textsuperscript{2+}. When a string of six histidines binds, two water molecules leave, which creates a strong and specific binding.

The dissociation constant ($K_D$) of 6His-tagged protein to Ni-NTA has been measured to be 10\textsuperscript{-13} M\textsuperscript{-1} at pH 8 [5]. This is stronger than most antibody bindings, which typically range from 10\textsuperscript{-6} M\textsuperscript{-1} to 10\textsuperscript{-9} M\textsuperscript{-1} (weak to high affinity), but can range from 10\textsuperscript{-5} M\textsuperscript{-1} to 10\textsuperscript{-12} M\textsuperscript{-1} [6]. The Ni-NTA-6His binding is weaker, however, than that of avidin-biotin, which has a $K_D$ of 10\textsuperscript{-15} M\textsuperscript{-1} [7]. It was also found that the 10-His segment binds even tighter, but then the side chains get unnecessarily long [8].

Since the binding between Ni\textsuperscript{2+} and nitrogen in the imidazole ring of histidine is ionic, binding can be stopped by protonating these nitrogens. The p\textsubscript{K}_a of histidines in ribonuclease was measured to be 6.5 [9]. A much better way to break the 6His – Ni-NTA interaction is to use imidazole since it competes with the imidazole groups in the histidines. This approach allows avoiding potential denaturing conditions of low pH.

Another interesting property of the interaction of 6His with Ni-NTA is that it is virtually unaffected by high salt concentrations (up to 1 M), nonionic detergents, organic solvents, ethanol or glycerol up to 30%, reducing agents (β-mercaptoethanol up to 10 mM
[10]) or highly denaturing conditions (such as 8 M urea or 6 M guanidine hydrochloride). Ni-NTA itself is stable over a pH range of 2.5-13 and withstands 2% sodium dodecyl sulfate and 100% ethanol.

2.1.1. Synthesis of molecules

2.1.1.1. NTA-amine.

NTA-amine synthesis presented no significant problems; however, the final product contained traces of NaOH and NaCl (Figure 2.1). The properties of this compound do not allow for aqueous extraction at basic pH because of higher solubility in water (due to three carboxylic groups) and low solubility in organic solvents prevent extraction from acidic solution (partition coefficient is unacceptable). However, since CDI coupling is achieved at pH~10 it was decided to allow for small quantities of NaOH and NaCl in the product.

*Synthesis of amine-terminated NTA*

- protected amine

\[
\text{BrOH} + \text{NTA-amine} \overset{(a)}{\rightarrow} \text{deprotected amine}
\]

- deprotected amine

\[
\text{NTA-amine} \overset{(b)}{\rightarrow} \text{NTA-amino acid}
\]

\[\text{a) } 0^\circ\text{C for 2h, room temp. for 12h, } 50^\circ\text{C for 2h, b) } \text{H}_2, 10\% \text{ Pd/C, 72h}\]

*Figure 2.1. NTA-amine Synthetic Scheme*
2.1.1.2. NTA-thioacetate.

NTA-thioacetate synthesis was a two step process. First step involved a coupling between NTA-amine and CDI-activated PEG-olefin. The product was subsequently purified and irradiated under Medium Pressure Mercury Vapor Lamp with thioacetic acid (Figure 2.2). After the UV irradiation step, the mixture was purified as discussed previously [11]. However, contrary to the literature, trituration with hexane resulted in no precipitation. It was hypothesized that impurities in the mixture and the structure of the product prevented its precipitation from the reaction mixture. Other purification techniques were attempted to obtain the pure compound, including MPLC with reverse-phase absorbent. Two different lengths of carbon chains were used (C₈ and C₁₈) but no acceptable results were obtained.

Synthesis of olefin-NTA compound

![Synthesis of olefin-NTA compound](image)

Synthesis of NTA-thioacetate

![Synthesis of NTA-thioacetate](image)

a) H₂O-DMF, pH=10.2, 12h; b) hv, AIBN, 4h

Figure 2.2. NTA-thioacetate Synthetic Scheme
2.1.1.3. NTA-thiol.

Failure to purify NTA-thioacetate led to a decision to proceed directly to the deprotection step with no further purification (Figure 2.3). The acetate is removed via disulfide formation with I$_2$ and oxygen. The reaction was monitored with TLC and NMR was used to confirm the structure of the product. To achieve reduction of the disulfide to a thiol, tri-n-butylphosphine was used in a water-acetone mixture (1:10). Unfortunately, yields were unacceptable averaging less than 5% and purification gave unsatisfactory results in spite of multiple attempts. Overall, the synthetic approach for making the NTA-terminated thiol became sufficiently challenging and was abandoned.

\textit{Deprotection of NTA-thioacetate}

\begin{center}
\includegraphics[width=0.8\textwidth]{figure2.3.png}
\end{center}

\text{a) NaOH, O$_2$, H$_2$O-DME b) P-Bu$_3$}

\textit{Figure 2.3. NTA-thiol Synthetic Scheme}

2.1.2. System assembly

The inability to obtain NTA-thiol forced us to revise our approach. The suitability of NTA molecule was exceptional, therefore it was decided to assemble a PEG-terminated monolayer first and then functionalize it \textit{in situ} with NTA amine via CDI coupling (Figure 2.1). This approach mimics the literature process [11], but has an explicit benefit of avoiding synthesis and purification steps. The changes in the procedure were easily accommodated as PEG-olefin and NTA-amine were previously synthesized (Figures 2.1 and 2.11).
Modifications included thioacetate attachment to PEG-olefin and deprotection of the resulting thioacetate to a thiol. Both procedures had literature precedence [12] and after small changes to the protocol, namely solvent, irradiation and deprotection times, they were carried out as described in the experimental section. Reaction schemes are shown in Figures 2.11 and 2.12. It should also be mentioned that self-assembling a PEG-terminated monolayer and subsequent activation creates a functionalized single-component SAM that is simultaneously inert and protects the gold surface from non-specific binding.

Figure 2.4 Ni-NTA System Step-by-Step Assembly
2.1.3. Protein binding

As mentioned before, NTA moiety has an intrinsic property of binding proteins terminated with 6-His sequence. Schematic view of this interaction is shown in Figure 2.5. Protein is not shown to scale, however the proper “head-up” orientation of the protein is depicted. In this study a wide variety of proteins were used. In initial tests of specific binding, His-DHP was used. The same protein in its His- and non-His-tagged form was used in preliminary non-specific binding studies. When elucidation of non-specific binding was undertaken the pool of the proteins was extended by using His-H93G mutant of myoglobin, lysozyme, bovine serum albumin (BSA), trypsin and pepsin in addition to His-DHP. Those experiments were conducted at very high protein concentrations of 1mM. Most of the proteins were later used in similar non-specific binding studies, but at much lower 1µM concentrations. The effect of His-tag on non-specific binding issue was also evaluated by comparing 3 pairs of His- and non-His tagged proteins. These proteins included Dehaloperoxidase (DHP), H93G Myoglobin and Sperm Whale Myoglobin (SWWT – Sperm Whale Wild Type).

Figure 2.5 Protein Binding Scheme
2.2. Characterization using IR

2.2.1. Step-by-step analysis

2.2.1.1. NTA on PEG-SAM

The presence of the assembled monolayer and functional group after coupling is investigated with PMIRRAS. Figures 2.6, 2.7 and 2.8 show an overlay of IR spectra obtained after each step of the assembly process. The spectrum of a PEG-terminated monolayer before any modifications is presented in black. Peaks seen at 2858 and 2926 cm\(^{-1}\) are due to symmetric and asymmetric methylene stretching, respectively. Literature reports methylene symmetric and asymmetric peaks between 2846 – 2850 cm\(^{-1}\) and 2915 – 2918 cm\(^{-1}\) for trans extended chains and at approximately 2860 and 2928 cm\(^{-1}\) for disordered chains in liquid-like phase. Based on these numbers it was concluded that the monolayer is not well ordered and its properties fall between liquid and solid phase. The other major feature of the PEG-terminated SAM is a peak visible at 1134 cm\(^{-1}\) denoting C-O stretching in the ethylene glycol units of the molecule.
After activating the PEG-terminated monolayer with CDI the spectroscopic picture changes significantly (red). Since the lower segments of the chains (methylene units) are not affected by the coupling there are no changes apparent in the peak positions or intensities for the methylene region. The carbonyl peak from CDI appears at 1770 cm\(^{-1}\). In addition, there is also a dramatic change in the C-O stretching band from 1134 to 1110 cm\(^{-1}\) and also a decrease in intensity. These factors are consistent with the suspected alteration of the orientation of ethylene glycol units upon CDI coupling. Other major peaks present at 1471,
1399, 1292 and 1240 cm\(^{-1}\) arise from an aromatic heterocyclic ring present in the CDI (C=C and C-N modes).

![Absorbance vs Wavenumber](image)

**Figure 2.7. IR Surface Characterization of CDI- and NTA-SAM**

Upon addition of NTA-amine, there are more significant changes to the IR spectrum (green). In the methylene region, there is a shift from 2926 to 2918 cm\(^{-1}\) and from 2858 to 2852 cm\(^{-1}\) suggesting increased ordering and change in orientation of the SAM forced by NTA-amine coupling. There is also a considerable increase in intensity caused by the addition of four methylene units present in the NTA molecule. Further spectroscopic proof of NTA coupling is present in the carbonyl stretching region. The quantitative shift of the carbonyl peak can be
explained by two factors. The shift of carbonyl stretching present in the CDI is due to a change in the functionality from carbonyl-aromatic to carbonyl-amide stretching. That result is a shift to lower wavenumbers. However, there are new stretching modes present in the carbonyl stretching region already. They arise from 3 carboxylic groups present in the NTA. Thus, it was concluded that the intense peak present at 1738 cm\(^{-1}\) is a signature of both C=O carbamate and C=O carboxylic acid stretching present in the PEG-NTA adduct. O-H bending modes at 1540 cm\(^{-1}\) and other modes at 1469, 1401, 1301 and 1182 cm\(^{-1}\) can also be distinguished from impossible to differentiate C-N, C-O stretching present in the molecules. The final step involves “charging” NTA with Ni\(^{2+}\). This does not alter the IR signature dramatically (blue) due to their similar structure, however changes are distinct enough to distinguish NTA from Ni-NTA. Since there is a change in the conformation of the carboxylic acid groups, some change in the carbonyl signal was expected. This change manifests itself in a slight decrease in intensity of the carbonyl stretching. The final step was also confirmed by an increase in the intensity of the O-H bending peaks and some minor changes in the intensities of the lower frequency region peaks.
2.2.1.2. NTA on MCH-SAM

A similar study was conducted for mercaptohexanol (MCH) SAM as shown in Figure 2.9. MCH was chosen as it was hypothesized that PEG-thiol may be too long and not allow for tunneling of the electrons in the electrochemical experiments with different transition metals (study described in Chapter 4). Low coverage and ordering of MCH SAM was expected since it only consists of six methylene units. However, the short chain length was to
be used as an advantage in our experiments. It would allow ferrocenemethylamine to rest relatively close to the surface and unambiguously confirm if coupling was taking place.

Figure 2.9 IR Surface Characterization of CDI-activated MCH Monolayer

The spectrum shown in red depicts MCH after self-assembly on gold. Low intensities of methylene stretching region is noted along with peak present at 2876 and 2929 cm\(^{-1}\). This confirms liquid-like conformation of the chains and low order achieved by the short chained monolayer on the gold surface. Some C-H bending modes in the lower frequency region of the spectrum were observed. After CDI coupling (black), intense peak at 1772 cm\(^{-1}\) appears. The high intensity of the carbonyl stretching band suggests some CDI physisorbed to the gold surface. This is possible considering only partial and imperfect monolayer coverage.
Analogously to the PEG-CDI spectrum, peaks due to stretching modes in the aromatic ring (C-N, C=C) are present at lower wavenumbers.

Spectroscopic handles intrinsically built in the system allowed to study the system at each stage of the assembly. IR gave expected high intensity peaks of amide I and II stretching making it easy to spot and analyze. Those peaks originate from C=O stretching at about 1650 cm\(^{-1}\) in the case of amide I and from C-N stretching with N-H bending between 1520 and 1545 cm\(^{-1}\) for the amide II. Figure 2.5 shows a basic layout of the protein binding experiments. The NTA surface is activated by first immersing it for 5 minutes in 100 mM NaOH aqueous solution (not shown) and then after brief rinse with water its placement in 40 mM solution of Ni\(^{2+}\) in water. As a result, Ni\(^{2+}\) creates an octahedral complex with 3 carboxylic groups in the NTA (with one nitrogen and two carbonyl oxygen atoms). The remaining 2 coordination spaces on Ni are occupied by water molecules in the absence of protein or imidazole. After exposure of NTA surface to any His-tagged protein, there is a fast replacement of water molecules by proteins. Since there are 2 sites in each Ni-NTA molecule and each protein contains 6 His tags, it takes 3 Ni-NTA molecules to bind each protein (not shown). In order to remove the protein from the surface aqueous imidazole solution is used. Imidazole is known to have a higher affinity for Ni-NTA sites and replaces the proteins on the surface [13].

2.2.2. DFT-calculations

Our analysis of the IR spectra for the step-by-step surface assembly has been confirmed by DFT calculations. DFT-calculated frequencies and intensities have been plotted and lower regions of PEG-, PEG-CDI- and NTA-thiol spectra are shown in Figure 2.10.
The black spectrum shows an IR image of PEG-terminated thiol. As expected for this molecule there is only one distinct feature in the lower region of IR spectrum. It is a C-O stretching mode present around 1100 cm\(^{-1}\). However, when CDI-PEG molecule was modeled (as shown in green), a sharp and distinct peak is present below 1750 cm\(^{-1}\) which represents C=O stretching. Also, as seen on the experimental spectra there multiple other peaks are present between 1200 and 1500 cm\(^{-1}\) which are attributed to heterocyclic aromatic ring in the CDI molecule and its C=C and C-N bonds. IR spectrum of NTA-thiol is depicted by the black line. Compared to CDI spectrum 2 major peaks are detected in the carbonyl region. One is present above 1750 cm\(^{-1}\). An overlapping peak can also be observed with maximum intensity around 1730 cm\(^{-1}\). The second peak is present around 1675 cm\(^{-1}\). These peaks arise from multiple C=O and N-H bonds present in the NTA-PEG molecule. There are several more peaks present between 1250 and 1500 cm\(^{-1}\). All peaks shown in Figure 2.10 for all modeled molecules are in agreement with experimental data. DFT calculations confirmed that the \textit{in-situ} system assembly occurs and that there are enough IR handles present in the system validate the use of IR spectroscopy as the exclusive technique in detection and confirmation of surface structures.
2.3. Conclusions

Ni-NTA system was assembled *in-situ* on the analytical surface via CDI-mediated coupling of surface bound PEG-thiol with NTA-amine. Assembly process was controlled at each step with surface IR. Intrinsic IR handles allow for a definite identification of the surface structures. Accurate assessment was confirmed by DFT calculations done for PEG-, PEG-CDI- and NTA-PEG-thiol molecules. The universal coupling system employed in this project offers an advantage of using a other activating agents or head groups (other then CDI and NTA, respectively). Additional proteins or molecules can then be captured specifically.
2.4. Experimental

2.4.1. Reagents

Chemicals were purchased from Aldrich or Acros and were used as received unless otherwise indicated. 11-bromo-1-undecyl was purchased from Lancaster. NTA-terminated thiol 1, NTA-amine 2 and PEG-terminated thiol 3 were prepared as discussed previously [2, 11, 12]. Proteins were used as received (Sigma). His-tagged proteins were obtained according to unpublished work of Belyea et al with the help of Franzen Group at NC State University. 200 proof ethyl alcohol (Aaper Alcohol and Chemical Co.) was used to prepare thiol solutions. Hepes-buffered saline (HBS) is 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 150 mM NaCl in water adjusted to pH 7.2 with NaOH.

2.4.2. Polarization Modulation-Infrared Reflection Absorption Spectroscopy (PM-IRRAS)

The PM-IRRAS spectra were recorded on a Digilab FTS 7000 spectrometer equipped with a step scan interferometer, a liquid nitrogen cooled narrow band MCT detector, a globar source, and a UDR-4 filter. The IR radiation was typically phase modulated at frequencies of 400 or 800 Hz, at an amplitude of 1.0 or 2.0 \( \lambda \) HeNe while stepping at 0.5 – 2.5 Hz. A gold grid polarizer was used to obtain p-polarized radiation, which was then modulated by a Hinds ZnSe PEM operating at 37 kHz and an amplitude of 0.5 \( \lambda \) (strain axis 45\(^{\circ}\) to the polarizer) before reflecting off the sample at an incident angle of 80\(^{\circ}\) from the surface normal. All spectra were recorded at room temperature at a resolution of 4 cm\(^{-1}\) and were a result of one (unless otherwise noted) scans with a spectral range of 650 – 4000 cm\(^{-1}\). The digital signal processing (DSP) algorithm incorporated into the Digilab spectrometer software was used to
obtain the spectra. The instrument allowed the gold slide containing the monolayer to act as the reference to obtain absorption spectra. An IR microscope (UMA-500) attached to a Digilab FTS 6000 FTIR spectrometer equipped with a Cassegranian objective containing a germanium crystal for single-pass ATR was used to obtain solution single-pass ATR-FTIR spectrum of NTA-amine. Solution spectrum of ferrocenemethylamine was obtained using an FTS 3100 spectrometer with Pike single-pass ATR attachment. Both spectrometers use a liquid nitrogen cooled MCT/A detector. Spectra were recorded at room temperature as an average of 64 scans recorded at a resolution of 4 cm\(^{-1}\) in a range of 650 – 4000 cm\(^{-1}\).

2.4.3. DFT Calculations

The geometry optimization and vibrational frequency calculations of the individual thiolated molecules were done using the MSI (Molecular Simulations, Inc.) quantum chemistry software program DMol3 at the North Carolina Supercomputer Center (NCSC) on the IBM RS/6000 SP. DMol3 is an \textit{ab initio} (first principles) software package that utilizes density functional theory [31]. These calculations were done in the gas phase using the DNP basis set, the GGA functional, and the method of finite differences for calculating the vibrational frequencies. The MSI software Insight II was used to build the models and to visualize the eigenvector projections of the vibrational modes of the models.

2.4.4. Self-Assembled Monolayers Preparation

SAMs were prepared on polycrystalline gold deposited on a glass slide with a titanium passivation layer (1000 Å of gold and 50 Å of titanium) (Evaporated Metal Films, Inc.). Gold was cleaned with piranha solution (70% concentrated H\(_2\)SO\(_4\)/30% H\(_2\)O\(_2\) (30%)), rinsed with Millipore 18 MΩ deionized water (Barnstead E-PURE) and dried with N\(_2\) gas.
PEG-terminated thiol was deposited from a 1 mM ethanolic solution for 12 hours. The gold slides were then rinsed with absolute ethanol, dried with N₂ gas and placed in a 100 mM solution of CDI in methylene chloride (anhydrous) for 2 hours. For protein absorption, NTA-amine was coupled with PEG-terminated monolayer overnight from 100 mM aqueous solution at pH 9. Protein binding studies were conducted in HBS buffer with protein deposition times of 1 hour for both specific and non-specific investigations. Removal of proteins from the surface was achieved by rinsing with HBS buffer (non-specific binding) and 100 mM aqueous imidazole solution (specific binding). The IR spectrum of ferrocenemethylamine coupled to PEG-terminated SAM was obtained by immersing a CDI-activated monolayer into a 100 mM solution of ferrocenemethylamine in anhydrous methylene chloride.

2.4.5. Synthetic Procedures

PEG-thioacetate. Previously synthesized and purified PEG-olefin was dissolved in anhydrous THF or methanol and 40 mg of 2,2'-azo-bis-isobutyrylnitrile (AIBN) was added. System was then purged with nitrogen for 20 minutes followed by addition of thioacetic acid. The mixture was irradiated for 4 to 6 hours with a medium-pressure mercury lamp (Ace Glass) equipped with pyrex filter. Purification was achieved using a silica column chromatography with ethyl acetate as an eluent. NMR showed pure thioacetate and was consistent with the literature.
**Synthesis of PEG-terminated thioacetate**

![Synthetic Scheme](image)

a) hv, AIBN, 4h

Figure 2.11. PEG-thioacetate Synthetic Scheme

**PEG-thiol.** PEG-thioacetate was dissolved in 20 ml of 0.5 M solution of HCl in methanol and the resulting mixture was refluxed for 6 hours under neutral atmosphere (N$_2$). The reaction was monitored via TLC and crude product was purified using silica gel column chromatography. The NMR was consistent with the literature [13], with the exception of the presence of olefin peaks. The PEG-olefin was appeared to co-elute with both PEG-thioacetate and PEG-thiol during corresponding purifications. Since there are no active sites for surface interaction (no thiol or thioacetate group), we decided to carry-on without further purification, making certain that correction was made for its amount (less then 4% by NMR) in the calculations for proper molarity of the ethanolic solutions.

**Deprotection of PEG-terminated thioacetate**

![Synthetic Scheme](image)

a) 0.5M HCl/MeOH

Figure 2.12. PEG-thiol Synthetic Scheme

### 2.5. References


CHAPTER III – SPECIFIC AND NON-SPECIFIC PROTEIN BINDING
3.1. Specific protein binding

After resolving multiple issues with obtaining and processing spectra (i.e. baseline correction), protein binding experiments were conducted. It was decided to use His-tagged dehaloperoxidase (DHP) as the native protein (non-His-tagged) was used in our group before. The protein immobilization was done under the same conditions as previously described [1] in order to directly compare results. The only variation was the use of static vials instead of flow-through cells and the use of IR instead of SPR to study the protein binding. Figure 3.1 displays an overlay of 4 spectra obtained in the process of testing the specific binding of His-DHP protein. The dark blue line shows a spectrum obtained for a Ni-NTA surface. By comparing it with the independent spectra obtained in the earlier experiments we confirmed the presence of Ni-NTA groups and exposed to the surface to a solution containing His-DHP. As expected we observe strong peaks at 1662 and 1547 cm\(^{-1}\) corresponding to amide I and amide II bands, respectively. It should be mentioned that their intensity, as expected from multiple amide bonds present in the relatively large protein, is much higher (~ 10 times) than the methylene stretching from the PEG molecule. We also noticed that the methylene region also increased in intensity for both symmetric and asymmetric modes. The increase however, is not as dramatic (~ 3 times) of an increase. Interestingly almost no change is apparent in the C-O stretching region at 1134 cm\(^{-1}\) except for a small increase in intensity. These observations confirm the presence of the protein on the surface.
In a subsequent step an attempt was made to remove the protein by rinsing it off with imidazole solution. After rinsing and drying the slide another spectrum was obtained, displayed as a green line in Figure 3.1. Amide I and II modes were immediately apparent. Their intensity dropped approximately 30% but, nonetheless displayed protein still bound to the surface. However, a peak appeared at 1265 cm\(^{-1}\) corresponding to C-N stretching present in the imidazole aromatic ring. Based on these observations, it was concluded that the protein bound specifically by NTA His-tag interaction was successfully removed. The rest of the protein that appears on the spectrum after washing is probably bound non-specifically and lays denatured over the surface. To confirm this, another spectrum was obtained after we
immersed the same slide in the imidazole solution for 1 hour. The hypothesis of non-specific binding was confirmed when a small decrease in the intensity of the amide I and II stretching bands was observed (~10 and 15 %, respectively). In addition, the imidazole peak (C-N stretching present at 1265 cm\(^{-1}\)) increased in intensity by approximately 18%. It was determined that the remaining protein on the surface was non-specifically bound and likely denatured.

The same set of data was obtained for another His-tagged protein. In this case, a mutant of hemoglobin, H93G, was used. The data showed similar results. The study was repeated again for both proteins to confirm the observations and exclude any systematic errors in the procedure. As expected, the experiments provided comparable data. In all cases, similar amounts of non-specifically bound protein were observed and unable to remove by rinsing with imidazole and buffer solution.

### 3.2. Non-specific protein binding

A way to eliminate non-specific binding needed to be found to continue with further experiments. Previous work showed that PEG-terminated molecules could effectively prevent any non-specific binding [5], however the amount of NTA molecules that coupled to the surface was unknown. If the entire surface was activated by CDI and NTA coupling was highly efficient, the proteins would not actually “see” a PEG-terminated SAM, but one that was NTA-terminated. This would result in non-specific binding due to the inability of the remaining PEG to passivate the surface.

To test for surface passivation by a PEG-terminated monolayer, two systems were assembled. The first displayed in Figure 3.2 in dark blue consists of gold slide with a PEG-terminated SAM exposed to a His-DHP. After an hour of incubation and rinsing with a buffer
solution, an IR spectrum was obtained. Again, substantial amount of protein on the surface was observed. It was concluded that all binding had to be non-specific as there were no active sites for the protein to bind to. Another experiment, shown in violet, was to study the amount of the coupling occurring on a NTA-terminated monolayer without exposing the surface to Ni\(^{2+}\) solution. No specific binding was expected. Comparing these results with the results obtained in the specific-binding experiments displays the actual amount of non-specific binding to be about 50% smaller on a PEG- than on a Ni-NTA-terminated monolayer and about 40% smaller than on a NTA-terminated monolayer. However, this result is still unacceptable as much lower values were expected, based on the result in the literature for PEG-terminated monolayers [5]. In order to arrive at any conclusive results, data obtained for more proteins were needed.

Figure 3.2. Non-specific binding of His-DHP to PEG- (dark blue) and NTA-terminated monolayer. Proteins rinsed off the surface with buffer solution.
3.2.1. Multi-protein effort to find trends in non-specific binding

(1mM concentration)

For testing non-specific binding, size and pI were the focus when determining other protein candidates. 4 additional proteins were studied by repeating the experiments already conducted with His-DHP (MW 46000, pI 4.2) and His-H93G (MW 17800, pI 7.2). The other proteins used were lysozyme (MW 14388, pI 11), bovine serum albumin (BSA) (MW 66430, pI 4.8), pepsin (MW 35000, pI 1) and trypsin (MW 23800, pI 10.5). The proteins vary in molecular weight from ~14400 to 66430 Daltons and in pI from 1 to ~11.

The data obtained in the non-specific binding experiments is shown in Figure 3.3. The experiments were run in duplicate in order to reduce experimental error. Both runs...
displayed similar trends. First, the influence of molecular weight of the proteins was examined. The two largest proteins, BSA and His-DHP, showed no non-specific binding. In addition, the smallest protein, lysozyme, did not show any non-specific binding. The other tested proteins, His-H93G, Trypsin and Pepsin, with molecular weights falling in the middle of the range (17800, 23800, 35000 Daltons, respectively), showed significant amount of non-specific binding. Of these three, trypsin and His-H93G showed the same amount of binding as determined by the intensity of amide I. Pepsin showed about 30% less binding, again as compared by amide I stretching peak intensity. In terms of a different pI values, no clear trend could be distinguished. The protein with the highest pI, lysozyme, shows no non-specific binding. However, the two proteins: trypsin and His-H93G, with the second and third highest pI, respectively, show non-specific binding. For the next two highest pI point proteins, BSA and His-DHP there is no binding detected. Trypsin, the protein with the lowest pI among the ones studied, shows specific binding. No conclusive statements could be made about the correlation of non-specific binding with pI from these data. The trend that was seen when comparing the molecular mass and non-specific binding was also hard to evaluate. The smallest and largest proteins show no non-specific binding while proteins with intermediate molecular weights do not. In addition, due to a lack of different His-tagged proteins, nothing can be said about the role of a His-tag and non-specific binding.

3.2.2. Variation of Protein Incubation Time

Another factor to analyze concerning non-specific binding was the protein incubation time. Ni-NTA surfaces were studied to determine the amount of a specific and non-specific binding when varying protein coupling time. His-DHP protein was chosen as it showed no non-specific binding on a PEG-terminated monolayer. This was unexpected on Ni-NTA
surface as most of the PEG was converted to NTA and the surface developed substantial amount of non-specific binding. Figures 3.4, 3.5, 3.6 and 3.7 show spectra obtained when varying the protein incubation time. Spectra of Ni-NTA-terminated monolayers are shown in dark blue and are consistent with previously obtained results. Violet depicts monolayers exposed to protein solution. Orange and light blue show the spectra after washing and immersing slides in imidazole solutions, respectively. Time of immersion was kept constant at 1 hour. Figure 3.4 shows the spectra obtained after 1 minute of protein incubation. After such a short incubation time, the amount of protein binding is comparable to that on a PEG-terminated monolayer. To check whether this binding was specific, slides were washed with imidazole. The result was interesting as the amount of binding indicated by a decrease of amide I peak intensity was small and didn’t exceed 10%. There is however approximately 50% increase in C-N stretching at 1266 cm⁻¹. Although these results were hard to correlate, it was concluded that most of the protein binding after 1 minute incubation was non-specific. Immersion in the imidazole solution resulted in a further decrease in amide stretching by 15% (amide I shows a slight increase which is attributed to baseline correction issues). Figure 3.5 shows the results obtained for 5 minute protein incubation time. Due to longer incubation times, changes are more dramatic here. The binding of the protein is increased by 20% determined from the amide I stretching band. Upon washing, less protein on the surface was noted which would suggest more specific binding after longer incubation. The amount of protein rinsed from the surface by imidazole was roughly 16% (50% more than after 1 minute incubation). Unfortunately, amide II stretching does not follow the trend as attributed to inconsistencies in baseline correction. After 30 minute incubation time, there was
insignificant change in the overall protein binding (Figure 3.6). It was decided that surface coverage was getting close to the point of saturating the surface with protein. However, a dramatic difference was apparent after washing the monolayer with imidazole. Comparing the amide I peak intensities a 60% decrease was found, while the amide II stretching decreased only by 40%. However, after immersing the surface in the imidazole solution, no noticeable change in amide I region is observed. In addition there was supplementary decrease in the amide II region by 25%. The overall change of both amide I and amide II stretching after rinsing and immersing was estimated to be 60%. These results suggested that most binding at a 30 minute protein incubation time appears to be specific. Figure 3.7 shows that after exposing the Ni-NTA surface to protein, larger changes were observed. First, the overall protein binding increased by 25%. After washing with imidazole, the change for both amide I and amide II stretching was not as pronounced as for the 30 minute incubation. The decrease was approximately equal for both at 20%. However, the most noticeable difference occurred after immersing the monolayer in imidazole solution. A decrease in amide I and II stretching was noted by an additional 50 and 35%, respectively. The amount of protein remaining on the surface and, therefore non-specifically bound, was estimated to be approximately 35% of the initial value. Based on the results, it was concluded that non-specific binding occurs faster than specific-binding and simple rinsing is not enough to remove even specifically-bound protein by a significant amount. The ideal conditions were established to be a 1 hour protein deposition, followed by a rinsing with the buffer solution and immersion in imidazole solution for 1 hour.
Figure 3.4 Non-specific binding of His-DHP to PEG-terminated monolayer. After 1 min incubation time. Proteins rinsed off the surface with imidazole wash (red) and imidazole immersion (light blue).

Figure 3.5 Non-specific binding of His-DHP to PEG-terminated monolayer. After 5 min incubation time. Proteins rinsed off the surface with imidazole wash (red) and imidazole immersion (light blue).
Figure 3.6 Non-specific binding of His-DHP to PEG-terminated monolayer. After 30 min incubation time. Proteins rinsed off the surface with imidazole wash (red) and imidazole immersion (light blue).

Figure 3.7 Non-specific binding of His-DHP to PEG-terminated monolayer. After 1 hr incubation time. Proteins rinsed off the surface with imidazole wash (red) and imidazole immersion (light blue).
3.2.3. Removal of denatured proteins with Sodium Dodecyl Sulfate

Another attempt to remove the protein from the surface was undertaken with the use of highly concentrated (0.5M) aqueous solution of Sodium Dodecyl Sulfate (SDS). The protocol was to expose the PEG-terminated monolayer to a 1 mM solution of DHP and obtain a IR spectrum to confirm and evaluate protein coverage. Next the same surface was rinsed with copious amounts of SDS solution. As shown in Figure 3.8 under extremely denaturing conditions only a small amount of the protein already bound non-specifically to the surface is removed. This experiment has no practical use for a specifically bound proteins as it would remove it, however it proved that once the protein denatures and covers the surface it is tremendously hard to remove.

![Figure 3.8: Non-specific binding of DHP on PEG-terminated monolayer before and after rinsing with 500 mM solution of SDS.](image)

Figure 3.8 Non-specific binding of DHP on PEG-terminated monolayer before and after rinsing with 500 mM solution of SDS.
3.2.4. Variation of CDI coupling time and concentration

Bases on previous observations it was also concluded that surface activation occurred too rapidly. As a result, most of the hydroxyl groups reacted with CDI leading to an excess of NTA groups on the surface. As a result proteins were freely binding non-specifically to the NTA surface since there was not enough PEG units remaining to passivate the remaining area. It was decided to shorten the CDI coupling step to achieve lower order of CDI-activated sites on the surface. This would provide larger areas of surface coverage in unaltered PEG-thiols preventing the protein from denaturing and binding non-specifically. To test the idea, the time of the CDI step was varied from 1 minute to 1 hour (Figure 3.9). The change in intensity of the carbonyl stretching present at 1774 cm$^{-1}$ was used as a spectroscopic handle. An increase of the intensity by 30%

![Figure 3.9 Spectra of CDI-activated PEG-terminated SAM after different CDI-coupling times.](image)
from 1 minute to 5 minutes was observed, however no further increase was noted after coupling for 1 hour. The result for the 30 minute time seems to be out of trend. However, there was an additional increase in many of the lower frequency region peaks between 1000 and 1500 cm\(^{-1}\). It should be noted that the highest intensity (approx. 30% higher) is always observed for the monolayer after 1 hour of the CDI coupling, but no coherent trend is observed in the spectra of other monolayers. The important observation made during these experiments was that CDI coupling kinetics are significantly fast even on the monolayer and a better handle had to be found in order to quantify the coupling.

### 3.2.5. Low concentration non-specific binding (1.2 µM)

1 mM protein concentration non-specific binding studies proved that even inert surface like PEG-terminated SAM cannot prevent unwanted protein denaturation and subsequent surface coverage. Moreover, 1 mM concentration was shown to lead to extremely high non-specific protein coverage. The amount of the protein varied slightly depending on the type and size of the protein but in a majority of the cases it was overwhelming and precluded any specific binding experiments. Attempts were made to lower the non-specific binding to controllable levels. The way to achieve it was to simply change the concentration of the protein from 1 mM to 1 µM. This concentration turned out to be useful in two ways. The amount of non-specific binding was lowered significantly and experiments were much more efficient and a lot of material could be saved. It is worth mentioning that at the same time a special, custom-made incubation container was used that allowed to conduct 5 deposition experiments at the same time. The container also allowed to save huge amounts of
protein solutions as the slides needed to be covered with only miniscule quantity of the solution.

Results are shown in Figure 3.10. Two protocols for removing excess protein from a slide were used for BSA, lysozyme and DHP. The first one was already used in previous studies and involved removing a slide after incubation period from solution using tweezers and then rinsing with buffer solution followed by rinsing with DI water. To avoid exposure to air and possible additional denaturation of the proteins at the air-solution interface the second protocol was devised. In this case a slide was not removed from deposition solution but that solution was diluted with buffer to remove the protein without exposing the slide to air. After dilution each slide was removed from the buffer and rinsed with DI water. IR spectra were then obtained and compared to study the effect of the differed protocol on the amount of non-specific binding.

BSA did not show any non-specific binding after both rinsing and diluting. Lysozyme showed a small amount of non-specific binding after rinsing. However the second result, obtained after dilution appeared to be anomalous as there was more protein binding than in the first case. Horseradish Peroxidase followed expected trend showing some amount of non-specific binding after traditional rinsing. This amount was later practically reduced to nothing after the alternative protocol of diluting the protein solution was used on a separate slide. Horse Heart Myoglobin and Cytochrome C did not show any non-specific binding after rinsing the slides. Further experiments were abandoned as the amount of non-specific binding was minimal or none in most cases and no real trends could be discovered.
3.2.6. Influence of His-tag on non-specific binding

Ni-NTA strategy allows to bind His-tagged proteins to analytical surfaces. Since non-specific binding showed up as a huge problem one has to consider the influence of the His-tag on the surface. Histidine is a H-bond donor and the PEG-terminated surface is an excellent H-bond acceptor due to its hydroxyl groups. This can lead to non-desired interaction between the surface and the protein.

A series of experiments were designed to elucidate this issue. DHP, SWWT and H93G and their respective His-tagged clones were incubated over PEG-terminated SAM for 1 hr and subsequently IR spectra were obtained. Results are showed in Figures 3.11, 3.12 and 3.13. Due to low protein concentration only small amount of non-specifically bound protein
is present. In case of DHP no detectable amounts of proteins were present. This was also a case with SWWT. The only protein that showed non-specific binding was His-tagged H93G. As it was the only case no trends could be discriminated.

![Graph showing comparison of non-specific binding for DHP (pink) and His-DHP (yellow). PEG spectrum is shown in blue.](image-url)

**Figure 3.11** Comparison of non-specific binding for DHP (pink) and His-DHP (yellow). PEG spectrum is shown in blue.
Figure 3.12 Comparison of non-specific binding for SWWT (pink) and His-SWWT (yellow). PEG spectrum is shown in blue.

Figure 3.13 Comparison of non-specific binding for H93G (pink) and His-H93G (yellow). PEG spectrum is shown in blue.
3.3. Final non-specific experiments.

The non-specific binding experiment to study the influence of His tag on protein absorption was conducted again and expanded to include NTA surface with DHP as protein of choice. The spectrometer used was Digilab FTS-3000 with grazing angle attachment. The results proved to be inconsistent with those previously obtained and discussed above. Fresh protein was used in this test and probably was a deciding factor in the result. The previously used protein was stored for a few days in a general use fridge and both factors (storage time and contamination) probably contributed to protein decomposition and inconsistent results. New data is shown in Figures 3.14 and 3.15. It shows results obtained for spectra of DHP and His-tagged DHP after incubation over PEG and NTA, respectively. Both cases demonstrate significantly higher amounts of DHP on the surface. It counterintuitive as DHP is smaller and doesn’t have a His tag which was hypothesized to have some affinity to both PEG and NTA surfaces due to it’s H-bonding properties.
Figure 3.14 Comparison of non-specific binding for DHP (black) and His-DHP (red) on PEG.

Figure 3.15 Comparison of non-specific binding for DHP (black) and His-DHP (red) on NTA
3.4. Final specific experiments.

Non-specific binding results with 1.2 µM proteins solutions showed virtually no non-specific binding. These results led to another set a set of experiments. His-DHP was used at 1.2 µM and incubated over Ni-NTA surface and NTA surface as control. The control did not show any non-specific binding as was expected based on previous studies. There was a small amount of His-DHP binding to Ni-NTA. Upon rinsing the surface with imidazole the protein was successfully removed. Because of use of FTS-3000 which is inferior to PM-IRRAS previously employed to collect data, spectra presented in Figures 3.16 and 3.17 are very noisy with poor resolution. However, the observation of successful specific binding and removing of His-tagged protein is presented in enough detail to support the assertion.

Figure 3.16 Non-specific binding of His-DHP (black) and His-DHP after imidazole rinsing (red) on NTA at 1.2 µM.
3.5. Conclusions

Protein binding experiments were conducted with His-tagged proteins to establish protocols for detection and evaluation of surface coverage using IR spectroscopy. Initial findings established high protein binding, but further experiments showed the coverage to be mixed between specific and non-specific. A comprehensive study was then conducted to ascertain factors aiding non-specific binding. The comparison experiment was set up to check for differences of protein binding to PEG-terminated and NTA-terminated SAMs. This study showed significant amounts of non-specific binding on both surfaces. This proved that...
PEG-surface was not inert enough to protect the surface from unwanted protein interaction. More experiments were conducted for multiple proteins to find any correlations between their size, structure and properties and non-specific binding. Protein incubation time was also varied in another series of experiments. Activation of surface with CDI reagent was also studied and proved that the coupling step was extremely fast and established that control over this step was crucial in achieving the goal of low surface coverage of Ni-NTA and surface inertness at the same time. Low concentration protein binding experiments were conducted and as predicted they showed significant correlation between low concentration of the protein in the solution and the prevention of non-specific binding. To conclude non-specific studies last batch of experiments was conducted that involved testing pairs of His- and non-His-tagged proteins. Although these experiments did not yield any significant trends they confirmed low amount of non-specific binding that occurred at low concentration of protein in the incubating solutions. Similar experiments at low concentration showed successful specific binding of His-DHP to Ni-NTA and their protein removal with imidazole.

### 3.6. References

CHAPTER IV – ELECTROCHEMICAL STUDY OF CDI-ACTIVATED SURFACE
4.1. Ferrocene-amine – attempts to optimize CDI coupling

4.1.1. Direct Measurement

The monolayers were formed by deposition of PEG-terminated thiol in solution. Activation is achieved by immersing SAMs in CDI solution. The purpose of the electrochemical experiments is to study the kinetics of binding and assess the amount of coupling of the active element on the surface. It was initially proposed to use NTA with different metals to do a direct electrochemical study of the surface. Four possibilities NTA-compatible metals were used: Co$^{2+}$, Fe$^{2+}$, Fe$^{3+}$ and Ni$^{2+}$. Electrochemical experiments were then carried out and involved exposure of the NTA-functionalized surface to the respective metal followed by analysis using cyclic voltammetry. Unfortunately, NTA surfaces did not show any electroactive species present. Co$^{2+}$- and Fe$^{2+}$-NTA complexes are not characterized in the literature and was hypothesized that they may not exist or may have no electroactive properties. Fe$^{3+}$- and Ni$^{2+}$-NTA complexes are known, however no signal was obtained which suggesting poor electrochemistry while immobilized on the surface, presumably due to low detection limits. Figure 4.1 shown an overlay of cyclic voltammograms obtained during this experiment.
4.1.2. MCH- and PEG-terminated SAM study

Since direct measurements on the NTA-terminated monolayer were not possible, electroactive species were synthesized. Ferrocenemethylamine was chosen, due to the group’s experience with the electrochemistry of similar derivatives of ferrocene bound to the surface. It had been postulated to use a shorter chain molecule. The rationale was that long alkyl chains molecule (i.e. PEG-terminated thiol) may place ferrocene too far from the gold surface. Since gold is the working electrode, it may not be possible to transfer electrons over such a great distance and as a result, no signal would be observed. To test this hypothesis,
two different SAMs were prepared. One consisted of a regular PEG-terminated monolayer, as used for protein studies, and the second of mercaptohexanol. Both were assembled on gold surfaces and then activated by immersion in CDI solution. Subsequently, both were exposed to a solution containing ferrocenemethylamine. After switching the organic solvent and finding a proper electrolyte cyclic voltammograms were obtained.

Figure 4.2 shows cyclic voltammograms obtained using PEG-terminated monolayer and MCH SAM functionalized with ferrocenemethylamine. To show changes in capacitance as the SAMs form on the surface, three CVs are overlaid. The black curve shows a CV of bare gold. As the PEG-terminated layer is assembled, the double layer capacitance decreases significantly as shown in green. Both PEG and MCH SAMs are not electrochemically active until it is coupled with ferrocenemethylamine. The red curves show redox behavior of coupled ferrocenemethylamine. In both cases, a decrease of the double-layer capacitance is observed from bare gold to an assembled monolayer. In addition, the capacitance is slightly higher for MCH as it is thinner and more disorganized, thus creating less resistance for the electrons. As we couple ferrocenemethylamine to the SAMs, we observe the appearance of the expected, electrochemical signal shown in orange. The oxidation wave at 428 mV and the reduction wave at 368 mV for the PEG-terminated monolayer and at 437 mV and 363 mV for MCH SAM were present, respectively. The differences in these values can be attributed to different packing and coupling efficiencies as both monolayers and the chemical environment for ferrocene species on both SAMs are different. The values agree with the literature [1] allowing for small differences due the different molecule. It should also be noted that surface coverage for both PEG and MCH SAMs is similar. This is explained by partial packing and ordering of MCH. This allows ferrocenemethylamine molecules to
interact non-covalently with methylene units of the carbon chain and may allow for physisorption to gold.

Control experiment was performed on bare gold that was exposed to CDI solution in methylene chloride. Subsequent cyclic voltammogram was obtained. It showed significant amount of ferrocene species on the gold surface. The amount was estimated to be an order of magnitude higher than a monolayer coverage.

![Figure 4.2 Cyclic voltammetry of ferrocenemethylamine bound to MCH- and PEG-SAM](image)

**4.2. Time- and concentration-controlled experiments**

To properly adjust the CDI coverage it was decided to vary both concentration and time of CDI coupling. Series of experiments were planned. Starting concentrations were set to 1, 10 and 50 mM and time of coupling to 1 hr. After the experiments were finished cyclic voltammograms were obtained for all samples. Unfortunately, calculated coverage was similar for all cells and approximately equal to that of a monolayer of ferrocene. It was decided at that point that time of coupling was to be reduced to 1 and 10 minutes with the same concentrations as in previous experiment. After obtaining cyclic voltammogram and calculating surface coverage the result turned out to be the same with coverage hovering around a single layer of ferrocene molecules on the surface. The next logical step was then
undertaken by cutting the concentration to 1, 10 and 100 µM. Deposition time was 1 hr. Surprisingly, similar results were obtained again. To confirm these unexpected results another experiment was conducted with overnight coupling of CDI to SAM at 1, 10 and 50 mM concentration. After obtaining cyclic voltammograms and calculating coverage it was discovered that same amount of molecules were present at the surface as previously. There was only one explanation – non-covalent interaction between the surface and ferrocene molecules. To substantiate this finding more control experiments were conducted. This time after CDI binding step surface was reacted with ethanolamine. Ethanolamine would then bind to CDI-PEG molecules on the surface and no active sites would be left for subsequent deposition of ferrocenemethylamine. If cyclic voltammetry showed any electroactive moieties on the surface at that point, it would confirm our conclusions about non-covalent bonding of ferrocenemethylamine and the surface. Another control was run at the same time with just PEG-SAM on the surface that was exposed to ferrocenemethylamine for 1 hr at 50mM. Both these controls showed non-covalently bound ferrocene species on the surface. At this point it was concluded that ferrocene-based probe was unsuitable on our surface and further experiments were halted. Some representative results are shown in Figures 4.3 and 4.4.
Figure 4.3 Cyclic voltammetry of ferrocenemethylamine on PEG-terminated monolayer in a time-controlled experiment.

Figure 4.4 Cyclic voltammetry of ferrocenemethylamine on PEG-terminated monolayer in a concentration controlled experiment.
4.3. Direct DHP detection

Efforts were undertaken with collaboration with Jennifer Belyea to establish a protocol for a direct electrochemical detection of DHP on Ni-NTA surface. After Ni-NTA surface was prepared, His-tagged DHP was incubated over it. Cyclic voltammogram was subsequently obtained after purging the cell with argon as shown in Figure 4.5. Surprisingly, both anodic and cathodic peaks are extremely narrow. Another interesting feature is a coverage. It was calculated to be less than a monolayer which is unusual. Due to low rate of success with direct detection of DHP (less than 50% reproducibility) these experiments are not continued at this time.

Figure 4.5 Cyclic voltammetry of His-DHP bound to Ni-NTA-SAM
4.4. Conclusions

Ferrocene-based probe was synthesized and used in an effort to optimize CDI coupling. The amount of surface activation is crucial and has to be strictly controlled to obtain desired NTA coverage on the surface and prevent non-specific protein binding at the same time. Unfortunately, ferrocenemethylamine proved to be ineffective as a surface electrochemical probe due to its non-covalent interaction with the surface. Some runs had been tried with dopamine, however the kinetics proved to be too slow to obtain any satisfactory data. These efforts are not wasted as many other probes may be synthesized to be tested at a later stage. There is also a chance that dopamine may be use with success with the help of Osteryoung Square Wave Voltammetry (OSWV), which would resolve slow kinetics issue. The same technique may be used for a direct NTA detection using transition metals like Co and Fe. Some preliminary attempts had been made but again with using OSWV no useful data was obtained. Electrochemical sensing of CDI-surface coverage remains an open option.

4.5. Experimental

4.5.1. Synthesis of Ferrocenethylamine.

Modified procedure [2] was used as follows. 1M Borane-THF was added to a cooled solution (0°C) of ferrocenecarboxaldehyde (2.14 g, 10 mmol) in THF (10ml). After stirring for 48h, the volume of the solution was reduced in vacuo to about 20 ml and water (10 ml) was added. The pH of the solution was adjusted with 1 N HCl to pH 2. Then solid KOH was added until pH 8 was reached. The resulting dark-brown solution was extracted with CH₂Cl₂ (4 x 100 ml). The organic phase was collected, washed with water (3 x 100 ml), dried over
anhydrous MgSO₄ and then pumped to dryness. The crude product was redissolved in MeOH (30 ml) and 1 N HCl (15 ml) was added and then pumped to dryness. The material was dissolved in a minimal amount of CH₂Cl₂/MeOH (3:1) and transferred onto an alumina column (30 cm). Product was eluted with CH₂Cl₂ followed by MeOH. After evaporation of solvent in vacuo, ferrocenethylamine was obtained as yellow microcrystalline solid. Yield: 72.8% (1.82 g); NMR and GC-MS data was obtained and confirmed the structure of obtained product).

**Synthesis of ferrocenethylamine**

\[
\text{Fe} \quad \text{CN} \quad \text{Fe} \quad \text{NH}_3^+\text{Cl}^- \\
\text{Fe} \quad \text{NH}_3^+\text{Cl}^- \\
\text{Fe} \quad \text{CN} \quad \text{Fe} \\
\]

(a) BH₃xTHF, b) HCl

Figure 4.6. Ferrocenethylamine Synthetic Scheme

### 4.5.2. Cyclic Voltammetry.

Experiments were carried out on a Bioanalytical Systems CV-50W Voltammetric Analyzer. The electrochemical cell consisted of an aqueous Ag/AgNO₃ reference electrode (Microelectrodes, Inc.), a Pt wire counter electrode, and a gold working electrode. All measurements for SAMs were obtained using a 0.1 M aqueous solution of NaClO₄ as supporting electrolyte at a scan rate of 50 mV/s. Experiments were carried out under ambient conditions. Gold slides were cleaned with Piranha solution (30 s) followed by electrochemical stripping accomplished by cycling from 0 to 1400 mV for 5 complete cycles.
in a 0.1 M KCl/0.01 H₂SO₄ solution. The cells were rinsed 5 times with Millipore 18 MΩ water followed by 5 rinses with absolute ethanol. 1 mM ethanolic solution of PEG-terminated thiol was then added to the cell. After 12 hours the cells were rinsed 5 times with absolute ethanol and 10 times with anhydrous methylene chloride. 100 mM solution of CDI in anhydrous methylene chloride was immediately introduced to the gold slides and allowed to stand for two hours. This was followed by a thorough rinse with anhydrous methylene chloride and subsequent addition of 100 mM of ferrocenemethylamine solution in anhydrous methylene chloride. Cyclic voltammograms were obtained after each step to confirm the assembly process and establish surface coverage of ferrocenemethylamine coupled to PEG-terminated monolayer.

4.5.3. GC-MS.

GC-MS analysis was obtained on a Hewlett Packard GCD Model G1800A. The instrument is equipped with a J&W DB-5MS 30m 0.25 mm capillary column, and the mass spectrometer is operating in Electron Ionization mode. Sample was injected from methylene chloride solution (Optima grade) and heated at 80°C for 2 minutes, followed by a temperature increase from 80°C to 300°C at 10°C per minute with a 2 minute wait time at 300°C. The MS detection range was set from 40 to 425 Daltons.

4.6. References


Novel protein immobilization technique was established and discussed in this thesis. It allows to capture proteins on analytical surfaces without losing physiological properties and subsequently express their functions. Use of Ni-NTA head group was facilitated along with the use of His-tagged proteins. Gold was used as a primary surface with PEG-terminated SAM as a linking point and an inert background at the same time. Surface IR spectroscopy was employed as primary visualization and evaluation tool. Intrinsic IR handles already present in the system allowed for a quick and reliable recognition of structures on the surface after each coupling step. Step-by-step strategy proved to be quick and efficient way to assembly desired system.

Protein binding experiments were conducted. Specific binding was detected but significant amount of non-specific was also shown. Multiple experiments were set up to establish deciding factors favoring non-specific interaction between the proteins and the surface. Lowering the concentration of protein in the buffer proved to be sufficient measure to control the non-specific binding on PEG-terminated surface. In the case of NTA terminated surface CDI coupling was established as a crucial step and a careful control over this step was needed in order to achieve desired results. Lowering the protein concentration to 1 µM solved the non-specific binding problem and allowed to demonstrate successful specific binding of His-DHP to Ni-NTA monolayer. Protein was later removed by rinsing with imidazole solution.

Several attempts were undertaken to directly detect the amount of NTA binding sited on the surface. These included employing transition metals in place of Ni to obtain an electroactive complex. Indirect method was also tried with dopamine as a probe. All these efforts proved to be unsuccessful due to unfavorable kinetics. Ferrocenemethylamine was
synthesized based on previous experience in our group with this molecule and its electroactive properties. However, its use in our setup was limited due to its non-covalent interaction with the system.

Potential applications of this technique involve *in-situ* experiments not possible before. Excellent example is Reaction Center that in conjunction with Cytochrome C. This system plays important role as it transfers electrons. Surface immobilization with specific orientation of the protein will allow to study it with techniques that couldn’t be used before like Double Resonant SFG (DR-SFG). More general possible applications include molecular machines and sensing devices.
APPENDIX I – NMR and GC-MS Data