Manipulating Electron Transfer Reactions from Micro- to Preparative Scale

Matthew Duane Graaf
Washington University in St. Louis

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Manipulating Electron Transfer Reactions from Micro- to Preparative Scale

by

Matthew Duane Graaf

A dissertation presented to the
Graduate School of Arts & Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2015
St. Louis, Missouri
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<tr>
<td>AIBN</td>
<td>2,2’-azobis(2-methylpropionitrile)</td>
</tr>
<tr>
<td>BET</td>
<td>Back Electron Transfer</td>
</tr>
<tr>
<td>Bpy</td>
<td>Bipyridine</td>
</tr>
<tr>
<td>Bpz</td>
<td>Bipyrazine</td>
</tr>
<tr>
<td>BrSt</td>
<td>4-bromostyrene</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CEMA</td>
<td>2-cinnamoyloxyethyl methacrylate</td>
</tr>
<tr>
<td>CV</td>
<td>cyclic voltammetry/cyclic voltammogram</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DFT</td>
<td>Density Functional Theory</td>
</tr>
<tr>
<td>DMF</td>
<td>N, N’-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-dicyclohexyl carbodiimide</td>
</tr>
<tr>
<td>EDC</td>
<td>N-(3-dimethylaminopropyl)-N-ethyl carbodiimide</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>Fc</td>
<td>ferrocene</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>HEMA</td>
<td>2-hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>HRMS</td>
<td>high resolution mass spectrometry</td>
</tr>
<tr>
<td>hv</td>
<td>light</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>MEK</td>
<td>methyl ethyl ketone</td>
</tr>
<tr>
<td>MeNO₂</td>
<td>Nitromethane</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>pBoSt</td>
<td>pinacol 4-styreneboronic ester</td>
</tr>
<tr>
<td>PBrSt</td>
<td>poly(4-bromostyrene)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PCEMA</td>
<td>poly(2-cinnamoyloxyethyl methacrylate)</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PHEMA</td>
<td>poly(2-hydroxyethyl methacrylate)</td>
</tr>
<tr>
<td>PSt</td>
<td>polystyrene</td>
</tr>
<tr>
<td>RAFT</td>
<td>reversible addition-fragmentation chain transfer polymerization</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>TBAB</td>
<td>tetrabutylammonium bromide</td>
</tr>
<tr>
<td>TEMPO</td>
<td>(2,2,6,6-Tetramethylpiperidin-1-yl)oxidanyl</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
</tr>
<tr>
<td>TOF-SIMS</td>
<td>time of flight secondary ionization mass spectrometry</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VB₁₂</td>
<td>vitamin B₁₂</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
</tbody>
</table>
Acknowledgments

I would like to thank my advisor and mentor, Dr. Kevin D. Moeller, for the opportunity to work with him on the science presented in this thesis. His knowledge of chemistry and drive to pass it onto his students is what fuels the work in his lab and the future success of his students. His enthusiasm for science is contagious and I can only hope to hold onto a fraction of that passion.

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you have always supported my love for science, even when others didn’t and your strength &
determination through difficult times have been an inspiration. Finally, Grandpa Hackett, you
taught me so much and always stood up for me. I miss you.

Matthew Duane Graaf

Washington University in St. Louis

August 2015
Dedicated to my wife Elizabeth
and my daughter Lucy.
ABSTRACT OF THE DISSERTATION

Manipulating Electron Transfer Reactions From Micro- To Preparative Scale

by

Matthew Duane Graaf

Doctor of Philosophy in Chemistry

Washington University in St. Louis, 2015

Professor Kevin D. Moeller, Chair

The utilization of electron transfer reactions has allowed the transformation of a variety of substrates from polymer surfaces to highly functionalized organic molecules. The intricacies of these electron transfer reactions have been utilized to develop a new platform for building and studying addressable molecular libraries as they interact with biological targets. As a low cost, reusable alternative to other methods of these studying binding interactions, microelectrode arrays are beginning to gain traction with the scientific community as we continually push the limits of their potential. Additionally, the electron transfer techniques used to build the molecular libraries can be expanded to the preparative-scale synthesis of complex molecules. The goal of this thesis is to demonstrate the significance of the advancements made towards these areas.

First, we discuss the development of a tunable polymer surface by taking advantage of a rapid ligand exchange on a boronic ester polymer. This allows us to reversibly modify the surface of an electrode for pacification towards any biological system being monitored. This is followed by the development of a copper-mediated, chemoselective coupling of peptides to the surface of the polymer-coated electrode. This method builds a molecular library with a selective orientation, which is critical for understanding structure-activity relationships of molecule
coupled to a surface. Additionally, we demonstrate the ability to directly couple peptides that contain common bioorthogonal functionalities such as an azide and acetylene for “click” chemistry. These techniques further expand our extensive synthetic toolbox for building molecular libraries.

This work of building molecules and selectively modifying electrode surfaces comes to fruition with the analysis of a cyclic peptide, v107 against a vascular endothelial growth factor protein that has been difficult to detect by other analytical techniques. This exciting work helps cement the efforts made by our group over the last several years in developing the microelectrode array-based binding studies as a viable alternative.

Finally, the utility of electron transfer reactions is shifted towards preparative-scale synthesis of highly functionalized ring system. After the improved synthesis of a widely used ligand, we apply photoredox catalysis to our previous work on anodic oxidation chemistry to better understand the mechanism of radical cyclizations.
Chapter One

Introduction

1.1 Electron Transfer Reactions and Microelectrode Arrays

Electron transfer reactions began to emerge as a facet of chemistry during the late 1940s. The field was driven by the interest in understanding isotope exchange electron transfer reactions involving radioactive elements, which became widely available after World War II\(^1\). The self-exchange reactions, like that in equation \(1\) (* = radioactivity), represent one of the simplest reactions in chemistry. These reactions eliminated the elements of bond breaking and bond making from reaction rate by keeping the reactants and products essentially identical\(^1\). Within the next couple of decades, the development of new instrumentation, lasers in particular, allowed the pico- and subpicosecond study of electron transfer processes. This new technology allowed scientists to expand the concept of electron transfer mechanisms into other areas of chemistry. In the field of inorganic and biochemistry, Gray and co-workers applied this technique to study the electron transfer processes of metalloproteins\(^2\). In conjunction with site-directed mutagenesis, they and other research groups have studied the dependence of the protein’s secondary and tertiary structure on the rate of electron transfer\(^3\). At its core, this work is employing the principles of electron transfer to map the relationship between a molecule’s structure and function.

For over the last decade, our group has strived to harness the potential of electron transfer reactions, in order to develop technology complementary yet orthogonal to that of Gray and

\[\text{(1)} \quad \text{Ce}^{2+} + \text{Ce}^{*4+} \rightarrow \text{Ce}^{4+} + \text{Ce}^{*2+}\]
others\textsuperscript{2}. Our efforts have been focused on using the synthetic capabilities of electron transfer reactions to construct not only individual molecules but also addressable libraries of molecules. In addition, we have been working towards the development of technology that uses electron transfer chemistry to indirectly monitor the interaction between a molecule of interest and a biological target. We hope that the marriage of the synthetic capabilities of electron transfer to construct molecular libraries with its ability to indirectly monitor their interactions will allow us to rapidly chart molecules structure with its activity or function.

The utilization of molecular libraries to rapid screen a series of molecules has been a well-established technique for several years. Libraries consisting of natural products and their synthetic analogies have been studied for their interaction with biological targets in micro-well plates in solution or on solid supports as molecular arrays\textsuperscript{4-7}. These techniques allow for high-throughput screening against numerous targets allowing for an efficient mapping of a compounds structure activity relationship.

These techniques often suffer from one or more problems that limit their generality. For example, one major disadvantage is the requirement to label the target protein for detection. This is often done with either a fluorescent or a radioactive marker on either the target itself or an antibody of the target, which is introduced as a means of secondary labeling\textsuperscript{8}. In either case, the labeling effort makes the technique expensive, and the label is an unnatural modification to the target protein. Although no clear evidence has been found, it cannot be ignored that such a modification to its natural structure may alter the binding characteristics of that system. Additionally, these experiments often require rigorous washing steps to ensure that only the remaining markers are from strong positive interactions. However, this has an inherent drawback by washing away weaker binders to the target. Although the weaker binding may not make it an
immediate drug candidate, it certainly holds useful structure information that may be critical for drug optimization. Finally, these methods typically do not allow for quality control of the library. How does one know that a molecule being studied is what it is supposed to be, especially if the library is used multiple times over a given period of time?

There are techniques currently being used that eliminate the need for a fluorescent or radioactive label. They include mass spectrometry, reflectometry, and surface plasmon resonance (SPR) among others\textsuperscript{9-11}. The advantages of these techniques are not only the absence of a label but also the ability to monitor a binding event in “real-time”. This allows for the detection of both strong and weak binding events. However, they are not without their own drawbacks. For example, the above-mentioned reflectometry and SPR techniques require a careful construction of the molecular libraries including the synthesis of the molecules prior to their placement onto a solid support. In addition, once the library is built onto the surface for analysis, the library cannot be modified or recovered. This extensive preparation of the single use libraries hinders the advancement of drug discovery while consuming valuable resources.

For these reasons, our group has been developing a cheaper, simpler and reusable method to build/modify molecular libraries in order to rapidly screen them against biological targets in “real-time” and with a high-degree of sensitivity. In addition, the technique must be compatible with full characterization of the molecules in the library. To accomplish this endeavor, we employ the use of microelectrode array technology. These microelectrode arrays, provided to us by the CombiMatrix Corporation (now CustomArray), provide a durable platform to build molecular libraries and assess the binding of each library member through the individually addressable electrodes. The microelectrodes used in our lab consist of two varieties, a 1K and a 12K array. The 1K array (Figure 1.1a)\textsuperscript{12} consists of 1,024 individually addressable electrodes.
which are ~95 µm in diameter and spaced 200-300 µm apart from one another. The more widely used 12K array (Figure 1.1b)\textsuperscript{12} contains 12,544 electrodes per cm\textsuperscript{2}, all ~45 µm in diameter and ~20 µm apart\textsuperscript{13}. Due to the additional instrumentation required to operate the individual arrays\textsuperscript{14}, the 12K arrays are used for all analytical studies. This also allows us to take advantage of the smaller electrode and reaction chamber size (100 µL compared to 1 mL for 1K arrays) to minimize the amount of protein needed for a particular binding study.

1.2 Binding Experiments

The foundation of using the microelectrode arrays to conduct binding experiments between a molecular library and a biological target lies with the ability to individually monitor an interaction in an indirect fashion. The general scheme for these binding experiments (Figure 1.2)\textsuperscript{15} consists of placing an individual member of the library proximal to an addressable electrode in the array. Once the library is built on the array it’s incubated in a solution containing a redox mediator, typically Fe\textsuperscript{2+}/Fe\textsuperscript{3+} based. An electrical current is established by cycling the iron mediator between selected array electrodes and an adjacent counter electrode via cyclic...
voltammetry. This current is monitored as the biological target is introduced above the array over a range of concentrations. If a binding event occurs, it is detected as a change in current due to a shift in the capacitance of the surface (Figure 1.3). Simply put, the presence of the receptor on the surface of the electrode alters the ability of the iron to reach the electrode below. The dependence of this change on the concentration of receptor in solution can be translated into a traditional binding curve for each library member analyzed. This procedure will be discussed in more detail in chapter 5.
1.3 Polymer Reaction Surface

The ability to monitor binding interactions between a biological target and an entire library using a microelectrode array is dependent on being able to affix each member of the library to an individual electrode or set of electrodes in the array. This requires a surface coating on the electrodes that can be chemically modified yet stable to the chemistry employed. Additionally, the surface must be porous enough to allow any mediator to reach the electrode yet protect the molecular library and targets from electrochemical degradation. After initially conducting experiments on arrays that were polymer coated with agarose and sucrose, a more stable di-block copolymer was developed\(^\text{16}\) (Figure 1.4). The di-block copolymer structure

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**Figure 1.3: Example of Cyclic Voltammograms Taken During A Binding Study**

A cyclic voltammogram (CV) is acquired at a particular set of electrodes for an individual concentration of biological target. Upon increasing the concentration of the target, a change in the total current (height of the wave around zero volts) is observed. This corresponds to an interaction between the biological target and a molecule on the array.
allows us to partition the polymer with different functionalities; some to be used in stabilizing the polymer and some to be used to add the molecular library to the polymer. Once the array is coated with the di-block copolymer shown, the first block containing the cinnamic ester moiety is photocrosslinked under a 100W Hg lamp. This adds stability to the surface and allows us to control the porosity of the polymer. The second block (bromostyrene) provides a handle to couple molecules to the polymer surface through a variety of synthetic transformations\textsuperscript{16}. These di-block copolymers have proven to be very stable and compatible with a wide range of experiments. Further advancements to the polymer surface are discussed in the next chapter.

1.4 Array-based Electrochemical Reactions

1.4.1 Site-selective Chemistry and Evaluation

As mentioned earlier, a major hurdle of performing a binding study using any of the aforementioned techniques, is constructing the addressable molecular library to be analyzed against a biological target. One advantage of using a microelectrode array is the ability to
perform electrochemical transformations adjacent to any selected electrode in the array. This allows one to place or build the molecules in the library directly on the surface of the electrodes to be used to monitor their binding properties. This is accomplished by incubating the array in a solution containing the substrate, an inert pre-catalyst and an electrolyte (typically a tetrabutylammonium salt), and then electrochemically converting the pre-catalyst into the reactive catalyst (or reagent) at selected sites in the array in order to perform the desired chemical reaction (Scheme 1.1). This technique is beautifully demonstrated by the electrochemical reduction of copper sulfate to the reactive copper(I) species in the presence of a pyrene-labeled thiol as illustrated in Scheme 1.2. The site-selective generation of the active catalyst resulted in the coupling of the thiol with the aryl bromide surface. The resulting array was evaluated with the use of a fluorescence microscope in order to obtain the image shown. This method of site-selectively generating an active reagent is proving to be quite general, and

Scheme 1.1: General Scheme for Array-based Reactions

A microelectrode array is incubated in a solution containing a “Pre-catalyst” or “Pre-substrate A”. A potential is applied to an individual electrode or set of electrodes generating a reactive species to couple molecule “A” to the polymer above the desired electrode. The procedure may be repeated to coupling a different molecule “B” above an adjacent electrode.
we have available a growing number of synthetic transformations that can be used to build molecular libraries on the array. In each case, conducting the reaction at various sites on the array allows the molecules in a library to be placed on the array so that each unique member of the library is isolated to a unique addressable location in the array. To date, acyl-transfer reactions, Heck reactions, Wacker oxidations, Suzuki reactions, “click” reactions, hetero-Michael reactions, Diels-Alder reactions, various oxidations and reductive amination reactions have all been successfully performed on the arrays. These methods take advantage of the electrochemical generation of acids, bases, Pd(0)-catalysts, Pd(II)-catalysts, oxidants, Lewis-acids, and Cu(I)-catalysts. In later chapters, we expand this extensive list with new reactions and surface modifications that are directly related to the building of peptide-based libraries on the arrays.
It is important to take note that the reactions conducted on the microelectrode arrays are evaluated by fluorescence microscopy. The substrates are fluorescently tagged and upon reaction completion, the array is analyzed to assess the success of said reaction. There are alternative methods of characterizing the product formed on the surface of the array, such as chemically cleavable linkers\textsuperscript{19}, the use of TOF-SIMS cleavable linkers\textsuperscript{20}, and a newly discovered method of reversibly functionalizing the surface is discussed in the next chapter. However, when it comes to assessing the site-selectivity of the reactions on the arrays, fluorescence microscopy is indispensible. For these efforts, pyrene is typically used as the fluorophore. The relative abundance of pyrene derivatives and its synthetic compatibility make it an excellent choice. However, it is not without its drawbacks. As the concentration of pyrene rises, it readily forms excimers with itself, resulting in a redshift in the fluorescence spectrum\textsuperscript{21}. Due to this phenomenon, it is very difficult to quantify the fluorescence observed on the surface of the array. However, we can obtain a sense of the quality of a particular reaction based on the amount of redshift in the fluorescence. The images you will see throughout this work range in color due to this shift. They vary between blue, green and red, corresponding to a moderate to high level of pyrene on the surface, respectively.

1.4.2 Reaction Confinement

The use of microelectrode arrays provides a significant advantage toward both building molecular libraries directly onto the polymer coated electrode and the ability to indirectly monitor binding events in “real-time”. However, given the size and proximity of the electrodes, one important aspect of building the molecular libraries is ensuring the precise location of each member. This is what was meant above by the site-selectivity of the reactions. The array’s
capability to selectively activate any number of electrodes ensures that the reactive species is only generated at those particular electrodes. Nevertheless, if the reactive species is not immediately consumed it has the opportunity to migrate away from the electrode where it was generated and trigger a reaction at unintended locations on the array. In order to avoid this scenario, a confining strategy is used.

Figure 1.5: Suzuki Reaction at Various Potentials

A Suzuki coupling was ran at an individual electrode at various electrode potentials. By decreasing the potential, from -2.4 V to 1.4 V, the reaction becomes more confined to the selected electrode. This is demonstrated by the attached fluorescence images (a, b, and c).
The general concept of a confining strategy is maintaining a balance between the rate at which a reactive species is generated at the electrodes in the array and the rate that it is consumed in the solution above the array. There are two main methods for achieving reaction confinement. The first method consists of adjusting the potential applied to the electrode, which in turn, alters the rate of reactive species generation. This was demonstrated nicely when a Suzuki reaction, between the aryl bromide surface and a pyreneboronic acid, was performed at various potentials (Figure 1.5)\(^{22}\). The electrochemical reduction of palladium(II) to palladium(0) at the potential of -2.4V, relative to the platinum counter electrode, (Figure 1.5a) produces a significantly large fluorescent spot although only a single electrode was activated. In addition, there is fluorescence by every electrode in the array. By lowering the potential of the electrode to -1.7 V (Figure 1.5b) and -1.4 V (Figure 1.5c), the size of the fluorescent spot observed decreases, and the reaction is confined to the single electrode used for generation of the Pd(0)-catalyst. This is due to the lower rate of palladium(0) generation relative to the rate of consumption by the Suzuki reaction or reoxidation by residual oxygen in solution.

The second method of achieving an optimal level of confinement involves playing with the amount and nature of the confining agent in solution. This alters the rate at which the reactive reagent is consumed. This type of reaction confinement is showcased in the scandium(III) catalyzed Diels-Alder reaction (Scheme 1.3)\(^{23}\). After functionalizing the array surface with an N-alkylmaleimide dienophile, the array was incubated in a solution containing the ammonium salt electrolyte, scandium(I) triflate, a pyrene labeled diene and 2-arylbenzothiazole. Upon applying a positive potential to a select pattern of electrodes, the scandium(I) was oxidized to scandium(III) serving as a Lewis acid for the Diels-Alder reaction. The benzothiazole was chosen because it is a known reducing agent for Sc(III). This ensured that the reactive scandium species did not
migrate away from the desired electrodes. The success of the confining reagent strategy can be seen in the fluorescence images taken after the Diels-Alder reaction was ran on both the 1K and 12K arrays (Scheme 1.3). The observed fluorescence is localized to only the selected electrodes. These methods for optimizing confinement, often used in tandem, are a critical component to the success of building molecular libraries on the array surface.

Scheme 1.3: Sc(III) Catalyzed Diels-Alder Reaction

A lewis-acid catalyzed Diels-Alder reaction was performed on an agarose surface functionalized with a dienophile. The attached fluorescence images indicate the successful coupling of the pyrene-labeled diene to a 1-K (left) and 12-K (right) array.
1.5 Preparative-scale Electron Transfer Reactions

In many ways, the synthetic chemistry conducted on the arrays take advantage of the same electrochemical opportunities seen in preparative scale electrochemical methods. If one ignores the array specific features of the reactions above like the polymer coating and the confinement strategy, then the transformations involve electron-transfer reactions and umpolung chemistry. In other words, they add or remove electrons from molecules in a manner that reverses the polarity of a species in solution (Scheme 1.4). This can allow coupling reactions between two nucleophiles or electrophiles\(^{24}\). In addition, the use of an electrode means that these transformations can be performed without the need for chemical oxidants or reductants providing a significant synthetic advantage.

**Scheme 1.4: Umpolung Chemistry**

![Scheme 1.4: Umpolung Chemistry](image)

*Umpolung chemistry consists of reversing the polarity of a functional group. By reducing (top) or oxidizing (bottom) a molecule and invoking Umpolung chemistry, to electrostatically similar functional groups may be coupled together.*
Our group has utilized this technique to perform numerous cyclizations on a variety of substrates\textsuperscript{25-29} (Scheme 1.5). In general, a molecule containing an electron-rich olefin is electrochemically oxidized to the corresponding radical cation. Upon formation of this radical cation, a tethered nucleophile undergoes an intramolecular cyclization capable of forming a variety of ring sizes. The remaining radical is then oxidized again to the cation, which is trapped by the solvent, typically methanol. As a result, this transformation efficiently couples two nucleophiles, a feat by more traditional synthetic means.

**Scheme 1.5: Oxidative Cyclizations**

![Scheme 1.5: Oxidative Cyclizations](image)

Electron-rich Olefins:

- \(\text{CH}_{2}\text{CHMe}\)
- \(\text{CH}_{2}\text{COOMe}\)
- \(\text{CH}_{2}\text{Ar}\)
- \(\text{CH}_{2}\text{SiMe}_{3}\)
- \(\text{CH}_{2}\text{NR}_{2}\)
- \(\text{CH}_{2}\text{Ar}\)

Nucleophiles:

- \(\text{CH}_{2}\text{OH}\)
- \(\text{CH}_{2}\text{EWG}\)
- \(\text{CH}_{2}\text{COOH}\)
- \(\text{CH}_{2}\text{CONH}_{2}\)
- \(\text{CH}_{2}\text{OCH}_{3}\)
- \(\text{CH}_{2}\text{SiMe}_{3}\)
- \(\text{CH}_{2}\text{Ar}\)

The versatility of this technique is complemented by its simplicity. The implementation of these experiments involves a solution containing the substrate of interest and an electrolyte in a common round bottom flask (Figure 1.6)\textsuperscript{30}. Two electrodes are then inserted into the solution
and connected to a power source. The substrate is oxidized at the anode, in order to initiate the chemistry described above, and the circuit is completed with the reduction of methanol to hydrogen gas and methoxide at the cathode. The methoxide neutralizes acid generated at the anode so that the reaction is net neutral in terms of pH. Furthermore, the reaction can be ran with a simple photovoltaic making this technique a significantly greener approach to building complex molecules\textsuperscript{31-33}.

In chapter 7, work to explore the mechanism of these reactions and their dependence on the method used to generate the radical cation will be discussed.
1.6 References


18. For a comprehensive look at the array instrumentation and general reaction protocol: Fellet, M. S.; Bartels, J. L.; Bi, B. and Moeller, K. D. “Site-Selective Chemistry and the Attachment of Peptides to the Surface of a Microelectrode Array”, *Journal of the American Chemical Society*, **2012**, 134, 16891-16898


Chapter Two

Development of a Tunable Polymer Surface

2.1 Introduction

The utilization of microelectrode array for monitoring biological binding event in real time, among other uses, is greatly dependent on the quality of the surface. The polymer coating placed on the array surface must fulfill several requirements. The surface must be able to tolerate a variety of electrochemically-mediated reactions used to build a molecular library on the array. Thus far, transition-metal catalysts, oxidation reactions, reduction reactions, nucleophilic additions, electrophilic additions, acids, bases, and Lewis acids have all been used to conduct synthetic reactions on the arrays. As mentioned in Chapter 1, the development of a diblock copolymer (Scheme 2.1, \( X = \text{Br} \)) as a porous reaction surface greatly improved the performance of the array towards these reaction conditions compared to the previous agarose and sucrose polymers.\(^1\) The polymer must also be stable for long periods of time including several uses and

Scheme 2.1: Diblock Copolymer Structure
storing. This allows the same microelectrode array to be used to study a biological target while new molecules are added to the library with each binding experiment. This is crucial to ensure consistency and accuracy during the optimization of a molecule’s binding to a specific target.

In addition to the modifications of the molecular library during the course of a binding study, the polymer surface may also need to be modified to decrease nonspecific binding. The initial attempt, done by Dr. Libo Hu, PEGylated the aryl bromide di-block copolymer surface in order to decrease binding with bovine serum albumin (BSA)\textsuperscript{2}. Although this technique was not successful, the experiment revealed the need of a method for rapid manipulation of the polymer surface. To this end, attention was turned toward the use of a boronic acid polymer. Known as the “sweet-tooth” polymer, Sumerlin and coworkers demonstrated the polymers affinity for free

**Scheme 2.2: Utility of a Boronic Acid Surface**

A boronic acid based polymer possesses several useful attributes including an affinity for sugars, possible site-selectively functionalize through transition metal chemistry, and alternative functionalization with vicinal diols.
hydroxyls on sugars and sterically hindered 1,2-diols. Additionally, the polymer would have the ability to be functionalized with a variety of transition metal couplings (Scheme 2.2). The five-membered ring borate ester would also be stable in water providing a surface that is compatible with the signaling experiments to be conducted on the arrays after synthetic efforts are complete.

A pinacol boronic ester (BPin) derivative of our diblock copolymer (Scheme 2.1, X = BPin) was synthesized by Dr. Hu and shown to be compatible with the binding experiment conditions. We have since utilized this polymer to develop a reaction surface that can be readily and reversibly functionalized. In this chapter, we discuss the work behind the formation of this tunable surface.

2.2 Results and Discussion

2.2.1 Selective Deprotection of the Boronic Ester Polymer

During the course of his preliminary investigation into the boronic acid polymer, Dr. Hu observed that the surface was more stable towards the iron redox mediator used in signaling studies on the arrays when the boronic acid was pinacol protected. The unprotected polymer was prone to bind the iron resulting in an unstable flux in current. When the boronic acid was protected, signaling studies on the surface had no such problem. The question became, how do we build molecular libraries on this surface?

In order to address this issue, we initially believed that we needed a method to site-selectively deprotect the surface. Most synthetic methods developed for arylboronates took advantage of the unprotected boronic acid group. The polymer was synthesized using a reversible addition-fragmentation chain transfer (RAFT) polymerization technique from the
styrenyl boronic ester then successfully deprotected in solution with acid$^2$ (Scheme 2.3). Since we were interested in the protected polymer for its enhanced stability, we needed to only deprotect the polymer on the surface by electrodes where we wanted to do chemistry. Hence, the acid needed for the deprotection needed to be generated at only selected electrodes in the array. Work by Maurer and coworkers at CombiMatrix Corporation showed that acid could be generated proximal to selected electrodes in an array by using the electrode to oxidize diphenylhydrazine (Scheme 2.4). Maurer and our group utilized this technique to perform a site-selective deprotection of tert-butoxycarbonyl protected amines on the surface of an array$^{4,5}$.
With the site-selective acid chemistry all ready in place, the direct deprotection of the boronic ester surface was attempted. A 1-K microelectrode array was spin coated with the boronic ester polymer and photocrosslinked under a 100W Hg lamp. The array was then placed in methanol solution of phenylhydrazine, tetrabutylammonium hexafluorophosphate electrolyte and water. A potential of +3.0 V was applied to select electrodes in a checkerboard pattern for 900 cycles (cycle = 0.5 sec on and 0.1 sec off). The chip was then subject to a Suzuki coupling with bromopyrene at all of the electrodes on the array. At the completion of the reaction, the chip was analyzed under a fluorescence microscope. The resulting image (Scheme 2.5) indicates that the coupling only occurred at the previously deprotected electrodes\(^2\). This verified that we could selectively functionalize the surface of an electrode in the array while retaining the protecting groups over the remainder of the surface.

Although the deprotection technique for coupling molecules to the array surface proved to be successful on the 1-K arrays, it appeared to be unique to this type of microarray. As discussed in Chapter 1, one of the defining characteristics of the microelectrode arrays is the spacing between the array surface and the counter electrode. This interelectrode distance lends the 1-K and 12-K arrays to operate as a divided and undivided cell respectively, altering the mechanism of a particular reaction\(^6\). This was also observed when the selective deprotection was transferred to the 12-K array. The reaction was run by first spin coating and crosslinking the

\[ \text{Scheme 2.4: Electrochemical Generation of Acid} \]

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\begin{align*}
\text{Scheme 2.4: Electrochemical Generation of Acid} \\
\begin{array}{c}
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25
polymer onto the array in the same manner as before. The chip was then incubated in the same diphenylhydrazine reaction solution and a potential of +2.4 V was applied to electrodes in a ‘D’ pattern across the entire array. Also as before, a Suzuki reaction was run by applying a -2.0 V potential to the array but in this case, the electrodes selected were in a split pattern. Half of the array consisted of all the electrodes being turned on, while on the other half of the array the electrodes used were the same D-pattern electrodes used for the deprotection. Based on this set of patterns, if only the deprotected electrodes were reactive then both halves of the array will fluoresce in a ‘D’ pattern. However, the fluorescence image (Scheme 2.6) shows no difference in
reactivity between the protected and unprotected electrodes. The lack of selectivity observed on the half of the array with all of the electrodes used for the Suzuki reaction may be due to the higher rate of catalyst production in a undivided cell. This may allow for a higher rate of reaction, but the specific mechanistic explanation is unknown. Nevertheless, the important result from this experiment is that the surface does not require prior deprotection to perform coupling reactions. In retrospect, this outcome is optimal. Realistically, if the surface was electrochemically deprotected, one could not assume that every subsequent coupling reaction would react with 100% completion. The remaining unreacted boronic acids would result in the same complications during binding experiment that were discussed earlier. The ability to conduct the Suzuki reaction on the unprotected surface removes this problem.

**Scheme 2.6: Deprotection and Suzuki Coupling on 12-K Array**

The site-selective deprotection of the pinacol borate ester surface was performed across the entire array in a “D” pattern. This was followed by a Suzuki coupling with all of the electrodes activated on half of the array and only in a “D” pattern on the other half. The lack of symmetry across the array demonstrates that the deprotection step is not necessary.
2.2.2 Acid Catalyzed Ligand Exchange

Upon determining the compatibility of the polymer surface with electrochemical coupling reactions, attention was turned towards the reversible modification of the surface. This was important for tuning the surface an array to minimize non-specific binding interactions once the molecules to be studied had been added to the array. As mentioned earlier, one of the major characteristics of a boronic acid based polymer is its affinity for vicinal diols\(^3\) (Scheme 2.2). To this end, our approach for developing a tunable surface consisted utilizing the site-selective acid reaction in the presence of a vicinal diol to exchange the ligand on the boron, in this case, pinacol (Scheme 2.7). The reactivity of the surface demonstrated earlier with the 12-K arrays suggests that the exchange reaction should perform quite well.

The first step in accomplishing this feat was to develop a method of qualitatively determining the success of the experiment. As mentioned in Chapter 1, upon performing a
coupling reaction or other various transformations, the success of the reaction is determined by taking advantage of a fluorescent label in the molecule used for the reaction. For the current reaction, this meant that we needed a fluorescently labeled diol (Scheme 2.8). This was accomplished by first performing a Swern oxidation on the commercially available pyrene butanol. The resulting aldehyde was subject to a Wittig reaction with methyltriphenylphosphonium bromide to yield the terminal alkene in a 76% yield over the two steps. The final product was obtained in 66% yield from a cis-dihydroxylation of the alkene with AD-Mix-β.

**Scheme 2.8: Synthesis of Pyrene labeled diol**

The synthesized diol was used to explore the ligand exchange technique. A 12-K microelectrode array was spin coated with the boronic ester polymer and crosslinked in the usual fashion. The chip was then incubated in solution containing the diol, electrolyte and diphenylhydrazine as the acid source. The site-selective oxidation of diphenylhydrazine was run for 300 seconds and analyzed under a fluorescence microscope. The image (Scheme 2.9, (a)) shows the successful exchange of the pinacol for the pyrene labeled diol. However, the electrodes were not completely covered. This suggested that the reaction had not reached
Completion. In order to achieve greater coverage of the electrodes, the experiment was repeated on a newly coated chip with the reaction time increased to 600 seconds. The fluorescence image (Scheme 2.9, (b)) indicated that the extended time did indeed yield a greater amount of ligand exchange, but it also led to a loss of confinement. This was evident by the fluorescence between the blocks of electrodes where the reaction was run. Even though this was an unfortunate result, it was not completely unexpected.

As mentioned earlier (Chapter 1), an important aspect of running electrochemically-mediated transformations on the microelectrode arrays is the concept of a confining reagent. If
the reactive species, formed proximal to a designated electrode, does not react quickly, it must be consumed by a sacrificial reagent to avoid unwarranted migration to other areas of the array. The intention of the confining reagent is to render the reactive species inert. In the site-selective generation of acid, the confining strategy was scavenging of unreacted protons by diphenylhydrazine or the oxidized form, azobenzene. In either case, the protonated form is still a weak acid. Thus over time, the accumulation of protonated hydrazine would actually contribute to the loss of confinement rather than prevent it. This is represented in Scheme 2.9, image (b) by the diffuse fluorescence around the blocks of electrodes. In order to counteract this issue a weak base, pyridine, was added to the reaction solution. Although still not a traditional confining reagent, it was hypothesized that the additional base would help scavenge the unreacted protons long enough for the ligand exchange reaction to progress to further completion. This was attempted by repeating the reaction on an unused surface for 600 seconds. As predicted, the presence of the weak base allowed the ligand exchange to occur with excellent coverage of the electrodes and a high degree of confinement (Scheme 2.9, (c)).

This outcome is a step forward toward our ultimate goal of developing a cheap, reusable platform for monitoring biological binding interactions in “real-time”. It completes one of the synthetic tools needed to build a library of molecules on a tunable arrays surface. For such a library, irreversible reactions are required for the placement of the molecules to be studied onto the arrays. On the other hand, a reversible reaction is needed to selectively alter the surface in order to maintain compatibility with various biological targets. Since the acid catalyzed ligand exchange is just an equilibrium between the two ligands, this transformation can be repeated numerous times. Thus, the surface of the array can be altered at will.
2.2.3. Reversible Peptide Coupling

The ability to tune the surface of an array by exchanging the ligands on the boron was a significant discovery. The original intention of the project was to facilitate means to render the surface inert to specific biological targets. However, as with many synthetic methods new avenues of utilization arose. For example, could we apply this reversible technique to the molecular library itself? If successful, this would allow us to build a library on the array surface, conduct necessary binding experiments, then easily remove select molecules for further characterization and analysis. In addition, if a biological study damaged one of the molecules on the array, then it could be replace without having to rebuild the entire library.

![Scheme 2.10: “Safety-catch” Linker Strategy](image)

This is not the first time a method for cleaving molecules off of the array surface had been developed. Previous work from our group by Dr. Bo Bi developed a “safety-catch” linker strategy to recover molecules coupled to the polymer surface (Scheme 2.10). Electrochemically-mediated deprotection of a nucleophile triggered an intramolecular cyclization resulting in a cleavage of the molecule from the electrode. This strategy was used to successfully cleave and analyze the products of a Diels-Alder reaction performed on the array.
Our technique aimed to accomplish the same goals in addition to including a fluorescent linker strategy also previously developed in our lab. Drs. Tanabe and Bi synthesized an amino acid derived fluorescent linker to couple molecules of interest to the array. The fluorophore portion of the linker was used to verify the placement of the molecule using the fluorescence microscopy technique discussed earlier. The tryptophan derivative (Scheme 2.11, (a)) was attached to the aryl bromide surface via a copper mediated coupling through the N-terminus and the C-terminus was utilized as the connecting point for biotin\(^7\). Our strategy involved the same technique but we modified the linker with a diol (Scheme 2.11, (b)) in order to take advantage of the ligand exchange chemistry discussed in Section 2.2.2.

The synthesis of the new linker (Scheme 2.11, (c)) consisted of a copper catalyzed coupling between the fluorescent linker (a) and a protected diol containing an aryl bromide (b) with subsequent deprotection. This route was chosen since the linker had shown successful coupling to aryl bromides in solution and onto the array\(^7\). Before this particular coupling could take place, the aryl bromide diol was made. The synthesis was done by performing a dihydroxylation of p-bromostyrene followed by protection of the diol to form the acetal. This protection was done to avoid potential complications from the chelation of the copper.

**Scheme 2.11: Proposed Synthesis of Second Generation Fluorescent Linker**
The copper couplings used to synthesize the new linker were developed by Buchwald and coworkers (Scheme 2.12)\textsuperscript{8,9}. These couplings and variations thereof have been previously utilized by our group both in solution phase reaction and on the array surface\textsuperscript{6,7}. However, when these techniques were applied to this particular system no product was obtained, only recovered starting material was observed. Adjustments to the reaction conditions including the catalyst amount, ligand, base, solvent, temperature, and reaction time resulted in no improvement of the product yield. The next step was to modify the substrates to improve reaction performance. Attention was turned toward the aryl halide.

In an effort to make the aryl halide more reactive, the bromide was converted to an iodide by utilizing an aryl-Finkelstein reaction developed by Klapars and Buchwald\textsuperscript{10}. The copper catalyzed halide exchange reaction was performed with the racemic trans-N,N’-dimethyl-1,2-cyclohexanediame ligand which is synthesized from the formylation and subsequent reduction of racemic trans-1,2-cyclohexanediame. This reaction produced the aryl iodide of the protected diol in a 74% yield (Scheme 2.13).

Scheme 2.12: General Schemes of Buchwald’s Copper Catalyzed N-Arylation
The new aryl iodide substrate was subject to the same nitrogen arylation reaction with the fluorescent linker. However, there was still no product obtained, even after the numerous modifications to the reaction conditions mentioned earlier. This observation was quite puzzling for a couple of reasons. Firstly, the insertion of the copper metal into the carbon-halogen bond is believed to be an important step in the mechanism. The more polarizable carbon-iodine bond is more reactive towards this step and as such, has been shown to be selective over other aryl-halide bonds in previous copper couplings. Secondly, as mentioned earlier, the copper coupling with this fluorescent linker and an aryl halide was already shown to be successful. In an effort to solve this dilemma, attention was again turned toward the aryl halide substrate.

Scheme 2.13: Aryl Finkelstein Reaction

![Scheme 2.13: Aryl Finkelstein Reaction](image)

The general reactivity of the aryl halide was investigated by attempting to perform the copper coupling with a simple alkyl amine. Propyl amine was treated with both the aryl bromide and the aryl iodide but still provided no coupling product. This showed that the aryl halide was the impedance to the success of the copper coupling reaction. The recovered starting material suggested that the insertion of the metal into the carbon-halide bond was not occurring. If the
copper had inserted into the carbon-halide bond, then we would expect to see loss of the starting material as the copper complex or other byproduct after an aqueous workup. To this end, the influence of the para dioxolane ring on the carbon-halogen bond was investigated.

Density functional theory (DFT) calculations were conducted to compute an electrostatic potential map for a variety of aryl halide derivatives. As control examples, the potential maps for bromobenzene and iodobenzene were calculated (Scheme 2.14, (a)). The distribution of electron density (red: high density, blue: low density) between the ring and the halogen atoms represents the dipole of that bond. This is even more apparent in the iodobenzene example. The contour map showed a larger separation in electron density. This was attributed to the greater polarizability of the carbon-iodine bond. The electrostatic potential maps of the dioxolane substrates (Scheme 2.14, (b)) showed something completely different. The low electron density on the halide atoms and the relatively uniform distribution of electrons across the carbon-halide bond suggests that the dioxolane ring drastically decreased the dipole of the bond. A lack of a dipole would impede the insertion of the copper metal into the bond. This was our explanation for the lack of reactivity.

As a comparison, parabromo- and paraiodo-acetophenone electrostatic potential maps were also calculated (Scheme 2.14, (c)). The structures showed little electron density on the halides due to the electron-withdrawing group on the ring. Interestingly, with the paraiodo-acetophenone, there appeared to be a slight dipole present in the halide bond. Again, this is seen by the change in the contours in the potential map as one moves along the carbon-halide bond.

The DFT calculations provided an explanation to the lack of reactivity of the dioxane containing substrate but it still remained a synthetic problem. Since the dioxane was originally formed from the dihydroxylation product of the styrene derivative, an alternative synthetic route
was hypothesized. The styrene could be coupled using the same copper chemistry followed by a
dihydroxylation to provide the necessary diol (Scheme 2.11, (c)). In order to support this route,
the same DFT calculations were conducted on the parabromo- and paraiodo-styrenes. The
electrostatic potential maps (Scheme 2.14, (d)) showed a strong dipole compared to the
dioxolane substrates. This did indeed support the proposed synthetic route but the practicality of
this new method fell short. The styrene starting material would be prone to polymerization,
especially with the presence of copper, as this is well known technique of radical
polymerization\textsuperscript{12}. Also, conducting a selective oxidation on the styrene in the presence of the
electron-rich tryptophan portion of the molecule would be synthetically challenging. To this end,
the synthesis of the diol-fluorescent linker was abandoned. However, the collapse of this project
left us with an interesting question. Can we couple peptides and proteins directly to the surface
of the array without the need of any linker? This concept was explored and the details of which
are discussed in chapter 3.
Scheme 2.14: Electrostatic Potentials of Various Aryl Halides

a) 

b)
2.3 Conclusion

Earlier work done by Dr. Hu demonstrated the utility of the newly formed boronic acid polymer and its stability when pinacol protected. Our efforts focused on functionalization of the boronic ester surface while preserving the polymer’s stability. We successfully demonstrated that the surface can be selectively and reversibly modified by utilizing a technique for electrochemically generating acid. The surface was also selectively deprotected for transition metal coupling chemistry although, in the instance of the 12-K array, deprotection is not needed. This work culminated in the development of a tunable polymer surface for analyzing molecular libraries.

The success of the ligand exchange reaction was planned for use in the reversible coupling of peptides via a fluorescent linker. However, the synthesis of the new linker proved to be a challenge. The attempted copper coupling was unsuccessful despite the work done by Buchwald and others mentioned earlier that has demonstrated the overwhelming success and diversity of these copper-coupling reactions. DFT calculations on the coupling substrates were able to shed light on the failure of the reactions by pointing to a lack of a bond dipole necessary for metal insertion. This however created a new avenue of building molecular libraries on the arrays.
2.4 Experimental Procedure

2.4.1. General Information

Materials were purchased from Sigma-Aldrich and used without further purification unless otherwise indicated.

Fluorescence microscopy was carried out with a Nikon Eclipse E200 microscope connected to a Boyce Scientific, M-100 burner and a Nikon D5000 camera. Optical filters used: CFW-BP01-Clinical-000 (Semrock) filter cube excitation 380-395 nm/emission 420-470 nm, ET - GFP (FITC/Cy2) (Chroma) filter cube excitation 450-490 nm, emission 500-550 nm and TxRed-A-Basic-000 (Semrock) filter cube excitation 540-580 nm, emission 590-670.

2.4.2. Sample procedure for spin-coating arrays with the diblock copolymer:

The microelectrode arrays were coated with a spin-coater MODEL WS-400B-6NPP/LITE. The chip was inserted into a socket in the spinner and adjusted to be horizontal, then three drops of 0.03 g/mL block copolymer solution (For PBrSt-b-CEMA in 1:1 p-xylene /THF; for PCEMA-b-PEGMA in DMF; for PCEMA-b-BSt in 9:1 1,4-Dixoane /water; for PCEMA-b-pBSt in 4:1.5 DMF/THF) were added onto the chip in order to cover the entire electrode area. The chip was then spun 1000 rpm for 40 seconds. The coating was allowed to dry for 15 min and subjected to irradiation using a 100 W Hg lamp for 20 min before use.
2.4.3. RAFT polymerization of PHEMA-b-pBSt:

The following are typical reaction conditions. In a 25 mL round-bottom Schlenk flask, 1.30 g (10.0 mmol) of HEMA, 34 mg (0.1 mmol) of 2-cyano-2-propyl dodecyl trithiocarbonate, 2.8 mg (0.017 mmol) of AIBN and solvent (DMF vs monomer v/v=1:1) were added and degassed with 5 cycles of freeze-pump-thaw (F-P-T). After the final thawing, the flask was injected with argon and was kept at 90 °C. At time intervals, samples were taken by syringe. The percent conversion was measured by proton NMR with the solvent serving as an internal standard. After the conversion reached 80%, the second monomer pinacol protected 4-styreneboronic acid (2.30 g, 0.010 mol) was diluted in DMF (v/v=1:1) and was degassed with 5 cycles of F-P-T. The mixture was then injected into the polymerization mixture and the temperature was raised to 110 °C. The reaction was stopped after the conversion of the second monomer reached 80% by cooling the reaction mixture to RT and opened to the atmosphere. The mixture was used directly for the post polymerization DCC coupling reaction without removal of the DMF solvent.
Post-polymerization modification of PHEMA-b-pBSt with DCC coupling to cinnamic acid:

In a 100 mL round-bottom flask, the reaction mixture obtained from the above procedure was diluted with 40 mL DMF, 1.64 g (0.011 mol) cinnamic acid, 2.48 g (0.012 mol) DCC, and 60 mg (5.0 mmol) DMAP was added into the solution and the mixture was protected from light and was allowed to stir under room temperature for 48 hrs. After the reaction was finished, the mixture was filtered and the polymer could be precipitated in methanol. The fluffy polymer was collected by centrifuge. The obtained polymer was dissolved in THF and precipitated again in methanol to further purify, and was collected by centrifuge and dried over vacuum.

2.4.4. Microelectrode Array Reactions:

Inverted Suzuki reaction on the PCEMA-b-BSt block copolymer:

A mixture of 0.18 mg Pd(OAc)$_2$, 0.63 mg PPh$_3$, 20.0 mg Bu$_4$NBr, 5.0 mg 1-bromopyrene, 28.0 $\mu$L Et$_3$N and 100.0 $\mu$L allyl acetate was dissolved in a 2:7:1 solution of DMF/MeCN/H$_2$O (1.5 mL). For the 1-K microelectrode arrays, the array coated with the PCEMA-b-BSt block copolymer was submerged in the solution and then selected electrodes used as cathodes by pulsing them at a voltage of −2.4 V for 600 cycles (0.5 sec on and 0.1 sec off). Once complete, the chip was repeatedly washed with ethanol and prepared for pyrene-based fluorescent analysis. For the 12-K microelectrode arrays, the array was coated with the block copolymer and then incubated in 100 $\mu$L of the solution prepared above. Selected electrodes were used as cathodes by
holding them at a voltage of $-2.4$ V for two 90-second intervals. The array was then repeatedly washed with ethanol before examination using a fluorescence microscope.

**Electrochemical deprotection of the PCEMA-b-pBSt surface:**

This procedure for electrochemically-generating acid was modified from a previously reported method$^4$. Bu$_4$NPF$_6$ (25 mg) and 40 mg diphenylhydrazine were dissolved in 1.5 mL methanol and 50.0 $\mu$L water. For the 1-K microelectrode arrays, the array coated with PCEMA-b-pBSt was submerged in the solution. Selected electrodes were then pulsed at $+3.0$V for 900 cycles (0.5s on and 0.1s off). The array was then washed with ethanol before additional use.

**Diol exchange on the PCEMA-b-pBSt surface:**

$\text{5-(pyren-1-yl)pentane-1,2-diol (10 mg), 80 mg Bu}_4\text{NPF}_6$ and 50 mg diphenylhydrazine were dissolved in 1.5 mL methanol with 100 $\mu$L pyridine. For the 12-K microelectrode arrays, the array coated with PCEMA-b-pBSt was incubated in 100 $\mu$L of the solution. Selected electrodes were then pulsed at $+2.4$V for 20 cycles (30s on and 10s off). The array was then washed with ethanol and examined using a fluorescence microscope.
2.4.5. Synthesis of 5-(pyren-1-yl)pentane-1,2-diol

1) In a 25 mL, flame-dried, round bottom flask, 0.09mL (1.0 mmol) oxalyl chloride was dissolved in 2.5 mL anhydrous dichloromethane while under an Argon atmosphere and cooled to -78°C. 0.15 mL (2.0 mmol) dimethylsulfoxide was added slowly to the solution. After stirring for 2 min, 250 mg (0.91 mmol) 1-pyrene-butanol (dissolved in 1.0 mL anhydrous dichloromethane) was slowly added to the flask. After the addition was complete, the solution was allowed to stir at -78°C for 15 minutes. 0.65 mL (4.6 mmol) triethylamine was added and stirred for 5 minutes. The solution was warmed to room temperature and quenched with 5.0 mL H₂O. The mixture was extracted with DCM (3x 10mL). The organic layers were dried with MgSO₄, filtered and rotavapped. The crude aldehyde was used without further purification.

2) In a 25 mL, flame dried, round bottom flask, 0.357 g (1.0 mmol) Methyltriphenylphosphonium bromide was dissolved in 10 mL anhydrous THF. The solution was cooled to 0°C, 0.112g (1.0 mmol) Potassium tert-butoxide was added and stirred for 2 hours. The crude aldehyde was dissolved in 2 mL THF and added slowly to the flask. The solution was held at 0°C for 1 hour then warmed to room temperature and stirred overnight. The reaction was
quenched with conc. NH₄Cl(aq) and extracted with diethylether (3x 20mL). The organic layers were dried with MgSO₄, filtered and rotavapped. The compound was purified via column chromatography (7:1 Hexanes/EtOAc). 187 mg (76% yield over two steps) of product was obtained. ¹H NMR (CDCl₃/300MHz) δ8.10 (d, 2H), 7.92 (m, 7H), 7.69 (d, 1H) 5.82 (ddt, X in ABX, 1H), 5.00 (2d, A and B in ABX, 2H), 3.18 (t, 2H), 2.12 (q, 2H), 1.83 (p, 2H) ppm; ¹³C NMR (CDCl₃/300 MHz) δ138.7, 136.9, 131.6, 131.1, 129.9, 128.8, 127.7, 127.4, 127.2, 126.7, 125.9, 125.3, 125.2, 124.9, 124.8, 123.4, 115.2, 33.9, 33.0, 31.1 ppm; IR (KBr) 3040, 2974, 2931, 2859, 1639, 1603, 1509, 1434, 1182, 991, 910, 840, 756, 707 cm⁻¹; HREI MS m/z [M]⁺ found: 270.1398, calculated: 270.1409

5-(pyren-1-yl)pentane-1,2-diol

In a 25 mL round bottom flask, 0.54 g AD-Mix-β was dissolved in 5 mL 1:1 tBuOH/H₂O. The solution was cooled to 0°C and 104 mg (0.385 mmol) 1-(pent-4-en-1-yl)pyrene was added. The reaction was held at 0°C while stirring overnight. 0.590 g sodium sulfite was added to the reaction, allowed to warm to room temperature, and stirred for 30 minutes. The suspension was extracted with DCM (3x 20 mL). The organic layers were dried with MgSO₄, filtered and the solvent was remove in vacuo. The crude compound was purified via column chromatography (3:1 EtOAc/Hexanes used). The reaction afforded 77 mg product (66% yield). ¹H NMR (CD₂Cl₂/300 MHz) δ8.32 (d, 2H), 8.09 (m, 7H), 3.74 (A in ABX, 1H), 3.62 (B in
ABX, 1H), 3.41 (m, X in ABX and 2H), 1.97 (m, 4H), 1.59 (td, 2H) ppm; $^{13}$C NMR (CD$_2$Cl$_2$/300 MHz) δ 136.8, 131.4, 130.9, 129.7, 128.6, 127.4, 127.3, 127.1, 126.5, 125.8, 125.0, 124.9, 124.8, 124.7, 124.6, 123.4, 72.1, 66.7, 33.3, 33.0, 27.7 ppm; IR (KBr) 3233(br), 3036, 2937, 2861, 1601, 1461, 1432, 1180, 1105, 1084, 1029, 984 cm$^{-1}$; HRESI MS m/z [M+Na]$^+$ found: 327.1356, calculated: 327.1363

2.4.6 Synthesis of 4-(4-bromophenyl)-2,2-dimethyl-1,3-dioxolane

1) In a 100 mL round bottom flask, 3.85 g AD-Mix-β was dissolved in 30 ml of a 1:1 H$_2$O:tBuOH solution. The mixture was cooled to 0°C before 500 mg (2.73 mmol) p-bromostyrene was slowly added. The reaction was allowed to warm to room temperature and stirred overnight. 4.20 g sodium sulfite was added to the solution and stirred for 30 minutes. The mixture was extracted with dichloromethane and the organic layers were dried over magnesium sulfate, filtered and rotavapped. The crude mixture was carried on to the next step without further purification. $^1$H NMR of the crude product showed a high level of purity.

2) The crude diol was protected by dissolving it in 27 mL dimethylformamide in a flame dried 50 mL round bottom flask followed by the addition of 2,2-dimethoxypropane (0.84 ml,
6.83 mmol) and p-toluenesulfonic acid (109 mg, 0.55 mmol). The mixture was stirred overnight before quenching with water and extracting with dichloromethane. The organic layers were dried over Mg$_2$SO$_4$, filtered and rotavapped. The crude mixture by purified by column chromatography using 5:1 Hexanes:EtOAc. 590 mg (84%) of the desired dioxolane product was obtained over the both steps. $^1$H NMR (CD$_2$Cl$_2$/300 MHz) $\delta$7.48 (d, 2H), 7.24 (d, 2H), 5.02 (dd, 1H), 4.29 (dd, 1H), 3.65 (t, 1H), 1.54 (s, 3H), 1.48 ppm (s, 3H)

2.4.7. Aryl Finkelstein Reaction

![Diagram of reaction](image)

Reaction conditions were taken from a known literature procedure$^{10}$. A flame dried 25 mL round bottom flask 416 mg (1.62 mmol) 4-(4-bromophenyl)-2,2-dimethyl-1,3-dioxolane, 310 mg (1.63 mmol) copper(I) iodide and 486 mg (3.24 mmol) sodium iodide was added. The flask was evacuated and filled with argon gas. The solids were dissolved in 10 ml dioxane followed by the addition of the trans-N,N’-dimethyl-1,2-cyclohexanediamine ligand$^{14}$ (0.56 ml, 3.24 mmol). The solution was refluxed overnight. The reaction mixture was quenched with 5 ml ammonium hydroxide followed by the addition of 20 ml water and subsequent extraction with dichloromethane. The organic layers were dried over Mg$_2$SO$_4$, filtered and rotavapped. The crude mixture by purified by column chromatography using 1:1 DCM:EtOAc. The aryl iodide
product was obtained in a 74% yield. $^1$H NMR (CD$_2$Cl$_2$/300 MHz) δ7.68 (d, 2H), 7.11 (d, 2H), 5.00 (dd, 1H), 4.29 (dd, 1H), 3.64 (t, 1H), 1.53 (s, 3H), 1.47 ppm (s, 3H).
Br

HO

OH

BromosyleneD of Col. - Spot 2 - 04/03/2012
File: exp
Pulse Sequence: s2pul
Solvent: odc13
Ambient temperature
Operator: vmnt_kem
Mercury-309 Thalii.wust1.edu

Relay delay 1.000 sec
Pulse 31.8 degrees
Acq. time 1.998 sec
Width 3011.0 Hz
8 repetitions
OBSERVE: H1, 200.1176015 MHz
DATA PROCESSING
FT size 16384
Total time 8 min, 25 sec
file: exp
Pulse Sequence: s2pu1
Solvent: cdcl3
Ambient Temperature
Operator: vmm_kdm
Mercury-300 "nathl.wustl.edu"

Relax, delay 1.000 sec
Pulse 21.2 degrees
Acq. time 1.396 sec
Width 2806.8 Hz
8 repetitions
OBSERVE H1, 308.1176097 MHz
DATA PROCESSING
FT size 32768
Total time 8 min, 25 sec
2.5 References


Chapter Three

N vs S vs O: Chemoselective Coupling of Peptides onto a Polymer-coated Microelectrode Array

3.1 Introduction

As mentioned at the end of chapter 2, the inability to synthesize the necessary diol containing fluorescent linker caused us to step back and take another look at the system we were attempting to develop. The original intention was to create a method to reversibly couple molecules to the surface and verify their placement via fluorescence. However, if one were to commit the resources necessary to build a larger molecular library, a more permanent attachment would be desired. This would ensure that members of the library did not migrate to other locations on the array or disconnect entirely.

In addition to the benefit of permanent attachment of peptides to the array surface, we also sought to develop a method to couple the library without the need of a specialized linker. During our some of our initial binding studies, our group utilized an amino-acid derived linker (discussed in Chapter 2) to couple biotin to the polymer array surface and verify its placement via fluorescence. A binding study was performed between the surface bound biotin and streptavidin in solution. Although the results of the study showed the specific binding of streptavidin with biotin (Figure 3.1, red line), there was a significant interaction detected between streptavidin and the linker itself (green line). This non-specific binding was not a concern since it was significantly weaker than the binding between streptavidin and biotin. However, if a weaker binding system were being investigated, any non-specific binding with the linker may be problematic. Thus, it would be advantageous for us to either avoid the use of a
linker or capitalize on an existing linker strategy that is already known to have minimal non-specific binding with a protein being targeted.

As the streptavidin/biotin binding study demonstrated the importance of excluding a specialized linker in the molecular library, another binding study also provided support for efforts to expand and better understand the methods we use for placing peptides onto the arrays. This study consisted of a surface bound RGD-peptide binding to an integrin receptor in solution. The resulting binding curve from this experiment (Figure 3.2) showed binding of integrin to the RGD peptide (black line) preferentially to a non-RGD peptide (green line) and the
unfunctionalized aryl bromide surface (red line). This was another important proof of concept for this microarray-based technique; however, a closer look led to several important questions. The coupling method used to place the RGD peptide onto the surface of the array is known to be successful for a variety of nucleophiles. The structure of the peptide (Figure 3.2) shows several nucleophiles including a thiol, amine and alcohol, which have all been shown to successfully couple to the aryl bromide polymer. Based on the relative nucleophilicities of the heteroatoms, it was presumed that the coupling of the RGD peptide to the array involved the thiol group. But was this true? Binding of the N-terminus to the array would lead to an inactive molecule. So how much of the peptide placed on the array contributed to the signal observed. In addition, knowing the surface orientation of a member in a molecular library is crucial for building a three-

Figure 3.2: RDG-Peptide/Integrin Binding Study Data

The binding data obtained from monitoring the interaction between an RGD peptide and integrin shows a strong binding event (black line) at approximately $10^{-12}$M. A non-RGD peptide (green line) and unfunctionalized surface (red line) were used as controls, each demonstrating little to no interaction with the integrin peptide.
dimensional image of it binding to a biological target.

The importance of answering questions about how the molecule is placed onto the array is highlighted by the binding constant observed for the integrin/RGD-peptide observed on the array (Figure 3.2). The results of this study show a picomolar binding constant between the surface-bound RGD peptide and the integrin receptor in solution. However, previous literature reports only a nanomolar binding constant for this system\(^2\). This enhancement in the binding of the RGD peptide to integrin may be an artifact of the array or a multi-dentate effect from a localized concentration of substrate on the surface. To answer this requires building an array with various concentrations of substrate. How can this be done if we do not understand how much of the molecule placed on the array is active? This supports the need to confirm the orientation of a molecular library member and the presence of the active portion of the molecule.

In this chapter, I will discuss the efforts taken to address these issues, and highlight the chemoselective coupling of peptides to the surface of a microelectrode array.
3.2 Results and Discussion

As discussed in Chapter 2, the tunability of the newly developed boronic ester polymer was a significant advance towards utilizing microelectrode arrays to monitor biological systems. As a result, efforts to chemoselectively couple peptides to the surface of an array took advantage of the boronic ester derived surface. This required the development of a new method to perform heteroatom couplings on the arrays. As mentioned in section 3.1, the Cu(I) mediated method was quite successful on the aryl bromide surface. It was compatible with the use of peptide reagents, and unlike the use of palladium on the arrays, its use on the arrays did not interfere with subsequent signaling studies. For this reason, a copper-catalyzed method was also sought for use with the borate ester surfaces. Upon investigating the literature, the widely successful Chan-Lam coupling (Scheme 3.1) appeared promising. This Cu(II)-based technique was shown to couple a variety of alkyl and aryl substituted heteroatoms with aryl boronic acids under oxidative conditions. A study of various boronic acid derivatives did show a significant drop in yield when using a protected boron acid. However, as mentioned in Chapter 2, there is no significant difference between a protected and unprotected surface when conducting coupling experiments on the microelectrode arrays. Thus, the copper mediated Chan-Lam coupling was investigated as means to chemoselectively couple peptides to the array surface.

Scheme 3.1: Chan-Lam Coupling

\[ \text{X} = \text{NR', O, S, NCO'R', or NSO}_2\text{R'} \]
\[ \text{R/R'} = \text{Alkyl or Aryl} \]
Since this type of oxidative copper coupling had not yet been conducted on the arrays, an assessment of how the reaction worked with different heteroatom nucleophiles was performed. The first substrate tested was a pyrene-labeled amine. The commercially available 1-pyrenemethylamine was placed in a DMF solution containing copper(II) acetate and the tetrabutylammonium hexafluorophosphate electrolyte. A 12-K array, spin-coated with the boronic ester polymer discussed in Chapter 2, was incubated in the reaction solution and a series of electrodes in a checkerboard pattern were used as an anode for 20 cycles (30 sec. on, 10 sec. off). Upon completion of the experiment, the array was washed with ethanol and analyzed under

Scheme 3.2: Amine Coupling Reaction

The copper-mediated coupling of a pyrene-labeled amine proved to be unsuccessful. The resulting fluorescence image shows a relatively low level of pyrene on the surface as well as no confinement to the selected electrodes.
a fluorescence microscope. The array image (Scheme 3.2) shows only a relatively small amount of pyrene coupled to the surface and no confinement to the selected electrodes. Although this initial experiment was not a success, the presence of some coupled product supported the notion that the Chan-Lam technique might be workable with other nucleophiles.

The Chan-Lam coupling was carried out on a pyrene-labeled thiol\(^5\) using identical reaction conditions as the amine coupling experiment. The resulting fluorescence image (Scheme 3.3) shows a high degree of pyrene coupled to the surface as well as very good confinement to the selected electrodes. The coupling experiment was also performed with a pyrene-labeled alcohol. In this example, the reaction was also successful leading to coupling of the nucleophile at the desired electrodes and excellent confinement in the selected "O"-pattern.

With the exception of the amine coupling experiment, the copper-mediated Chan-Lam technique proved to be a successful route to coupling heteroatoms to the array surface. However, from a mechanistic point of view the reactions were rather puzzling. The proposed mechanism for the original, preparative scale Chan-Lam coupling involves the coordination of the heteroatom nucleophile to the Cu(II) acetate followed by a transmetallation with the aryl boronic acid. The newly formed copper complex is oxidized to a copper(III) species, which undergoes reductive elimination to produce the desired coupling product and copper(I). The copper(II) is then regenerated by a second oxidation from molecular oxygen present in the reaction mixture. If this mechanism is translated to the microelectrode array, then the anodic oxidation is only necessary for the final copper complex and there is no confining strategy for this reaction. As a result, one would expect to see fluorescence over the entire array. However, since this is not the case (Scheme 3.3), there must be an alternative mechanism. The key step of the reaction that
The Chan-Lam coupling of a pyrene-labeled thiol (left) indicates a high degree of pyrene on the surface of the array and excellent confinement. The same coupling of a pyrene-labeled alcohol (right) also yielded a high degree of coupling with excellent confinement.

would explain this phenomenon appears to be the transmetallation. For an unknown reason, the transmetallation step appears to be dramatically accelerated by the electrochemical oxidation. Although this was not explored further, it may have significant synthetic implication by dramatically increasing the rate of reaction and possibly aid in the regeneration of the transition metal catalyst.
Upon assessing the individual coupling of amine, thiol and alcohol substrates to the aryl boronic ester surface, the next step was to analyze the selectivity between the different nucleophiles while present in the same molecule. The competition experiments began with comparing nitrogen versus sulfur coupling. Since the amine coupling was very poor compared to the thiol and alcohol coupling, it was predicted that the thiol would outcompete the amine. However, it was unknown if or how the amine might interfere with the reaction. Thus, a pyrene-labeled cysteine was synthesized for use in assessing the overall success of the reaction before analyzing the possible chemoselectivity. The initial route to synthesize the labeled cysteine consisted of an amidation between a protected cysteine and pyrenemethylamine with subsequent deprotection (Scheme 3.4). The amidation was performed successfully in a 92% yield, however, the deprotection step was a problem. Treatment of the molecule with trifluoroacetic acid and triethyl silane removed the trityl group from the thiol but not remove the Boc group from the amine. Even after several iterations of treating the molecule with trifluoroacetic acid, the amine still remained Boc-protected. Analysis of the compound by $^1$H NMR showed an interesting
change in the Boc methyl and pyrene aromatic peaks. The deprotection of the trityl-thiol allowed
the molecule to change conformations such that the Boc peak appeared as two singlets rather
than one. This splitting, along a change in the aromatic multiplet, suggested an interaction
between the two groups. DFT calculations conducted using Spartan led to a slightly lower energy
(~5 kcal/mol) folded conformer (Figure 3.3) versus the linear counterpart. The presence of the
folded and linear conformers could possibly explain the splitting of the Boc group peak in the
NMR, but this structure was not investigated further.

**Figure 3.3: Folded Cysteine Conformer**

*A possible conformer of the trityl-deprotected substrate in which the tBoc amine lies above the planar, polycyclic pyrene*

Extending the carbon chain between the pyrene and amide circumvented the issue of not
being able to deprotect the amine. In order to achieve this extension, pyrenebutanol was
converted to the amine before coupling with cysteine (Scheme 3.5). This was accomplished with
a Mitsunobu reaction followed by reduction of the azide. The subsequent coupling and deprotection successfully provided the fluorescently labeled cysteine.

The validity of coupling a molecule with multiple unprotected heteroatoms was investigated by performing the Chan-Lam coupling with the newly synthesized pyrene-labeled cysteine. The copper-mediated coupling was run under identical oxidation conditions as the previous examples (Scheme 3.6). The fluorescence image showed a high degree of coupling to the surface with excellent confinement to only the selected electrodes. This demonstrated the compatibility of having multiple heteroatoms present while performing a Chan-Lam coupling.

The cysteine coupling was quite successful, but it still does not show whether the coupling occurs via the amine, the thiol or both groups. In order to elucidate this problem, the group that had reacted during the Chan-Lam coupling was determined by detecting the presence of the unreacted heteroatom. The first step was conducting a control experiment to confirm the ability to detect free thiols on the array through the formation of a disulfide bond (Scheme 3.7).
Butanediithiol was coupled to the polymer surface in an “S” pattern. The array was then incubated in a solution containing copper(II) acetate and a pyrene-labeled thiol for 12 hours. Upon completion, the array was analyzed using a fluorescence microscope. The image of the array (Scheme 3.7) shows fluorescence in the same pattern in which the dithiol was previously coupled indicated the successful formation of a disulfide bond with the free thiol on the surface.

This technique was applied to a cysteine methyl ester coupled to the surface to potentially detect a free thiol. A cysteine methyl ester was coupled to the polymer via the Chan-Lam procedure in an “S” pattern before incubating the array in the same manner as before. The
resulting fluorescent image (Scheme 3.8) revealed several interesting characteristics about the copper-mediated Chan-Lam coupling. First, the image shows no fluorescence in the “S” pattern in which the cysteine was previously coupled. This result, along with the earlier mentioned control experiment, verifies the absence of free cysteine thiols on the surface of the array. Second, the array contains fluorescence around the electrodes containing cysteine. This background fluorescence is attributed to small amounts of Chan-Lam coupling between the pyrene thiol and the boronic ester surface given the excess of reagents and long incubation time in an air atmosphere that insured the presence of Cu(II). However, the image shows no

Scheme 3.7: Thiol Detection Control Experiment

Dithiolbutane was coupled to the surface of the array using the Chan-Lam procedure. The functionalized array was incubated in a solution containing Cu(OAc)$_2$ and a pyrene-labeled thiol. The resulting fluorescence image indicates successful disulfide formation.
fluorescence at the electrodes containing cysteine. This indicates the absence of free thiols as well as a very high degree of electrode coverage with cysteine resulting in no background fluorescence.

The thiol detection experiment showed no free thiols present when cysteine was coupled to the surface. Verifying the presence of a free amine would corroborate this chemoselectivity. This was initially attempted by performing an electrochemical amidation with a pyrene-labeled N-hydroxysuccinamide ester. However, an unexpected outcome of the reaction was that the

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*The disulfide bond formation technique was applied to an array functionalized with cysteine-methyl ester in an “S” pattern. The attached fluorescence image shows that no free thiols from cysteine. The residual fluorescence around the electrodes is from background Chan-Lam coupling.*
activated ester reacted with the borate ester surface. This reaction will be discussed in detail in Chapter 4. For the present experiment, this technique was deemed unreliable for the detection of amines on the surface of the electrode. This issue was resolved with the use of an acid fluoride, which are known to be superior to other acid halides or esters in amidation reactions. A chip coated with the boronic ester polymer was functionalized with cysteine in an “N” pattern under identical Chan-Lam conditions. The array was then incubated in a solution containing a pyrene-labeled acid fluoride (synthesized from the corresponding carboxylic acid). After several

**Scheme 3.9: Amine Detection Experiment**

An amine detection reaction using a pyrene-labeled acid fluoride was performed on an array functionalized with cysteine (left) and serine (right). Both fluorescence images indicate that free amines are present from both molecules.
minutes, analysis of the array revealed prominent fluorescence at the electrodes functionalized with cysteine (Scheme 3.9, X = S).

This detection of the unreacted amine confirmed the chemoselectivity of the array-based Chan-Lam reaction coupling through the thiol. Additionally, this set of results has important synthetic implications. Not only can a peptide be chemoselectively coupled to the boronic ester surface through a thiol, the N-terminus does not need to be protected. This dramatically simplifies the process of building a molecular library onto the surface of an array. Furthermore, the unprotected amine can be functionalized through an amidation reaction leading to opportunities to rapidly form peptide libraries on the arrays using existing parallel synthesis strategies.

Just as cysteine was used as to compare the selectivity of thiols vs. amines, serine was investigated to compare the relative reaction rates of alcohol and amine nucleophiles in the Chan-Lam coupling. The results from coupling the three heteroatoms individually and the thiol/amine competition experiment led to the prediction of the alcohol out competing the amine. To this end, the amine detection experiment was conducted to verify the presence of the free amine. Serine was coupled to the polymer surface in an “N” pattern using the Chan-Lam procedure. As before, the array was incubated in a solution containing the pyrene-labeled acid fluoride. The resulting fluorescence image (Scheme 3.9, X = O) indicated the presence of free amines.

In order to fully verify the selectivity between alcohols and amines, detection of any free alcohols was performed. The technique used came from the previous work of Dr. Bichlien Nguyen in our group\(^7\). She developed a method to electrochemically generate TEMPO in order to oxidize surface bound alcohols. A reductive amination between the resulting carbonyl and a
fluorescently labeled amine was used to identify the presence of the original alcohol (Scheme 3.10). The success of this technique and its selectivity for alcohols made it ideal for detecting any potential free alcohols from serine coupled to the surface.

Any residual alcohols present, after the Chan-Lam coupling of serine methyl ester to the polymer surface, were oxidized using the TEMPO procedure discussed above. The array was then incubated in a Texas Red hydrazide solution in order to trap any aldehydes that were produced. Analysis under a fluorescence microscope (Scheme 3.11) revealed no significant fluorescence above background. This concluded that the alcohol is selectively coupled to the boronic ester surface when in competition with a free amine. Also, as with the thiol/amine
Thus far, the competition experiments have only investigated the chemoselectivity between two heteroatoms, N vs. S and N vs. O. However, many biologic or biomimetic systems will contain all three of the heteroatoms found in the various natural amino acids. So the obvious question was, “What is the chemoselectivity of the Chan-Lam coupling when all three unprotected heteroatoms are present?” This was an intriguing matter to consider given the individual success of the thiol and alcohol coupling and their selectivity over amines. In order to investigate this problem a simple cysteine-serine dimer was synthesized through a standard peptide coupling technique and complete deprotection exposed the necessary heteroatoms. The dimer was then coupled to the surface of the array as done previously. Subsequently, the detection experiments mentioned above were performed in order to confirm the presence or absence of each heteroatom. The results of the study (Figure 3.4) show the presence of all three

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**Scheme 3.11: Alcohol Detection Experiment Data**

The Chan-Lam coupling of serine-methyl ester was performed on the array surface followed by the alcohol detection procedure. The fluorescence image indicates that no free alcohols are present.
heteroatoms on the surface of the electrode. Given the lack of reactivity seen in previous reactions with the amine, the most reasonable explanation for the observed result is that the reaction rates of the alcohol and thiol reactions are competitive. It is not known if the close proximity of the thiol and alcohol influences the result observed. However, at the present it appears that if one wants to place a peptide on an array using a thiol nucleophile, any free alcohol groups would need to be protected. Of course, the opposite would be true as well.

**Figure 3.4: N vs. S vs. O Selectivity Study Results**

![Thiol Detection](image1)
![Amine Detection](image2)
![Alcohol Detection](image3)

*The images shown were taken after conducting the thiol, amine, and alcohol detection experiments on individual arrays, each functionalized with the cysteine-serine dimer. The fluorescence in the respective “N”, “S”, and “O” patterns indicate the presence of each nucleophile.*

The ability to place both thiol and alcohol nucleophiles onto a borate ester coated array in high yield using the Chan-Lam coupling method allowed us to investigate if the amount of the
material placed on the surface of the electrode could be controlled. This would permit us to test the multidentate effect discussed earlier by conducting binding experiment with various degrees of ligand present on the surface. To test this idea, a peptide containing a serine group was placed onto an array along with a fluorescent molecule, calcoflour white (Scheme 3.12). The alcohol nucleophile was used for the placement reactions because it was already present in the

Scheme 3.12 : Peptide Gradient Strategy

The hexapeptide was coupled to various polymer-coated electrodes across the array, incrementally increasing the length of reaction time. The identical electrodes were then functionalized with the calcofluor white fluorophore.
fluorescent dye. In the experiment, the reaction used to place the peptide on the array was allowed to proceed for varying time periods. The reactions are run with the selected electrode being cycled on for a period of 30 s and then off for a period of 10s. This helps with confinement of the reagent by slowing the rate of active Cu(II) generated. The length of the reaction was then altered by varying the number of cycles used. After the peptide placement reaction, the fluorescent group was added to the array until coverage of the electrodes was complete (60 cycles). The relative amount of peptide placed on the surface was determined by measuring the amount of fluorescence on the electrode following the second reaction.

As can be seen in Figure 3.5, the amount of fluorescence decreased in a linear fashion as the length of time for the peptide placement reaction increased. This indicated that there was a linear increase in the amount of peptide coupled to the electrodes as the time for the experiment increased. Each experiment was conducted at three sites on the array, and the error bars reflect the spread in the data at those three sites.

After 25 cycles, the amount of peptide placed on the array leveled off suggesting that at that point, the peptide placement reaction had proceeded to completion. It was clear that the relative amount of a peptide placed on the array could be controlled in a predictable fashion. Hence, we should be able to place peptides on the surface in a gradient concentration.
Quantification of the fluorescence from the calcofluor white probe was plotted against the corresponding number of reaction cycles in which the hexapeptide coupling was performed. The linear curve demonstrates a successful peptide gradient formed on the array surface.
3.3 Conclusion

The chemoselective coupling of peptides to the surface of a polymer-coated microelectrode array was investigated. The newly formed aryl boronic ester polymer required a new method for coupling peptide directly to the surface. As a result, the copper-mediated Chan-Lam coupling was adapted to the microelectrode arrays. This technique proved to be sufficient for the individual coupling of thiols and alcohols. However, amine coupling was inadequate. This was reflected in the competition experiments, which showed chemoselectivity towards thiol and alcohols over amines. Another important synthetic aspect of this study is the ability to selectively couple peptides without the need of a protecting group on the N-terminus.
3.4 Experimental Procedure

3.4.1 General Information

Materials were purchased from Sigma-Aldrich and used without further purification unless otherwise indicated. Amino Acids were purchased from Advanced Chemtech and used without further purification.

Fluorescence microscopy was carried out with a Nikon Eclipse E200 microscope connected to a Boyce Scientific, M-100 burner and a Nikon D5000 camera. Optical filters used: CFW-BP01-Clinical-000 (Semrock) filter cube excitation 380-395 nm/emission 420-470 nm, ET - GFP (FITC/Cy2) (Chroma) filter cube excitation 450-490 nm, emission 500-550 nm and TxRed-A-Basic-000 (Semrock) filter cube excitation 540-580 nm, emission 590-670.

The measurement and analysis of the fluorescence intensities was performed using the bio-imaging software Icy, version 1.6.1.1 (http://icy.bioimageanalysis.org). Six equiradial circular segments encompassing the functionalized electrodes were selected (below) and the average intensity value was computed. A baseline fluorescence was determined by using six equally sized segments of an unfunctionalized portion.
3.4.2 Sample procedure for spin-coating arrays with the diblock copolymer

The microelectrode arrays were coated with a spin-coater MODEL WS-400B-6NPP/LITE. The chip was inserted into a socket in the spinner and adjusted to be horizontal, then three drops of 0.03 g/mL PCEMA-b-pBSt solution (4:1.5 DMF/THF) were added onto the chip in order to cover the entire electrode area. The chip was then spun 1000 rpm for 40 seconds. The coating was allowed to dry for 15 min and subjected to irradiation using a 100 W Hg lamp for 20 min before use.

3.4.3 Array based Chan-Lam Coupling

10 mg 5-(pyren-1-yl)pentane-1,2-diol, 80 mg Bu₄NPF₆ and 50 mg diphenylhydrazine were dissolved in 1.5 mL methanol with 100 µL pyridine. For the 12-K microelectrode arrays, the array coated with PCEMA-b-pBSt was incubated in 100 µL of the solution. Selected electrodes were then pulsed at +2.4V for 20 cycles (30s on and 10s off). The array was then washed with ethanol and examined using a fluorescence microscope.

3.4.4 Incubation Experiments

All of the incubation experiments discussed in section 3.2 were conducted using the following general procedure. The microelectrode array was placed flat in a petri dish with the electrodes (red box) facing upward (Figure 3.5). 10 mL of the appropriate reaction solution (below) was added onto the array while elevated at one end to ensure the array portion remained
submerged. Once complete, the array was washed with methanol, dried and analyzed under the fluorescence microscope.

Figure 3.5: Incubation Example

Reaction Solutions

Thiol Detection Solution

50 mg 3-mercapto-N-(pyren-1-ylmethyl)propanamide\(^5\); 100 mg Cu(OAc)\(_2\); 5 mL dichloromethane; 5 mL methanol

Amine Detection Solution

50 mg 1-pyrenebutyryl fluoride; 10 mL dichloromethane

Alcohol Detection Solution

50 mg Texas Red hydrazide; 10 mL methanol
3.4.5 Synthesis of Boc-Cysteine(Trt)-NH-methylpyrene

In a flame-dried 25 mL round bottom flask, 673 mg (1.45 mmol) Boc-Cysteine(Trt)-OH, 305 mg \textit{N}-\textit{Ethyl-\textit{N}′-(3-dimethylaminopropyl)carbodiimide hydrochloride} (EDC), 206 mg \textit{Ethyl cyano(hydroxyimino)acetate} (Oxyma), and 420 mg 1-pyrenemethylamine hydrochloride was dissolved in 7 ml anhydrous DMF. 0.7 ml \textit{N}-methylmorpholine was added to the solution and stirred overnight. The reaction solution was quenched with brine and extracted with ethyl acetate. Organic layers were dried, rotavapped and purified via column chromatography using 1:1 ethyl acetate:hexanes. The coupling product was obtained in a 92% yield (902mg, MW = 676.88 g/mol). $^1$H-NMR (300 MHz; CDCl$_3$): \( \delta \) 8.19-7.95 (m, 8H), 7.89 (d, \( J = 7.8 \) Hz, 1H), 7.36 (dd, \( J = 7.9, 1.6 \) Hz, 6H), 7.21-7.11 (m, 9H), 6.32 (s, 1H), 5.05 (d, \( J = 5.3 \) Hz, 2H), 5.05 (d, \( J = 5.3 \) Hz, 2H), 4.90-4.83 (m, 1H), 3.89 (d, \( J = 5.3 \) Hz, 1H), 2.83 (dd, \( J = 12.6, 6.8 \) Hz, 1H), 2.58 (dd, \( J = 12.8, 5.4 \) Hz, 1H), 1.26 ppm (d, \( J = 7.2 \) Hz, 9H); HRESI MS: \textit{C}_{44}\textit{H}_{40}\textit{N}_{2}\textit{O}_{3}\textit{S}, m/z [M+Na]$^+$ found: 699.2507, calculated: 699.27

3.4.6 Amination of Pyrenebutanol

![Diagram of the amination process](image)
Azidobutylpyrene

In a flame-dried 100 mL round bottom flask, 653 mg (2.38 mmol) 1-pyrenebutanol and 686 mg triphenylphosphine were dissolved in 24 mL anhydrous THF. 1.2 mL of a diethyl azodicarboxylate solution (40 wt. % in toluene) and 0.6 mL diphenylphosphoryl azide were added to the solution and allowed to stir overnight. Upon completion, the solvent was removed in vacuo and the crude product was purified by column chromatography using 3:1 hexanes:ethyl acetate as the eluent. The azide product was obtained in a 92% yield (658 mg, MW = 299.37).

$^1$H-NMR (300 MHz; CDCl$_3$): δ 8.25 (d, $J = 9.3$ Hz, 1H), 8.19-8.10 (m, 4H), 8.06-7.97 (m, 3H), 7.86 (d, $J = 7.8$ Hz, 1H), 3.35 (dt, $J = 13.8, 7.0$ Hz, 4H), 2.01-1.90 (m, 2H), 1.82-1.72 ppm (m, 2H); $^{13}$C NMR (CDCl$_3$/75 MHz) δ 136.1, 131.5, 131.0, 130.0, 128.6, 127.6, 127.4, 127.2, 126.8, 126.0, 125.2, 125.1, 125.0, 124.9, 124.8, 123.2, 51.4, 32.9, 28.9, 28.8 ppm; IR (KBr) 3044, 2963, 2935, 2922, 2141, 2102, 1599, 1464, 1307, 1278, 1247 cm$^{-1}$; HRESI MS: C$_{20}$H$_{17}$N$_3$, m/z [M+Na]$^+$ found: 322.1305, calculated: 322.1322.

Pyrenebutylamine

In a 100 mL round bottom flask, 650 mg (2.17 mmol) azidobutylpyrene and 1.7 g triphenylphosphine was dissolved in 18 mL THF and 13 mL water. The solution was refluxed
overnight before being quenched with 1N HCl. The organic solvent was removed in vacuo and the resulting ammonium salt was filtered and dried. The product was used without further purification.

3.4.7 Synthesis of Cysteine-NH-butylpyrene

In a flame-dried 25 mL round bottom flask, 1.07 g Boc-Cysteine(Trt)-OH, 488 mg N-Ethyl-N"-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 330 mg Ethyl cyano(hydroxyimino)acetate (Oxyma), and the previously formed pyrenebutylamine hydrochloride was dissolved in 12 ml anhydrous DMF. 0.85 ml N-methylmorpholine was added to the solution and stirred overnight. The reaction solution was quenched with brine and extracted with ethyl acetate. Organic layers were dried, rotavapped and purified via column chromatography using 1:1 ethyl acetate:hexanes. The coupling product was obtained in a 64% yield over two steps (998 mg, MW = 718.96 g/mol). $^1$H NMR (CD$_2$Cl$_2$/300 MHz) $\delta$8.23 (d, $J =$
9.3 Hz, 1H), 8.16 (d, J = 7.3 Hz, 2H), 8.04 (m, 5H), 7.82 (d, J = 7.8 Hz, 1H), 7.40 (d, J = 7.2 Hz, 6H), 7.27-7.16 (m, 11H), 6.00-5.91 (m, 1H), 4.77-4.69 (m, 1H), 3.85-3.77 (m, 1H), 3.33 (t, J = 7.7 Hz, 2H), 3.26 (m, 2H), 2.77-2.67 (dd, J = 12.6, 7.0 Hz, 1H), 2.49 (dd, J = 12.7, 4.8 Hz, 1H), 1.90-1.80 (m, 2H), 1.63 (dt, J = 15.1, 7.0 Hz, 2H), 1.35 (s, 9H) ppm; $^{13}$C NMR (CD$_2$Cl$_2$/75 MHz) $\delta$ 170.5, 155.5, 144.6, 136.4, 131.5, 130.9, 129.8, 129.7, 128.6, 128.1, 127.6, 127.33, 127.28, 126.9, 126.7, 125.9, 125.10, 125.07, 124.93, 124.88, 124.8, 123.4, 80.1, 67.2, 39.4, 34.2, 33.0, 29.5, 28.9, 28.4 ppm; IR (KBr) 3561, 3428, 3047, 2930, 2253, 2123, 1716, 1680, 1665, 1555, 1502, 1365, 1247, 1166 cm$^{-1}$; HRESI MS: C$_{47}$H$_{46}$N$_2$O$_3$S, m/z [M+Na]$^+$ found: 741.3121, calculated: 741.3129.

**Cysteine-NH-butylpyrene**

![Cysteine-NH-butylpyrene](image)

In a flame-dried 25 mL round bottom flask, 970 mg (1.35 mmol) of Boc-Cys(Trt)-NH-butylpyrene was dissolved in 5 mL dichloromethane 1.0 mL triethylsilane and 0.25 mL trifluoroacetic acid was added to the reaction flask. The deprotection was monitored via TLC. Upon completion, the solution was quenched with saturated sodium bicarbonate solution and extracted with dichloromethane. The organic layers were dried and rotavapped. The crude mixture was purified via column chromatography. 1:1 ethyl acetate:hexanes was used to remove impurities before switching to 1:1 DCM:Methanol to acquire the desired product in a 90% yield (457 mg; MW = 376.52 g/mol). $^1$H NMR (CD$_2$Cl$_2$/300 MHz) $\delta$8.23-7.91 (m, 8H), 7.81 (m, 1H), 7.51-7.44 (m, 1H), 3.62-3.55 (m, 1H), 3.36-3.20 (m, 5H), 3.17 (d, J = 4.2 Hz, 1H), 2.69 (dt, J =
13.9, 7.1 Hz, 1H), 1.83 (dt, J = 14.6, 7.4 Hz, 4H), 1.62 ppm (m, 2H); $^{13}$C NMR (75 MHz; CDCl$_3$): δ 173.1, 136.4, 131.3, 130.8, 129.7, 128.5, 127.4, 127.2, 126.6, 125.8, 124.98, 124.92, 124.84, 124.75, 124.68, 123.3, 53.8, 43.8, 39.10, 39.06, 33.0, 29.4, 29.0 ppm; IR (KBr) 3284, 3044, 2935, 2863, 2359, 1918, 1651, 1531, 1437, 1181, 1120, 845 cm$^{-1}$; HRESI MS m/z [M+Na]$^+$ found: 399.1507, calculated: 399.1509.

### 3.4.8 Synthesis of 1-pyrenebutyryl fluoride

![Chemical Structure](image)

In a flame-dried 25 mL round bottom flask, 288 mg (1mmol) 1-pyrenebutyric acid was dissolved in 6.8 mL anhydrous dichloromethane followed by the addition of 0.1 mL pyridine. Separately, 393 mg (1.2 mmol) Fluoro-$N,N,N',N'$-tetramethylformamidinium hexafluorophosphate (TFFH) was dissolved in 4.5 mL DCM. The TFFH solution was then added dropwise to the butyric acid solution and allowed to stir for three hours. Upon completion, the reaction was quenched with 10 mL ice water and 10 mL DCM. The organic layer was removed and washed with ice water (2x 10 mL). The organic layer was then dried over MgSO$_4$, filtered and rotavapped. The crude acid fluoride was used without further purification. 1H NMR of the crude mixture is provided.
3.4.9 Synthesis of Cysteine-Serine Dimer

In a flame-dried 25 mL round bottom flask, 927 mg (2.0 mmol) Boc-Cys(Trt)-OH, 420 mg N-Ethyl-N’-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 284 mg Ethyl cyano(hydroxyimino)acetate (Oxyma) was dissolved in 6 mL anhydrous DMF. 0.75 mL N-methylmorpholine was added to the solution before the addition of a 2 mL DMF solution containing 423 mg (2 mmol) H-Ser(tBu)-OMe (HCl) and stirred overnight. Upon completion, DMF was removed in vacuo and the crude mixture was purified by column chromatography using 3:1 Hexanes:Ethyl Acetate. The desired coupling product was obtained in a 74% yield (918 mg; MW = 620.8 g/mol). $^1$H-NMR (300 MHz; CDCl$_3$): δ 7.42 (d, $J$ = 5.2 Hz, 6H), 7.31-7.19 (m, 9H), 6.71 (d, $J$ = 8.2 Hz, 1H), 4.81-4.77 (m, 1H), 4.61 (dq, $J$ = 7.9, 3.9 Hz, 1H), 3.93-3.89 (m, 1H), 3.77 (dt, $J$ = 9.0, 2.4 Hz, 1H), 3.69 (s, 3H), 3.50 (ddd, $J$ = 9.3, 6.8, 2.9 Hz, 1H),
2.76-2.70 (m, 1H), 2.56 (dt, \( J = 12.3, 6.0 \text{ Hz}, 1H \)), 1.42 (s, 9H), 1.09 ppm (s, 9H); \(^{13}\text{C} \text{NMR} \) (75 MHz; CDCl\(_3\)): \( \delta \) 170.37, 170.23, 144.4, 129.6, 128.0, 126.8, 73.3, 67.1, 61.8, 53.4, 53.0, 52.2, 34.0, 28.3, 27.27, 27.22 ppm; IR (KBr) 3500, 3415, 3056, 2976, 1747, 1669, 1508, 1489, 1363, 1245, 1167, 1099, 1020 cm\(^{-1}\); HRESI MS m/z [M+Na\(^+\)] found: 643.2800, calculated: 643.2820.

**Cys-Ser-OMe**

\[
\begin{array}{c}
\text{H}_2\text{N} \\
\text{O} \\
\text{SH} \\
\text{O} \\
\text{OH} \\
\end{array}
\]

In a flame-dried 25 mL round bottom flask, 745 mg (1.20 mmol) of Boc-Cys(Trt)-Ser(tBu)-OMe was dissolved in 5 mL dichloromethane. 1.0 mL triethylsilane and 0.50 mL trifluoroacetic acid was added to the reaction flask. The deprotection was monitored via TLC. Upon completion, the solution was quenched with saturated sodium bicarbonate solution and extracted with ethyl acetate. The organic layers were dried and rotavapped. The crude mixture was purified via column chromatography. 3:1 DCM:Acetone to acquire the desired product in a 90% yield (240 mg; MW = 222.26 g/mol). Spectral data is consistent with previously published data\(^8\).
3.5 References


Chapter Four

Site-Selective Placement of a Peptide Library

4.1 Introduction

The utilization of microelectrode array technology towards monitoring binding interactions between a biological receptor and a molecular library has been the focus of this thesis thus far. The work discussed in the previous chapter involved the development of a chemoselective method of coupling peptides to the surface of the array through thiol or alcohol functionalizations. Since thiol functionalizations are common moieties for placing peptides or peptidomimetics onto solid supports\(^1\), this was a significant advancement for the microelectrode arrays in becoming a comparable method to other, more traditional binding assays. Continuing along this trend, it was desirable to investigate the compatibility of the polymer-coated arrays towards other common techniques for synthesizing peptide-bioconjugates. One of the more popular techniques is the use of “click” chemistry for the formation of stable triazole rings\(^2,3\). Efforts towards translating this chemistry to the microelectrode arrays was previously investigated by our group and was quite successful\(^4\). Dr. Bartels and others functionalized an agarose coated microelectrode array with a terminal alkyne through an esterification with the activated ester (Scheme 4.1). They then successful conducted a site-selective, copper-mediated “click” reaction with a fluorescently labeled azide. The triazole product was later used as part of a mass spectrometry cleavable linker strategy that helped illustrate the versatility of the microelectrode arrays towards building molecular libraries.
The ability to place molecules onto the surface of an electrode via the biocompatible "click" reaction was indeed another significant advancement, however, the chemistry was performed on an agarose surface. Agarose is a nice "practice polymer" for coating arrays because it is not stable and allows for easy recycling of the arrays. It is not a useful platform for building a stable molecular library on an array. As a result, this chemistry must be transferred to the newly formed boronic ester polymer. This "click" chemistry, in addition to the chemoselective peptide coupling discussed in chapter three, would provide a wide range of methods to place molecules of interest onto the surface of an electrode. More importantly, these techniques would

Upon functionalizing an agarose-coated microelectrode array with an alkyne, a copper-mediated "click" reaction was performed. The attached fluorescence image shows the successful coupling.

Scheme 4.1: Previous Array based “Click” Reactions
take advantage of common biomimetic functionalizations. In this way, the microelectrode arrays could be used in conjunction with other methods without any need to resynthesize the molecules being studied. This chapter will discuss the development of the “click” reaction on the borate ester surface along with other methods to build peptide libraries on microelectrode arrays.

4.2 Results and Discussion

4.2.1 Site-Selective “Click” Chemistry

As discussed in section 4.1, the use of "click" chemistry on the borate ester surface is at first glance a simple extension of the site-selective Cu(I) chemistry already developed for the arrays. The first challenge associated with this extension was determining whether the azide or the alkyne would be bound to the surface. In our group’s previous work⁴, the alkyne was coupled to the surface through an esterification reaction then treated with an azide in solution (Scheme 4.1). In regards to only the “click” reaction itself, the orientation of the alkyne and azide is moot. However, when considering the synthesis and stability of the reagents involved in the “click” reaction, in particular the peptide/protein members of the molecular library, a clear preference starts to surface. The use of a propargylic arginine derivative has already been widely used in performing “click” chemistry on bioactive molecules⁵ and has been widely used in the synthesis of natural products and peptide-based materials⁶. To this end, we focused on developing the “click” chemistry where a surface bound azide would react with a substrate containing the propargylic arginine modification.

The strategy to couple the acetylene-modified peptides began with a linker strategy to functionalize the surface of the array with the necessary azide. This route stemmed from the overwhelming success of the Chan-Lam coupling discussed in the previous chapter. In order to
utilize the copper-mediated coupling a cysteine was modified by performing a diazotransfer reaction onto the unprotected amine. A stable imidazole-sulfonyl azide reagent, developed by Goddard-Borger and Stick\textsuperscript{7}, along with a base and catalytic Cu\textsuperscript{2+} was used to perform the transformation on H-Cys(trt)-OH (Scheme 4.2). The resulting Azido-Cysteine was esterified and trityl deprotected to provide the necessary linker to perform the “click” reaction on the surface of the array.

**Scheme 4.2: Azido-Cysteine-Methyl Ester Synthesis**

Before the “click” reaction could be tested on the arrays with the azido-cysteine linker, a fluorescently labeled alkyne was needed to evaluate the success of the reaction. This alkyne started from the same pyrenebutanol starting material used several times before (Scheme 4.3). A Swern oxidation was performed on pyrenebutanol followed by a Wittig reaction with a dibromomethyl phosphonium ylide, produced in situ from tetrabromomethane and
triphenylphosphine. The resulting dibromoalkene was then treated with two equivalents of n-butyllithium to generate the terminal alkyne.

![Scheme 4.3: Synthesis of Pyrene-labeled Alkyne](image)

With both the azide and alkyne synthesized, the “click” reaction was tested on the 12-K arrays. To this end, a 12-K array as spin-coated with the aryl boronic ester polymer and cross-linked under a broad spectrum, 100W Hg lamp for 15 minutes. The array was incubated in a DMF solution containing the azido-cysteine, copper(II) acetate and tetrabutylammonium hexafluorophosphate electrolyte. Several 3x4 blocks of electrodes were selected and a potential of +2.4 V was applied for 20 cycles (cycle = 30s on, 10s off) resulting in the Chan-Lam coupling of the azido-cysteine (Scheme 4.4).
The “click” reaction was tested on the array by incubating the array in a 9:1 DMF:DCM solution of the pyrene-labeled alkyne, copper(II) sulfate, the sulfonated bathophenanthroline ligand and tetrabutylammonium bromide electrolyte. The blocks of electrodes containing the previously coupled azido-cysteine were selected and a voltage of -2.0 V was applied for 20 cycles. The array was then analyzed under the fluorescence microscope to evaluate the “click” reaction. The resulting image (Scheme 4.4) showed fluorescence around the electrodes but none

**Scheme 4.4: Array-based “Click” Chemistry Trial**

A “click” reaction was performed after functionalizing the array with the azido-cysteine linker. The fluorescence image shows no coupling at the selected electrodes but rather around the electrodes.
at the selected electrodes. This was an unusual outcome given the previous success of this reaction\textsuperscript{4} and at that time, there was no explanation for it.

In an effort to resolve this behavior, incremental adjustments to the individual reaction conditions were made. Throughout this study, the amount of copper, amount and type of ligand, electrolyte concentration, alkyne amount, voltage and reaction time were all varied with no improvement. This indicated that the failure in the “click” reaction did not involve the reaction conditions. After exploring all of these variables, the next attribute of the array-based reaction investigated was the azido-cysteine linker previously used to functionalize the surface.

As mentioned earlier, the chemoselectivity and overall success of the Chan-Lam coupling was the reasoning behind the azido-cysteine linker strategy. However, the capabilities of the copper-mediated coupling go beyond what was discussed in Chapter 3. It has been reported in

**Scheme 4.5: Azide Coupling**

![Scheme 4.5: Azide Coupling](image-url)
the literature that the Chan-Lam coupling has been used to couple alkyl and sulfonyl azides with aryl boronic acids to produce an aryl amine\textsuperscript{8,9}. This coupling was tested on the arrays with a pyrene-labeled alkyl azide using the identical Chan-Lam conditions as before. The fluorescence image taken after performing the azide coupling experiment verified their ability to couple to the boronic ester surface (Scheme 4.5). As this began to shed light onto why the initial “click” reaction failed, this also provided yet another tool to build molecular libraries on the surface of an array.

The ability to couple azides to the surface meant that one could possibly circumvent the complications of the click reaction by directly coupling the substrate to the array surface. However, this would only be beneficial if an acetylene substrate could also be directly coupled to the surface. As mentioned previously, most biomolecules placed on supports with the use of click-chemistry functionalize the molecule with the acetylene. Thus, the coupling of acetylene to the boronic ester polymer was explored. The reaction of acetylenes under Chan-Lam type conditions had not been previously performed. However, other aryl couplings of acetylenes using Cu(II) under oxidative conditions are known\textsuperscript{10,11}. On the microelectrode array (Scheme 4.6), the copper mediated Chan-Lam addition of the acetylene to the borate surface worked beautifully.

The ability to successfully couple both components of a “click” reaction, the azide and acetylene, to the array provides a significant strategy for building a molecular library. This allows us to circumvent any need to pre-modify the array surface. A biomolecule functionalized with either an azide or acetylene in connection with another study can simply be added to the array as well. These results are discussed in the next chapter.

Although we had found an alternative method to place the peptides that had been previously functionalized for “click” chemistry on the array surface, we still did not know why
the click reaction on the array surface failed. Certainly, the loss of confinement and fluorescence away from the electrodes containing the azide could be explained by the addition the acetylene directly to the borate ester surface even in the absence of the azide. But why was there no "click" reaction on the surface of the electrodes that were functionalized with the azide? Given the success of the azide coupling discussed above, it was hypothesized that the azide outcompeted the thiol for the borate ester surface during the initial Chan-Lam coupling reaction. If this were true, then we would be able to detect the residual free thiols on the surface of the electrode in the same manner as discussed in Chapter 3. This concept was tested by coupling the azido-cysteine linker to the surface then incubating the array in order to form fluorescently labeled disulfide bonds (Scheme 4.7). However, upon completion of this experiment, no fluorescence was seen. This meant that the linker was indeed being coupled through the thiol. However, since the linker
is selectively being coupled to the surface through the thiol as originally intended, why does the “click” reaction not work?

To help answer this question we took a closer look at the azide coupling reaction. As mentioned earlier, the product of the azide/boronic ester coupling is not an azide product but

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### Scheme 4.7: Thiol detection on “click” linker

![Scheme 4.7: Thiol detection on “click” linker](image)

Cu(OAc)$_2$, Bu$_4$NP, DMF, +2.4 V
20 Cycles (30s on, 10s off)
3x4 Blocks

---

### Scheme 4.8: Proposed Route of Cysteine Reduction and Coupling

![Scheme 4.8: Proposed Route of Cysteine Reduction and Coupling](image)

Cu$_2$(OAc)$_2$, MeOH, 12hrs
No Fluorescence

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rather an amine. This implies that at some point during the reaction the azide is reduced to the amine. Our initial assessment was that the reduction was occurring in solution before any interaction with the surface (Scheme 4.8). The newly formed amido-cuprate would then undergo a metal transfer to the thiol resulting in the chemoselective coupling of the thiol. As discussed in Chapter 3, any competition between a thiol and an amine for the surface in the Chan-Lam coupling would lead to selective coupling of the thiol. The result here would be neither a free azide nor a free thiol on the surface of the array.

Scheme 4.9: Detection of Amines from Coupled Azido-Cysteine Linker

The amine-trapping fluorescence image verifies the presence of amines. This is a result of the reduction of the azido-cysteine linker before coupling to the surface.
This theory was supported by performing the Chan-Lam coupling of azido-cysteine followed by the previously used method (Chapter 3) of introducing a pyrene-labeled acid fluoride to selectively detect any free amines on the array. The experiment (Scheme 4.9) was successful and the residual amines predicted from the proposed mechanism were detected.

The results discussed above from the analysis of coupling the azido-cysteine to the surface of the array indicate that the failure of the “click” reaction was due to the inadvertent reduction of the azide during the Chan-Lam procedure. Therefore, if the surface of the array

Scheme 4.10: “Click” Reaction on Benzylic Azide

The complications of using the azido-cysteine linker were circumvented by utilizing a benzylic azide to functionalize the surface before performing the “click” reaction.
were functionalized with an azide using a non copper-mediated method, the “click” reaction should be successful. This concept was tested by first performing a Suzuki coupling\textsuperscript{14} between the boronic ester surface and a para-bromobenzyl azide (Scheme 4.10) in order to functionalize the array without azide reduction. The “click” reaction was then repeated under the identical conditions as before. Analysis of the array using a fluorescence microscope showed that the “click” reaction was performed successfully. This outcome allows us to keep the “click” reaction in our synthetic toolbox while securing our previous assessment that the reduced azide is what led to the reaction’s initial failure.

4.2.2 Direct Esterification of the Borate Ester Surface

In addition to the “click” chemistry, another method of coupling peptides was discovered. While attempting to detect free amines as part of the chemoselective Chan-Lam coupling (Chapter 3), one of the initial trials consisted of an amidation reaction with a succinamide ester and an electrochemically-generated base. This was based on previous work by our group\textsuperscript{15} in which an electrochemically generated base, via the reduction of Vitamin B\textsubscript{12}, evoked an esterification with a surface bound nucleophile and an activated succinamide ester. Due to the success of this technique, it was chosen to conduct amidations on the surface in order to fluorescently label any free amines. Upon coupling cysteine methyl ester to the polymer surface in a “C” pattern, the amidation was performed by reducing vitamin B\textsubscript{12} at all of the electrodes while a fluorescently labeled succinamide ester was in solution. The result from analyzing the array under a fluorescence microscope was quite surprising (Scheme 4.11). The fluorescence appeared stronger at the electrodes that didn’t contain cysteine. This was an immediate failure in regards to detecting free amines due to the high background but showed also an intriguing
interaction with the polymer surface. The fluorescence at these electrodes pointed towards the ability to directly esterify the borate ester polymer.

**Scheme 4.11: Amidation Attempt of Surface Bound Amines**

An electrochemically-generated base reaction was attempted at every electrode on the array, which was previously functionalized with cysteine-methyl ester in a “C” pattern. The attached fluorescence image shows a higher degree of pyrene at the unfunctionalized electrodes than those with cysteine.

This ability to directly esterify the surface was explored further as an additional method to selectively modify the surface or couple peptides to the array via an activated C-terminus. This reaction was initially attempted by repeating the vitamin B$_{12}$ reduction in the presence of the pyrene labeled succinamide ester. The fluorescence on array (Scheme 4.12) did indeed appear at the designated blocks of electrodes, however a fluorescent halo was visible around the electrodes indicating a loss of confinement. This was attributed to a lack of confining reagent. The reaction
solution only contained the succinamide ester substrate, vitamin $\text{B}_{12}$ and the ammonium salt electrolyte all in methanol. This meant that once the base was generated by the reduction of vitamin $\text{B}_{12}$, it was free to migrate across the array before reacting.

In order to get around this issue, a lesson learned from a previous experiment resurfaced. As discussed in Chapter 2, the electrochemical acid generated reaction used to perform the ligand exchange on the surface also suffered from a loss of confinement (Scheme 2.9). This problem was solved by adding a mild base, pyridine, to the reaction solution. The added base served to confine the acid to the selected electrodes (Scheme 2.9). Thus, if a base confines an acid reaction, then an acid should help confine a base reaction. This logic was tested by performing the identical base-mediated esterification reaction with the addition of toluene-sulfonic acid (Scheme 4.13). As predicted, the acid appeared to help confine the reaction to only the selected blocks of electrodes. The confinement and overall success of this reaction adds to our growing list of techniques for modifying the electrode surface and building molecular libraries.
Looking back at the previous attempt to perform an amidation on the free amines from the coupling of cysteine to the polymer surface, it was intriguing to consider how the addition of acid as the confining reagent might influence the reaction. Once again the Chan-Lam coupling of cysteine to the boronic ester polymer was performed. Subsequently, the reduction of vitamin B$_{12}$ in the presence of the activated ester and toluene-sulfonic acid was performed at the same electrodes. The resulting fluorescence image (Scheme 4.14) was quite unexpected. Even though the amidation reaction was only run at the electrodes containing cysteine, the pyrene succinamide ester reacted everywhere but those electrodes. The best explanation for this outcome is that the toluene-sulfonic acid introduced into the solution protonated the surface-bound amines, effectively protecting them from amidation. This clearly did not improve the performance of the reaction as it had with direct esterification of the surface but it was an interesting result in itself.

Scheme 4.13: Confined Esterification Reaction

The loss of confinement was fixed by adding toluenesulfonic acid to the reaction mixture.
The technique of confining the esterification reaction by adding acid was applied to the amidation of surface-bound amines. However, the fluorescence image shows no coupling at the desired electrodes.
4.3 Conclusion

The formation and immobilization of peptides and small molecules on surfaces for the purpose of analyzing their interactions with biological targets has been under investigation from many research groups for several years\(^1\). One of the more widely used methods because of its efficiency and biocompatibility it’s the formation of triazole rings via “click” chemistry\(^2,3\). This technique was been successfully adapted to the microelectrode arrays coated with the boronic ester-based diblock copolymer. The initial design of an azido-cysteine linker to functionalize the surface was not successful as the azide was reduced under the Chan-Lam conditions. Utilizing a Suzuki coupling to couple a benzylic azide linker circumvented this issue allowing the “click” chemistry to occur.

Several new methods of functionalizing the array surface were also discovered during this project. While investigating the “click” reaction, the versatility of the Chan-Lam coupling was expanded by coupling both the azide and acetylene components of the “click” reaction directly to the array. The esterification of the array with an activated ester was also discovered, a finding that allows peptides to be coupled to the array through their C-termini. Together, these capabilities of placing peptides onto the surface of an array will change the way molecular libraries are built on the arrays.
4.4 Experimental Procedure

4.4.1 General Information

Materials were purchased from Sigma-Aldrich and used without further purification unless otherwise indicated. Amino Acids were purchased from Advanced Chemtech and used without further purification.

Fluorescence microscopy was carried out with a Nikon Eclipse E200 microscope connected to a Boyce Scientific, M-100 burner and a Nikon D5000 camera. Optical filters used: CFW-BP01-Clinical-000 (Semrock) filter cube excitation 380-395 nm/emission 420-470 nm, ET - GFP (FITC/Cy2) (Chroma) filter cube excitation 450-490 nm, emission 500-550 nm and TxRed-A-Basic-000 (Semrock) filter cube excitation 540-580 nm, emission 590-670.

The array-based Chan-Lam couplings and array incubations were performed following the procedures outlined in Chapter 3, Section 3.4.

The array-based “click” chemistry was performed following previously published procedures\(^4\).

4.4.2 Sample procedure for spin-coating arrays with the diblock copolymer

The microelectrode arrays were coated with a spin-coater MODEL WS-400B-6NPP/LITE. The chip was inserted into a socket in the spinner and adjusted to be horizontal, then three drops of 0.03 g/mL PCEMA-b-pBSt solution (4:1.5 DMF/THF) were added onto the chip in order to cover the entire electrode area. The chip was then spun 1000 rpm for 40 seconds. The coating was allowed to dry for 15 min and subjected to irradiation using a 100 W Hg lamp for 20 min before use.
4.4.3 Synthesis of Azido-Cysteine-methylester

In a 25 mL, flame-dried round bottom flask, 726 mg (2.0 mmol) H-Cys(Trt)-OH, 5 mg (0.020 mmol) CuSO₄, and 552 mg (4 mmol) K₂CO₃ were dissolved in 10 mL anhydrous methanol. The imidazole-sulfonyl-azide (HCL) diazotransfer agent (0.5 g, 2.4 mmol) was slowly added to the solution and allowed to stir overnight while open to the atmosphere. Upon completion, the methanol was removed and the crude mixture was suspended in H₂O before being acidified with concentrated HCl. The acidified mixture was extracted with ethyl acetate. All organic layers were dried with MgSO₄, filtered and rotavapped.
Esterification of the azido-cysteine was performed without further purification. The mixture was dissolved in 5.0 mL methanol and 0.05 mL thionyl chloride was added to the solution, being allowed to stir overnight. The crude mixture was purified via column chromatography using 9:1 Hexanes:Ethyl ether obtaining the product in a 69% yield (558 mg, MW = 403.5 g/mol). $^1$H-NMR (300 MHz; CDCl$_3$): $\delta$7.47-7.43 (m, 6H), 7.33-7.21 (m, 9H), 3.71 (s, 3H), 3.22 (dd, $J = 8.1$, 5.9 Hz, 1H), 2.71 (dd, $J = 13.4$, 5.9 Hz, 1H), 2.60-2.53 (dd, $J = 13.4$, 8.1 Hz, 1H); $^{13}$C NMR (75 MHz; CDCl$_3$): $\delta$169.3, 144.2, 129.5, 128.1, 126.9, 67.3, 61.3, 52.7, 33.2; IR (KBr) 3467, 3055, 2950, 2505, 2107, 1745, 1593, 1487, 1443, 1206, 740 cm$^{-1}$; HRESI MS m/z [M+Na$^+$] Found: 426.1244, Calculated: 426.1254

$\text{N}_3$-$\text{Cys}$-$\text{OMe}$

The trityl deprotection of the azido-cysteine was in a flamed-dried 25 mL round bottom flask. 530 mg $\text{N}_3$-$\text{Cys}$(Trt)$\text{-OMe}$ (1.31 mmol) was dissolved in 5 mL dichloromethane. 0.5 mL TFA and 0.3 mL Et$_3$SiH were added to the flask and allowed to stir overnight. Upon completion, the mixture was diluted with DCM and rotavapped. The crude mixture was purified via column chromatography using 9:1 Hexanes:Ethyl ether. The deprotected product was obtained in a 96% yield (203 mg, MW = 161.18 g/mol). $^1$H-NMR (300 MHz; CDCl$_3$): $\delta$4.25 (dd, $J = 8.0$, 5.3 Hz, 1H), 3.82-3.81 (s, 3H), 3.21 (dd, $J = 14.0$, 5.3 Hz, 1H), 3.00 (dd, $J = 14.0$, 8.1 Hz, 1H), 1.25-1.23 (s, 1H); $^{13}$C NMR (126 MHz; CDCl$_3$): $\delta$ 164.1, 55.8, 47.9, 34.8 ppm; IR (KBr) 3365, 2952,
In a flame-dried, 50 mL round bottom flask, 0.11 mL oxalyl chloride was dissolved in 3 mL dichloromethane and cooled to -78°C. 0.2 mL dimethylsulfoxide was added to the flask and stirred for two minutes. 1-pyrenebutanol (300mg, 1.09 mmol) was dissolved in 2 mL DCM and added slowly to the reaction solution and stirred for 15 minutes. 1 mL triethylamine was added and stirred for 5 minutes before warming to room temperature. The solution was quenched with water and extracted with DCM. The organic layers were dried over MgSO₄, filtered and rotavapped. No further purification was performed.

In a flame-dried, 50 mL round bottom flask, 362 mg carbon tetrabromide and 571 mg triphenylphosphine were dissolved in 10 mL DCM. The crude aldehyde from the previous Swern oxidation was dissolved in DCM, slowly added to the reaction solution and stirred for one hour. Upon completion, the reaction was quenched with water and extracted with DCM. The combined
organic layers were dried over MgSO$_4$, filtered and rotavapped. No further purification was performed.

The final elimination/dehalogenation step was performed on the Wittig crude. The dibromoalkene product was dissolved in 10 mL THF. Two equivalents of nBuLi (1.6M soln in hexanes) was slowly added to the flask and stirred for 10 minutes. The reaction was quenched with water and extracted with DCM. The organic layers were dried over MgSO$_4$, filtered and rotavapped. The product was purified via column chromatography using 95:5 Hexanes:Ethyl ether as the eluent. The pyrene-labeled alkyne was obtained in a 78% yield over the three steps (228 mg, MW = 268.36 g/mol). $^1$H-NMR (300 MHz; CDCl$_3$): $\delta$8.31 (d, $J$ = 9.3 Hz, 1H), 8.15 (dd, $J$ = 17.2, 7.5 Hz, 4H), 8.06-8.00 (m, 3H), 7.90 (d, $J$ = 7.8 Hz, 1H), 3.49 (t, $J$ = 7.6 Hz, 2H), 2.34 (td, $J$ = 6.9, 2.5 Hz, 2H), 2.15-2.05 (m, 3H) ppm; $^{13}$C NMR (75 MHz; CDCl$_3$): $\delta$135.8, 131.4, 130.9, 130.0, 128.7, 127.51, 127.37, 127.33, 126.7, 125.8, 125.01, 124.91, 124.81, 124.75, 123.3, 84.3, 69.1, 32.2, 30.3, 18.3 ppm; IR (KBr) 3509, 3296, 3037, 2939, 2113, 1918, 1648, 1601, 1456, 1181, 637 cm$^{-1}$; GC-HRMS m/z [M$^+$] Found: 268.1217, Calculated: 268.1252
4.5 References


5. Ogasawara, Y.; Murai, Y.; Yasuko Sakihama, Hashidoko, Y. and Hashimoto, M. “Quantitative analysis of Cu(I) concentration in click chemistry: biotinylation at side chain of propargylglycine using click chemistry under heating conditions”, *Heterocycles*, **2012**, *86*, 735-743


5.1 Introduction

The end goal of the work discussed thus far is to apply what we have learned about building molecular libraries and manipulating the polymer surface in an effort to detect and better understand the binding interactions of biological systems. The development of this microarray-based technique has been geared towards providing an alternative to other methods, which are often quite time and resource consuming. One particular biological target was brought to our attention courtesy of Dr. Suzanne Lapi and her research group from Washington University Medical School’s Division of Radiological Sciences. After several encounters with our group and becoming familiar with our research and its capabilities, they approached us for assistance in analyzing the binding interaction between the vascular endothelial growth factor (VEGF) protein and a known antagonist peptide, v107.

The VEGF protein is a potent endothelial cell-specific mediator of angiogenesis and vasculogenesis, the processes in which new blood vessels are formed from existing vessels or in avascular tissue. This formation is essential for the normal development and survival of mammalian cells, but it is also responsible for several pathological conditions including tumor growth, diabetic retinopathy, rheumatoid arthritis and psoriasis\(^1\). The latter conditions make the VEGF protein a clear target for clinical treatment.

Several monoclonal antibodies against VEGF have been discovered and have been shown to result in tumor shrinkage\(^1\). However, small molecule antagonists can provide greater
information towards the structure/function relationship of the VEGF protein. This is especially true in the general area of imaging. While antibodies can have high selectivity for a protein and bind with exceptional affinity, they are large molecules and a radio-labeled antibody is often slowly cleared from the body. This leads to high levels of background signal. Small molecules that are cleared from tissue more rapidly can serve as much more effective imaging agents. To this end, the 19-residue, cyclic v107 peptide (Figure 5.1) was discovered to bind to VEGF and inhibit interactions with its receptors. The v107 peptide ligand was found to bind to the human VEGF protein with a mild affinity of $\sim 10^{-6}$ M. For an effective imaging agent, it would be better
if an analog of v107 could be found with an affinity for VEGF that was on the order of $10^{-9}$ M or smaller. In order to reach this goal for the v107 peptide, structural analogues must be analyzed in order to hone the structure-activity relationship. Progress along these lines is being hindered by the expense and complexity of the biological assay currently used to evaluate v107/VEGF binding. In this chapter, we will discuss the collaboration with Dr. Lapi and her group towards utilizing the microelectrode arrays to monitor the binding interaction between v107 analogues and the human and mouse VEGF proteins.

5.2 Results and Discussion

5.2.1 Peptide Coupling Strategy

Before analysis of the binding interaction between v107 and VEGF could begin, a strategy for coupling the v107 peptide to the surface of the array was needed. Our initial approach was to apply the Chan-Lam coupling technique discussed in Chapter 3. However, we were concerned over disrupting the disulfide bond in v107, which has shown to be a critical component in antagonists of VEGF, and thus altering the conformation of the peptide. This led us to look for an alternative route. Given that this study took place before the discoveries discussed in Chapter 4, the next viable method was to couple the peptide to the surface via a “click” reaction. Given the success of the “click” reaction on earlier polymer surfaces, the v107 peptide was obtained from a commercial source with a PEG-4 propargylic arginine modified N-terminus. However, as discussed in chapter 4, the initial trial of the “click” reaction was a failure. As a result, the acetylene coupling reaction (Scheme 4.6) was discovered and optimized to use the acetylated peptide already in our possession. Although the “click” reaction has been shown to be successful with a benzylic azide, this discovery was made after any v107/VEGF binding
studies were conducted. Therefore, all binding experiments discussed in this chapter were conducted by coupling the v107 peptide to the aryl boronic acid surface via the copper-mediated acetylene coupling (Scheme 4.6).

5.2.2 Binding Data Acquisition and Processing

Before the results from the v107/VEGF experiments are revealed, it is important to discuss how the data from binding experiment is obtained and how it is translated into a binding curve. As mentioned in Chapter 1, the ability to indirectly detect the binding interaction of any biological system under investigation depends on monitoring the current associated with a redox mediator in the solution above the array. This is done by sweeping the potential of the array relative to a reference electrode and then recording the current for the redox mediator at each potential. This leads to a voltammetry wave as shown in Figure 5.2. As the biological target is introduced in solution above the array, the current associated with the mediator is measured at a set of individually addressable electrodes, typically twelve, which was previously functionalized with a molecular library member. This process is repeated as the concentration of the biological target in solution is varied with each voltammogram representing an individual concentration. The resulting data (Figure 5.2) shows the change in the current, represented by the height of each curve, as the concentration of the target is increased. For this particular set of data the current measured dropped as the target concentration increased. This observation is consistent with the binding event hindering approach of the redox mediator to the electrode. While such events are common, the current may increase as a function of target concentration if the binding event improves diffusion of the redox mediator to the surface of the electrode. An example of this type of change will be discussed later.
Translating the raw CV data obtained during the binding experiment into a binding curve consists of computing the change in the current at a particular electrode as a function of the target’s concentration. This begins by determining the total current that passed through the electrode for each CV acquired. This is accomplished by calculating the difference between the current at measured at the peak in the oxidative wave (Figure 5.3, Point 1) and the reductive wave (Figure 5.3, Point 2). It is important to point out that the microelectrode arrays do not contain a reference electrode. Therefore, the peak points used must be assessed for each electrode used in a binding experiment. It may be difficult to assign the “peak” potential for a
particular wave. For example, for the CV shown in Figure 5.3, the reductive wave does not appear to “peak”. Therefore, potential at point 2 in the CV was assigned where the curve begins to level off. This arbitrary peak assignment is sufficient as long as the potential points (1&2) used to measure the total current are consistent for each CV taken at a particular electrode. It is important to note that any variation in the peak assignment results in no statistical deviation, outside of experimental error, in the calculated binding data. This analysis is repeated for each voltammogram (Table 5.1, CVs 1-9) taken at a particular set of electrodes resulting in the total current measured at a specific target concentration.

**Figure 5.3: Sample Cyclic Voltammogram**
In order to compare the change in current from one set of electrodes to another, the current data must be normalized. During the course of investigating the binding of the v107/VEGF system, there was much debate as to how the data should be normalized. It was concluded that there are in fact two methods of normalization, each providing a unique perspective of the data, yet both are necessary for a complete analysis of the observed binding event. The first method, referred to as “self-normalization”, normalizes the change in the observed current on a scale of 0 to 1 relative to itself. The normalization is performed using equations 2 and 3. The normalized current value, $A_{Norm,X}$, for a particular curve is the difference between the current at the minimum concentration ($A_{min. conc}$) and the current at that CV ($A_X$) divided by the total current change (the difference between the current at the minimum and maximum concentrations measured).

For example, using the data in Table 5.1 and equations 2 and 3:

$$A_{Total} = 1.37e^{-6} - 5.62e^{-7} = 8.08e^{-7}$$
Completing the normalization for the remaining current values affords the data in Table 5.2. Again, this represents the relative change in current measured at each concentration of the biological target normalized between zero (lowest concentration) and one (highest concentration). Plotting the normalized data as a function of the logarithm of the target concentration yields the curve in Figure 5.4. Based on the sign of the differences calculated in equation 2, a positive change in the current corresponds to a drop in the current. This is caused by the biological target interacting with the surface-bound library member impeding the redox mediator from reaching the electrode surface. From this plot, we can say that an immediate change in current occurs (this is explained later in the chapter), but then remains relatively unchanged until a concentration of approximately one micromolar, at which point there is a dramatic change in the current. Based on the fundamentals of the binding experiments, as outlined in chapter one, we can conclude that a significant interaction between the biological target and the library member at this electrode had occurred beginning at a concentration of one micromolar.

<table>
<thead>
<tr>
<th>CV#</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. (M)</td>
<td>5.00E-09</td>
<td>1.00E-08</td>
<td>5.00E-08</td>
<td>1.00E-07</td>
<td>5.00E-07</td>
<td>1.00E-06</td>
<td>5.00E-06</td>
<td>1.00E-05</td>
<td>2.50E-05</td>
</tr>
<tr>
<td>Normalized Current (A)</td>
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<td>-7.43E-02</td>
<td>-1.36E-01</td>
<td>-9.90E-02</td>
<td>-1.11E-01</td>
<td>-8.66E-02</td>
<td>2.97E-01</td>
<td>5.07E-01</td>
<td>1.00</td>
</tr>
</tbody>
</table>
The result of the normalization method is to make all of the curves measured on an array the same size irrespective of the total change in current. This can help us identify the components of the curve (nonspecific and specific binding) that contribute to the overall picture. The method is not as effective for assessing relative binding events. A strong binding event on the surface of the array is expected to cause a much larger change in current than a weak-binding event. The self-normalization method removes this difference. Hence, a different normalization method is needed that preserves this information.

A plot of the normalized current data against the logarithm of the biological target concentration, allows us to verify the presence of an interaction between a target and molecular library member.
To this end, a second method of normalizing the current data, referred to as "reference-normalization" has been developed. The mathematical distinction for this method is that instead of dividing the difference in current by the total current change for that same set of electrodes, \( A_{Total} \), as in equation 2, it is divided by the total change in current measured for a reference molecule. This normalization method still sets the current measured at the minimum concentration as the zero-point, however there is no upper-limit. In this way, the total change in current is preserved. The significance of this method, compared to the self-normalization method is explained later in the chapter; as we look at the binding data obtain from the v107/VEGF system.

5.2.3: v107/Human VEGF Binding Experiments

The Human VEGF protein has already been identified as a clinical target for several pathological conditions and is known to posses micromolar binding against the v107 peptide\(^1\). However, the use of human VEGF protein in biological screen is expensive and therefore impractical in terms of continually screening potential drug candidates for activity. A VEGF protein from an alternate source but with similar binding properties could alleviate the need to use the human VEGF Protein. With this in mind, the murine VEGF protein was investigated as an alternative in collaboration with the Lapi group. It was hoped that both the murine and human VEGF would afford similar results on the arrays. This work started by verifying the ability to detect a binding interaction with VEGF by using the known v107/Human VEGF system as a standard.

The analysis of the v107/Human VEGF system began by coupling the v107 peptide to a microelectrode array that was polymer coated with the boronic ester polymer via the acetylene
coupling discussed earlier. Upon placement of the v107 peptide, the array was incubated in a 1xPBS buffer solution consisting of 8 mM Fe$^{2+}$/Fe$^{3+}$ redox mediator and the human VEGF protein at an initial concentration of 5.0x10$^{-9}$ M. The current was measured at several sets of electrodes via cyclic voltammetry before incrementally increasing the concentration of human VEGF. The current was analyzed over a range of human VEGF concentrations for electrodes functionalized with the v107 peptide as well as the unfunctionalized polymer before being translated into a binding curves as described above.

In this case, the overall change in current at the electrodes with v107 and the electrodes with the plain polymer was roughly equivalent (Figures 5.5 and 5.6). Hence, the two normalization methods give rise to roughly the same curve (the denominator in the normalization equation is the same). The change in current for the v107 relative to the peptide background can be seen nicely. The large decrease in current associated with both curves as the concentration of VEGF increases may well be due to precipitation of the protein from solution onto the electrode since the concentration of protein was reaching its solubility limit at that point.

The initial signaling experiment hinted towards the binding of v107 to human VEGF, but two issues remained. First, we wanted to reduce the non-specific binding event even more if possible, and second we needed to probe whether the v107 binding event was simply a non-specific event with the protein on the surface or truly characteristic of a specific binding event with v107. In other words, we needed to compare the binding of v107 to a negative control. In an effort to lower non-specific binding, a polymer coated-array was functionalized with v107 along with separate electrodes functionalized with a polyethylene glycol (PEG) chain. This functionalization was chosen after a recent finding by Sakshi Uppal in our lab, demonstrated the successful hindrance of bovine serum albumin (BSA) binding to a PEGylated polymer surface$^5$. 
Figure 5.5: v107/Human VEGF Binding Experiment #1 Self-Normalization Plot

Figure 5.6: v107/Human VEGF Binding Experiment #1 Reference-Normalization Plot
The functionalized array was subject to the same binding experiment as before and the resulting CV data was processed in the same manner. The only deviation from the previous binding experiment was the iron redox mediator. In the previous case, the mediator consisted of a mixture of potassium ferricyanide and potassium ferrocyanide. Due to incompatibilities of this redox mediator with the PEGylated surface\(^4\), the mediator was switched to ferrocenecarboxylic acid.

When the self-normalization method was used for plotting the data from this second binding experiment (Figure 5.7), little difference could be discerned between the v107 functionalized electrodes and the unfunctionalized electrodes. Remember that this method dampens out the total change in current and focuses on the nature of the binding event, which appears to be very similar for the two sets of electrodes. The data corresponding to the PEGylated electrodes hinted at a later occurring binding event, however, due to the dramatic change in current compared to the over curves the shift may be exaggerated. This dramatic current change is addressed later.

The importance of utilizing both normalization methods is demonstrated in this experiment. When the data is replotted using the reference-normalization technique (Figure 5.8), by setting the v107 electrodes as the reference point, we see a significant difference in the curves. At this point, the polymer and v107 curves begin to slightly differentiate themselves. The sudden rise in the two curves, earlier explained as being indicative of a binding event, are still quite close to one another rendering them statistically indistinguishable. However, the greater amount of total current change in the v107 electrodes compared to the polymer electrodes does hint at the interaction with v107. Interactions on a surface that occur with roughly equal binding constants will not necessarily change the current at the electrode in the same manner due to
Figure 5.7: v107/Human VEGF Binding Experiment #2 Self-Normalization Plot

Figure 5.8: v107/Human VEGF Binding Experiment #2 Reference-Normalization Plot
differences in the nature of the interaction. A binding event that leads to a spatially closer association of the protein with the surface will cause a greater change, etc. Overall, this experiment did not verify the detection of the v107/human VEGF interaction, but the subtleties in the curves did suggest that we are on the right track.

The immediate drop in the last set of binding curves (Figures 5.7 and 5.8), designating a sudden increase in current measured at the electrode surface, is a dramatic example of a phenomenon that occasionally occurs when performing a binding study. After observing this unusual characteristic over the course of several binding experiments performed by myself and other group members, it was determined to be a form of background noise. It appeared to be independent of the system under investigation, the starting concentration of the biological target and the mediator used. As mentioned in chapter 1, the size of the electrodes used are only ~45 microns in diameter. This corresponds to only a few femtomoles of material present on the surface. As a result, this initial drop in current became an important issue to resolve in order to maintain a sufficient signal-to-noise ratio.

Given that the issue occurred over a wide range of experimental conditions, this led us to believe that it was a flaw in the fundamental process in which the experiments were run. All signaling experiments run on the arrays utilize the same routine. A redox mediator containing solution is introduced above the array surface, a current is measured, then the solution is replaced and the process repeats itself. At first glance, it seems as if all of the iron mediator is removed from the array then reintroduced with the next solution. However, we know that once the mediator migrates into the polymer it may become trapped\textsuperscript{4}. As a result when the mediator solution is removed it now contains 8-x mM iron, where x is the amount trapped in the polymer. Now when a new solution is introduced, the total amount of iron in the system is 8+x mM. This
net increase in the iron present should cause an increase in the current. Depending on the thickness of the polymer, the polymer functionalization and the mediator, a wide range of current change could be observed. Following this logic, if the iron solution replacement procedure was conducted numerous times, the polymer surface would essentially become saturated and the current would stabilize. This thought experiment was put to the test. A polymer-coated array, functionalized with the v107 peptide, was incubated in the standard 8 mM ferri/ferrocyanide mediator solution. The current was measured at a set of electrodes, and then the solution was replaced before the current was measured again. As illustrated in Figure 5.9, the currents measured quickly converged after only three iterations of replacing the iron solution.

Once the issue of the initial flux in current was resolved, this technique was applied to a binding experiment between v107 and human VEGF. As with the previous binding experiment, a

Figure 5.9: Current Stabilization Experiment

Replacing the redox mediator solution and remeasuring the current allowed the system to quickly equilibrate, creating a stable background for subsequent binding studies.
microelectrode array was coated and cross-linked with the boronic ester polymer then functionalized with the v107 peptide and PEG at separate locations on the array. In an effort to further reduce any non-specific binding to the VEGF protein, an additional set of electrodes were functionalized with v107 followed by PEGylation of the same electrodes. The PEGylation of any residual polymer present after the v107 coupling would, hopefully, help pacify the surface and reduce background binding.

The binding experiment was performed against human VEGF after the current stabilization method was conducted. The CV data was self-normalized and plotted (Figure 5.10). In each case, the immediate drop in current did not occur indicating that the current stabilization method did work. With the "artificial" change in current removed, we were able to better analyze the data. In each case, the data showed an overlap in the binding curves that suggest again that the background binding event to the surface and binding to v107 had roughly equal binding constants. However, as before, the difference in the curves allowed us to detect the presence of the v107 on the surface. In this case, the initial drop in the current at the electrodes functionalized with only v107 is matched by a very rapid rise to a point higher that any of the currents. The presence of the v107 peptide altered the nature of the binding event. Interestingly, the presence of the PEG on the surface with the v107 interfered with this change in the signal. It appears that PEGylation of the surface does not significantly reduce the background binding of human VEGF compared to the unfunctionalized polymer electrodes. In fact, the amount of current change (designated by the height of the curves about the binding event), indicates a greater interaction with PEG than the unfunctionalized surface. Even when you consider the amount of current change from the zero-point of each curve to the point when the curves levels off, \( \sim 10^{-6} \) M, there is little influence by PEGylating the surface.
This analysis of the magnitude of current change at the binding event reaffirmed our feeling that we were detecting the interaction between v107 and VEGF. This was even true for the current measured at the electrodes with both PEG and v107 (measured relative to PEG alone), although the effect was dampened. However, to be comfortable with the detection method, we needed to tune the method so that we could also see a binding event with a greater binding constant than background. In other words, we needed to reach a point where we had a horizontal differentiation between the CV waves on the basis of VEGF concentration.
Since the PEGylation of the surface did not appear to be applicable to this particular system, the strategy was abandoned along with the use of ferrocenecarboxylic acid as the mediator. Throughout these studies, it has been observed that the ferri/ferrocyanide mediator has greater permeability into the polymer surface due to its ionic character compared to the ferrocene derivative. This provides a more intense signal for observing this intricate v107/VEGF system. The second modification made to the binding experiment was the use of a negative control, a molecule that is not known to bind to VEGF. The molecule used for this study was recommended and provided by Dr. Lapi’s research group. The negative control, tetraazacyclododecane-tetraacetic acid (DOTA) modified Octreotide peptide (Figure 5.11), was easily coupled to the polymer surface using the Chan-Lam alcohol coupling procedure discussed.

**Figure 5.11: Structure of the Negative Control DOTA-Octreotide**
in chapter 3. After coupling the v107 peptide to the surface of the same array in the manner
described before, the current stabilization procedure was performed before once again
conducting a binding study with human VEGF.

The results of this particular binding study were quite fruitful. Analyzing the self-
normalized plot (Figure 5.12) of the binding data demonstrated that the current stabilization
process was very effective. The binding curves obtained for the v107 and DOTA-Octreotide
peptides were steady at the beginning of the study. The curve corresponding to the polymer-only
electrodes (green line) starts out steady but begins to deviate well before the other curves.
However, this can still be attributed to an interaction between the surface and VEGF given the
steady start. The curves for the other electrodes show a similar, but much smaller effect.
However, the curves for the v107 and negative control functionalized electrodes begin to turn
positive at a lower concentration of the receptor, with the v107 electrode derived curve changing
first.

Upon considering the dynamic interactions occurring on the surface of the array and what
we have learned thus far about the behavior of the polymer during these interactions, we can
begin to draw a lot of information from the shape of the curves themselves. As mentioned, earlier
when the polymer is functionalized the surface swells, allowing more a greater amount of
mediator to penetrate the polymer and therefore observing a change in the current. One can
conclude that a particular interaction with a surface will have a greater effect on an
unfunctionalized surface compared to a “pre-swollen” functionalized surface. We attribute to the
large dip in the polymer curve (Figure 5.12, green line) to this “enhanced” signal. Furthermore,
by normalizing all of the curves to the same zero to one scale, this feature is exaggerated even
more. In practice, the actual current change for the unfunctionalized electrode is relatively small.
Figure 5.12: v107/Human VEGF Binding Experiment #4 Self-Normalization Plot

Figure 5.13: v107/Human VEGF Binding Experiment #4 Reference-Normalization Plot
This is why the inclusion of the reference-normalized plot (Figure 5.13) is important. By setting the v107 peptide curve (blue line) as the reference point, we can compare the effect of a particular interaction more accurately by examining the size of the current change in addition to the location of the wave. The magnitude of the dip in the polymer curve (green line) and the v107 curve (blue line) is statistically the same. However, now you can see that the dip in the polymer curve (green line) is considerably wider than that of the v107 curve (blue line). We attribute the shape of this initial dip to the nature of the interaction occurring on the surface. As the amount of VEGF on the surface increases, the current flow then decreases eventually to a point where it reaches saturation. Comparatively, the curve associated with the v107 functionalized electrodes (Figure 5.13, blue line) appears to accumulate the VEGF receptor at a much faster rate. In fact, the sharper transition to a curve moving in the positive direction indicates a clear binding event that does not occur in the absence of v107. When compared with the DOTA-octreotide functionalized electrodes, this binding event with v107 occurs at a lower concentration leading to a faster and overall larger drop in current.

Finally, an analysis of the inflection points on the binding curves again indicates the binding of human VEGF to v107 on the array. In other words, we can see the horizontal shift we were hoping for. The concentration corresponding to the inflection point in the v107 curve (Figure 5.12, blue line) is approximately one micromolar. This is consistent with previously published results\(^1\), thus confirming our ability to detect this interaction. It is also worth mentioning that that the human/VEGF does appear to bind to the DOTA-Octreotide, although with a weaker binding constant. To our knowledge this peptide was not previously known to bind human/VEGF. Regardless, the ability to detect the binding event between v107 and human VEGF allowed us to continue with the plan to determine if both human and murine VEGF can be
used interchangeably to probe binding interactions between peptide ligands and the VEGF receptor.

5.2.4 v107/Murine VEGF Binding Experiments

As mentioned earlier, the affinity of the v107 peptide for the murine VEGF receptor has not been confirmed. With this in mind, we subjected the array-based v107 peptide to the murine VEGF receptor using the same analytical method developed for the human VEGF receptor. A microelectrode array was coated with the boronic ester polymer and photo-crosslinked. Analysis of the murine VEGF was performed at electrode sites functionalized with v107, electrode sites functionalize with the negative control DOTA-Octreotide, and electrodes sites the retained the unfunctionalized polymer.

The data from obtained from this binding study was quite promising. Shown in Figure 5.14, the binding curve generated for the v107 peptide shows steady current until a strong interaction was observed beginning at approximately $10^{-7}$ M. The inflection point for this curve puts the binding constant at $\sim$300 nM, almost an order of magnitude stronger than that observed for the human VEGF. Unfortunately, the negative control, DOTA-Octreotide, also appears to bind to the murine VEGF at approximately the same concentration. It was becoming clear that our "negative" control wasn't one. An alternate negative control was needed. The interaction between the polymer and murine VEGF produced current data that was inconsistent across the array, thus its absence from Figure 1.4. This observation is not uncommon. The binding of a receptor to the surface is susceptible to variations in the surface to a greater extent than is binding of the receptor to a molecule that is removed from the surface by a linker. Hence, a less
than uniform polymer coating will strongly interfere with binding to the polymer in a manner that does not with binding to the peptide ligands on the array.

As an alternative negative-control, we decided to move towards a structurally different, shorter, linear hexapeptide, GRDGSP. The strong interaction between the DOTA-Octreotide and the VEGF proteins may be due its similarity with v107, both consisting of disulfide-bridging cyclic structure. This linear peptide was also chosen due to its success in the peptide gradient experiment discussed in the previous chapter.

Once again, a boronic ester polymer-coated microelectrode array was functionalized with the v107 peptide and the GRDGSP peptide at separate locations on the array. A binding experiment was then performed using the same process as before. The self-normalized binding
Figure 5.15: v107/Murine VEGF Binding Experiment #2 Self-Normalization Plot

Figure 5.16: v107/Murine VEGF Binding Experiment #2 Reference-Normalization Plot
data (Figure 5.15) shows the same interaction between the v107 peptide (blue line) and murine VEGF as seen in the previous experiment at approximately 300 nM. The GRDGSP peptide curve (red line) also showcases an interaction, but at a higher concentration of murine VEGF. The final curve corresponding to the unfunctionalized polymer (green line) indicates a significant interaction with the VEGF protein almost immediately. However, as described earlier, the self-normalization technique highlights the subtle changes in the binding curves due to the dynamics of the surface interactions. It tells us that the nature of the binding interactions are different. However, it does not provide any feel for the relative size of the changes observed. When the data is analyzed using the reference-normalized technique (Figure 5.17), the relative intensities of the interactions are observed. Compared to murine VEGF binding to v107 (blue line), the interactions of the negative-control GRDGSP peptide (red line) and the polymer (green line) are significantly smaller. The binding event between v107 and murine VEGF can be readily observed, again with an affinity on the array of ca. 300 nM.
5.3 Conclusion

The human VEGF protein, which has become a target of interest to our collaborators due to its possible therapeutic capabilities, was identified to be inhibited by the cyclic peptide v107\textsuperscript{1,2}. In an effort to better understand the binding characteristics of this system, we analyzed the interaction between the VEGF protein and the v107 peptide utilizing the microelectrode array technology developed in our lab. After several trials, we were able to confirm the micromolar binding of v107 to human VEGF. Attention was then turned toward the murine VEGF protein as a possible alternative to the human protein. Once again the interaction between v107 and VEGF was observed at a slightly stronger binding constant of \(~300\) nM. This successfully confirmed the affinity of v107 for murine VEGF, which had been difficult using other experimental techniques. It is clear that the murine VEGF can be used as an alternative to the human VEGF in future studies.
5.4 Experimental Procedure

5.4.1 General Information

Materials were purchased from commercial sources and used without further purification.

5.4.2 Sample procedure for spin-coating arrays with the diblock copolymer

The microelectrode arrays were coated with a spin-coater MODEL WS-400B-6NPP/LITE. The chip was inserted into a socket in the spinner and adjusted to be horizontal, then three drops of 0.03 g/mL PCEMA-b-pBSt\(^6\) solution (4:1.5 DMF/THF) were added onto the chip in order to cover the entire electrode area. The chip was then spun 1000 rpm for 40 seconds. The coating was allowed to dry for 15 min and subjected to irradiation using a 100 W Hg lamp for 20 min before use.

5.4.3 Peptide coupling and signaling procedure

All compounds were coupled to the array surface using copper-mediated Chan-Lam coupling described in chapter 4.

All binding experiments were performed following the previously published procedure\(^7\).
5.5 References


Chapter Six

Improved Synthesis of the Bipyrazine Ligand

6.1 Introduction

Early into the mechanistic investigation of radical cations generated via photoredox catalysts (discussed in Chapter 7), we encountered a significant barrier. The ruthenium tris(bipyridine) catalyst (Scheme 6.1, 1) was initially chosen due to the extensive photophysical and chemical studies already performed\(^1\). It has also already been utilized in many photochemical transformations\(^2\). This catalyst, which possesses an oxidation potential of 0.77V in the Ru(II) photoexcited state\(^3\), was appropriate for my initial studies concerning the sulfonamide anion substrate (Scheme 6.1, 3) which has a potential of 0.69V. However, in order to investigate the remaining substrates (Scheme 6.1, 4-6), a photoredox catalyst with a higher oxidation potential was needed.

Scheme 6.1: Photoredox Catalysts and Substrates

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>(X)</th>
<th>(E_{p/2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>-NTs anion</td>
<td>0.69V</td>
</tr>
<tr>
<td>4</td>
<td>-OH</td>
<td>1.4V</td>
</tr>
<tr>
<td>5</td>
<td>-CH=CH-OMe</td>
<td>1.05V</td>
</tr>
<tr>
<td>6</td>
<td>-CH=CH-CH(_2)TMS</td>
<td>1.02V</td>
</tr>
</tbody>
</table>
The best alternative was the bipyrazine based ruthenium catalyst (Scheme 6.1, 2). This ruthenium derivative has been successfully utilized in various photochemical reactions and possesses an oxidation potential compatible with our substrates of interest. Another major advantage of the bipyrazine-based catalyst is the minimal deviation from the bipyridine catalyst. This allows us to directly compare photochemical reactions run with either catalyst. A significant impediment to the availability of this new catalyst is its synthesis and specifically the synthesis of the bipyrazine ligand itself. The most widely used approach was developed by Laffery and Case\(^4\) (Scheme 6.2). This method requires elevated temperatures and only produces the ligand in a 7\% yield. Recently, Yoon and coworkers have developed a biaryl coupling of chloropyrazine but utilizes an expensive Palladium catalyst\(^5\). To this end, I sought to find an efficient and scalable synthesis of bipyrazine that took advantage of an Earth abundant metal catalyst.

**Scheme 6.2: Lafferty and Case Synthesis of Bipyrazine**

![Scheme 6.2: Lafferty and Case Synthesis of Bipyrazine](image)
6.2 Results and Discussion

When contemplating a synthetic method towards bipyrazine the initial approach was to utilize known copper-based biaryl couplings developed by Lipshutz and coworkers\(^6\) (Scheme 6.3). This method creates a higher order cuprate between various aryllithiums (typically form from a metal halogen exchange reaction) and copper(I) cyanide. This complex is then oxidized at low temperature to produce a homocoupling or mixed biaryl species. This technique was applied to the formation of bipyrazine but unfortunately, did not proceed well. Methods for generation of the aryllithium, the nature of the oxidant used, and the time and temperature of the reaction were all varied. The maximum yield obtained was only 35%. The amount of recovered pyrazine halide starting material and results from subsequent experiments (discussed below) indicated the limiting factor was the formation of the aryllithium species, which in turn prevents the formation of the necessary cuprate.

In order to circumvent the issue of forming the necessary aryllithium, a modification of the Lipshutz cuprate was attempted. This technique was taken from the previous work of Wheatley, Uchimaya and coworkers. When copper(I) cyanide was treated with lithium tetramethylpiperidine (LiTMP) an amino-based cuprate was formed. It was then utilized for the direct ortho cupration of numerous substituted aromatics, which are trapped upon introduction of

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**Scheme 6.3: Lipshutz Biaryl Coupling**

\[
\begin{align*}
2 \text{ArLi} & \quad \text{CuCN} & \quad \text{Ar}_2\text{CuCNLi}_2 & \quad \text{O}_2 \\
& \quad \text{-125 °C} & \quad \rightarrow & \quad \text{Ar-Ar}
\end{align*}
\]
an electrophile. An interested observation from Wheatley, Uchimaya and coworkers was the formation of homodimers upon oxidation of the formed aryl cuprate\(^7\) (Scheme 6.4). This observation provided a promising solution forming the necessary aryllithium of pyrazine. Upon applying this technique towards the synthesis of bipyrazine, a yield of only 20\% was obtained. Even though the yield was lower than the previous Lipshutz example, this reaction was a success. The completion of with reaction only provided approximately 20\% recovered starting material. This suggested that the formation of the aryl cuprate as a result of direct deprotonation was no longer the major problem. The oxidation step to produce the homodimers was now the hindrance.

Further search into the literature revealed a variant of the direct cupration technique that was shown to be the first successful method for heteroaromatics. Nguyen and coworkers employed a Gilman-type reagent similar to the amino-based cuprate discussed earlier. They were able to perform a similar aromatic deprotonation with only one equivalent of cuprate. Also, the use of a stronger oxidant produced the homocoupling product on excellent yield\(^8\) (Scheme 6.5). The initial cuprate was formed in the same manner as the previous example, however the in-situ
production of copper(I) from the reduction of copper(II) chloride with n-butyllithium was employed rather than the use of commercially available copper(I) cyanide. Experimental results discussed later shows that this in-situ reduction to copper(I) is essential to the success of our pyrazine homocoupling.

When this method of deprotonative metalation with subsequent oxidation was applied to pyrazine the result was a homocoupling that yielded a 41% of the desired product (Table 6.1, Entry 1). Dinitrobenzene was used as an oxidant resulting a slightly lower yield and as a control no oxidant present yielded minimal product (Table 6.1, Entries 2,3). Throughout these experiments the recovered starting material ranged from 20-25%. This suggested that the oxidation step still needed to be the primary focus. Upon investigating alternative oxidants, quinone derivatives were the first to be considered. Not only had chloranil (tetrachloroquinone) produced the greatest yield for us to date, the work of Iyoda and coworkers suggested the importance of quinones. Their methodology took advantage of a Lipshutz cuprate to construct macrocycles. However, during the oxidation step they noted an important π–complex formed consisting of lithium-carbonyl and copper-olefin coordinations. They also showed that the use of duroquinone (tetramethylquinone) was the most efficient oxidant for the cuprate. Duroquinone
was used as an oxidant toward the synthesis of bipyrazine and led to a yield of 45% (Table 6.1, Entry 4). This was a slight advancement but there was still room for improvement.

### Table 6.1: Bipyrazine Coupling Data

<table>
<thead>
<tr>
<th>Entry</th>
<th>Oxidant (1.2 eq)</th>
<th>Conditions</th>
<th>Variation</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chloranil</td>
<td>60 °C, 16 h</td>
<td>--</td>
<td>41%</td>
</tr>
<tr>
<td>2</td>
<td>Dinitrobenzene</td>
<td>RT, 16 h</td>
<td>--</td>
<td>39%</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
<td>RT, 16 h</td>
<td>--</td>
<td>10%</td>
</tr>
<tr>
<td>4</td>
<td>Duroquinone</td>
<td>60 °C, 16 h</td>
<td>--</td>
<td>45%</td>
</tr>
<tr>
<td>5</td>
<td>Duroquinone</td>
<td>Reflux, 16 h</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Duroquinone</td>
<td>60 °C, 16 h</td>
<td>5.0 equiv</td>
<td>39%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TMEDA</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Duroquinone</td>
<td>Sonicated, 10 min</td>
<td>--</td>
<td>56%</td>
</tr>
<tr>
<td>8</td>
<td><strong>Duroquinone</strong></td>
<td><strong>Sonicated, 60 min</strong></td>
<td>--</td>
<td><strong>76%</strong></td>
</tr>
<tr>
<td>9</td>
<td>--</td>
<td>Sonicated, 60 min</td>
<td>--</td>
<td>18%</td>
</tr>
<tr>
<td>10</td>
<td>Duroquinone</td>
<td>Sonicated, 60 min</td>
<td>0.5 equiv oxidant</td>
<td>54%</td>
</tr>
<tr>
<td>11</td>
<td>2-Methylanthraquinone</td>
<td>Sonicated, 60 min</td>
<td>--</td>
<td>58%</td>
</tr>
<tr>
<td>12</td>
<td>Benzoquinone</td>
<td>Sonicated, 60 min</td>
<td>--</td>
<td>45%</td>
</tr>
<tr>
<td>13</td>
<td>Chloranil</td>
<td>Sonicated, 60 min</td>
<td>--</td>
<td>25%</td>
</tr>
<tr>
<td>14</td>
<td>Duroquinone</td>
<td>Sonicated, 60 min</td>
<td>1.25 equiv (TMP)$_2$CuLi</td>
<td>43%</td>
</tr>
<tr>
<td>15</td>
<td>Duroquinone</td>
<td>Sonicated, 60 min</td>
<td>0.5 equiv. (TMP)$_2$CuLi</td>
<td>50%</td>
</tr>
<tr>
<td>16</td>
<td>Duroquinone</td>
<td>Sonicated, 60 min</td>
<td>(TMP)$_2$Cu(CN)Li$_2$</td>
<td>25%</td>
</tr>
</tbody>
</table>
During the course of these reactions, an interesting observation was made. The reaction procedure (discussed in detail in Section 6.3) creates a THF solution of the amido-based cuprate then the pyrazine is slowly introduced. At this point, a suspension, presumably the aryl cuprate, would form. If this complex is only slightly soluble in THF, it could drastically impede the subsequent oxidation. To this end, several techniques were used to increase the solubility of this complex. The first tried was to increase the temperature of the oxidation step from 60°C to a mild reflux (Table 6.1, Entry 5). Interestingly, no product was obtained. Though not listed in Table 6.1, the reaction was run at lower concentration but had no improvement in yield. Another alternative came at the suggestion of Lipshutz and coworkers. They had noted that the introduction of several equivalents of N,N,N’,N’-tetramethylethylenediamine (TMEDA) serves as a lithium chelator to activate the complex for oxidation. This was attempted but no improvement was made (Table 6.1, Entry 6).

As a final attempt, the solution was sonicated for 10 minutes to break up the aggregated complex in order to increase solubility during oxidation. This lead to an increased yield of 56% (Table 6.1, Entry 7). With this step forward, the length of sonication was increased to 60 minutes. Remarkably, this technique provided a 76% yield of the bipyrazine ligand and 23% recovered pyrazine starting material (Table 6.1, Entry 8). As controls, the reaction was sonicated with no oxidant and only a 0.5 equivalent of oxidant yielding only 18% and 54% product, respectively (Table 6.1, Entries 9-10). This observation is quite intriguing given that in the previous case, when 1.2 equivalents of oxidant was used, 90% of the quinone was recovered. This phenomenon was also reported by Iyoda and co-workers. They report recovering 80-90% quinone with no hydroquinone observed during or after the reaction. The electron-transfer mechanism in this oxidation step remains unknown.
After the aggregation of the cuprate complex was resolved, the nature of both the oxidant and the cuprate were investigated further. The dependence on the potential of the oxidant used was probed by using several quinone derivatives. The potentials are listed in Table 6.2. The use of a weaker oxidant was shown to be quite successful however, a weaker oxidant, 2-methylanthaquinone, led to a decreased yield of 58% (Table 6.1, Entry 11). Shifting the potential in the other direction, the stronger oxidants benzoquinone and chloranil were used. In these reactions the yield fell off quickly, 45% and 25% respectively (Table 6.1, Entries 12-13).

As with Iyoda’s previously mentioned work, duroquinone appeared to be the best oxidant for the biaryl coupling of pyrazine. The nature of the cuprate was investigated first by increase the molar amount of the cuprate to 1.25 equivalents in hope of getting the 23% starting material previously recovered to react. The end result was a yield of 43% (Table 6.1, Entry 14). The amount of cuprate was then decreased to 0.5 equivalents, but gave only a 50% yield (Table 6.1, Entry 15). Lastly, after the oxidation procedure and the amounts of reagents had been optimized, the use of a Lipshutz cyanocuprate was reinvestigated. The cuprate was made in the same manner by introducing two equivalents of LiTMP to CuCN before the addition of the pyrazine. Nevertheless, the coupling only proceeded in a 25% yield. This evidence supports the earlier

<table>
<thead>
<tr>
<th>Compound</th>
<th>$E_{1/2}$ vs. Fe$^{3+}$/Fe (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Methylanthaquinone</td>
<td>-1.316</td>
</tr>
<tr>
<td>Duroquinone</td>
<td>-1.175</td>
</tr>
<tr>
<td>Benzoquinone</td>
<td>-0.851</td>
</tr>
<tr>
<td>Chloranil</td>
<td>-0.340</td>
</tr>
</tbody>
</table>
statement that the source of copper(I) can be critical to any particular coupling reaction. It is known that at temperatures of \(-78^\circ\text{C}\) and higher, the higher order Lipshutz-type cuprate disassociates into a lower order Gilman-type cuprate\(^{10,12}\) (Scheme 6.6). Given that in this work the reaction temperatures range from \(0^\circ\text{C}\) to room temperature, this suggests that the CuCN used in this coupling is merely a source of copper(I).

With the optimized conditions for the bipyrazine coupling in place, the final aspect was to scale up the synthesis. The reaction was run on a 40.0 mmol scale and the procedure was carried out in the same manner as the previously discussed optimized conditions (Table 6.1, Entry 8). The only deviation taken was the use of a Morton flask in order to maintain proper agitation and the sonication time was increased to 1.5 hrs to compensate for the increased reaction volume. This large scale synthesis yielded 2.0 grams (65\%) of bipyrazine product (Scheme 6.7).

\[
\text{Scheme 6.6: Temperature Dependent Cuprate Equilibrium}
\]

\[
\text{RR'Cu(CN)\text{Li}_2} \quad \text{High Temp} \quad \text{Low Temp} \quad \text{RR'CuLi + LiCN}
\]

\[
\text{R/R'} = \text{Aryl or Alkylithium}
\]
6.3 Conclusion

The bipyridine and bipyrazine based ruthenium catalysts have been used for several years by chemists to perform a variety photoredox transformations. Its use across the synthetic community is growing, including our research. However, limited availability of the bipyrazine ligand has been a fundamental hindrance towards our efforts. In order to solve this problem, we have developed a biaryl coupling technique that expands upon previously developed deprotonative metalation. Our reaction, utilizing an Earth abundant copper reagent, has provided the bipyrazine ligand in a 65-76% yield. This technique has also been shown to be successful on a multi-gram scale and is the first reported direct coupling of pyrazine to bipyrazine.
6.4 Experimental Procedure

6.4.1. General Information:

Tetrahydrofuran (THF) was dried by distilling over sodium and benzophenone. 2,2,6,6,-Tetramethylpiperidine (TMP) was dried over molecular sieves prior to use. All remaining reagents were purchased commercially and used without further purification.

6.4.2. Preparation of CuCl$_2$·TMEDA Complex:

Synthesis was taken and modified from previous literature procedure$^{13}$. In a flame dried 1000 mL round bottom flask, 23.86 g (140 mmol) Copper(II) Chloride dihydrate was suspended in 700 mL butanol and refluxed. 21 mL (140 mmol) N,N,N',N'-tetramethylethylenediamine (TMEDA) was dissolved in 50 mL butanol and added slowly to the refluxing copper solution. Refluxing continued for 15 minutes before cooling to room temperature. The resulting copper complex was precipitated with hexane, filtered and dried under vacuum. 74% yield (25.9 g) of CuCl$_2$·TMEDA was obtained.

6.4.3. Cuprate mediated synthesis of Bipyrazine

In a flame dried 25 mL round bottom flask, 500mg CuCl$_2$·TMEDA (2.0 mmol) was suspended in 5 mL THF and cooled to 0°C. The copper(II) suspension was reduced with the dropwise addition of 1.25 mL (2.0 mmol) n-Butyllithium solution (1.6M in hexanes) resulting in a light green Cu(I) solution. (Note: Butyllithium solution must be free of lithium salts. Also, it should be added slowly under sufficient stirring to avoid over reduction of copper).

In a separate, flame dried 10 mL round bottom, 0.63 mL (4.0 mmol) TMP was dissolved in 2 mL THF and cooled to 0°C. 1eq. n-Butyllithium was added before warming to room temperature.
The LiTMP was then added slowly to the Cu(I) mixture at 0°C yielding a dark yellow solution containing the necessary LiCu(TMP)$_2$ complex. 160 mg (2.0 mmol) pyrazine was dissolved in 1 mL THF before dropwise addition to the copper solution. Upon completion, the mixture was allowed to warm to room temperature and stir for 2 hours resulting in the formation of a dark brown precipitate. The cuprate suspension was oxidized with 394 mg (1.2 eq.) duroquinone while sonicated for 1 hour. The resulting mixture was quenched with water and extracted with dichloromethane. All organic layers were combined, dried over magnesium sulfate, filtered and solvent removed under reduced pressure. Crude product was purified by column chromatography using 1:1 Ethyl Acetate in Hexanes. 120 mg bipyrazine (76% yield, MW = 158.16 g/mol) was obtained with no further purification needed. $^1$H NMR: δ9.61 ppm (S, 2H), 8.76 ppm (S, 4H) Spectral data collected on product was consistent with previous literature$^{14}$. The included $^1$H NMR verifies the high purity of the product.

6.4.4. Large Scale Synthesis of Bipyrazine

In a flame dried 1000 mL Morton flask (to maintain sufficient agitation of reaction mixture), 10.0g CuCl$_2$·TMEDA (40.0 mmol) was suspended in 100 mL THF and cooled to 0°C. 25 mL (40.0 mmol) n-Butyllithium solution (1.6M in hexanes) was added dropwise yielding Cu(I) solution.

In a separate, flame dried 100 mL round bottom, 13.6 mL (80.0 mmol) TMP was dissolved in 40 mL THF and cooled to 0°C. 1eq. n-Butyllithium was added before warming to room temperature.

The LiTMP was then added slowly to the Cu(I) mixture at 0°C yielding a dark yellow solution containing the necessary LiCu(TMP)$_2$ complex. 3.2 g (40.0 mmol) pyrazine was dissolved in 10 mL THF before dropwise addition to the copper solution. Upon completion, the
mixture was allowed to warm to room temperature and stir for 2 hours. Oxidation occurred with the addition of 7.88 g (1.2 eq.) duroquinone and sonicated for 1.5 hours. The resulting mixture was quenched with water and extracted with dichloromethane. All organic layers were combined, dried, filtered and solvent removed in-vacuo. Crude product was purified by column chromatography using 1:1 Ethyl Acetate in Hexanes yielding 2.05 g bipyrazine (65%).
6.5 References


Chapter Seven

Application of Photoredox Catalysts to Oxidative Cyclizations

7.1 Introduction

The electrochemical formation of radical cations and their subsequent reactions has been a topic of study in our lab for many years.\textsuperscript{1,2} Taking advantage of the electrochemical set-up described in chapter 1, the radical cation intermediates have been generated from a wide variety of electron-rich olefins having a range of oxidation potentials. Much of the work has focused on understanding the mechanism of the oxidative cyclizations that follow radical cation formation. One notable study by Dr. John Campbell\textsuperscript{3} involved the competitive trapping of radical cations by two nucleophiles that were both tethered to the electron-rich olefin that was oxidized. Upon analyzing the products formed over a wide range of reaction conditions, the study revealed the mechanistic nature of alcohol and sulfonamide nucleophile trapping of the radical cations (Figure 7.1). The study concluded that alcohol nucleophiles trapped the radical cation in a fast, reversible manner. When the reactions were run under kinetic conditions, alcohol-trapping product were obtained. On the other hand, sulfonamide nucleophiles trapped the radical cation to generate the thermodynamic product. An additional study performed by Dr. Allison Redden\textsuperscript{4} revealed that the outcome of many radical cation reactions can be understood with the use of the Curtin-Hammett principle. As our understanding of the reactions improved, we began to ask if the method in which a radical cation is generated influences the subsequent mechanism of the cyclization and thus the products formed.
In an effort to answer this question and understand more about the nature of these oxidative cyclizations, we turned our attention toward the use of a photoredox catalysis to generate the radical cation intermediates.\(^5\,^6\) This technique utilizes the photophysical properties of a transition metal complex to produce a catalyst capable of undergoing electron transfer with a substrate. The general scheme for these photoredox reactions is outlined in Scheme 7.1\(^7\). A transition metal catalyst, typically ruthenium-based such as ruthenium(II) tris(bypyridine) chloride, is photoexcited from the ground state upon exposure to visible light. This photoexcited state is then capable of reducing or oxidizing a substrate to produce the corresponding radical.

*Figure 7.1: Energy Profile of Alcohol and Sulfonamide Cyclization*

The calculated energy profile of an alcohol/sulfonamide nucleophile competition shows that the sulfonamide cyclization is the thermodynamic product while the alcohol trapping is the kinetic product.
ion. The catalyst is then oxidized or reduced back to its original Ru\(^{2+}\) oxidation state by either the substrate or an additional “sacrificial” reagent. This technique has been utilized to perform a wide variety of radical induced transformations. An example of this type of transformation is illustrated in Scheme 7.2. The photoexcited ruthenium complex is reduced by the triethylamine additive to Ru\(^{1+}\). This allows that catalyst to achieve a higher reduction potential (Figure 7.1, -1.35 V vs -0.87 V) to reduce the α-bromo malonic ester. The malonic radical produced then cyclizes onto the indole, which is rearomatized upon oxidation by either the catalyst or the triethylamine radical cation. This example illustrates one of the many photoredox-mediated transformations.

In this chapter, we will discuss the application of photoredox catalysis to our nucleophilic cyclization substrates previously used in anodic oxidation studies.
Scheme 7.2: Example Mechanism of a Photoredox Reaction
7.2 Results and Discussion

Photoredox reactions were new territory for our group thus requiring the reproduction of a previously published reaction to verify our synthetic capabilities in this area. The reaction chosen was a cyclization accomplished by Stephenson and coworkers, consisting of the cyclization of a reduced α-bromo malonic ester onto indole\textsuperscript{8} discussed earlier. A DMF solution containing the cyclization substrate, the ruthenium catalyst, and triethylamine was illuminated with blue LEDs (450 nm) for 12 hours at room temperature. The resulting cyclized product was obtained in a 54\% yield (Scheme 7.3). Although the reported yield was 60\%, we were confident in our ability to perform the photoredox chemistry. Thus, we decided not to optimize the known reaction and began our investigation of photoredox generated radical cations.

\textbf{Scheme 7.3: Stephenson Malonic Ester Cyclization}

\begin{center}
\includegraphics[width=\textwidth]{Scheme_7.3.png}
\end{center}

7.7.1 Alcohol and Sulfonamide Nucleophiles

The first substrate analyzed under the photoredox conditions contained a ketene dithioacetal that was tethered to a sulfonamide nucleophile (Scheme 7.4). The substrate was dissolved in nitromethane, treated with the ruthenium tris(bipyridine) catalyst and air under anhydrous conditions, and illuminated for 16 hrs. In this case, oxygen was used as a sacrificial
oxidant for the reaction since the comparable electrochemical cyclization involved a net two electron oxidation of the substrate. Upon purifying the crude reaction mixture, an olefin cleavage product was obtained in 75% yield. This product was unexpected given that oxidation of the substrate had cleanly led to a cyclization product (Scheme 7.4) when the reaction was conducted with an electrolysis reaction. Of course, there were a number of changes to the reaction conditions that were associated with the use of the photoredox catalyst. The photochemical reaction was conducted in nitromethane due to the solubility of the various ruthenium catalysts used in this study. The electrochemical reaction had been run in methanol. In addition, the electrochemical reaction did benefit from the use of base to form a sulfonamide anion. The
addition of methanol to the photoredox initiated reaction did not alter the results. Methoxide was not added to the photochemical reaction because it would be expected to react with the catalyst.

As a control experiment, the photoredox-initiated reaction was repeated in the absence of oxygen. In this case, only starting material was recovered. Additional control experiments including the absence of light and ruthenium catalysts yielded only starting material. It is known that the presence of molecular oxygen aids in accelerating the rate of photoredox reaction, presumably by helping turnover the catalyst. However, in the case of the ketene dithioacetal substrate it played an additional role.

In order to help elucidate the mechanism of this transformation, an additional substrate was tested. To this end, the nucleophile tethered to the ketene dithioacetal was changed to an alcohol and the reaction was repeated. The alcohol trapping of ketene dithioacetal derived radical cations do not require the addition of base. As mentioned in the previous chapter, oxidation of the ketene dithioacetal in the absence of base requires an oxidation potential that is consistent with the use of the ruthenium tris(bipyrazine) catalyst. Upon illuminating the reaction solution in the presence of air for 16 hrs, a cyclic ether product was obtained in 67% yield (Figure 7.5). Although the substrate has cyclized in the same manner as the electrolysis product, the overall change in the molecules oxidation state was different. The electrolysis procedure produces a two-electron oxidized product but the cyclic product from this experiment was redox neutral. In other words, back electron-transfer from the reduced form of the catalyst to the substrate following the cyclization occurred faster than oxidation of the cyclic intermediate by oxygen.

The same control experiments were performed. When either the catalyst or light was excluded from the reaction, only starting material was recovered. However, in this case the
absence of oxygen afforded the same cyclic product but in a considerably higher yield of 83% (Figure 7.5). Notably, the reaction also took considerably longer (48 h) to reach completion.

**Scheme 7.5: Alcohol Nucleophile Photoredox Reaction**

![Scheme 7.5: Alcohol Nucleophile Photoredox Reaction](image)

Based on the data acquired thus far, we began to formulate a mechanism to explain the products observed from the two substrates studied (Figure 7.6). It appears that the photoexcited ruthenium complex does oxidize the substrate to the radical cation. The catalyst, now in the 1+ oxidation state is oxidized back to Ru$^{2+}$ by molecular oxygen. The superoxide radical anion produced then forms an intimate radical cation/radical anion pair with the oxidized substrate. It is at this point, where the nature of the nucleophile influences the product that is formed. If the
Scheme 7.6: Proposed Mechanism

Ru$^{2+}$ \text{hv} \rightarrow Ru^{2+*} 

O$_2$ 

Ru$^+$ 

O$_2^{--}$ 

\text{X} = \text{OH} \quad -H^+ 

\text{X} = \text{NHTs} 

\text{Retro [2+2] Cyclization} 

\text{Ketone NHTs}
nucleophile is an alcohol, the nucleophilic cyclization, previous shown by Dr. Campbell’s competition study to be fast but reversible\(^3\), breaks up the radical cation/ radical anion pair. Upon elimination of molecular oxygen and protonation, the observed product is formed.

Instead, if the nucleophile in the substrate is a sulfonamide, which is known to undergo a significantly slower cyclization\(^3\), the radical cation is not readily trapped. This allows time for the oxygen radical anion to add to the radical cation and then a retro [2+2] cyclization to afford the products from cleavage of the carbon-carbon double bond (Figure 7.6).

This mechanism is supported by the known characteristics of photoredox catalysts\(^5,6\) and the observations made when the reactions are performed in the absence of molecular oxygen. Similar to how the ruthenium catalyst reoxidized the indole substrate in the cyclization discussed earlier (Scheme 7.2), photoredox catalysts are capable of undergoing back electron transfer (BET) to an previously oxidized substrate\(^5,6\). In the case of our substrates, if molecular oxygen is not present to help recycle the catalyst and trap the radical cation of the substrate, the rate of

---

**Scheme 7.7: Photoredox Reactions in the Absence of Molecular Oxygen**

![Scheme 7.7: Photoredox Reactions in the Absence of Molecular Oxygen](image)
BET from the catalyst is greatly increased. In the event of the nucleophile being an alcohol (Scheme 7.7a), the nucleophilic cyclization is fast enough to compete with the BET resulting in the observed product. However, due to the increased rate of BET, the reaction time is dramatically longer. If the nucleophile was a sulfonamide (Scheme 7.7b), the cyclization is too slow to compete with the BET and the reaction leads to recovered starting material even when the reaction is conducted for long periods of time (48+ hours).

It is important to note that the reaction mixture must be completely anhydrous to achieve a high yield of product. This is not only due to the potential hydrolysis of the starting material or

Scheme 7.8: Photoredox Reaction in the Presence of Water

![Scheme 7.8: Photoredox Reaction in the Presence of Water](image-url)
dithiane products but altering the reaction scheme itself. If water is present in the reaction solution, we propose that it disrupts the radical cation/radical anion pair forming an epoxy-type intermediate (Scheme 7.8). Similar epoxy-type intermediates have been previously observed and synthetically utilized by Nicewicz and co-workers. If the epoxy-type intermediate contains an alcohol nucleophile, the rapid cyclization will open the epoxide forming a hydroxy dithiane. A second one electron oxidation and ring opening yields the thioester cyclized product in a 36% yield. Except for the hydrolysis of the starting material, this was the only product observed. The epoxy-type intermediated is believed to be in equilibrium with the superoxide counterpart. This is proposed based on the observed products (Scheme 7.8). Once again, other than hydrolyzed starting material the only product observed is the olefin cleavage product mentioned earlier but in a considerably lower yield of 26%. This suggests that the sulfonamide nucleophile is too slow to open the epoxide thus the equilibrium is drained towards the superoxide complex as the retro [2+2] cyclization cleaves the bond.

7.2.2 Carbon-Based Nucleophiles

The proposed mechanism to explain the observed products in the photoelectron transfer reactions requires the nucleophiles to trap the radical cations generated in a manner similar to that observed in the previous electrolysis experiments (sulfonamide cyclization is slow and alcohol cyclization is fast). If this is the case, then previously studied electrochemical cyclization reactions should provide us with a means of predicting the outcome of future photoelectron transfer initiated reactions. For example, a recent paper from our lab demonstrated the relative rates of cyclization for enol ether and allylsilane tapping of a ketene dithioacetal derived radical cation to generate a new carbon-carbon bond (Scheme 7.9). The results of this study revealed
that the enol ether cyclization was fast and the allylsilane was quite slow. In fact, when paired with the alcohol nucleophile, the enol ether out-competed the alcohol under all conditions. The allylsilane did not, and the competition study led to exclusive oxygen trapping under all conditions.

The study of these carbon-based nucleophiles using the photoredox catalysis was started but remains incomplete. Preliminary results suggest that the enol ether does successfully cyclize. This is as expected since we know that enol ether trapping of the radical cation is faster than alcohol trapping of the radical cation which is now known to be faster than trapping of the radical cation by the oxygen radical anion. To our knowledge, this is the first such trapping of a photoelectron transfer generated, ketene dithioacetal derived radical cation. Based on a private communication from the Stephenson group, we know it is not the first attempt. That attempt had used an allylsilane trapping group. However, we know that allylsilane trapping of the radical cation is much slower than the alcohol and enol ether. Does that reaction suffer from the same problems with oxygen radical anion trapping of the radical cation that we saw with the slower sulfonamide nucleophile? Work to answer this question is under way with Mr. Luis Gonzalez in

Scheme 7.9: Carbon-based Nucleophile Cyclization

![Scheme Diagram](image-url)
our lab. If this does turn out to be the case, then we will have further evidence that the extensive mechanistic studies conducted by our group on electrochemical oxidative cyclization reactions can serve as a guide not only for the design of future anodic transformations, but also for the design of future photoelectron-transfer based reactions.

### 7.3 Conclusion

The use of photoredox catalysts as a means of generating radical ions for organic synthesis has been under investigation for several years and is becoming a popular field of study\(^5\,^6\). This technique was paired with our electron-rich olefin substrates to compare the effects of how a radical is generated and add to our understanding of how they react. The characteristics of the alcohol and sulfonamide nucleophiles in anodic cyclization reactions appear to translate to the photoredox-initiated reactions. However, due to the more dynamic system and the capability of back electron transfer, the products obtained diverge. This provides an interesting route to obtain similarly cyclized products but with alternative functionalities based on the electron transfer method chosen.
7.4 Experiment Procedure

7.4.1 General Information

All reagents were purchased from commercial sources and used without further purification unless otherwise stated.

Methanol and nitromethane solvents were dried by distillation from magnesium sulfate and stored over activated molecular sieves.

Substrates were synthesized by following previously published procedures\textsuperscript{10}.

7.4.2 Photoreactor

The photoreactor used consisted of a 12 inch long (3 LEDs/inch) 12V high-density blue LED strip (450 nm output) purchased from Elemental LED\textsuperscript{®}, adhered to the inside of a 70 x 50mm recrystallizing dish. The reaction flask is suspended in the photoreactor as shown below.
### 7.4.3 Photoredox Reaction Procedure

The photoredox reactions were run using the following general procedure:

In a flame-dried 10 mL round bottom flask, 1 mmol substrate and 25-50 µmol Ruthenium catalyst were dissolved in 2 mL anhydrous solvent. The flask was filled with dry air and placed in the photoreactor. The reaction solution was stirred and illuminated overnight. Upon completion, the solvent was removed *in vacuo* and the crude mixture was purified via column chromatography.

\[
\begin{align*}
\text{H-NMR (300 MHz; CDCl}_3\text{): } & \delta 4.25 (s, 1H), 3.90 (\text{td, } J = 6.7, 2.0 \text{ Hz}, 2H), 2.89 (\text{dd, } J = 7.9, 3.1 \text{ Hz}, 4H), 2.23 (\text{dt, } J = 12.2, 8.1 \text{ Hz}, 1H), 2.11 (\text{dp, } J = 14.0, 3.4 \text{ Hz}, 1H), 1.97 (\text{p, } J = 6.7 \text{ Hz}, 2H), 1.87 (\text{dt, } J = 14.3, 7.1 \text{ Hz}, 1H), 1.74 (\text{td, } J = 12.4, 5.9 \text{ Hz}, 1H), 1.36 \text{ ppm (s, } 3H); \\
\text{C-NMR (75 MHz; CDCl}_3\text{): } & \delta 84.3, 68.3, 58.6, 35.6, 30.59, 30.50, 26.1, 25.8, 24.1 \text{ ppm; IR (KBr) } 2969, 2895, 1680, 1443, 1421, 1372, 1275, 1172, 1092, 1044, 783 \text{ cm}^{-1}; \text{HRESI MS m/z [M+Na]}^+ \text{ found: 227.0539, calculated: 227.0543.}
\end{align*}
\]
\[ \text{O} \quad \text{NHTs} \]

\[ \text{O} \quad \text{S} \]

\(^1\)H-NMR (300 MHz; CDCl\(_3\)): \( \delta \) 7.72 (d, \( J = 8.3 \) Hz, 2H), 7.30 (d, \( J = 8.0 \) Hz, 2H), 4.60 (t, \( J = 6.1 \) Hz, 1H), 2.95 (q, \( J = 6.5 \) Hz, 2H), 2.52 (t, \( J = 6.7 \) Hz, 2H), 2.42 (s, 3H), 2.12 (s, 3H), 1.74 ppm (p, \( J = 6.7 \) Hz, 2H); \(^{13}\)C-NMR (75 MHz; CDCl\(_3\)): \( \delta \) 208.5, 143.3, 136.9, 129.7, 127.0, 42.5, 40.2, 30.0, 23.2, 21.5 ppm; IR (KBr) 3512, 3284, 2928, 2872, 1709, 1596, 1425, 1358, 1322, 1221, 1091 cm\(^{-1}\); HRESI MS m/z [M+Na]\(^+\) found: 278.0813, calculated: 278.0829.

\[ \text{O} \quad \text{S} \]

\[^1\)H-NMR (300 MHz; CDCl\(_3\)): \( \delta \) 4.02 (t, \( J = 6.6 \) Hz, 2H), 2.91-2.86 (t, \( J = 7.3 \) Hz, 2H), 2.82-2.69 (m, \( J = 3.8 \) Hz, 4H), 2.31-2.23 (m, 1H), 2.10 (pd, \( J = 6.5 \), 2.2 Hz, 1H), 2.01-1.88 (m, 4H), 1.87-1.77 (m, 1H), 1.42 ppm (s, 3H); \(^{13}\)C-NMR (75 MHz; CDCl\(_3\)): \( \delta \) 206.7, 89.4, 69.5, 37.4, 36.98, 36.79, 28.8, 28.1, 26.8, 25.6, 24.6 ppm; IR (KBr) 2975, 2928, 2883, 2249, 1681, 1443, 1366, 1251, 1115, 1045, 731 cm\(^{-1}\); HRESI MS m/z [M+Na]\(^+\) found: 243.0492, calculated: 243.0495.
7.5 References


Chapter Eight
Conclusions and Future Directions

Over the course of this research, we have demonstrated the capabilities of utilizing microelectrode arrays to build molecular libraries and detect biological binding events in “real-time”. We successfully demonstrated that the surface can be selectively and reversibly modified by utilizing a technique for electrochemically generating acid. This work culminated in the development of a tunable polymer surface for analyzing molecular libraries. Additionally, it was discovered that the widely successful copper-mediated Chan-Lam coupling could be translated to the arrays to chemoselectively couple peptides to the polymer surface. This novel approach to building molecular libraries allows us to couple peptides through a thiol or alcohol without protecting the N-terminus. One caveat of this study is the lack of selectivity between the thiol and alcohol nucleophiles. One aspect that would be advantageous to investigate further is the ligand dependence on the selectivity between the two nucleophiles.

The culmination of the work to build a molecular library on a microelectrode array is to monitor the binding event between the members of the library and a biological target. As mentioned in Chapter 3, one characteristic of this microelectrode array-based system is the possibility of a multidentate effect when binding to a biological target. An initial experiment towards studying this effect was developing a peptide gradient on the surface of the array. The approach used depended on the reaction time to govern the amount of peptide coupled to the array followed by a separate reaction to quantify the negative space left from the unreacted polymer. In hindsight, a more beneficial method may consist of performing the coupling reaction
with a predetermined mixture of two molecules. By utilizing the same functional group on both compounds, the ratio in solution should reflect that on the polymer surface. This could provide a more accurate way of developing a peptide gradient on the array surface.

One of the many accomplishments of the work discussed in this thesis is the successful assessment of the human and murine VEGF protein binding against the v107 peptide. The micromolar binding of v107 with human VEGF was confirmed using the “real time” technique outlined in Chapter 1. Additionally, the murine VEGF binding to the v107 peptide was successfully detected at a slightly stronger binding constant of ~300 nM. The next major step in this study is investigating analogues of v107 in order to increase the binding affinity. An additional study that may prove to be beneficial in understanding the intricacies of the v107/VEGF system involves the kinetics of the binding interaction. As discussed in Chapter 5, the current measured from recycling the redox mediator will quickly stabilize by collecting consecutive CVs. If this technique were to be applied when the VEGF protein is introduced to a functionalized array, the rate at which the current stabilized may provide information toward the kinetics of the protein binding.