Spring 4-15-2019

Developing a synthetic model of the Candida albicans cell wall using Self-Assembled Monolayers to host Beta-glucan as ligands

Ushnik Ghosh
University of New Mexico

Ushnik Ghosh
Candidate

Biomedical Engineering
Department

This thesis is approved, and it is acceptable in quality and form for publication:

Approved by the Thesis Committee:

Aaron Neumann , Chairperson

David Whitten

Linnea Ista

DEVELOPING A SYNTHETIC MODEL OF THE CANDIDA ALBICANS CELL WALL USING SELF-ASSEMBLED MONOLAYERS TO HOST BETA GLUCAN AS LIGANDS

by

USHNIK GHOSH

B.S. BIOMEDICAL ENGINEERING, GEORGIA INSTITUTE OF TECHNOLOGY, 2013

THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Master's of Science
Biomedical Engineering

The University of New Mexico
Albuquerque, New Mexico

May 2019

ACKNOWLEDGEMENTS

I would like to thank Dr. Aaron Neumann for mentoring and giving guidance to me throughout the research that is performed in this thesis. His oversight has been invaluable to the steady progression of research that has been conducted in the two years I have been a Master's Degree student. I would also like to thank Dr. Dave Whitten and Dr. Linnea Ista for accepting the position of committee members for my thesis defense. I would like to thank the students in the Neumann Laboratory group, with whom I have worked alongside studying *Candida albicans*. I would also like to thank the University of New Mexico, specifically the Department of Biomedical Engineering and the Department of Pathology.

DEVELOPING A SYNTHETIC MODEL OF THE CANDIDA ALBICANS CELL WALL USING SELF-ASSEMBLED MONOLAYERS TO HOST BETA GLUCAN AS LIGANDS

by

Ushnik Ghosh

B.S. Biomedical Engineering, Georgia Institute of Techonology, 2013
M.S. Biomedical Engineering, University of New Mexico, 2019

ABSTRACT

The goal of this investigation is to study mechanisms the immune cell receptor, Dectin-1, uses to identify the fungal cell species, *Candida albicans*. Dectin-1 identifies extracellular matrix polysaccharides that *Candida albicans* express known as β-glucan. To study the interaction of β-glucan – Dectin-1 at the nanoscopic scale, the investigators of this project have endeavored to model the *Candida albicans* cell wall with engineered Self-Assembled Monolayers presenting β-glucan as a ligand for immobilized Dectin-1 receptors. By engineering a simplified ex-vivo model of the *Candida albicans* cell wall, the investigators of this study aim to gain precise control of the composition and structure of the delicate embedded nanostructured supra-molecular assemblies that define the cell wall of *Candida albicans*. The investigator has proposed developing the synthetic model of the cell wall using Self-Assembled Monolayers and Biotin-Streptavidin chemistry to host β-glucan polysaccharides as ligands.

Table of Contents

List of Figures

List of Tables

1.0. Introduction.

The goal of this investigation is to study mechanisms the immune cell receptor, Dectin-1, uses to identify the fungal cell species, *Candida albicans*. Dectin-1 identifies extracellular matrix polysaccharides that *Candida albicans* express known as β-glucan [1]. The challenge presented in this investigation is that *Candida albicans* incorporates a masking mechanism that conceals the β-glucan on its surface, and enables it to evade the Dectin-1 identification that can trigger immune responses [2]. This masking mechanism can cause diseases generated by *Candida albicans* to evade immune response. The effects of this β-glucan masking on the *Candida albicans* cell walls is manifested with intermittent expression of β-glucan in an abundance of mannan [1].

Studying the individual recognition events taking place is a difficult challenge given the complex composition of the *Candida albicans* cell surface. To study the cell-signaling of β-glucan – Dectin-1 interactions, the investigators of this project have endeavored to model the *Candida albicans* cell wall with engineered Self-Assembled Monolayers presenting β-glucan as a ligand for immobilized Dectin-1 receptors. In order to reduce the experimental variables inherent in studying a biological system, the major challenge of this investigation was to artificially model the cell wall of the *Candida albicans*.

Alkanethiols on gold and Silane functionalized PolyEthylene Glycol chains on glass is in many ways similar to the cell wall in that the Self-Assembled Monolayer can be engineered to have embedded nanostructured supramolecular assemblies such as proteins,

glycoproteins, polysaccharides on its surface. Previous studies [3, 4] have demonstrated that it is possible for Self-Assembled Monolayers made with Alkanethiols or Silane to be functionalized by attaching ligands to the ends of the Alkanethiols or Silanes. The embedded ligand nanostructures are formed by self-assembly, which makes the engineered model an authentic representative of the biological system. By engineering a simplified ex-vivo model of the *Candida albicans* cell wall, the investigators of this study aim to gain precise control of the composition and structure of the delicate embedded nanostructured supramolecular assemblies that define the cell wall of *Candida albicans*. The goal of this overarching investigation is to develop a synthetic model of the *Candida albicans* cell wall with the ability to precisely modulate the β-glucan densities for studying β-glucan – Dectin-1/Immune Cell interactions. This investigation will be conducted in two phases: Phase 1: Develop Self-Assembled Monolayers and develop a molecular complex to host β-glucan as ligands, and future Phase 2 studies: Conduct β-glucan + Dectin-1/Immune Cell interaction studies using the Self-Assembled Monolayer and molecular complex.

This thesis will discuss the Phase 1 of this investigation. The objective of Phase 1 is to develop a mixed Self-Assembled Monolayer consisting of HS-$(CH_2)_{11}$-EG_6-OH and HS-$(CH_2)_{11}$-EG_6-Biotin alkanethiols onto lithographed gold substrates. The investigator was able to demonstrate a dose-dependent response for surface Biotin to Streptavidin ratio as the concentration of HS-$(CH_2)_{11}$-EG_6-Biotin was increased from 0-60% and as Streptavidin concentration remained constant. However, clustering of Streptavidin to the Self-Assembled Monolayers with Biotin molecules, proportionate to Biotin

concentrations, was observed. Since the goal of the experiment is to develop a substrate that would enable the investigator to precisely control the concentration and homogeneity of the presented β-glucan ligand, the model was changed from Alkanethiol to Silane Self Assembled Monolayers.

The investigator was able to reduce the non-specific binding and clustering of Neutravidin molecules using the Silane Self-Assembled Monolayer. The Silane Self-Assembled Monolayer was composed of Silane mPEG and Silane-PEG-Biotin. Similarly to the alkanethiol based Self-Assembled Monolayers, the percentage of Silane-PEG-Biotin was modulated to study the differences in Neutravidin fluorescence density and intensity. The investigator has determined the necessary concentrations of Neutravidin, Silane-PEG-Biotin, and Silane mPEG that were needed to develop a substrate with homogeneous Biotin-Neutravidin binding without non-specific binding or clustering. The goal of developing the first stages of a homogeneous substrate were successful when Neutravidin was immobilized at a concentration of 4-8 μg/ml, on a 5-10% range of Silane-PEG-Biotin Self-Assembled Monolayers. Silane Self-Assembled Monolayers with higher percentage of Silane-PEG-Biotin and higher concentrations of Neutravidin formed clusters, which is an indication of non-specific binding between Biotin-Neutravidin.

The ultimate goal of developing this substrate model is to host β-glucan polysaccharides at varying densities on the Self-Assembled Monolayer. Towards this goal, Biotin-PEG-Cy5 is used as an analog to study the binding properties of the Biotin-Neutravidin complex. The Neutravidin with Oregon Green 488 fluorescent tag and Biotin-PEG-Cy5

enabled the investigator to study the binding ratios of the two molecules as Silane-PEG-Biotin and Silane mPEG ratios were modulated. A pilot Experiment is currently being conducted and preliminary results yield co-localized Silane-PEG-Biotin, Neutravidin, and Biotin-PEG-Cy5 binding. This indicates that the Silane Self-Assembled Monolayer based model can be used to successfully modulate densities of the ligand that is presented for future Phase 2 studies.

1.1. Background.

Candida albicans is a common human pathogen fungal cell that causes fungal infections, otherwise known as Candidiasis. Candidiasis affects ~60000 patients every year in the U.S., has a 30-40% mortality rate, and costs $2-4 billion in care [5, 6]. Dendritic Cells provide the primary means of innate immune defense against *Candida albicans.* The fungal cell wall of *Candida albicans* express the polysaccharides mannan, β-glucan, and chitin [7, 8] (Figure 1). Dendritic Cells use the C-type lectins, DC-SIGN and CD206 to bind to mannan, and Dectin-1 to recognize β-glucan [1].

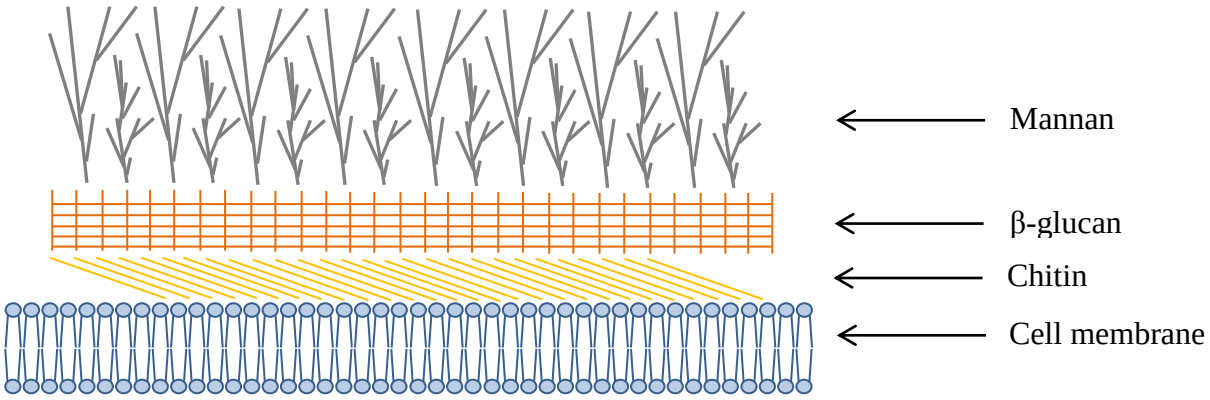


Figure 1: Cell wall of *Candida albicans.*

Previous studies have demonstrated that the spatial organization of the polysaccharide ligands play an important role in Dendritic Cell immune recognition or evation [9, 10]. Dentritic Cells express the immune receptor, Dectin-1, on its cell membrane which detects β-glucans on *Candida albicans.* Dectin-1 has a HemITAM (hemi-immunoreceptor tyrosine-based activation motif) domain, and when the Dectin-1 receptor makes contact with a Beta-Glucan molecule, the HemITAM domain undergoes phosphorylation [11]. In cases where the two HemITAM domain of two Dectin-1 receptors in close proximity un-

dergo phosphorylation, Spleen Tyrosine Kinase (Syk) can bind to the phosphate groups, thus initiating downstream signaling processes that activates a wide range of immune responses that cause the immune cell to activate its immune responses (Figure 2). The downstream signaling processes that mediate immune cell response are not observed when isolated Dectin-1 receptors, with no other Dectin-1 receptors in close proximity, bind to β-glucan. This is because the Syk protein only triggers downstream signaling processes when both of its binding sites are occupied by Phosphate groups.

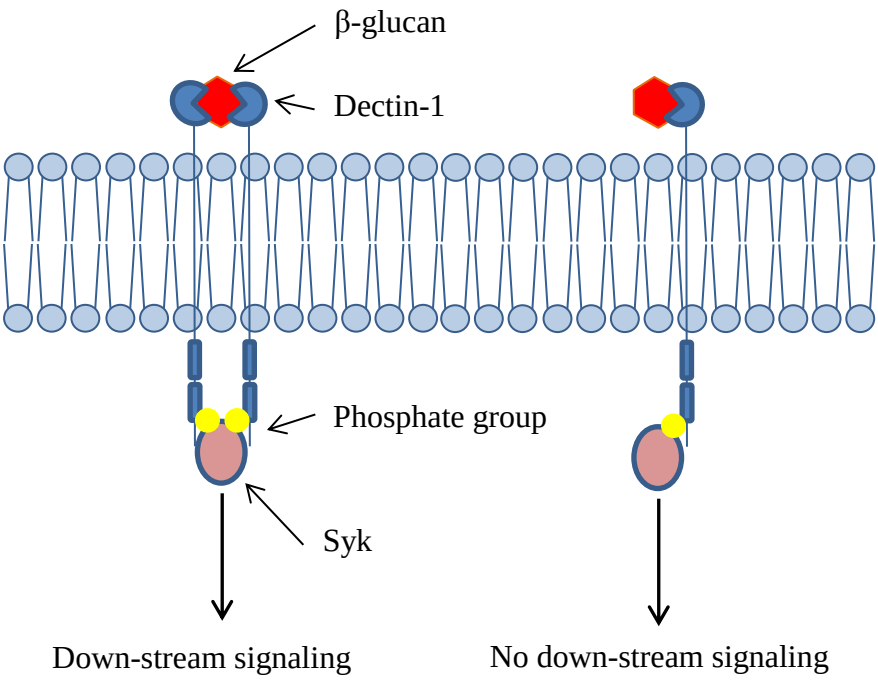


Figure 2: Immune Cell – fungal cell interaction and Immune Cell cell-signaling process.

This cellular process motivates the investigators to study the role the density of β-glucan can have on Immune Cell and Dectin-1 response. The investigator would like test the hypothesis of if the density of β-glucan expression on *Candida albicans* plays a role in Dectin-1 identification and Syk activation. The basis for this hypothesis is that if the number

of Dectin-1 receptors of the immune cell are held constant, the magnitude of the down-stream signaling processing initiated by Syk will be greater for the Dectin-1 receptors binding to the higher density β-glucan ligands (Figure 3).

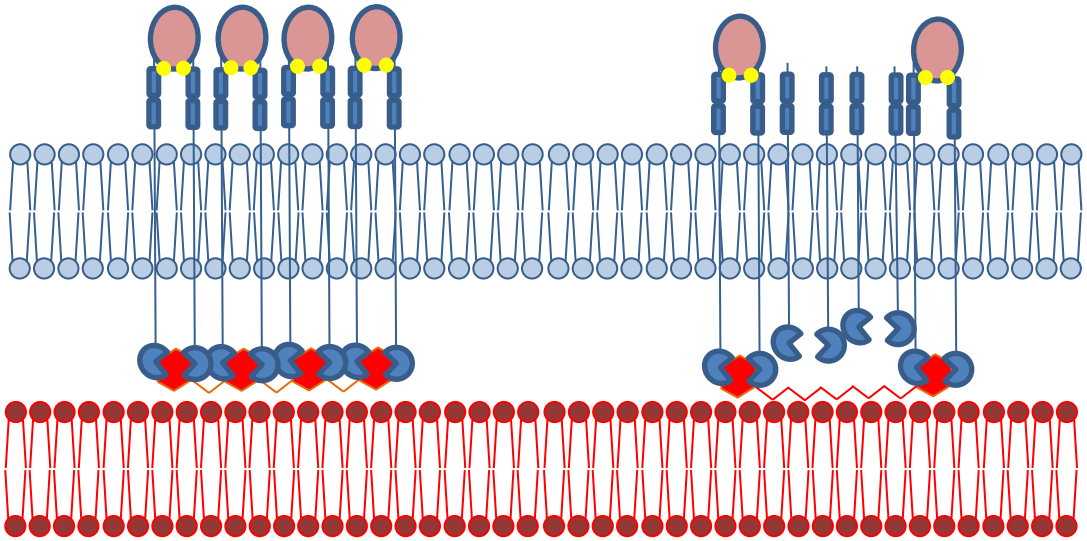


Figure 3: The magnitude of down-stream cellular processes initiated by Syk will be greater for Dectin-1 receptors binding to higher density Beta-Glucan ligands.

Towards the goal of elucidating this mechanistic basis of *Candida albicans* recognition by Dectin-1, the investigator will develop a synthetic model of the cell wall of the *Candida albicans*, and design it such that the β-glucan ligand density can be precisely controlled. Future studies investigating the Dectin-1 and Immune Cell responses to the synthetic substrates with varying densities of β-glucan can enable investigators to test this hypothesis.

Engineered Self-Assembled Monolayers can be used to model the cell wall of the *Candida albicans* cell for investigations seeking to study the binding interactions taking place

between β-glucan – Dectin-1 in a reduced variable, environment. This reduced variable environment can provide the investigators a greater level of acumen for determining the mechanisms of β-glucan – Dectin-1interactions. By developing a system that can enable a better elucidation process for studying the mechanisms of β-glucan – Dectin-1 identification, avenues for developing novel Candidiasis pharmaceutical therapeutics that incorporate the knowledge of β-glucan nanostructures can be furthered. Candidiasis affects ~60000 U.S. patients annually, cost $2-4 billion in care and inflicts 30-40% mortality [12].

1.2. Criteria and constraints.

Criteria:

1. Host β-glucan polysaccharides on Self-Assembled Monolayers
2. Be able to precisely modulate density of β-glucan on Self-Assembled Monolayers.

Constraints:

1. β-glucan ligands must be uniform and homogeneous throughout the substrate.
2. Density of β-glucan ligands must be quantifiable using Confocal Fluorescent Microscopy.

2.0. Develop Self-Assembled Monolayers.

The objective of Phase 1 of this investigation is to develop a mixed Self-Assembled Monolayer consisting of HS-$(CH_2)_{11}$-EG_6-OH and HS-$(CH_2)_{11}$-EG_6-Biotin alkanethiols onto gold substrates (Figure 4) and Silane Self-Assembled Monolayers consisting of Silane-PEG-Biotin and Silane mPEG onto glass substrates (Figure 5). Section 2.1 discusses the parameters that will be taken into consideration when the Self-Assembled Monolayers are being developed: 1) Substrate structure, 2) Cleanliness of substrate/Oxidation state, 3) Alkane chain length, 4) Non-specific protein adsorption, 5) Purity of thiols for Alkanethiols, 6) Solvent, 7) Concentration of Alkanethiol/Silane, 8) Time length of immersion, 9) Temperature. The procedure presented in Section 2.0.2. incorporates the parametric requirements for Self-Assembled Monolayer development discussed in Section 2.1, and are congruent with procedures used in the literature.

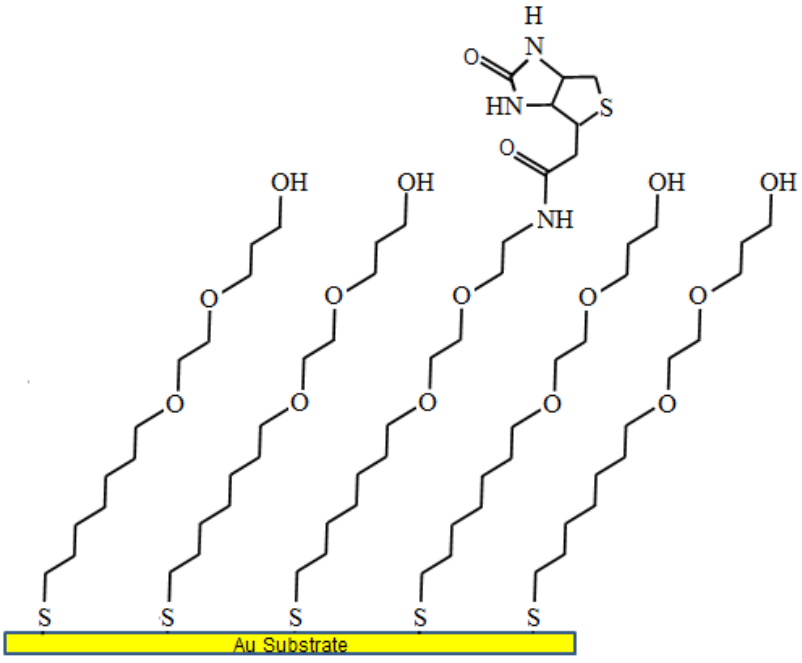


Figure 4: A mixed Self-Assembled Monolayer of HS-(CH2)11-EG6-OH and HS-(CH2)11-EG6-Biotin.

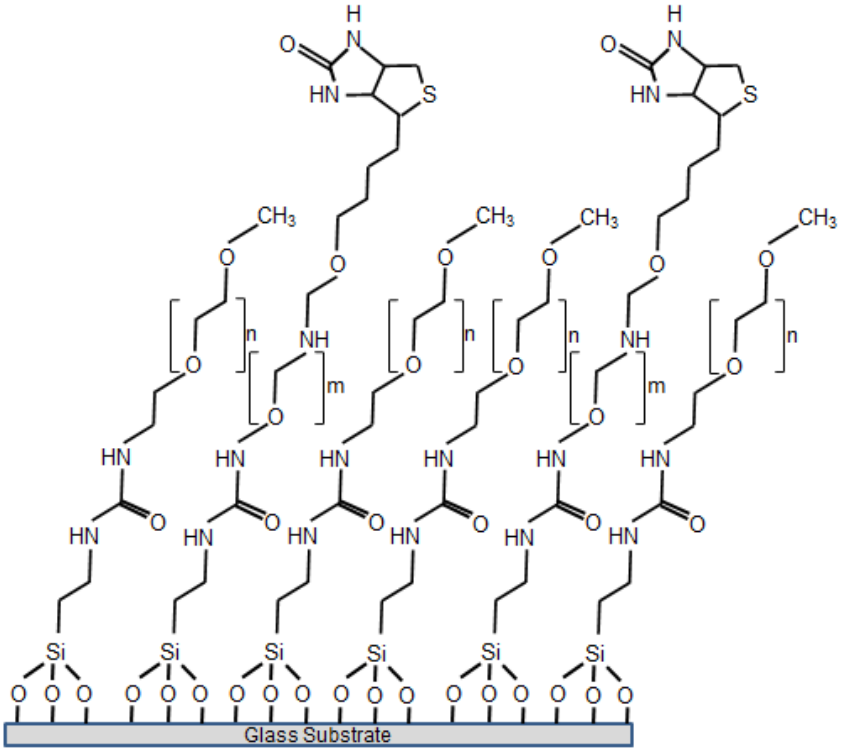


Figure 5: A mixed Silane Self-Assembled Monolayer composed of Silane mPEG and Silane-PEG-Biotin.

2.0.1. Defining the parameters for developing Self-Assembled Monolayers.

Substrate structure: The most common type of substrate used to develop Self-Assembled Monolayers of Alkanethiols is Gold that is deposited onto glass slides via Physical or Chemical Vapor deposition. Gold films (~15nm thick) are deposited on glass slides are typically used as substrates for Biological investigations [13]. Silane Self-Assembled Monolayers are developed on glass substrates.

Cleanliness of substrate/Oxidation state: Developing reproducible Self-Assembled Monolayers is observed when the substrate is oxidized within 1 hour of preparation via UV ozone plasma treatment [13]. For Alkanethiols, substrates that are pretreated to have an oxidized state demonstrate stronger individual Gold-Sulfur bonds [14]. Self-Assembled Monolayers that are developed at low pH conditions have a higher proportion of Gold-Sulfur coordinate bonds than Self-Assembled Monolayers that are developed at higher pH conditions, where the Gold-Sulfur bonds become more covalent. Silane Self-Assembled Monolayers are formed with Oxygen bonding to the glass substrate and do not require surface oxidation.

Alkane chain length: The properties of alkane chains of Self-Assembled Monolayers change with as the *n* (the length) of $(CH_2)_nCH_3$ increases. As *n* increases the structure is expected to become less flexible; Self-Assembled Monolayers with longer *n* assemble in a crystalline-like state, where Self Assembled Monolayers with short *n* assemble in a more disordered state. This is attributed to stronger van der Waals interactions in the alkane chains of the SAMs with longer *n* [15]. Raman Shift profiles analyzing low

frequency vibration (<500cm^{-1}) where Gold-Sulfur bond stretch occurs demonstrate increasing vibration with increasing *n*. Although the change in magnitude is very little, this indicates that there is very little differences in chain length vibration as *n* increases [16]. Measurements of angles at which alkane chains bend normal to the substrate are measured for varying length of n as a droplet of water is put on top of the SAMs, demonstrate very little change in angles with increasing *n* [17]. Alkane chains tilt approximately 30° normal to the surface [18]. Taken altogether, developing Self-Assembled Monolayers with long alkane chains will yield a more structured and dense monolayer that will have little disadvantage of bending and vibration of the alkane chains.

Non-specific protein adsorption: Alkane chains with Poly-Ethylene Glycol (PEG) chains (with length of 6) that have the ability to resist non-specific adsorption of proteins will be used to form Self-Assembled Monolayers. The ability of the substrate to resist adsorption from proteins increases with both density and length of the Poly-Ethylene Glycol group and is attributed to “steric stabilization”. Ellipsometry and Surface Plasmon Resonance spectroscopy studies have demonstrated that the Poly(ethylene glycol) chains in alkanes of Self-Assembled Monolayers are responsible for resisting adsorption of protein [18].

Purity of thiols: Oxidation can cause Sulfhydrl groups at the terminated ends of the Alkane chains to become Di-sulfide bonds, thus reducing the quantity of Au-S bonds. Degassing the solvent with inert gas prior to preparing an Alkanethiol solution improves the reproducibility of the procedure. Sulfhydryl groups in the Self-Assembled Monolayers get oxidized 1-7 days once they are exposed to ambient temperature [13].

Silane groups are less prone to oxidation, and thus have less demanding requirements for storage and use.

Solvent: The most commonly used solvent for preparing Self-Assembled Monolayers is a 1-10mM ethanol solution with thiols/silane. Ethanol is widely used because it solvates a variety of alkanethiols, it is inexpensive, it is available in high purity, and it has low toxicity. Non-polar solvents promote densely packed monolayers [13].

Concentration of Alkanes: 1-10mM of alkanethiols/silanes in ethanol solution yield Self-Assembled Monolayers with an average density of $4.5*10^{14}$ molecules/cm^2 [13]. Concentration of solution and the time length of immersion are inversely related to one another.

Time length of immersion: Most commonly used time immersion lengths used range from 12 hours to 18 hours. Dense coverages of alkanethiols on the gold substrate form in the first minutes of the immersion; a second slower reorganization process that maximize density of molecules and minimize defects in the Self-Assembled Monolayer takes place in a time-span on the order of hours [13]. Self-Assembled Monolayers developed through an immersion period of 12-18 hours were stronger than those that were developed over 1-7days. Silane Self-Assembled Monolayers have a similar process of organization, take less time to develop, and are formed in 30 minutes in PEGylation solution.
Temperature: Room temperature (25°C) is most frequently used. Elevated temperatures increate rate of desorption [13].

2.0.2. Procedure:

Alkanethiol Self-Assembled Monolayers:

Substrate preparation: Gold will be deposited onto glass slides using Physical Vapor deposition. Gold substrates will undergo UV ozone plasma treatment for 1 hour prior to being immersed in alkanethiol solution. 10mM Alkanethiol in ethanol solutions will be deoxygenated with inert gas (nitrogen) to minimize oxidation of Sulfhydryl groups. Oxidized gold substrates will be immersed in 10mM alkanethiol-ethanol solution for 18 hours at 25°C [13]. The effectiveness of a final rinse and dry step will be assessed. Contact angle Goniometry will be used to characterize Self-Assembled Monolayers.

Silane Self-Assembled Monolayers:

Sterile glass bottom dishes will be used to develop Silane-Self-Assembled Monolayers. Silane mPEG and Silane-PEG-Biotin molecules will be immersed in 95% Ethanol – 5% water solution at 13mg/ml concentration to form a PEGylation solution. The PEGylation solution will be put onto the glass bottom dishes, where it will form the Silane Self-Assembled Monolayers in agitated conditions for 30 minutes in room temperature.

2.1. Developing molecular complex to host β-glucan as ligand on Self-Assembled Monolayers.

With the goal of gaining precise control of the density of the β-glucan ligand presentation, the potential for using different Alkanethiols have been assessed. For each Alkanethiol, the investigator and the Principal Investigator have taken into account the chemical reactions that would be needed to host β-glucans on Self-Assembled Monolayers. The following Alkanethiols and reactions for developing the Self-Assembled Monolayers were considered: HS-$(CH_2)_{11}$-NH-C(O)-Biotin, HS-$(CH_2)_m$-COO-NHS, and HS-(CH2)m-COOH.

HS-$(CH_2)_{11}$-NH-C(O)-Biotin and Silane-PEG-Biotin: This Biotin terminated Alkanethiol can be used to bind Streptavidin to its surface[19, 20]. Biotin-Streptavidin chemistry is used widely in protein chip technology. This reaction is very convenient, as Streptavidin has a very high affinity for Biotin, and is one of the strongest non-covalent interactions known in nature. Streptavidin has 4 binding sites. The Biotin-terminated alkanethiols would occupy one binding site, leaving 3 binding sites available for 3 more biotin molecules. Thus the Biotin-Streptavidin complex has high versatility in that it can enable the investigator to host up to 4 different reactions using the same chemistry. A disadvantage of using this alkanethiol is that it is very expensive.

HS-$(CH_2)_m$-COO-NHS and Silane-PEG-NHS: This NHS terminated alkanethiol can be used to form an amide bond between the NHS ester group and free amino group of the molecule to be attached. The NHS ester coupling reaction is easy to carry out and has

excellent yield. Using this protocol would result in a Self-Assembled Monolayer with top layer of reactive NHS ester. In a typical NHS ester reaction, the NHS terminated Self-Assembled Monolayer would be immersed in a Methylene Chloride solution the molecule with a free amino group. The primary disadvantage of pursuing this reaction is that NHS esters will hydrolyze if there is any water in the solution. Self-Assembled Monolayers develop in ethanol solutions, which is very hydroscopic. This would require developing the Self-Assembled Monolayer in a dry and desiccated environment.

HS-(CH2)m-COOH and Silane-PEG-COOH: This Carboxyl-terminated alkanethiol is used widely throughout Self-Assembled Monolayer development literature for a wide range of applications [21]. The Carboxyl group can be coupled to a molecule containing a free amine group. The 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide is the carboxyl activating agent and yield is amide bonds. This alkanethiol and reaction can be used to covalently couple Streptavidin to the top of the Self-Assembled Monolayer.

For this project, the investigator has selected the HS-$(CH_2)_{11}$-NH-C(O)-Biotin (for Alkanethiol Self-Assembled Monolayers) and Silane-PEG-Biotin molecules (for Silane Self-Assembled Monolayers) for hosting Biotin-Streptavidin chemistry. This reaction has two primary advantages, its convenience, and in Streptavidin's ability to host multiple biotinylated molecules with high affinity.

A Biotin-Streptavidin complex will be used to host the β-glucan + Dectin-1/Immune Cell interactions (Figure 6). The mixed Alkanethiol Self-Assembled Monolayer will expose biotin at the surface as a terminal functional group which will act as a ligand to form a

complex with Streptavidin. Streptavidin can then form a complex with up to three other biotin terminated molecules [22]. The remaining three binding sites of Streptavidin can be used for hosting β-glucan – Dectin-1 interactions for cell-signaling studies and/or biotinylated fluorescent molecules for imaging studies. Parameters such as the β-glucan presentation density, as well as the ratio of $HS\text{-}(CH_2)_{11}\text{-}EG_6\text{-}OH$ and $HS\text{-}(CH_2)_{11}\text{-}EG_6$-Biotin alkanethiols of the Self-Assembled Monolayer will be modulated to study associated changes in β-glucan density. Previous studies in the literature [3, 4] also perform similar capture ligand immobilization methods that modulate the selectivity of the immobilized protein. They highlight the importance of presenting the proteins in a well-defined environment (addressing factors such as steric hindrance).

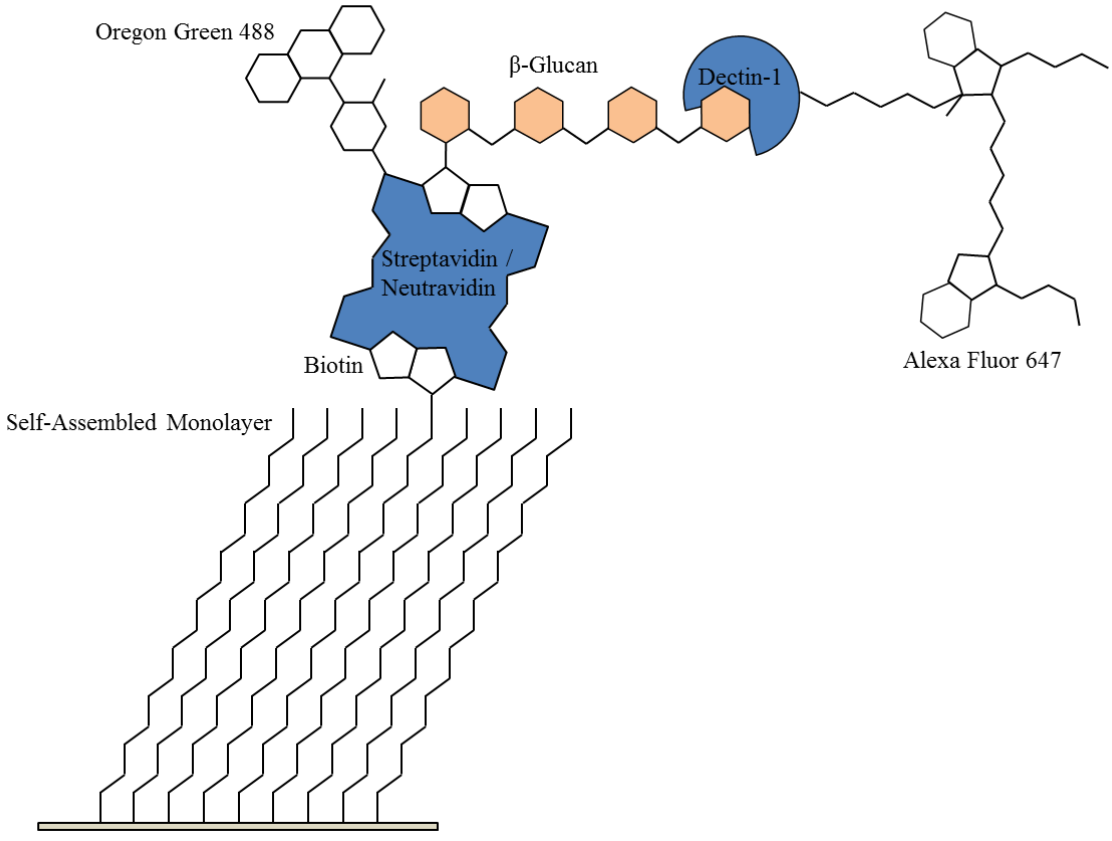


Figure 6: Proposed Self-Assembled Monolayer with Biotin-Streptavidin complex hosting β-glucan.

2.1.1. Defining the criteria for developing Self-Assembled Monolayer & β-glucan complex.

Nonspecific Protein adsorption: A requirement of this study will be to prevent the non-specific adsorption of Dectin-1 with the substrate, and ensure that the Dectin-1 interacts with only the β-glucan ligands thus ensuring significant β-glucan – Dectin-1 interactions. Self-Assembled Monolayers with alkane chains containing Poly-Ethylene Glycol chains are able to fulfill this requirement [18]. This criterion is also discussed for Phase 1 parameters (Section 2.0.1.).

Prevent non-specific binding with blocking buffers: The effectiveness of blocking non-specific Streptavidin, Neutravidin, and Biotin-PEG-Cy5 to Silane Self-Assembled Monolayers will be assessed using 5% BSA, 5% FBS, 5% Milk, and Tween-20 buffers. These blocking buffers are used widely in Biochemistry investigations [23].

Modulating HS-$(CH_2)_{11}$-EG_6-OH and HS-$(CH_2)_{11}$-EG_6-Biotin ratios: The density of the β-glucan ligands presented to the surface of the Alkanethiol Self-Assembled Monolayer will be achieved by modulating the ratios of HS-$(CH_2)_{11}$-EG_6-OH and HS-$(CH_2)_{11}$-EG_6-Biotin alkanethiol chains. For example if the investigators wish for 10% of the Alkanethiol chains in the Self-Assembled Monolayer to present β-glucan as a ligand, 10% of the Self-Assembled Monolayer solution will contain β-glucan terminated Alkanethiol chains, and 90% of the Self-Assembled Monolayer solution will contain hydroxide terminated Alkanethiol chains. A specific molar ratio between the Biotin, Streptavidin, and β-glucan will be established. The end result will be a mixed Self-Assembled

Monolayer with intermittent expression of β-glucan similar to that of a *Candida albicans* cell.

Alkane chain length: The length of alkanethiol chains forming the Self-Assembled Monolayers plays a role in determining how well β-glucan can be presented for Dectin-1 immobilization. The alkane chains HS-$(CH_2)_{11}$-EG_6-OH and HS-$(CH_2)_{11}$-EG_6-Biotin are almost identical, with the terminal group being the only difference. This identical chain length can cause steric hindrance during the Streptavidin binding to the Biotin on the surface of the Self-Assembled Monolayer [3, 4]. Shorter HS-$(CH_2)_m$-EG_n-OH or longer HS-$(CH_2)_m$-EG_n-Biotin alkanethiol chains can be used; however developing the Mixed Self-Assembled Monolayer using alkanethiol chains with different lengths can cause the longer alkanethiol chains to deform and bend under its own weight, causing the monolayer to be more disordered and less dense [13]. This parameter is also discussed for Phase 1 parameters (Section 2.1.).

Developing mixed Self-Assembled Monolayers: Single molecule Self-Assembled Monolayers introduce steric hindrance between immobilized Streptavidin molecules on the surface [24, 25]. Mixed Self-Assembled Monolayer has been shown to minimize non-specific binding and reduce steric hindrance of the Streptavidin molecules, thereby increasing the stability of binding to the Self-Assembled Monolayer.

2.1.2. Procedure.

As described in the Phase 1 procedure (Section 2.2), the first step is to develop a mixed Self-Assembled Monolayer of HS-C11-EG6-OH and the HS-C11-EG6-Biotin alkanethiols. The mixed Self-Assembled Monolayer will then be the host for immobilized Streptavidin for forming the Biotin-Streptavidin complex. Biotin terminated β-glucan will be immobilized to form Streptavidin – Biotin – β-glucan complex.

3.0. Experiments:

The proposed investigation consists of the following experiments (Table 1). The first five experiments have been successfully completed and are discussed in this thesis.

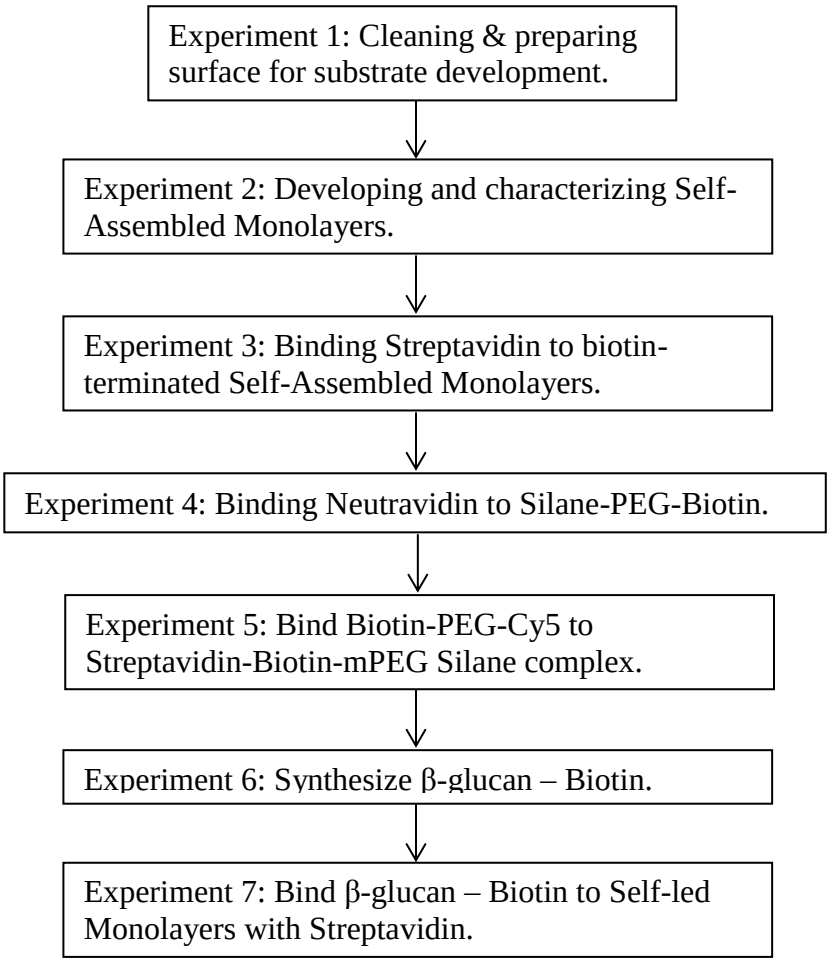


Table 1: Flowchart of the experiments that will be conducted in this investigation. Of the seven proposed experiments, the first five were completed.

3.1. Materials and Instruments.

Materials:

Glass coverslips.

Glass bottom dishes.

Gold alloy.

HS-$(CH_2)_{11}$-EG_6-OH alkanethiols.

HS-$(CH_2)_{11}$-EG_6-Biotin alkanethiols.

Silane mPEG silane (5k).

Silane PEG Biotin (5k).

Streptavidin-Alexa Fluor 647.

Neutravidin-Oregon green 488.

Biotin-PEG-Cy5.

Instruments:

Physical Vapor Deposition.

Confocal Microscope.

UVO Ozone oxidizer.

3.2. Experimental Design.

The following experiments were conducted towards the goal of developing the system elucidated in Section 2.1:

1. Experiment 1: Cleaning & preparing surface for substrate development.
2. Experiment 2: Developing and characterizing Self-Assembled Monolayers.
3. Experiment 3: Binding Streptavidin to biotin-terminated alkanethiols. See Table 2 for the Test Metrics of this experiment.
    1. Experiment 3.1: Measuring Streptavidin binding by varying percentages of HS-$(CH_2)_{11}$-EG_6-Biotin.
    2. Experiment 3.2: Factoring binding time of Streptavidin to HS-$(CH_2)_{11}$-EG_6-Biotin.
    3. Experiment 3.3: Analyzing Streptavidin stock solution for clustering.
    4. Experiment 3.4: Measuring Streptavidin binding with blocking buffers to address clustering of Streptavidin.
    5. Experiment 3.5: Substituting Streptavidin with Neutravidin.
4. Experiment 4: Binding Neutravidin to Silane-PEG-Biotin. See Table 3 for the Test Metrics of this experiment.
    1. Experiment 4.1: Binding Neutravidin to Biotin-terminated Silane Self Assembled Monolayers.
    2. Experiment 4.2: Preventing non-specific Neutravidin binding to Silane Self-Assembled Monolayer.
    3. Experiment 4.3: Developing homogeneous surfaces with Silane mPEG and Neutravidin.

5. Experiment 5: Bind Biotin-PEG-Cy5 to Streptavidin-Biotin-mPEG Silane complex. See Table 3 for the Test Metrics of this experiment.
    1. Experiment 5.1: Non-specific Binding experiment for Silane-PEG-Biotin – Neutravidin – Biotin-PEG-CY5.
    2. Experiment 5.2: Co-localized Neutravidin-OG488 and Biotin-PEG-Cy5 binding.

Table 2: Test-metrics for experiments consisting Alkanethiol Self-Assembled Monolayers. Experiments 3.1, 3.2, 3.4, 3.5.

Exp #	Self-Assembled Monolayer composition				Streptavidin / Neutravidin		Binding time		Blocking		
	100% OH	5-20% Biotin, 95-80% OH	25-60% Biotin, 75-40% OH	0% Biotin, 0% OH	Streptavidin	Neutravidin	10 min	30 min – 1 hr	5% BSA	5% FBS	5% Milk
3.1	X	X	X	X	X		X				
3.2	X	X		X	X		X	X			
3.4	X	X		X	X			X	X	X	X
3.5	X	X		X	X	X		X		X	

Table 3: Test-metrics for experiments consisting Silane Self-Assembled Monolayers. Experiments 4.1, 4.2, 4.3, 5.1, 5.2.

Exp #	Silane Self Assembled Monolayer composition				Neutravidin concentration		Biotin-PEG-Cy5	Blocking		
	100% CH_3	5-20% Biotin, 95-80% CH_3	25-75% Biotin, 75-25% CH_3	0% Biotin, 0% CH_3	16, 32, 128, 256, 1024, 2048 µg/ml	4-8µg/ml		5% BSA	5% FBS	5% Milk
4.1.	X		X	X	X	X				
4.2.	X					X		X	X	X
4.3.	X	X		X		X			X	
5.1.	X			X			X	X	X	X
5.2.	X	X		X		X	X		X	

4.0. Results.

The investigator has developed a working protocol to clean glass substrates using UVO Ozone cleaning, use Plasma Vapor Deposition to layer 35nm of Gold. These glass surfaces were then used to develop Self-Assembled Monolayers, which were characterized using a Contact-Angle Goniometer.

In Experiment 3, the investigator was able to demonstrate a dose-dependent response for surface Biotin to Streptavidin ratio as the concentration of HS-$(CH_2)_{11}$-EG_6-Biotin was increased from 0-60% and as Streptavidin concentration remained constant. However, clustering of Streptavidin to the Self-Assembled Monolayers with Biotin molecules, proportionate to Biotin concentrations, was observed. Effects of binding time of Streptavidin also played a role in the formation of Streptavidin clusters. The investigator did a test and verified the stock Streptavidin solution had no clusters in its purchased form, thus verifying the aforementioned theory. 5%BSA, 5%FBS, 5%Milk, Tween-20 blocking solutions were added to the Streptavidin solution in an effort to reduce the clustering non-specific binding with no success. Finally, Neutravidin, an analog to Streptavidin with a lower isoelectric point, was used in an effort to reduce/eliminate the clustering non-specific binding, and was also not successful.

The goal of this experiment is to develop a substrate that can act as a model of the *Candida albicans* cell wall. Before Silane Self-Assembled Monolayers was used to constitute the cell wall model, the investigator proposed using Self-Assembled Monolayers consti-

tuted of alkanethiols to model the cell wall. Since the goal of the experiment is to develop a substrate that would enable the investigator to precisely control the concentration and homogeneity of the presented β-glucan ligand, the model was changed from Alkanethiol Self-Assembled Monolayers to Silane Self Assembled Monolayers. The Silane Self-Assembled Monolayer was composed of Silane mPEG and Silane-PEG-Biotin. Similarly to the alkanethiol based Self-Assembled Monolayers, the percentage of Silane-PEG-Biotin was modulated to study the differences in Neutravidin fluorescence density and intensity. The goal of Experiment 4.1 was to determine the minimum concentrations of Neutravidin that was necessary to observe a difference in fluorescence when Silane-PEG-Biotin concentrations are modulated. At higher concentrations of Neutravidin, little or no difference in fluorescence intensity was observed as Silane-PEG-Biotin percentages increased from 0-100%, indicating too much Neutravidin can lead to non-specific binding, and not reflect Biotin-Neutravidin specific binding. At a concentration of 4-8 µg/ml, a difference in fluorescence intensity is observed as Silane-PEG-Biotin percentages are increased. Experiment 4.2 successfully demonstrated blocking non-specific binding of Neutravidin to the Silane Self-Assembled Monolayer using 5% BSA, 5% FBS, or 5% Milk buffers, thus validating using Silane Self-Assembled Monolayers over Alkanethiol Self-Assembled Monolayers. The goal of Experiment 4.3 was to determine what concentrations of Neutravidin, Silane-PEG-Biotin, and Silane mPEG were needed to develop a substrate with homogeneous non-specific Biotin-Neutravidin binding.

The ultimate goal of developing this substrate model is to host β-glucan polysaccharides at varying densities on the Self-Assembled Monolayer. Towards this goal, Biotin-PEG-

Cy5 is used as an analog to study the binding properties of the Biotin-Neutravidin complex. This experiment studies the binding interactions of Silane-PEG-Biotin, Neutravidin, and Biotin-PEG-Cy5. The Neutravidin with Oregon Green 488 fluorescent tag and Biotin-PEG-Cy5 enabled the investigator to study the binding ratios of the two molecules as Silane-PEG-Biotin and Silane mPEG ratios were modulated. Experiment 5.1. has successfully demonstrated the blocking of Biotin-PEG-Cy5 to 100% Silane mPEG Self-Assembled Monolayers using 5% BSA, 5% FBS, and 5%Milk. A pilot Experiment 5.2 is currently being conducted. Preliminary results yield co-localized Silane-PEG-Biotin, Neutravidin, and Biotin-PEG-Cy5 binding. The variability and reproducibility of these results is currently being investigated.

4.1. Substrate cleaning & preparation.

The first step taken towards developing a substrate model of the *Candida albicans* cell wall is to ensure the surface of the glass cover-slips are clean and can provide a suitable environment to host the immune cell – β-glucan experiments. For the scope of this experiment cleanliness and oxidative/reductive states of the glass surfaces have been characterized. For the purposes of the developing Self-Assembled Monolayers on the glass cover-slips, the investigator has assessed effects oxidation, ethanol treatment, and nitrogen gas stream drying have on the cover slips. The cleanliness of the glass cover-slips before and after exposure to PBS buffer and alkanethiols has also been characterized.

The investigator has assessed two primary means of cleaning cover-slips for preparing the substrates: 1) UVO Ozone cleaning, and 2) Sonication. UVO ozone cleaning was a preferred method for cleaning the glass-cover slips over sonication due to the fact that UVO ozone cleaning was a more gentle method for cleaning glass cover slips, and it oxidized the surface and therefore provided a helpful environment for the Gold-thiol reactions that would take place when the alkanethiols would bind with the gold substrate.

Figure 7 shows the cleanliness of the substrates under various conditions. Figure 7.A shows the glass substrate cleanliness without any treatment. Figure 7.B. shows a glass slide immersed in PBS buffer. Figure 7.C. shows a glass slide that has been immersed in ethanol for 18 hours without been stream dried. Figure 7.D. shows a glass slide that has been immersed in ethanol for 18 hours and has been NO_2 stream dried. Figure 7.E. shows a glass slide that has been treated with UVO Ozone oxidation. Figure 7.F. shows a glass

slide with 35nm gold deposition following 30 minutes of sonication. Figure 7.G. shows a glass slide with 35nm gold deposition and with Self-Assembled Monolayer formation. These results demonstrate that these cover-slip preparation methods are appropriate means of cleaning substrates for developing Self-Assembled Monolayers.

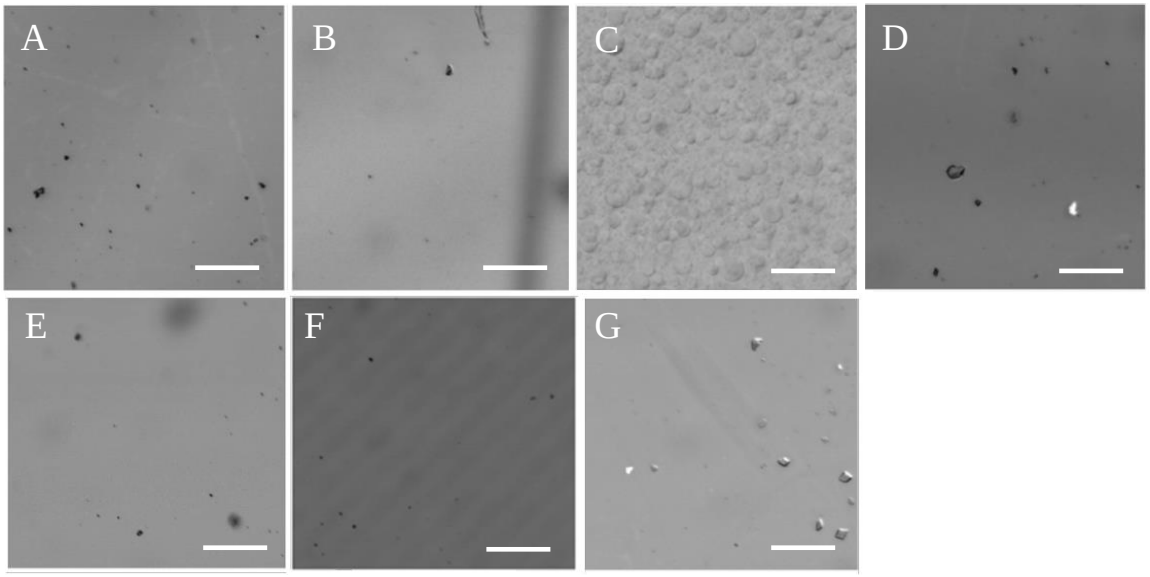


Figure 7: Confocal Microscope image of glass cover-slip under different cleaning conditions. Scale bars represent 100µm. (A) Glass slide with no treatment. (B) Glass slide with PBS buffer. (C) Glass slide with ethanol, no stream dry. (D) Glass slide with ethanol, NO_2 stream dried. (E) Glass slide with UVO Ozone oxidation. (F) Sonicated glass slide with gold deposition. (G) Glass slide with gold deposition and Self-Assembled Monolayer formation.

4.2. Developing and characterizing Self-Assembled Monolayers.

Self-Assembled Monolayers were developed based on the procedure described in Section 2 of the Goals section of this thesis. Table 4 shows compositions of Self-Assembled Monolayer at varying percentages of OH terminated and Biotin terminated alkanethiols:

Procedure:

1. Physical vapor deposition to deposit a thin homogeneous layer of 35nm of Gold onto glass cover-slip.
    1. Image to check for cleanliness.
2. UVO oxidation for1 hour to clean and oxidize the surface.
3. Self-Assembled Monolayer formation. Table 4 shows the composition of developing Self-Assembled Monolayer at varying Hydroxide - biotin terminated alkanethiol ratios. Coverslips were immersed in alkanethiol and ethanol solution overnight (18 hours).
4. Wash & Stream dry.
    1. Remove Alkanethiol-Ethanol solution.
    2. Wash with ethanol 3X.
    3. Stream dry with nitrogen gas.
5. Streptavidin binding for 30 minutes.
6. PBS rinse to wash non-specifically bound streptavidin.
    1. PBS was filtered through a 0.2µm filter.
    2. Substrate is kept wet to prevent streptavidin from denaturing.
7. Image with confocal microscope.

Table 4: Self-Assembled Monolayer compositions.

Self-Assembled Monolayer	OH (µL)	Biotin (mg)
100% OH	2.09 µL OH	0
90% OH 10% Biotin	1.88 µL OH	.26 mg Biotin
80% OH 20% Biotin	1.67 µL OH	.52 mg Biotin
70% OH 30% Biotin	1.46 µL OH	.78 mg Biotin
60% OH 40% Biotin	1.25 µL OH	1.04 mg Biotin
50% OH 50% Biotin	1.05 µL OH	1.3 mg Biotin
40% OH 60% Biotin	0.84 µL OH	1.56 mg Biotin
0% OH 0% Biotin	0	0

Following the development of the Self-Assembled Monolayers, Contact angle goniometry was used to characterize the surface. A Contact Angle Goniometer is an instrument that is used to quantify the hydrophilicity/hydrophobicity of a solid-liquid interface [26, 27]. This method can be used to verify whether Self-Assembled Monolayers have formed on the gold deposited glass surfaces. Figure 8 shows the Contact Angle readings for five samples. Figure 8.D. and 8.E. show the contact angles of the Self-Assembled Monolayer samples to be ~31º, and is congruent with measurements made in literature [9].

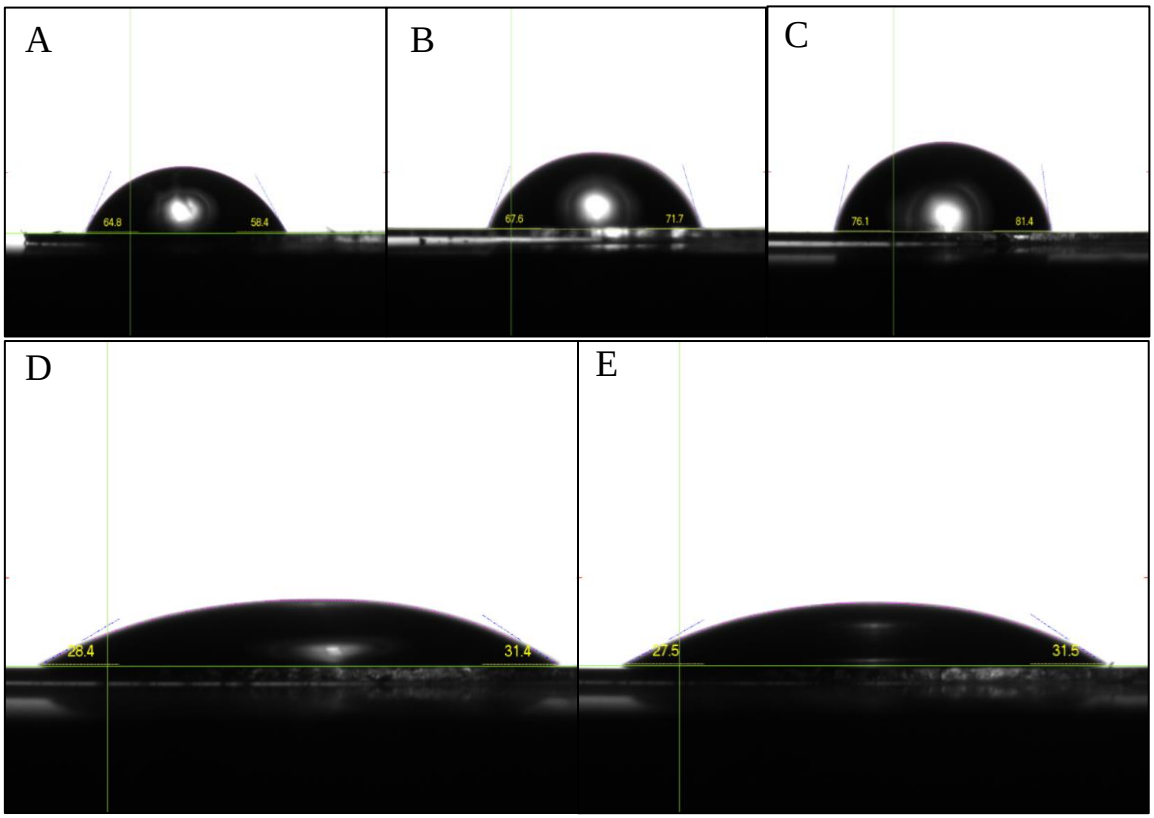


Figure 8: Contact angle goniometry measurements of glass slides with ethanol treatment, gold deposition, Self-Assembled Monolayer formation (1mM and 10mM). (A) Glass slide control, Contact angles 64.8° & 58.4º. (B) Glass slide treated with ethanol, Contact angles 67.6° & 71.7º. (C) Gold substrate treated with ethanol, Contact angles 76.1° & 81.4º. (D) Gold substrate + 1mM alkanethiol in ethanol solution. Contact angles 28.4º & 31.4º. (E) Gold substrate + 10mM alkanethiol in ethanol solution. Contact angles 27.5º & 31.5º.

4.3. Binding Streptavidin to Self-Assembled Monolayers.

Following successful development of the Self-Assembled Monolayers on gold substrates, Streptavidin binding to varying % HS-$(CH_2)_{11}$-EG_6-Biotin terminated Self-Assembled Monolayers was quantified. The goal of this experiment is to determine how Streptavidin density relates to % HS-$(CH_2)_{11}$-EG_6-Biotin. Streptavidin conjugated with Alexa-Fluor 647 is used to quantify the streptavidin concentrations. In this experiment % HS-$(CH_2)_{11}$-EG_6-Biotin v.s. Streptavidin (fluorescently labelled) will be plotted to understand the relationship between the two molecules. It is hypothesized that Streptavidin will increase until it plateaus at a saturation point. The goal is to determine at what % HS-$(CH_2)_{11}$-EG_6-Biotin v.s. Streptavidin yields the maximum Streptavidin binding.

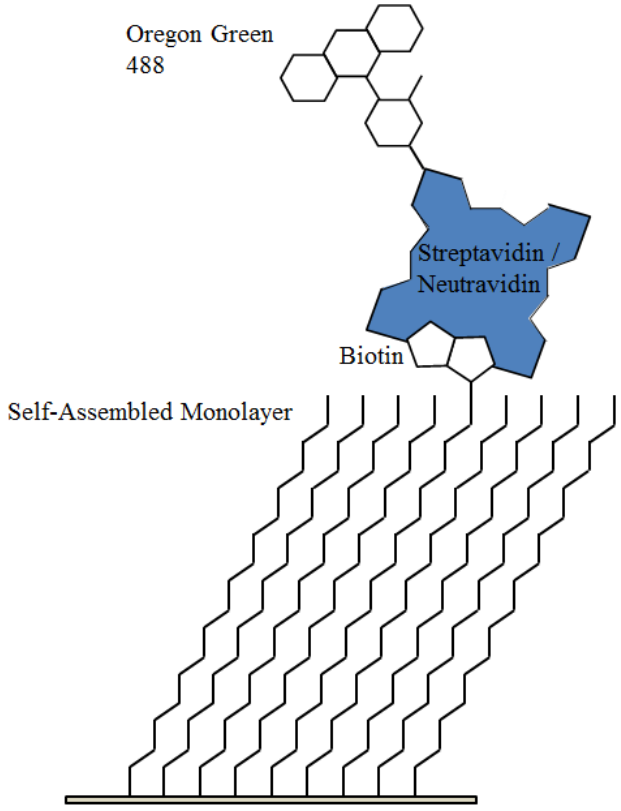


Figure 9: Binding Streptavidin-Alexa Fluor-647 or Neutravidin-Oregon Green 488 to Self-Assembled Monolayers with Biotin.

A challenge that was encountered in this experiment was streptavidin aggregating into clusters. The goal of this experiment is to develop a substrate with homogeneous Streptavidin binding to the HS-$(CH_2)_{11}$-EG_6-Biotin, so that as percentage of biotin increases, a homogeneous surface with modifiable amounts of β-glucan can be presented. If the final model has not accounted for Streptavidin clusters, then the advantage of having precise control of presented β-glucan will be lost.

The five following experiments that were conducted to study how Streptavidin binds to Self-Assembled Monolayers:

1. 4.3.1: Measuring Streptavidin binding by varying % HS-$(CH_2)_{11}$-EG_6-Biotin.
2. 4.3.2: Factoring binding time of Streptavidin to HS-$(CH_2)_{11}$-EG_6-Biotin.
3. 4.3.3: Checking Streptavidin stock solution for clumping.
4. 4.3.4: Measuring Streptavidin binding with blocking reagents to address clustering of Streptavidin.
5. 4.3.5: Substituting Streptavidin with Neutravidin.

4.3.1. Measuring Streptavidin binding by varying percentages of HS-$(CH_2)_{11}$-EG_6-Biotin.

The goal of this experiment is to measure Streptavidin binding and fluorescence with varying percentages of HS-$(CH_2)_{11}$-EG_6-Biotin on the Self-Assembled Monolayers. Streptavidin binding to Self-Assembled Monolayers with HS-$(CH_2)_{11}$-EG_6-Biotin percentages ranging from 10%-60% are shown in Figure 10.A – 10.F. The background Streptavidin binding fluorescence for each sample is shown to increase from 10% to 30% HS-$(CH_2)_{11}$-EG_6-Biotin, with the maximum binding fluorescence at 30%, and decays from 40% to 60% HS-$(CH_2)_{11}$-EG_6-Biotin (Figure 11). This result is congruent with the expected outcome of observing a maximum Streptavidin binding at 20% or 30% due to steric hindrance.

A second type of fluorescence is observed in this experiment, and this is the clustered Streptavidin fluorescence. Unlike background Streptavidin binding fluorescence, clustered streptavidin fluorescence increases linearly as the percent of HS-$(CH_2)_{11}$-EG_6-Biotin increases. Because the goal of this experiment is to develop a surface with homogeneous Streptavidin binding, the clustering of streptavidin is an unwanted artifact and has posed a challenge to this investigation.

The formation of Streptavidin clusters in studies using biotinylated alkanethiols is a phenomenon that is observed in literature [28]. Streptavidin, once adsorbed onto the Self-Assembled Monolayer, can reorganize to form more energetically favorable, crystal-like,

supramolecular architectures that can be described by the Lifschitz-Slyozov Law [29, 30]. The reorganization of Streptavidin layer on the Self-Assembled Monolayer is a result of the Streptavidin changing its viscoelastic properties from a soft state to a more ridgid state as Streptavidin molecules form hydrogen bonds with lateral Streptavidin [29]. Towards this goal, diluting Biotinylated Alkanethiols and Streptavidin concentrations are required for homogeneous Biotin-Streptavidin binding on Self-Assembled Monolayers.

It is hypothesized that the clustering of Streptavidin can be controlled by the amount of time Streptavidin is allowed to bind to the HS-$(CH_2)_{11}$-EG_6-Biotin on the substrate. This hypothesis will be tested in 4.3.2: Factoring binding time of Streptavidin to HS-$(CH_2)_{11}$-EG_6-Biotin.

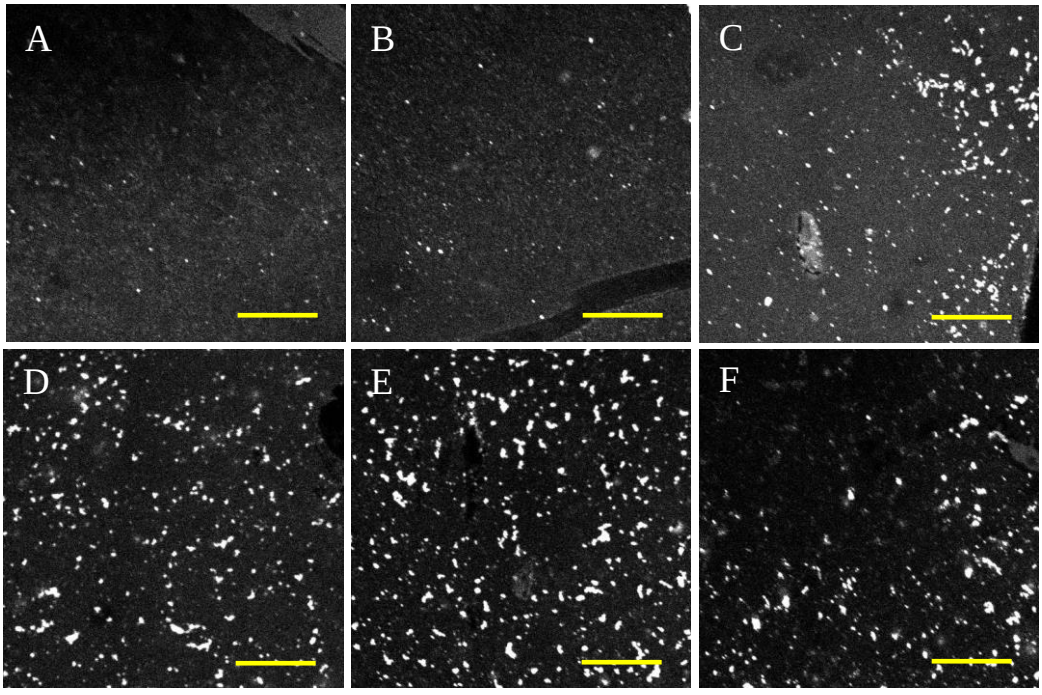


Figure 10: Streptavidin-AF647 fluorescence on Self-Assembled Monolayers with varying percent biotin. Scale bars represent 100µm. An increase in background Streptavidin-AF647 fluorescence is observed when percent Biotin increases from 10% to 30%, and a decrease is observed as percent Biotin increases from 40% to 60%. Streptavidin clusters increase as percent biotin increase from 10% - 60%. (A) 10% HS-$(CH_2)_{11}$-EG_6-Biotin SAM. (B) 20% HS-$(CH_2)_{11}$-EG_6-Biotin SAM. (C) 30% HS-$(CH_2)_{11}$-EG_6-Biotin SAM. (D) 40% HS-$(CH_2)_{11}$-EG_6-Biotin SAM. (E) 50% HS-$(CH_2)_{11}$-EG_6-Biotin SAM. (F) 60% HS-$(CH_2)_{11}$-EG_6-Biotin SAM.

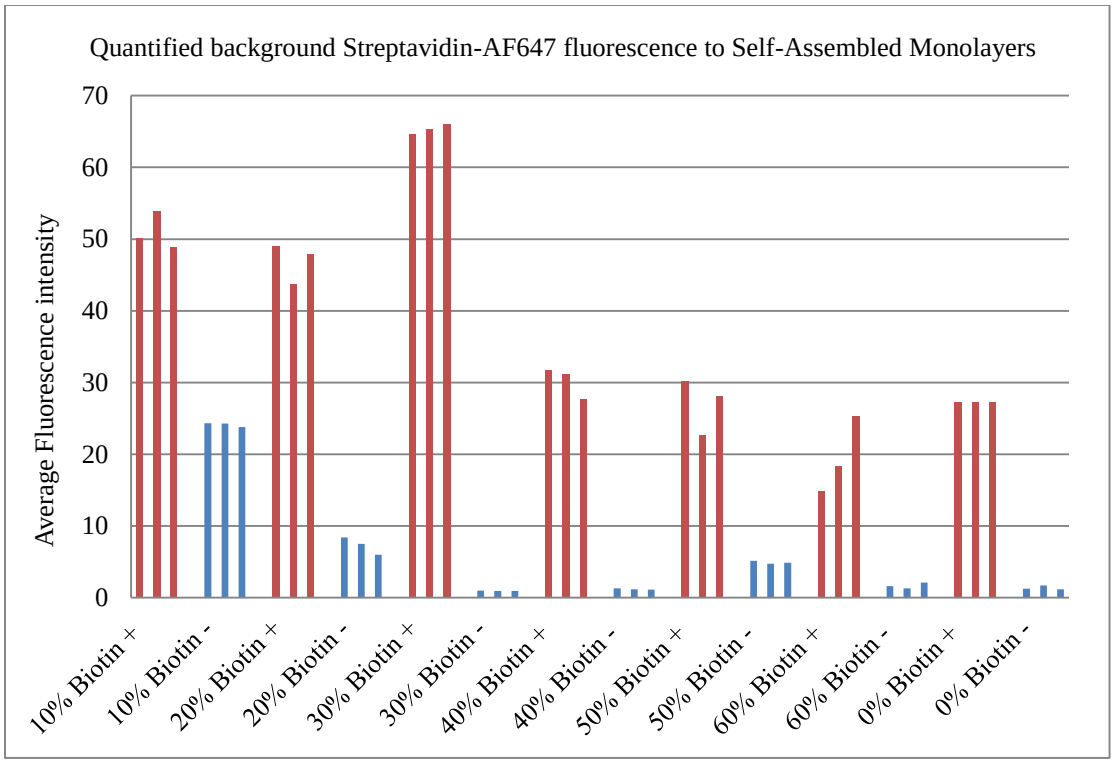


Figure 11: Quantified background Streptavidin-AF647 fluorescence (red) as percent HS-$(CH_2)_{11}$-EG_6-Biotin increase from 0% -60%. (+, red bars) indicates with Streptavidin, (-, blue bars) indicates without Streptavidin.

4.3.2. Factoring binding time of Streptavidin to HS-$(CH_2)_{11}$-EG_6-Biotin.

The goal of this experiment is to test Streptavidin binding to 30% HS-$(CH_2)_{11}$-EG_6-Biotin Self-Assembled Monolayers at different binding time lengths and streptavidin concentrations. Figure 12 demonstrates that the concentrated clusters of streptavidin form over time. The background fluorescence decreases as the length of streptavidin binding progresses (as seen in Figures 12.A→12.B, and in Figures 12.C→12.D→12.E). This progression demonstrates that the Streptavidin goes from a more spread out distribution and aggregates to a more concentrated distribution. The negative controls (Figures 12.F, 12.G, and, 12.H) demonstrate the concentrated regions of streptavidin binding are a property of Biotin-Streptavidin binding on the SAMs, and are not due to aggregations on Streptavidin in its purchased form (this claim is verified in 5.3.3).

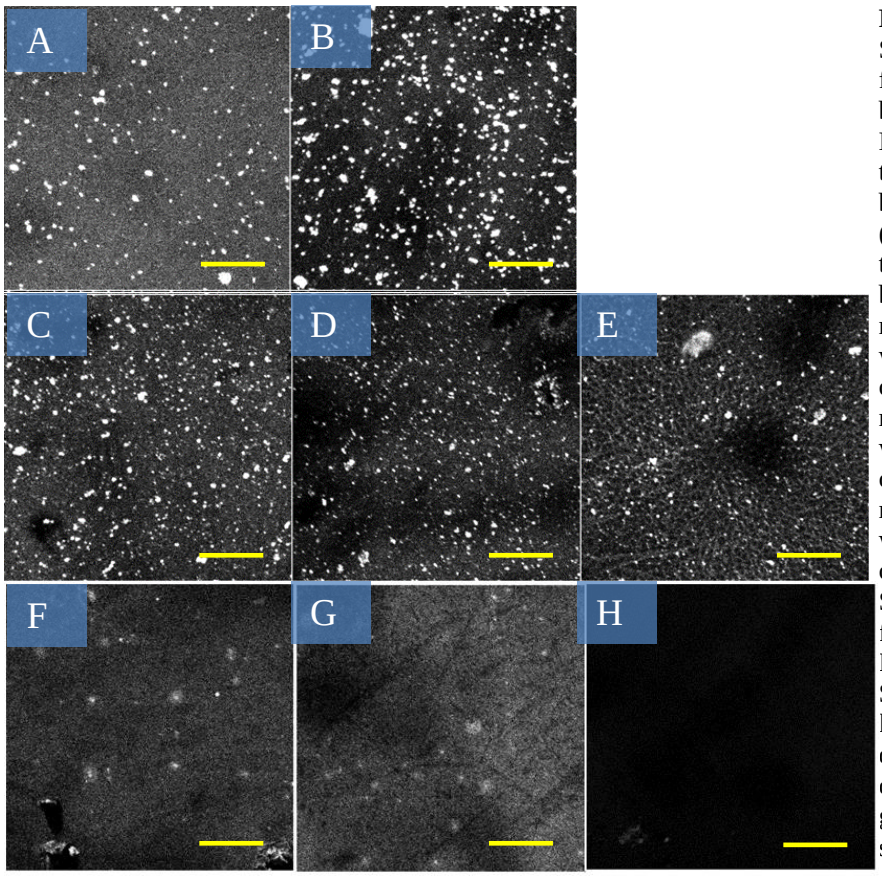


Figure 12: (A-E) Streptavidin-AF647 fluorescence on 20% biotin Self-Assembled Monolayers as binding time is varied. Scale bars represent 100µm. (A) 10 minutes binding time. (B) 30 minutes binding time. (C) 10 minutes binding time with 0.5X Streptavidin concentration. (D) 30 minutes binding time with 0.5X Streptavidin concentration. (E) 60 minutes binding time with 0.5X Streptavidin concentration. (F) Streptavidin-AF647 fluorescence to 100% HS-$(CH_2)_{11}$-EG_6-OH Self-Assembled Monolayers. (G) Streptavidin-Af647 fluorescence to gold deposited glass surfaces. (H) No streptavidin control.

4.3.3. Analyzing Streptavidin stock solution for clustering.

In this experiment, a line imaging scan of a droplet of Streptavidin conjugated with Alexa Fluor 647 was measured to clustering of streptavidin. The line scan indicates that there is a heterogeneous distribution of Streptavidin + Alexa Fluor 647 molecules in the 1% BSA-PBS buffer (Figure 13) that is not observed in the negative control (1% BSA-PBS buffer) (not shown). If the Streptavidin in the stock solution was forming clusters, the line scan image would show streaks of fluorescence. Since there are no observable streaks in the image, the investigator has concluded that the clustered regions of Streptavidin observed in 5.3.1 & 5.3.2 are not caused due to clustered Streptavidin particles in its purchased form. This experiment validates the hypothesis made in 5.3.2 that the concentrated clusters of streptavidin binding are caused by the biotin on the SAMs, and are not due to aggregations of Streptavidin in its stock form.

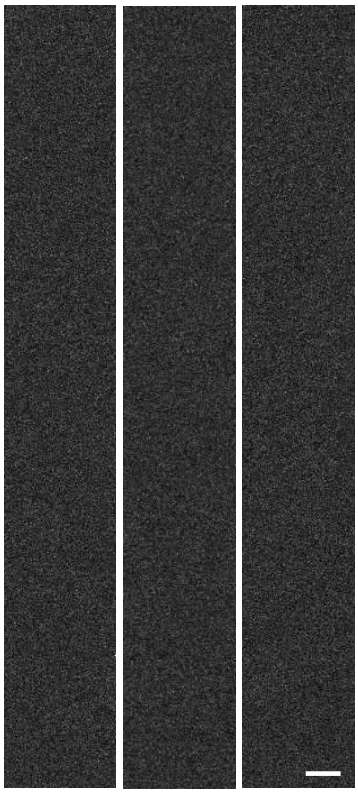


Figure 13: Line scan image of stock Streptavidin-AF647 in 5% BSA-PBS buffer to test for Streptavidin clusters. Scale bar represents 150µm.

4.3.4. Measuring Streptavidin binding with blocking buffers to address clustering of Streptavidin.

The goal of this experiment is to use blocking buffers to reduce or remove the clustering of Streptavidin on the Self-Assembled Monolayers. Bovine Serum Albumin (BSA), Fetal Bovine Serum (FBS), Non-fat Milk, and Tween-20 buffers are used extensively in molecular biology to block non-specific protein adsorption to substrates [31]. The effectiveness of three blocking reagents to reduce non-specific protein adsorption was considered for this experiment: 5% BSA+ 0.1% Tween20 in PBS buffer, 5% FBS+ 0.1% Tween20 in PBS buffer, 5% Milk+ 0.1% Tween20 in PBS buffer. Confocal Microscope images show that 5% FBS+ 0.1% Tween20 in PBS buffer (Figure 14.F) was most effective at blocking clustering of Streptavidin, but was not able to fully remove the clustering of Streptavidin. The clustering of Streptavidin poses a significant challenge towards developing the final model. The clustering of Streptavidin is not observed in the positive control (Figure 14.A), or negative controls (Figures 14.B and 14.C).

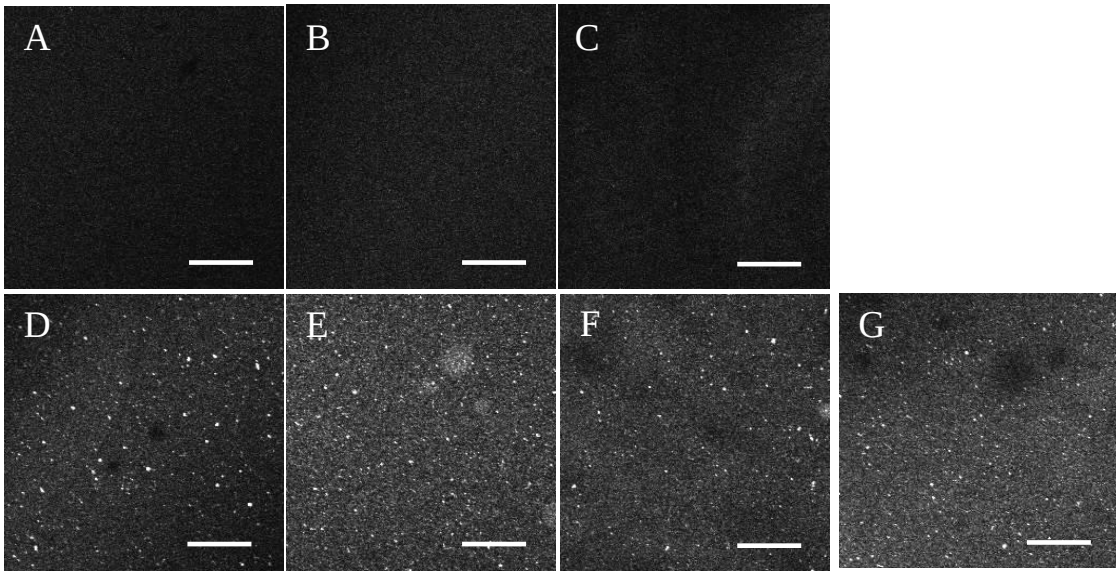


Figure 14: Confocal Microscope images of Streptavidin-AF647 to Self-Assembled Monolayers under different blocking conditions. Scale bar represents 100µm. (A) Gold deposited glass surface control. (B) 100% HS-$(CH_2)_{11}$-EG_6-OH Self Assembled Monolayer without Streptavidin. (C) 100% HS-$(CH_2)_{11}$-EG_6-OH Self Assembled Monolayer with Streptavidin. (D) 20% HS-$(CH_2)_{11}$-EG_6-Biotin Self Assembled Monolayer with Streptavidin. (E) 20% HS-$(CH_2)_{11}$-EG_6-Biotin Self Assembled Monolayer with Streptavidin blocked with 5% BSA + Tween-20. (F) 20% HS-$(CH_2)_{11}$-EG_6-Biotin Self Assembled Monolayer with Streptavidin blocked with 5% FBS + Tween-20. (G) 20% HS-$(CH_2)_{11}$-EG_6-Biotin Self Assembled Monolayer with Streptavidin blocked with 5% Milk + Tween-20.

4.3.5. Substituting Streptavidin with Neutravidin.

The clustering of Streptavidin that is observed in the previous experiments is a form of non-specific binding of Streptavidin to the biotinylated Self Assembled Monolayers. The investigator was curious to see if using Neutravidin, an alternative to Streptavidin, would address the non-specific binding challenge. Neutravidin is a deglycosylated native avidin from egg whites, has excess carbohydrate removed that yields a protein with a more neutral isoelectric point, which promotes to less non-specific binding. Removing the glycosylation of Streptavidin reduces carbohydrate-based lectin binding to undetectable levels without altering the biotin-binding affinity [32].

In this experiment, seven samples were developed. Figures 15.B-15.D shows Streptavidin-Af647 binding to a gold substrate (B), a 20% biotin – 80% hydroxide terminated Self-Assembled Monolayer (C), and a 20% biotin – 80% hydroxide terminated Self-Assembled Monolayer with 5% FBS blocking buffer (D). Figure 15.E-15.G shows Neutravidin binding to a gold substrate (E), a 20% biotin – 80% hydroxide terminated Self-Assembled Monolayer (F), and a 20% biotin – 80% hydroxide terminated Self-Assembled Monolayer with 5% FBS blocking buffer (G). The Neutravidin samples show a greater amount of protein clustering, but yields a more uniform fluorescence than the Streptavidin samples. Since the clustering of Streptavidin and Neutravidin is observed only on samples with terminated Self-Assembled Monolayers, and not in blank gold substrates, it is concluded the clustering of the Streptavidin/Neutravidin is due to biotin-avidin binding properties.

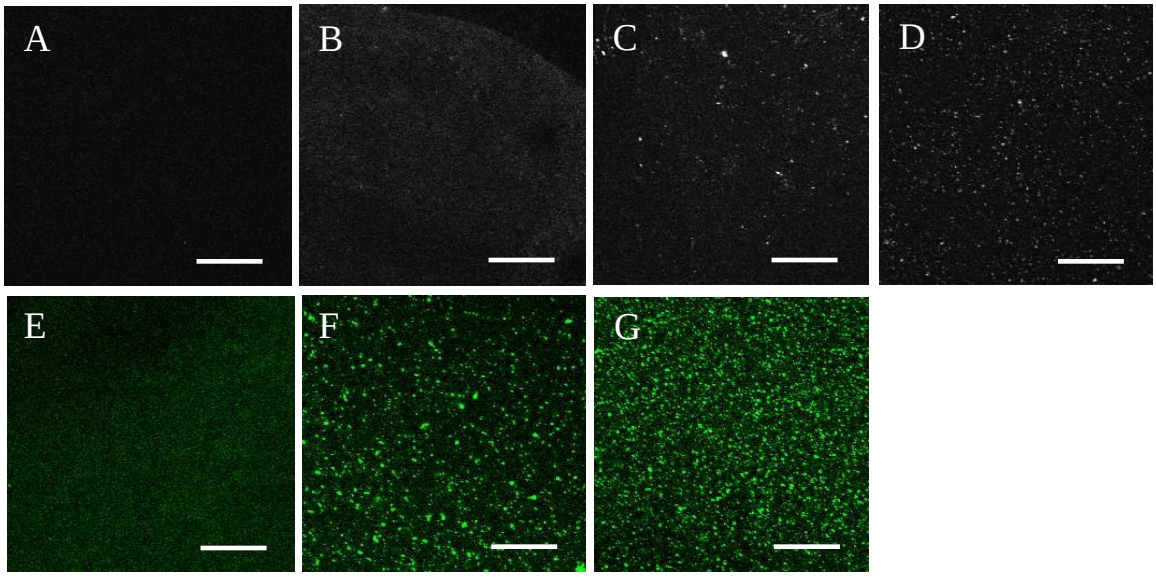


Figure 15: Confocal Microscope images of Streptavidin-AF647 and Neutravidin-OG488 to Self-Assembled Monolayers with 5% FBS blocking buffer. Scale bar represents 100µm. (A) Gold deposited glass surface control. (B) 100% HS-$(CH_2)_{11}$-EG_6-OH Self Assembled Monolayer with Streptavidin-AF647. (C) 20% HS-$(CH_2)_{11}$-EG_6-Biotin Self Assembled Monolayer with Streptavidin-AF647. (D) 20% HS-$(CH_2)_{11}$-EG_6-Biotin Self Assembled Monolayer with Streptavidin-AF647 and 5% FBS blocking buffer. (E) 100% HS-$(CH_2)_{11}$-EG_6-OH Self Assembled Monolayer with Neutravidin-OG488. (F) 20% HS-$(CH_2)_{11}$-EG_6-Biotin Self Assembled Monolayer with Neutravidin-OG488. (G) 20% HS-$(CH_2)_{11}$-EG_6-Biotin Self Assembled Monolayer with Neutravidin-OG488 and 5% FBS blocking buffer.

4.4. Silane m-PEG Self-Assembled Monolayer experiments.

The results from Experiment 3 indicate that the clustering of Streptavidin/Neutravidin on biotinylated Alkanethiol Self-Assembled Monolayers are properties of Biotin-Streptavidin/Neutravidin binding. The investigator proposed changing the model from Alkanethiol-based Self-Assembled Monolayers to Silane-based Self-Assembled Monolayers to test if this would address the clustering of Streptavidin. In Experiment 4.4.1, mixed Silane-based based Self-Assembled Monolayers were synthesized on glass substrates (see Figure 16). The Silane Self-Assembled Monolayer meets the criteria defined in Section 2.1 of this thesis, and introduces small changes to the procedure to synthesize a monolayer. A mixed Silane Self-Assembled Monolayer is composed of 5k Methoxy PEG Silane (mPEG-Silane) and 5k Silane PEG Biotin molecules.

In the experiments discussed in this section, a significant improvement of non-specific binding of Neutravidin to the Biotin-terminated Silane Self-Assembled Monolayers was observed, and thus justified changing the model. Another significant advantage of using this model is the use of the glass substrate instead of a gold substrate. The use of a glass substrate removed the 35nm of gold deposition from the previous model, excluded the need for additional brightness adjustment during imaging and significantly improved the quality of images.

The following experiments using the Silane Self-Assembled Monolayers were conducted:

1. 4.4.1: Binding Neutravidin to Biotin-terminated Silane Self-Assembled Monolayers.
2. 4.4.2: Blocking non-specific Neutravidin binding to Silane Self Assembled Monolayers
3. 4.4.2: Developing homogeneous surfaces with Silane mPEG and Neutravidin.

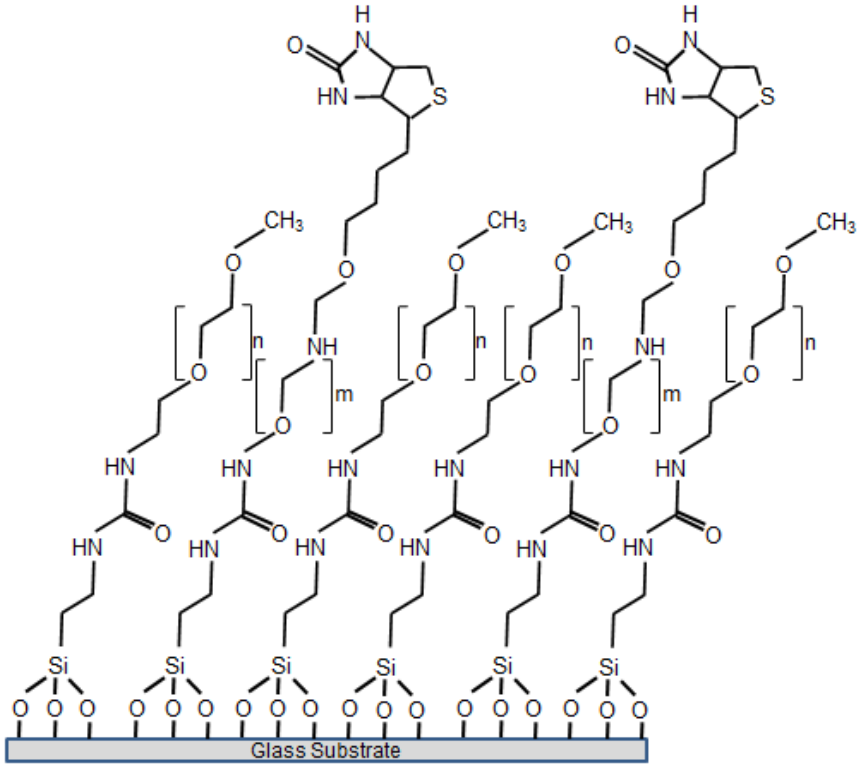


Figure 16: Silane Self-Assembled Monolayer.

4.4.1. Binding Neutravidin to Biotin-terminated Silane Self Assembled Monolayers.

A thorough experiment is conducted where Self-Assembled Monolayers with varying Silane-PEG-Biotin and Silane mPEG ratios are developed to determine what concentration of Neutravidin is necessary to see a density response in Silane-PEG-Biotin and Neutravidin binding. Neutravidin binding at 4, 8, 16, 32, 128, 256, 1024, and 2048 µg/ml concentrations are conducted on 0%/100%, 25%/75%, 50%/50%, 75%/25%, and 100%/0%, Silane-PEG-Biotin/Silane mPEG Self-Assembled Monolayers. The goal of this experiment is to see at what concentrations of the 3 molecules yields a dose-dependent fluorescence. In an experiment with 84 samples, Neutravidin concentrations varying from 16-2048 µg/ml did not yield any difference in fluorescence intensity as the ratio of Silane-PEG-Biotin and Silane mPEG changed. Figure 17.A-17.H show Neutravidin-OG488 fluorescence at 16-32 µg/ml concentrations as %Silane-PEG-Biotin are increased in 25% increments from 0-100%, and show no difference in fluorescence intensity. Figure 17.I-17.P show Neutravidin-OG488 fluorescence at 4-8 µg/ml concentrations as %Silane-PEG-Biotin are increased in 25% increments from 0-100%, and a yields differences in fluorescence intensities as ratio of Silane-PEG-Biotin and Silane mPEG changed.

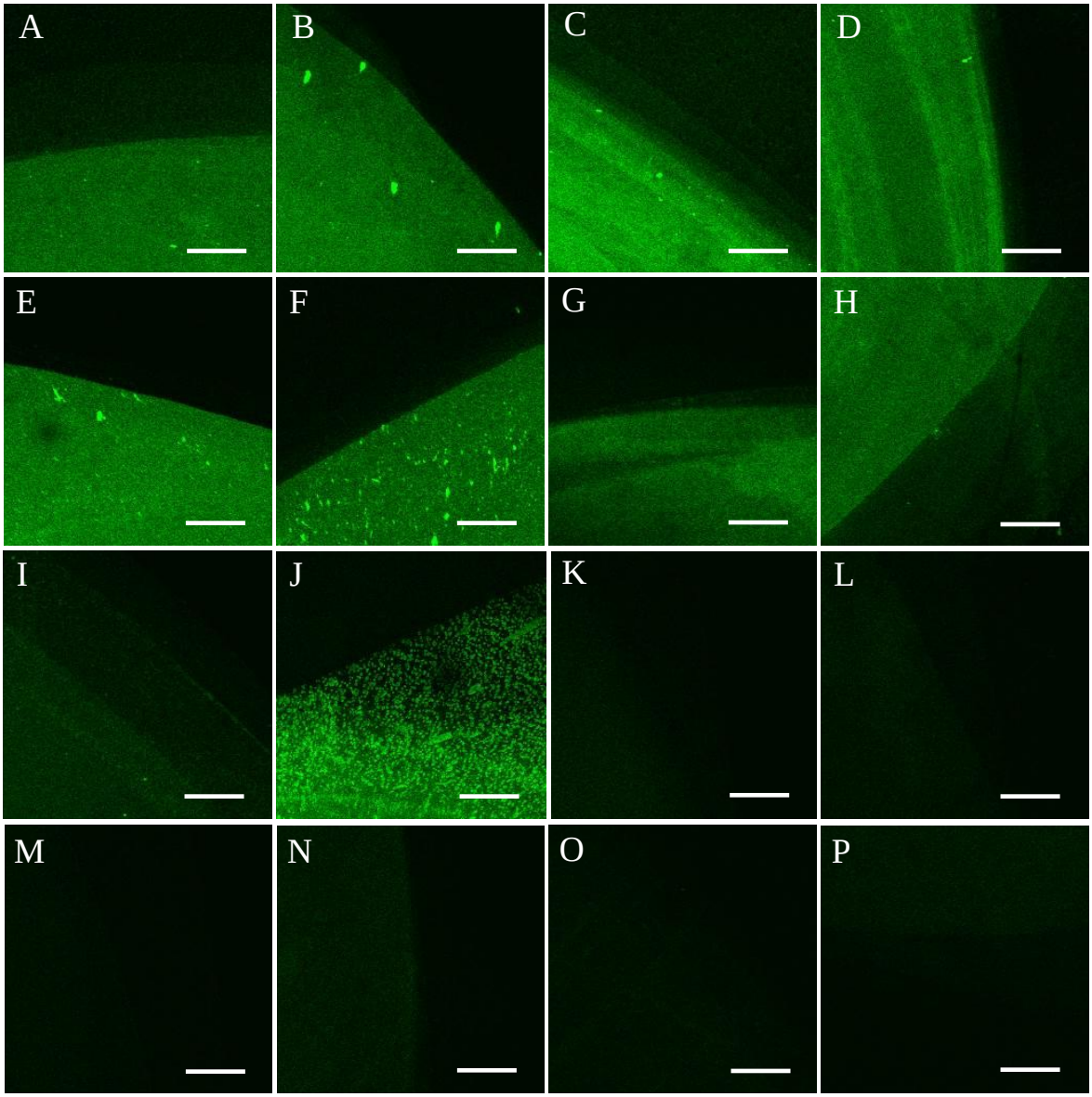


Figure 17: Neutravidin-OG488 fluorescence to Silane Self-Assembled Monolayers. (A) 100% Silane mPEG with 32µg/ml Neutravidin-OG488. (B) 100% Silane mPEG with 16µg/ml Neutravidin-OG488. (C) 75% Silane-PEG-Biotin with 32µg/ml Neutravidin-OG488. (D) 75% Silane-PEG-Biotin with 16µg/ml Neutravidin-OG488. (E) 25% Silane-PEG-Biotin with 32µg/ml Neutravidin-OG488. (F) 25% Silane-PEG-Biotin with 16µg/ml Neutravidin-OG488. (G) 100% Silane-PEG-Biotin with 32µg/ml Neutravidin-OG488. (H) 100% Silane-PEG-Biotin with 16µg/ml Neutravidin-OG488.

(I) 100% Silane mPEG with 8µg/ml Neutravidin-OG488. (J) 100% Silane mPEG with 4µg/ml Neutravidin-OG488. (K) 75% Silane-PEG-Biotin with 8µg/ml Neutravidin-OG488. (L) 75% Silane-PEG-Biotin with 4µg/ml Neutravidin-OG488. (M) 25% Silane-PEG-Biotin with 8µg/ml Neutravidin-OG488. (N) 25% Silane-PEG-Biotin with 4µg/ml Neutravidin-OG488. (O) 100% Silane-PEG-Biotin with 8µg/ml Neutravidin-OG488. (P) 100% Silane-PEG-Biotin with 4µg/ml Neutravidin-OG488.

Scale bars represent 100µm.

4.4.2. Blocking non-specific Neutravidin binding to Silane Self Assembled Monolayers.

The first experiment that was conducted using the Silane Self-Assembled Monolayers was to test whether the use of BSA, FBS, and Milk blocking buffers would prevent the non-specific protein adsorption and formation of Neutravidin clusters to the substrate. Figures 18.A – 18.D demonstrate that non-specific Neutravidin clustering on the 100% mPEG-Silane Self-Assembled Monolayer can be prevented by using the blocking buffers. The results from this experiment validate the use of Silane Self-Assembled Monolayers as the primary method for modeling the cell wall. Although the clustering of Neutravidin is still observed in the Silane Self-Assembled Monolayer with no blocking, the all of the experiments that were performed here-on-after incorporated the blocking buffers into the procedure.

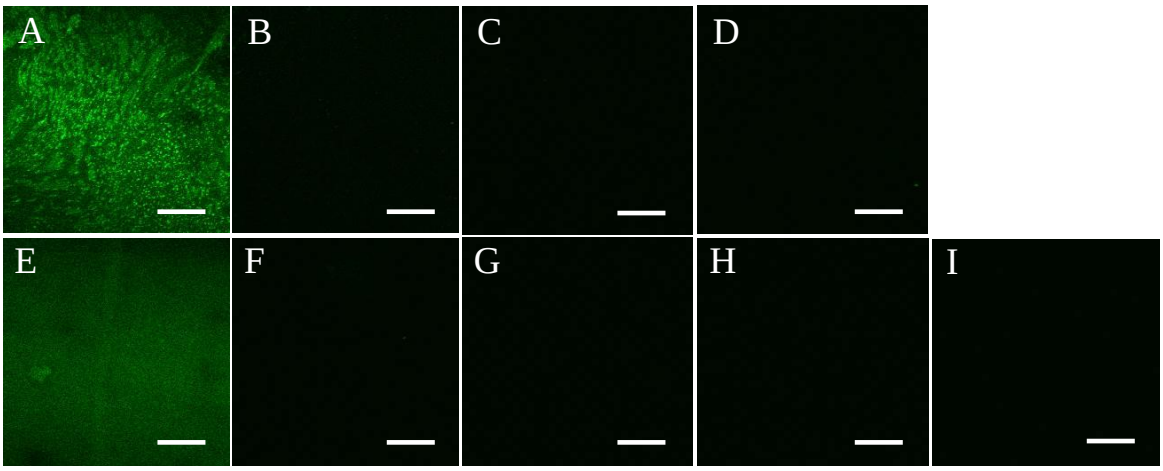


Figure 18: (A-D) 100% Silane mPEG Self-Assembled Monolayers with 4µg/ml Neutravidin-OG488 with different blocking buffers. Scale bars represent 100µm. (E-H) Glass surface with 4µg/ml Neutravidin-OG488 with different blocking buffers. (A) and (E) no blocking buffer. (B) and (F) 5%BSA + 0.1% Tween20 blocking buffer. (C) and (G) 5%FBS + 0.1% Tween20 blocking buffer. (D) and (H) 5%Milk + 0.1% Tween20 blocking buffer. (I) Glass slide control.

4.4.3. Developing homogeneous surfaces with Silane mPEG and Neutravidin.

The goal of this experiment was to develop a substrate with homogeneous streptavidin fluorescence. The results from 5.4.1 demonstrated that non-specific clustering of Neutravidin to the biotinylated Silane Self-Assembled Monolayers could be eliminated/reduced through the use of blocking buffers. A series of experiments were performed to identify the ideal concentrations of mPEG-Silane, Silane PEG Biotin, Neutravidin, and the blocking buffers, with the goal of developing a substrate the can host the β-glucan in the subsequent steps of the development process.

100% mPEG-Silane Self Assembled Monolayers with blocking buffer yield a very low density, homogeneous Neutravidin layer (Figure 19.A.), indicating that there is a very small amount of Neutravidin non-specifically binding to the mPEG-Silane molecules. Figures 19.B – 19.E shows Neutravidin fluorescence as % Silane PEG Biotin and Neutravidin concentrations are increased. There is a trend that is taking place when moving the concentration of % Silane PEG Biotin from 5-10%, and when moving the concentration of Neutravidin from 4µg/ml to 8µg/ml. When % Silane PEG Biotin is held constant at 5%, doubling the Neutravidin concentration does not yield a denser Neutravidin monolayer. However, when % Silane PEG Biotin is held constant at 10%, doubling the Neutravidin concentration yields a denser Neutravidin monolayer. The Neutravidin fluorescence is also very similar for 5 % Silane PEG Biotin and 10% Silane PEG Biotin monolayer at 4µg/ml Neutravidin; 10% Silane PEG Biotin monolayer, does however, have more Neutravidin cluster formation. The goal of developing a substrate with uniform Neutravidin binding is met best at 5% Silane PEG Biotin Self-Assembled Monolayers.

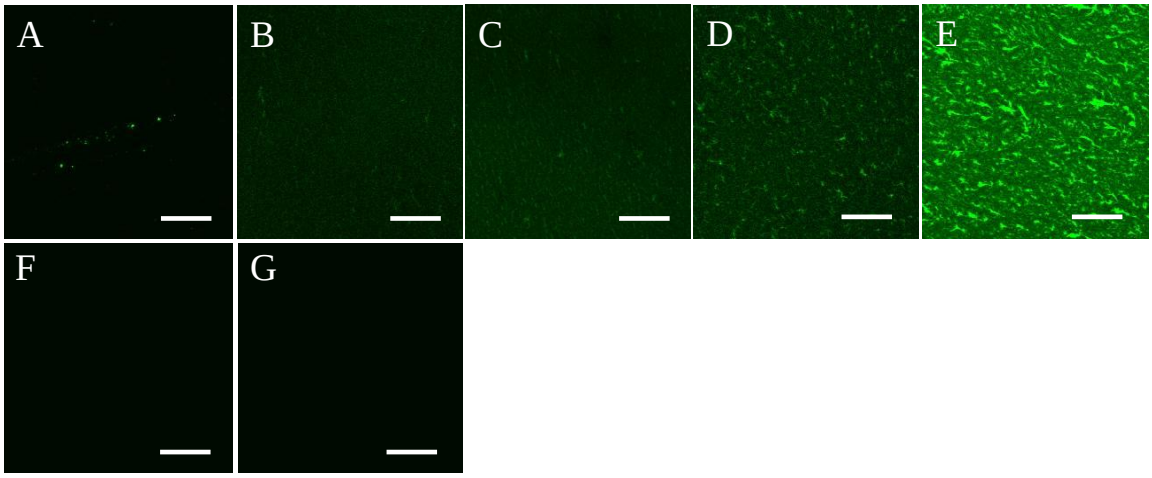


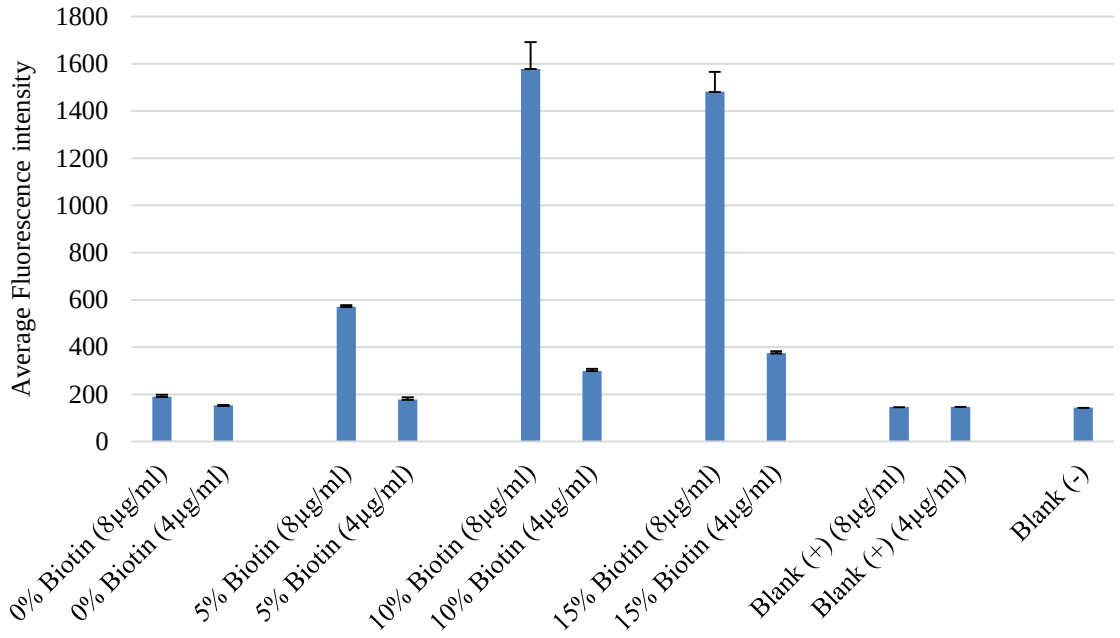
Figure 19: Varying Neutravidin-OG488 fluorescence on Silane Self-Assembled Monolayers with varying percent Silane-PEG-Biotin. Scale bars represent 100µm.
(A) 100% mPEG-Silane, 4µg/ml Neutravidin, 5% FBS+ 0.1% Tween20 + PBS.
(B) 5% Silane PEG Biotin, 95% mPEG-Silane, 4µg/ml Neutravidin, 5% FBS+ 0.1% Tween20 + PBS.
(C) 5% Silane PEG Biotin, 95% mPEG-Silane, 8µg/ml Neutravidin, 5% FBS+ 0.1% Tween20 + PBS.
(D) 10% Silane PEG Biotin, 90% mPEG-Silane, 4µg/ml Neutravidin, 5% FBS+ 0.1% Tween20 + PBS.
(E) 10% Silane PEG Biotin, 90% mPEG-Silane, 8µg/ml Neutravidin, 5% FBS+ 0.1% Tween20 + PBS.
(F) Glass Slide, 4µg/ml Neutravidin, 5% FBS+ 0.1% Tween20 + PBS.
(G) Glass Slide.

Several experiments were conducted to identify the ideal molecular concentration ratios needed to develop reproducible, homogeneous surfaces. The graphs from Figure 20 – 25 show the repeatability reproducing these results. Each bar in the graphs is an average Neutravidin fluorescence value from three distinct regions of the same Self-Assembled Monolayer sample. The x axis labels represents each sample, and the y axis is a value of Neutravidin fluorescence quantified using the ImageJ image processing software. Each bar has a standard deviation error bar.

Figure 20 compares the quantified Neutravidin fluorescence for Self-Assembled Monolayers with 0%, 5%, 10%, and 15% Silane-PEG-Biotin compositions and for Neutravidin concentrations of 4µg/ml to 8µg/ml. Each graph is the quantified fluorescence for one distinct batch of experiment. Figures 20 and 21 display that

Neutravidin with concentrations of 4µg/ml see an increase in fluorescence as Silane-PEG-Biotin concentrations in the Self-Assembled Monolayer increase from 0%-20%. Figures 22, 23, and 24 display that Neutravidin with concentrations of 8µg/ml see an increase in fluorescence as Silane-PEG-Biotin concentrations in the Self-Assembled Monolayer increase from 0%-20%, whereas figures 20 and 25 show experiments with quantified fluorescence trends that with more variation across samples. A 1-way ANOVA test was used to generate the p value for each experiment, and is listed on the title of each experiment. The fluorescence of blank samples (0% Silane-PEG-Biotin, 0% Silane-mPEG) with and without Neutravidin are also quantified, and they indicate that there is very little difference in the quantified fluorescent signals, and also that very little background fluorescence is seen.

Figure 20: Neutravidin (4 & 8 µg/ml) on Silane monolayer
p = <.0001 for both 4 & 8 µg/ml experiments



Figures 20 - 25: Average fluorescence Intensity values for Neutravidin and Silane Self-Assembled Monolayers. p value for each experiment is on listed on title. X axis is sample. Y axis is quantified average fluorescence intensity.

Figure 21: Neutravidin (4µg/ml) on Silane monolayer
p = 0.0076

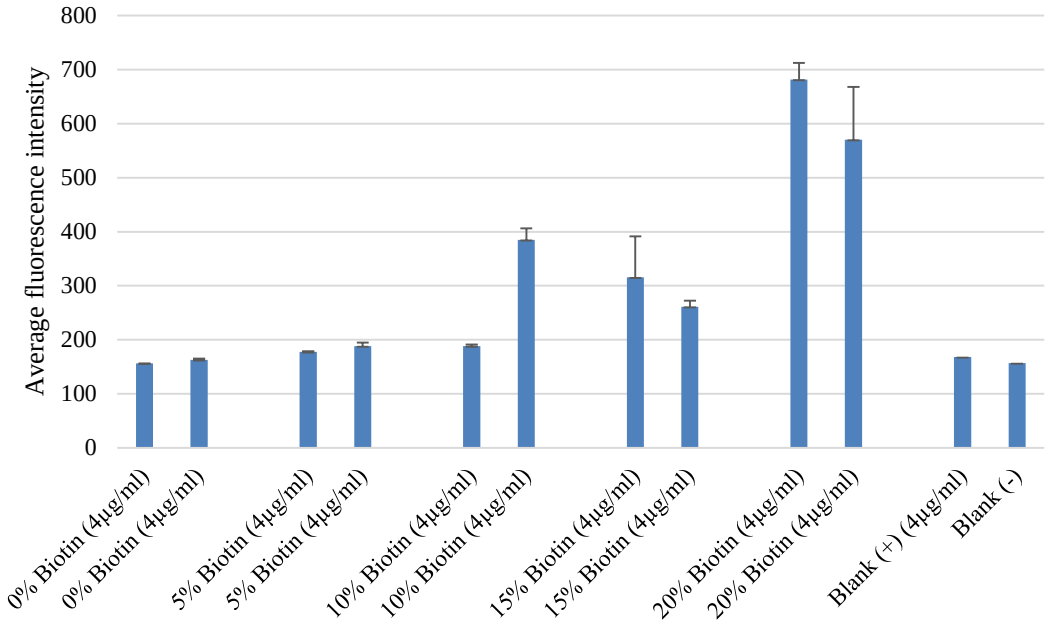


Figure 22: Neutravidin (8µg/ml) on Silane monolayer
p = 0.5189

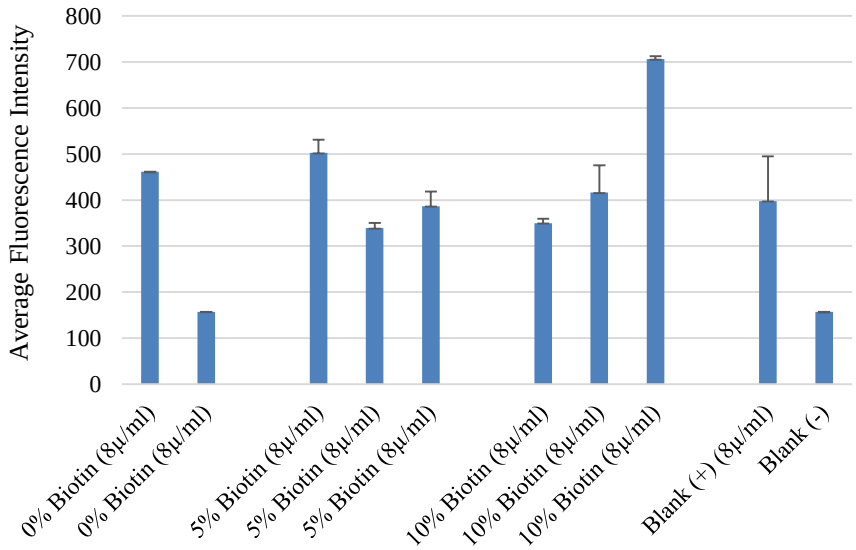


Figure 23: Neutravidin (8µg/ml) on Silane Monolayer
p = 0.053

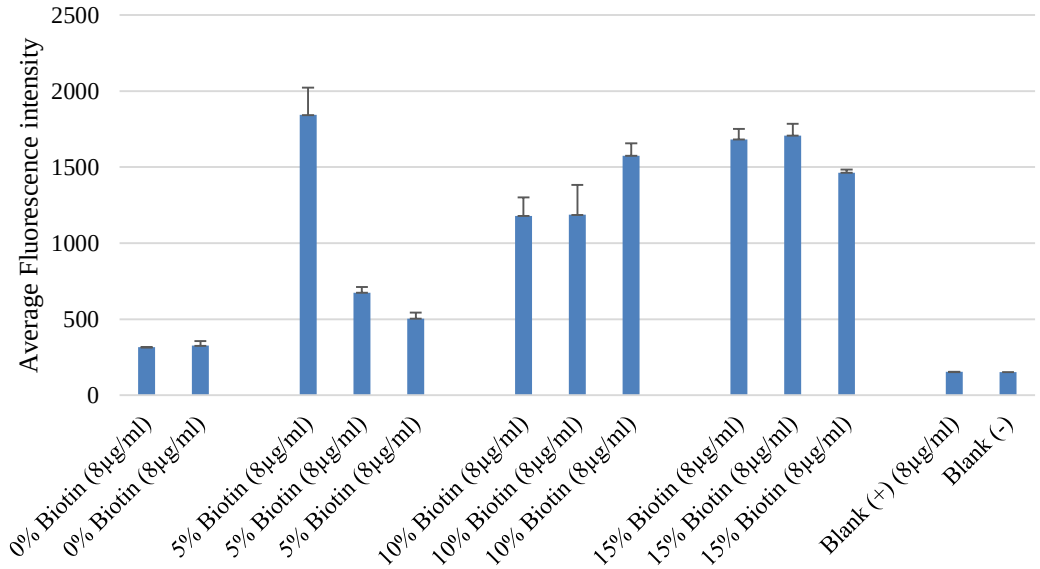


Figure 24: Neutravidin (8µg/ml) on Silane Monolayer
p = 0.0149

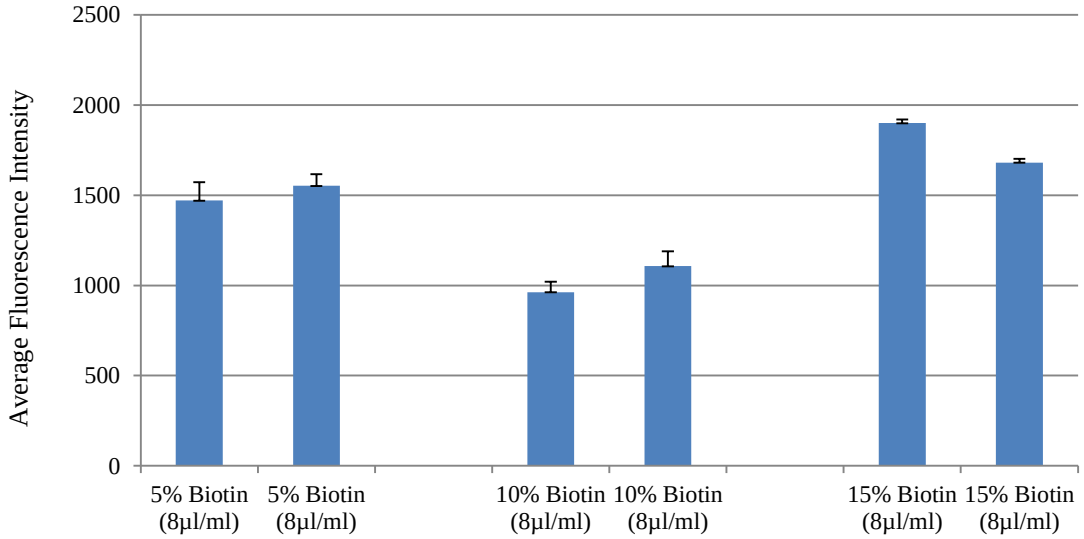
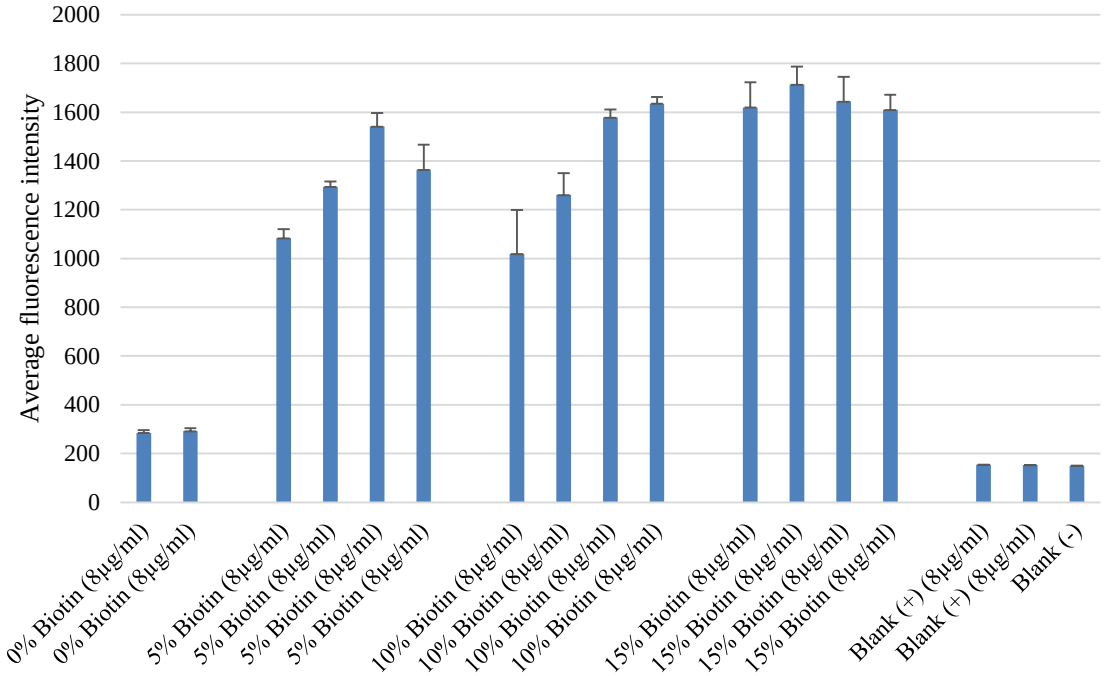


Figure 25: Neutravidin (8µg/ml) on Silane monolayer
p = <.0001



4.5. Binding Biotin-PEG-Cy5 to Neutravidin-Biotin-mPEG Silane complex.

Since the ultimate goal of developing this substrate model is to host β-glucan polysaccharides at varying densities on the Self-Assembled Monolayer, it is important to quantify the binding ratios of the Biotin-Neutravidin complex, and the binding efficiencies of Silane-PEG-Biotin and Neutravidin and Biotin- β-glucan must be determined. Towards this goal, Experiments 4.5.1. and 4.5.2. were conducted to determine the binding efficiencies.

The following experiments using Neutravidin and Biotin-PEG-Cy5 were conducted.

1. 4.5.1: Non-specific binding experiment for Biotin-PEG-Cy5.
2. 4.5.2: Co-localized Neutravidin-OG488 and Biotin-PEG-Cy5 binding.

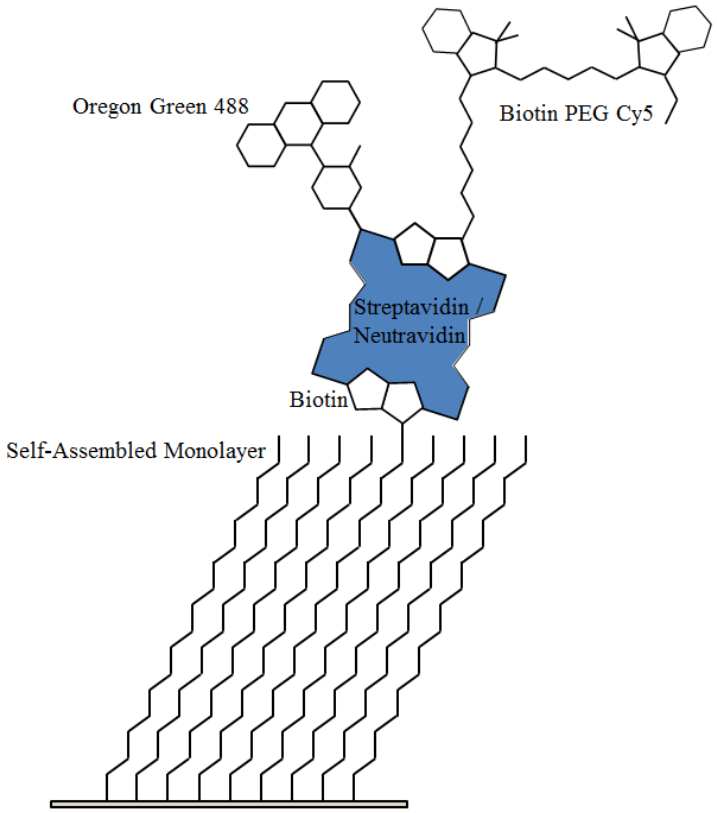


Figure 26: Self-Assembled Monolayer with Neutravidin-OG488 and Biotin-PEG-CY5.

4.5.1. Non-specific Binding experiment for Silane-PEG-Biotin – Neutravidin – Biotin-PEG-CY5.

The first Biotin-PEG-Cy5 experiment conducted checks to see if the Biotin-PEG-Cy5 molecules bind non-specifically to the substrate. Biotin-PEG-Cy5 molecules are immobilized to be bound on Silane Self-Assembled Monolayers and on glass substrates, and are blocked with 5% BSA, 5% FBS, and 5% Milk blocking buffers. Figures 27.A-27.D show that Biotin-PEG-Cy5 binding to the Silane Self-Assembled Monolayer can be virtually completely blocked using any of the three blocking buffers. Figures 27.E-27.H show that Biotin-PEG-Cy5 binding to glass substrates without Silane Self-Assembled Monolayers can be blocked using any of the three blocking buffers. The success of this experiment will enable the investigator to reliably use Biotin-PEG-Cy5 molecules as a way to test the Biotin-Neutravidin-Biotin binding that will lead to the final use of the Streptavidin complex.

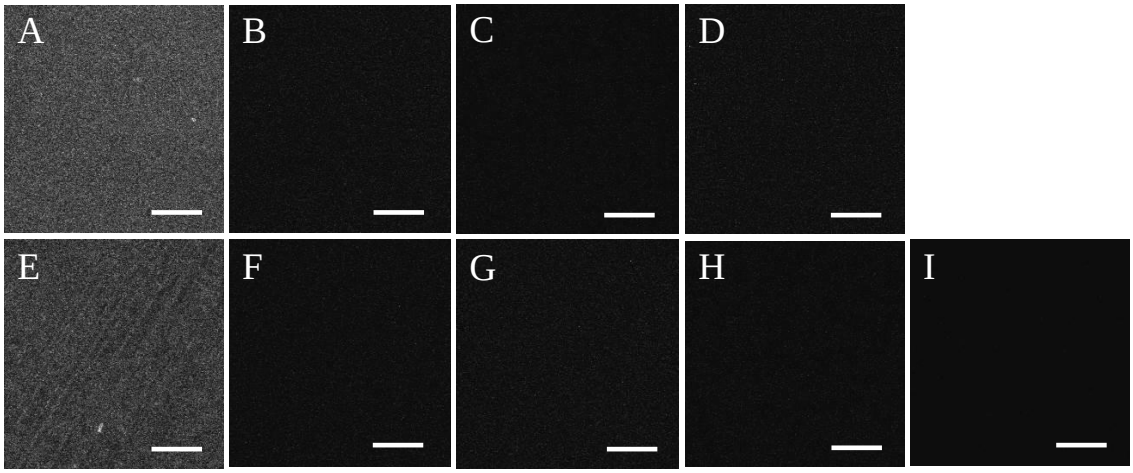


Figure 27: Confocal Fluorescent Microscope images of Biotin-PEG-CY5 binding to Silane-Self-Assebled Monolayers and glass surfaces with different blocking buffers. Scale bars represent 100µm. (A) 100% Silane mPEG with Biotin-PEG-Cy5, no blocking reagent. (B) 100% Silane mPEG with Biotin-PEG-Cy5 with 5% BSA + 0.1% Tween-20 blocking buffer. (C) 100% Silane mPEG with Biotin-PEG-Cy5 with 5% FBS + 0.1% Tween-20 blocking buffer. (D) 100% Silane mPEG with Biotin-PEG-Cy5 with 5% Milk + 0.1% Tween-20 blocking buffer. (E) Glass surface with Biotin-PEG-Cy5, no blocking reagent. (F) Glass surface with Biotin-PEG-Cy5 with 5% BSA + 0.1% Tween-20 blocking buffer. (G) Glass surface with Biotin-PEG-Cy5 with 5% FBS + 0.1% Tween-20 blocking buffer. (H) Glass surface with Biotin-PEG-Cy5 with 5% Milk + 0.1% Tween-20 blocking buffer. (I) Glass surface.

4.5.2. Co-localized Neutravidin-OG488 and Biotin-PEG-Cy5 binding.

In this experiment, the investigator tests the specificity of Biotin-PEG-Cy5 binding to Silane-PEG-Biotin molecules on Silane Self-Assembled Monolayers. The goal of this experiment is to see if the Biotin-PEG-Cy5 molecules bind to the Neutravidin molecules without binding non-specifically to the glass surface. Figures 28.A-28.C show Biotin-PEG-Cy5 binding to low density Neutravidin Self-Assembled Monolayer, and Figures 28.D-28.F show Biotin-PEG-Cy5 binding to high density Neutravidin Self-Assembled Monolayer.

Figures 28.A and 28.D show the superimposed fluorescence of Neutravidin with Oregon Green 488 (green-488nm) and Biotin-PEG-Cy5 (red-647nm). Figures 28.B and 28.E show green channel fluorescence of a low density Neutravidin monolayer, and figures 28.C and 28.F shows the red channel fluorescence of the Biotin-PEG-Cy5 that has bound to the Neutravidin. The Biotin-PEG-Cy5 binding to both low and high density Neutravidin molecules are more significant than the non-specific binding in regions without Neutravidin, and this difference in Biotin-PEG-Cy5 binding is distinct in regions with and without Neutravidin. Figures 28.A-28.F demonstrate the potential for using the Biotin-Neutravidin-Biotin complex with success for the overarching investigation is not yet fully elucidated, and the reproducibility of the experiment is currently being investigated.

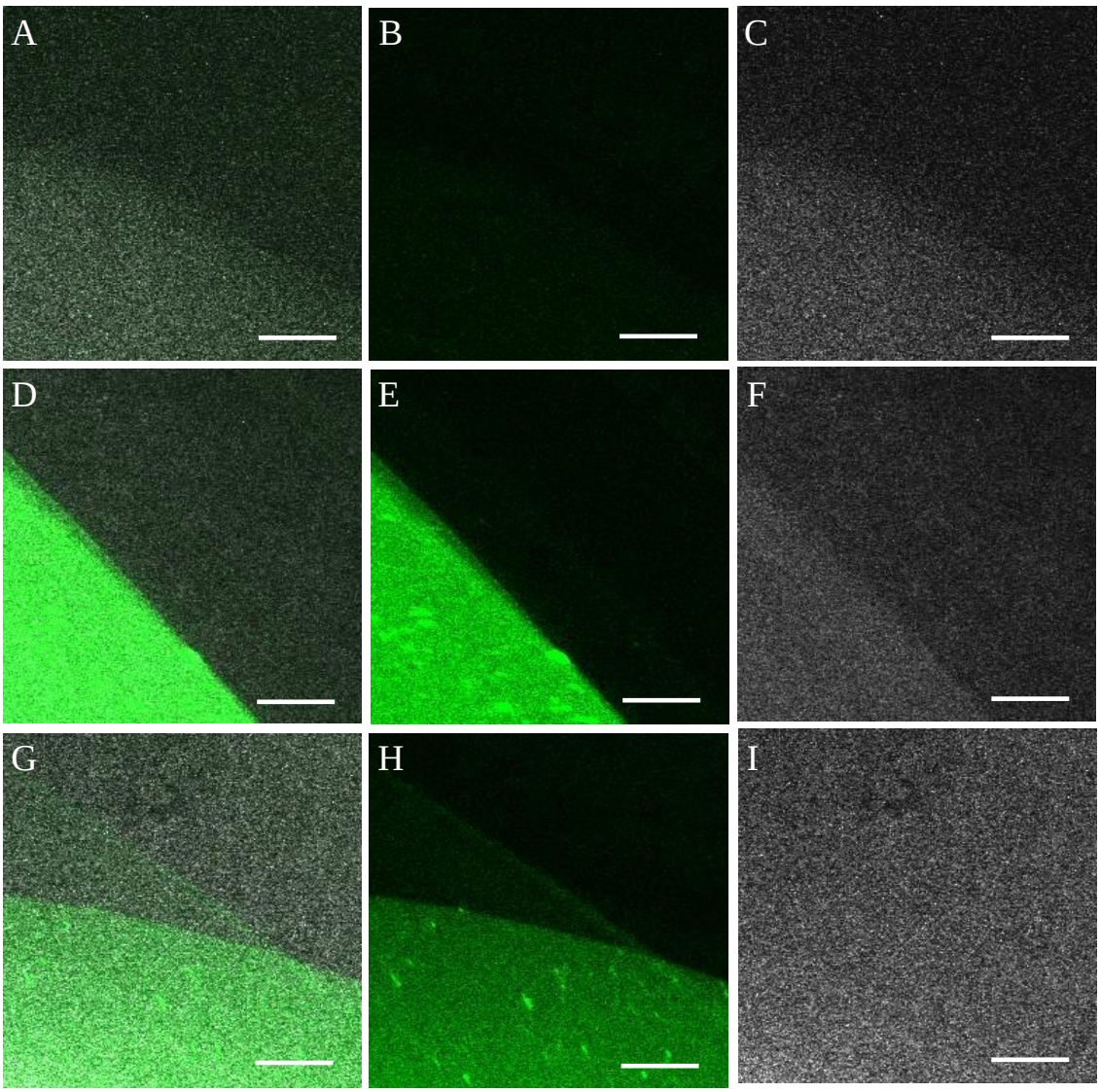


Figure 28: Co-localized Neutravidin-OG488and Biotin-PEG-Cy5 binding to low and high density Neutravidin-Silane Self-Assembled Monolayers. Scale bars represent 100µm. (A), (D), and (G) show overlayed Neutravidin-OG488 and Biotin-PEG-CY5 signals. (B), (E), (H) show Neutravidin-OG488 fluorescence. (C), (F), and (I) show Biotin-PEG-Cy5 fluorescence.

5.0. Discussion.

Previous studies have demonstrated that the spatial organization of the polysaccharide ligands play an important role in Dendritic Cell immune recognition [9, 10]. The Dectin-1 – β-glucan cell signaling process motivates the investigators to study the role of the density of β-glucan can have on Dectin-1 and Immune Cell response. To test the hypothesis of if the density of β-glucan expression on *Candida albicans* plays a role in Dectin-1 identification and Syk activation, this investigation has endeavored to develop a synthetic model of the cell wall of the *Candida albicans*, and design it such that the Beta-Glucan density can be precisely controlled. The investigator has initiated engineering Self-Assembled Monolayers to model the cell wall of the *Candida albicans* cell for investigations seeking to study the role density of β-glucan plays in the β-glucan – Dectin-1 interaction in a reduced variable, non-biological, environment. This reduced variable environment can provide the investigators a greater level of acumen for determining the critical mechanisms of β-glucan – Dectin-1interaction cell-signaling.

Although the development of this synthetic model is currently on-going, the investigator has demonstrated that it is possible to use Self-Assembled Monolayers to gain precise spatial control of the specific hosting of the ligands. Towards the goal of hosting β-glucan polysaccharides at varying densities on the Self-Assembled Monolayer, experiment 4.5.2. has demonstrated Biotin-PEG-Cy5 can be bound specifically to Neutravidin. However, whether the densities of Biotin-PEG-Cy5 can be modulated to reflect the Biotin-Neutravidin densities on the surface is still being investigated. The inter-substrate varia-

bility and reproducibility are also currently being investigated. The results discussed in thesis have developed the foundations for using Self-Assembled Monolayers to act as synthetic models of the *Candida albicans* cell wall for the goals of gaining control of how β-glucan is presented as a ligand on the surface.

The completion of this synthetic substrate can be completed by performing two more experiments. The first experiment is synthesizing biotinylated β-glucan. The second experiment is binding the biotinylated β-glucan to the Neutravidin complex on the Self-Assembled Monolayers, and studying the concentration ratios of Silane mPEG, Silane-PEG-Biotin, Neutravidin, and Biotin- β-glucan that are necessary to yield a substrate with modifiable and homogeneous β-glucan ligands. Various β-glucan – Dectin-1 and Immune Cell interaction experiments can be performed using a completed model for Phase 2 of this investigation.

The work presented in this thesis elucidates the demanding level of detail that is necessary for goals to engineer a synthetic model with precisely modifiable densities of β-glucan. Gaining the ability to precisely modulate densities of Beta-Glucan requires a gaining a working balance between Silane mPEG, Silane-PEG-Biotin, Neutravidin, and Beta-Glucan molecules. Experiment 4.4.3 serves as an excellent example for demonstrating the level of precision that is required to develop homogeneous substrates. The procedures listed in the appendix have been optimized to yield reproducible, homogeneous substrates.

6.0. Future Experiments - Phase 2: β-glucan + Dectin-1/Immune Cell interaction studies.

The objective of Phase 2 of this investigation is to use the Self-Assembled Monolayer developed in Phase 1 to study the cell-signaling of β-glucan – Dectin-1 interactions. The goal of this study will be to develop a method for immobilizing Dectin-1/Immune Cell to the surface of the Alkanethiol Self-Assembled Monolayers presenting β-glucan as a ligand. Specific dynamics of β-glucan – Dectin-1 interactions such as density of binding, time length of binding, reversibility/irreversibility of binding are topics of interest.

Phase 2 of this investigation using this model will consist mobilizing Dectin-1 to study Glucan & Dectin interaction cell-signaling to varying β-glucan density.

1. Specify the process by which Dectin-1 receptors are immobilized.
2. Characterize control of β-glucan density + spatial distribution.
    1. Modulate HS-$(CH_2)_{11}$-EG_6-OH and HS-$(CH_2)_{11}$-EG_6-Biotin ratios.
3. Possible experiments:
    1. Dectin-1 diffusion: Dectin-1 will interact with Glucan on the SAM surface, decreasing diffusion and Mobile fraction. Determine dependence of Dectin-1 mobile fraction on glucan surface density on SAM.
    2. Dectin-1 NFKB response: Reporter cells with NFKB-SEAP reporter will yield increased signal in response to increasing glucan density on SAM.
    3. Dectin-1 Ca^{2+} signaling: Study amplitude elevation duration or frequency of Ca^{2+} signal of Dectin-1 expressing cells dropped onto glucan SAM.

7.0. References.

[1] Geitenbeek TB, Gringhuis SI. Signaling through C-type lectin receptors: shaping immune responses. Nat Rev Immunol. 2009;9(7):465-79.

[2] Lin J, Wester MJ, Graus MS, Lidke KA, Neumann AK. Nanoscopic cell-wall architecture of an immunogenic ligand in candida albicans during antifungal drug treatment. Mol Biol Cell. 2016;27(6):1002-14.

[3] Hodneland CD, Lee YS, Min DH, Mrksich M. Selective immobilization of proteins to self-assembled monolayers presenting active site-directed capture ligands. Proc Natl Acad Sci USA. 2002;99(8):5048-52.

[4] Houseman B.T, Gawalt E.S, Mrksich M. Maleimide-Functionalized Self-Assembled Monolayers for the Preparation of Peptide and Carbohydrate Biochips. Langmuir. 2003; 19(5): 1522-1531.

[5] Banerjee SN, Emori TG, Culver DH, Gaynes RP, Jarvis WR, Horan T. Secular trends in nosocomial primary bloodstream infections in the Univted States, 1980-1989. National Nosocomial Infections Surveillance System. Am J Med. 1991;91(3B):86S-9S.

[6] Beck-Sague C, Jarvis WR. Secular trends in epidemiology of nosocomial fungal inefctions in the United States, 1980-1990. National Nosocomial Infections Surveillance System. J Infect Dis. 1993;167(5):1247-51.

[7] Gow NA, Hube B. Importance of the Candida albicans cell wall during commensalism and infection. Curr Opin Microbiol. 2012;15(4):406-12.

[8] Chattaway FW, Holmes MR, Barlow AJ. Cell wall composition of mycelial and blastopore forms of Candida albicans. J Gen Microbiol. 1968;51(3):367-76.

[9] Lipke PN, Garcia MC, Alsteens D, Ramsook CB, Klotz SA, Dufrene YF. Strengthening relationships: amyloids create adhesion nanodomains in yeasts. Trends Microbiol. 2012;20(2):59-65.

[10] Goodridge HS, Reyes CN, Becker CA, Katsumoto TR, Ma J, Wold AJ. Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. Nature. 2011;472(7344):471-5.

[11] Craig E. Hughes, Brenda A. Finney, Frank Koentgen, Kate L. Lowe, Steve P. Watson. The N-terminal SH2 domain of Syk is required for (hem)ITAM, but not integrin, signaling in mouse platelets. Blood. 2015 Jan 1; 125(1): 144–154.

[12] Hajjeh RA, Sofair AN, Harrison LH, Lyon GM, Arthington-Skaggs BA, Mirza SA, et al. Incidence of bloodstream infections due to Candida species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. J Clin Microbiol. 2004;42(4):1519-27.

[13] Love JC, Estroff LA, Kriebel JK, Nuzzo RG, Whitesides GM. Self-assembled monolayers of thiolates on metals as a form of nanotechnology. Chem Rev. 2005;105(4):1103-69.

[14] Xue Y, Li X, Li H, Zhang W, Quantifying Thiol-Gold interactions towards efficient strength control. Nature Communications. 2014; 5(4348).

[15] Nishi N, Hobara D, Yamamoto M, Kakiuchi T. Chain-length-dependent change in the structure of self-assembled monolayers of n-alkanethiols on Au(111) probed by broad-bandwidth sum frequency generation spectroscopy. Journal of Chemical Physics. 2003; 118(4):1904-1911.

[16] Levin CS, Janesko BG, Bardhan R, Scuseria GE, Hartgerink JD, Halas NJ. Chain-

length-dependent vibrational resonances in alkanethiol self-assembled monolayers observed on plasmonic nanoparticle substrates. Nano Lett. 2006;6(11):2617-21.

[17] Wang Y, Canchaya J. G, Dong W, Alcamí M, Busnengo H. F, Martín F. Chain-Length and Temperature Dependence of Self-Assembled Monolayers of Alkylthiolates on Au(111) and Ag(111) Surfaces. The Journal of Physical Chemistry A. 2014; 118(23), 4138-4146.

[18] Mrksich M, Whitesides G.M**.** Using Self-Assembled Monolayers that present Oligo(ethylene glycol) groups to control the interaction of proteins with surfaces. American Chemical Society. 1997; 680(23), 361-373.

[19] David S. Wilson, Steffen Nock. Recent developments in protein microarray technology. Angew Chem Int Ed Engl. 2003 Feb 3; 42(5): 494–500.

[20] Michael Schaeferling, Stefan Schiller, Hubert Paul, Margit Kruschina, Petra Pavlickova, Mark Meerkamp, Chiara Giammasi, Dev Kambhampati. Application of self-assembly techniques in the design of biocompatible protein microarray surfaces. Electrophoresis. 2002 Sep 19; 23(18):2097-105.\

[21] Ralph G. Nuzzo, Lawrence H. Dubois, David L. Allara. Fundamental studies of microscopic wetting on organic surfaces. 1. Formation and structural characterization of a self-consistent series of polyfunctional organic monolayers. *J. Am. Chem. Soc.*, 1990, *112*(2):558–69.

[22] Haeussling L, Ringsdorf H, Schmitt F.J, Knoll W. Biotin-Functionalized Self - Assembled Monolayers on Gold: Surface Plasmon Optical Studies of Specific Recognition Reactions. Langmuir. 1991; 7(9), 1837-1840.

[23] Buchwalow I, Samoilova V, Boecker W, Tiemann M. Non-specific binding of

antibodies in immunohistochemistry: fallacies and facts. Sci Rep. 2011;1:28.

[24] H. Kim, I.-H. Cho, J.H. Park, S. Kim, S.-H. Paek, J. Noh, H. Lee. Analysis of a non-labeling protein array on biotin modified gold surfaces using atomic force microscopy and surface plasmon resonance. Colloids and Surf. A: Physicochem. Eng. Aspects. 2008 Feb 1; 313:541-44.

[25] D.A. Offord, C.M. John, M.R. Linford, J.H. Griffin. Contact Angle Goniometry, Ellipsometry, and Time-of-Flight Secondary Ion Mass Spectrometry of Gold Supported, Mixed Self -Assembled Monolayers Formed from Alkyl Mercaptans. Langmuir. 1994; 10:883-889.

[26] K.E. Nelson, L. Gamble, L.S. Jung, M.S. Boeckl, E. Naeemi, S.L. Golledge, T. Sasaki, D.G. Castner, C.T. Campbell, P.S. Stayton. Surface characterization of mixed self-assembled monolayers designed for streptavidin immbolization. Langmuir. 2001 Mar 28; 17(9):2807-2816.

[27] G.M. Whitesites, P.E. Laibinis. Wet Chemical Approaches to the Characterization of Organic Surfaces: Self-Assembled Monolayers, Wetting, and the Physical-Organic Chemistry of the Solid-Liquid Interface. Langmuir. 1990; 6:87-96.

[28] J. Spinke, M. Liley, F.J. Schmitt. Molecular recognition at self-assembled monolayers: Optimization of surface functionalization. J. Chem. Phys. 1993; 99:7012.

[29] O. Azzaroni, M. Mir, W. Knoll. Supramolecular Architectures of Streptavidin on Biotinylated Self-Assembled Monolayers. Tracking Biomolecular Reorganization after Bioconjugation. J. Phys Chem. B. 2007; 111(48):13499-503.

[30] J. Hyun, Y. Zhu, A. Liebmann-Vinson, T.P. Beebe, A. Chilkoti. Microstamping on an Activated Polymer Surface: Patterning Biotin and Streptavidin onto Common Polymeric

Biomaterials. Langmuir. 2001; 17(20):6358-67.

[31] Y.L. Jeyachandran, J.A. Mielczarski, E. Mielczarski, B. Rai. Efficiency of blocking of non-specific interaction of different proteins by BSA adsorbed on hydrophobic and hydrophilic surfaces, Journal of Colloid and Interface Science. 2010; 341(1).

[32] Hiller Y, Gershoni JM, Bayer EA, Wilchek M. Biotin binding to avidin. Oligosaccharide side chain not required for ligand association. *Biochem J*. 1987;248(1):167-71.

8.0. Appendix A: Methods.

Experiment 4.3.1.

Procedure:

1. Physical vapor deposition.
    1. Image to check for dirt.
2. UVO oxidation (1 hour).
3. SAM formation. (overnight)
4. Wash & Stream dry.
    1. Remove Alkanethiol-Ethanol solution.
    2. Wash with ethanol 2X.
    3. Stream dry.
5. Streptavidin (1.5 hours).
6. PBS wash out (10 minutes).
    1. Use .2µm filter.
    2. Keep wet, do not dry.
    3. Do one sample with water.
7. Image. Do negative controls first to establish baseline fluorescence.

SAM	OH (µL)	Biotin (mg)	Streptavidin
100% OH	2.09 µL OH	0	+, 10µL (1024µg/mL)
100% OH	2.09 µL OH	0	-
90% OH 10% Biotin	1.88 µL OH	.26 mg Biotin	+, 10µL (1024µg/mL)
90% OH 10% Biotin	1.88 µL OH	.26 mg Biotin	-
80% OH 20% Biotin	1.67 µL OH	.52 mg Biotin	+, 10µL (1024µg/mL)
80% OH 20% Biotin	1.67 µL OH	.52 mg Biotin	-
70% OH 30% Biotin	1.46 µL OH	.78 mg Biotin	+, 10µL (1024µg/mL)
70% OH 30% Biotin	1.46 µL OH	.78 mg Biotin	-
60% OH 40% Biotin	1.25 µL OH	1.04 mg Biotin	+, 10µL (1024µg/mL)
60% OH 40% Biotin	1.25 µL OH	1.04 mg Biotin	-
50% OH 50% Biotin	1.05 µL OH	1.3 mg Biotin	+, 10µL (1024µg/mL)
50% OH 50% Biotin	1.05 µL OH	1.3 mg Biotin	-
40% OH 60% Biotin	0.84 µL OH	1.56 mg Biotin	+, 10µL (1024µg/mL)
40% OH 60% Biotin	0.84 µL OH	1.56 mg Biotin	-
0% OH 0% Biotin	0	0	+, 10µL (1024µg/mL)
0% OH 0% Biotin	0	0	-
80% OH 20% Biotin (H_2O wash)	1.67µL OH	.52 mg Biotin	+, 10µL (1024µg/mL)

Experiment 4.3.2.

Procedure:

1. Physical vapor deposition.
2. UVO oxidation (1 hour).
3. SAM formation. (overnight)
4. Wash & Stream dry.
    1. Remove Alkanethiol-Ethanol solution.
    2. Wash with ethanol 2X.
    3. Stream dry.
5. Streptavidin (10 minutes).
6. PBS wash out (10 minutes).
    1. Use .2µm filter.
    2. Keep wet, do not dry.
    3. Do one sample with water.
7. Image.
    1. Do negative controls first to establish baseline fluorescence.

SAM	OH (µL)	Biotin (mg)	Streptavidin	Binding t
100% OH	2.09 µL OH	0	+, 10µL (1024µg/mL)	10 min
100% OH	2.09 µL OH	0	+, 10µL (1024µg/mL)	30 min
100% OH	2.09 µL OH	0	0µL	0 min
90% OH, 10% Biotin	1.88 µL OH	.26 mg Biotin	+, 10µL (1024µg/mL)	10 min
90% OH, 10% Biotin	1.88 µL OH	.26 mg Biotin	+, 10µL (1024µg/mL)	30 min
90% OH, 10% Biotin	1.88 µL OH	.26 mg Biotin	0µL	0 min
80% OH, 20% Biotin	1.67 µL OH	.52 mg Biotin	+, 10µL (1024µg/mL)	10 min
80% OH, 20% Biotin	1.67 µL OH	.52 mg Biotin	+, 10µL (1024µg/mL)	30 min
80% OH, 20% Biotin	1.67 µL OH	.52 mg Biotin	0µL	0 min
70% OH, 30% Biotin	1.46 µL OH	.78 mg Biotin	+, 10µL (1024µg/mL)	10 min
70% OH, 30% Biotin	1.46 µL OH	.78 mg Biotin	+, 10µL (1024µg/mL)	30 min
70% OH, 30% Biotin	1.46 µL OH	.78 mg Biotin	0µL	0 min
0% OH, 0% Biotin	0	0	+, 10µL (1024µg/mL)	10 min
0% OH, 0% Biotin	0	0	+, 10µL (1024µg/mL)	30 min
0% OH, 0% Biotin	0	0	0µL	0 min

Experiment 4.3.4.

Procedure:

1. Physical vapor deposition.
2. UVO oxidation (1 hour).
3. SAM formation. (overnight)
4. Wash & Stream dry.
    1. Remove Alkanethiol-Ethanol solution.
    2. Wash with ethanol 2X.
    3. Stream dry.
5. Streptavidin (30 minutes).
6. PBS wash out (10 minutes).
    1. Use .2µm filter.
    2. Keep wet, do not dry.
7. Image.
    1. Do negative controls first to establish baseline fluorescence.
    2. Check the histogram values. Check if the Black, white values are the same.

SAM	OH (µL)	Biotin (mg)	Streptavidin	Binding t	Blocking
0% OH , 0% Biotin	0	0	0µL	0 min	-
0% OH , 0% Biotin	0	0	+, 5µL (1024µg/mL)	30 min	-
90% OH, 10% Biotin	1.88 µL OH	.26 mg Biotin	0µL	0 min	-
90% OH, 10% Biotin	1.88 µL OH	.26 mg Biotin	+, 5µL (1024µg/mL)	30 min	-
90% OH, 10% Biotin	1.88 µL OH	.26 mg Biotin	+, 5µL (1024µg/mL)	30 min	5% BSA
90% OH, 10% Biotin	1.88 µL OH	.26 mg Biotin	+, 5µL (1024µg/mL)	30 min	1% FBS
90% OH, 10% Biotin	1.88 µL OH	.26 mg Biotin	+, 5µL (1024µg/mL)	30 min	5% Milk

Experiment 4.3.5.

Procedure:

1. Physical vapor deposition.
2. UVO oxidation (1 hour).
3. Self-Assembled Monolayer formation. (overnight)
4. Wash & Stream dry.
    1. Remove Alkanethiol-Ethanol solution.
    2. Wash with ethanol 2X.
    3. Stream dry.
5. Streptavidin (30 minutes).
6. PBS wash out (10 minutes).
    1. Use .2µm filter.
    2. Keep wet, do not dry.
7. Image.
    1. Do negative controls first to establish baseline fluorescence.
    2. Check the histogram values. Check if the Black, white values are the same.

SAM	OH (µL)	Biotin (mg)	Strept/Neut	Binding t	Blocking
0% OH , 0% Biotin	0	0	0µL	0 min	-
0% OH , 0% Biotin	0	0	+, 5µL, Strept (1024µg/mL)	30 min	-
0% OH , 0% Biotin	0	0	+, 5µL, Neut (1024µg/mL)	30 min	-
80% OH, 20% Biotin	1.67 µL OH	.52 mg Biotin	0µL	0 min	-
80% OH, 20% Biotin	1.67 µL OH	.52 mg Biotin	+, 5µL, Strept (1024µg/mL)	30 min	-
80% OH, 20% Biotin	1.67 µL OH	.52 mg Biotin	+, 5µL, Neut (1024µg/mL)	30 min	-
80% OH, 20% Biotin	1.67 µL OH	.52 mg Biotin	+, 5µL, Strept (1024µg/mL)	30 min	5% FBS
80% OH, 20% Biotin	1.67 µL OH	.52 mg Biotin	+, 5µL, Neut (1024µg/mL)	30 min	5% FBS

Experiment 4.4.1. & 4.4.3.

Procedure:

1. Silane solution preparation. (30 minutes)
    1. Make PEGylation solution of Ethanol/H_2O (w/w 95%/5%)solution.
    2. Make Silane PEG solution: 13mg/mL of Silane PEG in PEGylation solution.
    3. Allow PEGylations solution to cover the surface of the Glass-bottom dish at Room temperature for 30 minutes.
    4. Wash out unreacted materials with water.
2. Streptavidin (30 minutes).
3. PBS wash out (30 minutes, 5X).
    1. Use .2µm filter.
    2. Keep wet, do not dry.
4. Image..
    1. Do negative controls first to establish baseline fluorescence.
    2. Check the histogram values. Check if the Black, white values are the same.

Composition	Silane (mg in µL)	Biotin (mg)	Strept/Neut	Blocking
100% Silane	1mg in 80µL	0	10µL (4µg/mL)	-
100% Silane	1mg in 80µL	0	10µL (8µg/mL)	5% FBS + 0.1% Tween20 + PBS
25% Silane, 75% Biotin	0.25mg in 20µL	0.75mg in 60µL	10µL (4µg/mL)	-
25% Silane, 75% Biotin	0.25mg in 20µL	0.75mg in 60µL	10µL (8µg/mL)	5% FBS + 0.1% Tween20 + PBS
50% Silane, 50% Biotin	0.5mg in 40µL	0.5mg in 40µL	10µL (4µg/mL)	-
50% Silane, 50% Biotin	0.5mg in 40µL	0.5mg in 40µL	10µL (8µg/mL)	5% FBS + 0.1% Tween20 + PBS
75% Silane, 25% Biotin	0.75mg in 60µL	0.25mg in 20µL	10µL (4µg/mL)	-
75% Silane, 25% Biotin	0.75mg in 60µL	0.25mg in 20µL	10µL (8µg/mL)	5% FBS + 0.1% Tween20 + PBS
100% Biotin	0	1mg in 80µL	10µL (4µg/mL)	-
100% Biotin	0	1mg in 80µL	10µL (8µg/mL)	5% FBS + 0.1% Tween20 + PBS
Blank	0	0	10µL (4µg/mL)	-
Blank	0	0	10µL (8µg/mL)	5% FBS + 0.1% Tween20 + PBS
Blank	0	0	0µL	-

Experiment 4.4.2.

Procedure:

1. Silane solution preparation. (30 minutes)
    1. Make PEGylation solution of Ethanol/H_2O (w/w 95%/5%)solution.
    2. Make Silane PEG solution: 13mg/mL of Silane PEG in PEGylation solution.
    3. Allow PEGylations solution to cover the surface of the Glass-bottom dish at Room temperature for 30 minutes. Use rotator to agitate.
    4. Wash out unreacted materials with water.
2. Streptavidin (30 minutes).
3. PBS wash out (30 minutes, 5X).
    1. Use .2µm filter.
    2. Keep wet, do not dry.
4. Image.
    1. Do negative controls first to establish baseline fluorescence.
    2. Check the histogram values. Check if the Black, white values are the same.

Composition	Silane (mg in µL)	Biotin (mg)	Strept/Neut	Blocking
100% Silane	1mg in 80µL	0	10µL (4µg/ml)	-
100% Silane	1mg in 80µL	0	10µL (4µg/ml)	5% BSA
100% Silane	1mg in 80µL	0	10µL (4µg/ml)	5% FBS
100% Silane	1mg in 80µL	0	10µL (4µg/ml)	5% Milk
Blank	0	0	10µL (4µg/ml)	-
Blank	0	0	10µL (4µg/ml)	5% BSA
Blank	0	0	10µL (4µg/ml)	5% FBS
Blank	0	0	10µL (4µg/ml)	5% Milk
Blank	0	0	0µL	-

Experiment 4.5.1.

Procedure:

1. Silane solution preparation. (30 minutes)
    1. Make PEGylation solution of Ethanol/H_2O (w/w 95%/5%) solution.
    2. Make Silane PEG solution: 13mg/mL of Silane PEG in PEGylation solution.
    3. Allow PEGylations solution to cover the surface of the Glass-bottom dish at Room temperature for 30 minutes. Use rotator to agitate. Wash out unreacted materials with water.
2. Biotin-PEG-Cy5, 8µg/ml after FBS. PBS wash out (30 minutes, 5X). Keep wet, do not dry. Block with 5%FBS + 0.1% Tween20.
3. Image.
    1. Do negative controls first to establish baseline fluorescence.
    2. Check the histogram values. Check if the Black, white values are the same.

Composition	Silane (mg in µL)	Biotin (mg)	PEG-Cy5	Blocking
100% Silane	1mg in 80µL	0	10µL (8µg/ml)	-
100% Silane	1mg in 80µL	0	10µL (8µg/ml)	5% BSA
100% Silane	1mg in 80µL	0	10µL (8µg/ml)	1% FBS
100% Silane	1mg in 80µL	0	10µL (8µg/ml)	5% Milk
Blank	0	0	10µL (8µg/ml)	-
Blank	0	0	10µL (8µg/ml)	5% BSA
Blank	0	0	10µL (8µg/ml)	1% FBS
Blank	0	0	10µL (8µg/ml)	5% Milk
Blank	0	0	0µL	-

Experiment 4.5.2.

Procedure:

1. Silane solution preparation. (30 minutes)
    1. Make PEGylation solution of Ethanol/H_2O (w/w 95%/5%)solution.
    2. Make Silane PEG solution: 13mg/mL of Silane PEG in PEGylation solution.
    3. Allow PEGylations solution to cover the surface of the Glass-bottom dish at Room temperature for 30 minutes. Use rotator to agitate. Wash out unreacted materials with water.
2. Streptavidin (30 minutes), 8µg/ml after FBS. PBS wash out (30 minutes, 5X). Keep wet, do not dry. Block with 5%FBS + 0.1% Tween20.
3. Biotin-PEG-Cy5, 8µg/ml after FBS. PBS wash out (30 minutes, 5X). Keep wet, do not dry. Block with 1%FBS + 0.1% Tween20.
4. Image.
    1. Do negative controls first to establish baseline fluorescence.
    2. Check the histogram values. Check if the Black, white values are the same.

Composition	Silane (mg in µL)	Biotin (mg in µL)	Neutravidin	Biot-Peg-Cy5	Blocking
100% Silane	1mg in 80µL	0	10µL (8µg/ml)	10µL (8µg/ml)	5% FBS
100% Silane	1mg in 80µL	0	10µL (8µg/ml)	10µL (8µg/ml)	5% FBS
100% Silane	1mg in 80µL	0	10µL (8µg/ml)	10µL (8µg/ml)	5% FBS
10% Biotin, 90% Silane	0.90mg in 72µL	0.1mg in 8µL	10µL (8µg/ml)	10µL (8µg/ml)	5% FBS
10% Biotin, 90% Silane	0.90mg in 72µL	0.1mg in 8µL	10µL (8µg/ml)	10µL (8µg/ml)	5% FBS
10% Biotin, 90% Silane	0.90mg in 72µL	0.1mg in 8µL	10µL (8µg/ml)	10µL (8µg/ml)	5% FBS
10% Biotin, 90% Silane	0.90mg in 72µL	0.1mg in 8µL	10µL (2048µg/ml)	10µL (8µg/ml)	5% FBS
10% Biotin, 90% Silane	0.90mg in 72µL	0.1mg in 8µL	10µL (2048µg/ml)	10µL (8µg/ml)	5% FBS
10% Biotin, 90% Silane	0.90mg in 72µL	0.1mg in 8µL	10µL (2048µg/ml)	10µL (8µg/ml)	5% FBS
Blank	0	0	10µL (8µg/ml)	10µL (8µg/ml)	5% FBS
Blank	0	0	0µL		-