GLYCOSYLATION OF SURFACES VIA POST-POLYMERIZATION MODIFICATION AND HYDRAZONE CONJUGATION

by

DEBORAH CAROLINE LEMAN

(Under the Direction of Jason Locklin)

ABSTRACT

As technologies for surface design and synthetic biomaterials advance, the development of cost-effective and simple methods for polymeric bioconjugated surfaces becomes increasingly important. Carbohydrate conjugation to surfaces has routinely been performed through small molecule attachments to complementary chemistries at the interface. This, however, lacks the advantages of polymeric materials and often requires synthetic modification to the sugar residue which may come at a time cost or loss of biological recognition. Polymer-based materials provide avenues to mimic cell membrane presentation of glycans in order to increase binding affinity. To design surfaces with variable density and ratio of functionalities and the ability to be patterned, a "grafted-to" polymeric activated ester, pentafluorophenyl acrylate (PFPA), was utilized. Post-polymerization modification of the surface-grafted polymer with hydrazine resulted in the ability to conjugate carbohydrates via their reducing end. This attachment chemistry was shown to retain the biorecognition by incubation of 3'-sialyllactose conjugated substrates in the presence of Mycoplasma pneumoniae. The bacteria displayed gliding motility comparable to that observed when exposed to sialic acid containing glycoproteins. This

process was optimized to achieve cost-effective and mild conditions as a route to conjugation of a wide variety of carbohydrates.

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DEBORAH CAROLINE LEMAN

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DEBORAH CAROLINE LEMAN

Major Professor: Jason Locklin

Committee: Geert-Jan Boons
Sergiy Minko

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
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DEDICATION

My thesis is dedicated to my late grandfather, Wesley Perschbacher. He was the inspiration for me to attend graduate school as I initially planned to seek a PhD. He is the only member of my family to complete a doctoral degree and taught as a Greek professor at Moody Bible Institute where my parents met and I was born. Although he had a high education, it was later in life that this was the case. He was a hard working pastor with six children who was academic and respected among many. He loved his wife greatly and served Jesus until his last breath. Combined with his influence and my father's academic interests, I have held a large respect for post-graduate education. I hope to use my education to better those around me while continually serving Jesus myself. One day I will see you again Grandpa and I hope to make you proud, not only in hard work but especially in the way I devote my life to Christ and loving others.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Bioconjugation of Carbohydrates

In the realm of science and technology, there lies bioconjugation as an interface between the natural and the synthetic. It is this juncture that provides a major platform for modern biomedical and biochemical development. Bioconjugation is defined simply as the attachment of two molecules, at least one of which is typically biologically derived, forming a complex that is most often made through a covalent bond, as pictorially described by Figure 1. There are countless areas of research and technology that utilize techniques for these types of attachments with goals ranging from drug development or disease diagnosis or simply explanation of natural phenomena. The joining of two types of molecules with very different properties can allow for a combination of both properties within the same complex[1].

Figure 1.1: Cartoon representation of the basic principle of bioconjugation as the attachment of two molecules with different properties by some linking chemical bond.

There are a vast number of applications for each major class of biomolecule and its attachment to either a synthetic molecule or another natural component. Bioconjugation encompasses linkages involving nucleic acids and oligonucleotides[2, 3], amino acids and proteins[2, 4, 5], enzymes, carbohydrates[6, 7], biotinylation, antibodies[8-10], and more. Some

areas of interest include fluorescent labelling[11], detection, diagnostics[3, 11, 12], immunoassays, cellular and *in vivo* imaging[6, 13], protein assays, vaccine development[14], drug delivery[6, 7, 9], affinity purification and capture[15], and biomaterials[6, 16]. An example of its use in drug delivery is the conjugation of alkyne-modified doxorubicin to azide functionalized glycans found on IgG antibodies via strain-promoted cycloaddition and the demonstration of its dose-dependent cytotoxicity as an anticancer agent by the Boons group[17]. The attachment of oligonucleotides to fluorescent probes provides an avenue for diagnostics related to genetics and DNA sequencing[3]. Lukhtanov et al showed the attachment of fluorescein and other similar dyes through an amide linkage to oligonucleotides and their successful hybridization with DNA segments[12]. These are just a few examples of the importance and utility of bioconjugates in a growing technological field.

The toolbox of chemistries for conjugation linkages is extensive and often provides many routes to the same complex formation. A strategy can be developed for each conjugation to allow the best combination of selectivity, bioorthogonality, efficiency, ease, and sequence. Development of these techniques leads to the ability to control the attachments made and provide simple steps for a variety of ligands. The chemoselectivity can allow for investigation of specific interactions while having little to no effect on the biological activity of the molecule or its surroundings. It requires the presence of a moiety within the biomolecule structure, either natural or synthetic, that can serve as a reactive handle for covalent bond formation. Some commonly used biorthogonal chemistries are alkyne-azide cycloadditions, ester cleavages forming amides, oxime and hydrazone formations, reductive amination, disulfide linkages, thioester formations, thiol-ene

coupling, Staudinger ligations, Diels-Alder cyclizations, olefin metathesis, and many more[18].

Many times the chemistry of choice requires the addition of a reactive handle to the biomolecule of interest to increase reactivity and make the ligation more selective. For example, many carbohydrates are modified to contain a complementary reactive group for attachment to another molecule, support, or surface. Proteins can also be synthetically modified, but they serve as good examples of the utility of native moieties in that they naturally contain many complementary reactive groups for attachments[19]. Some of the most used are the thiol present in cysteine which can be used in disulfide and thio-ester bond formations[5], the amine present in lysine[20, 21], and the carboxylic acid found in glutamic acid residues. The common use of lysine is for amide linked complex formation as shown in Figure 2.

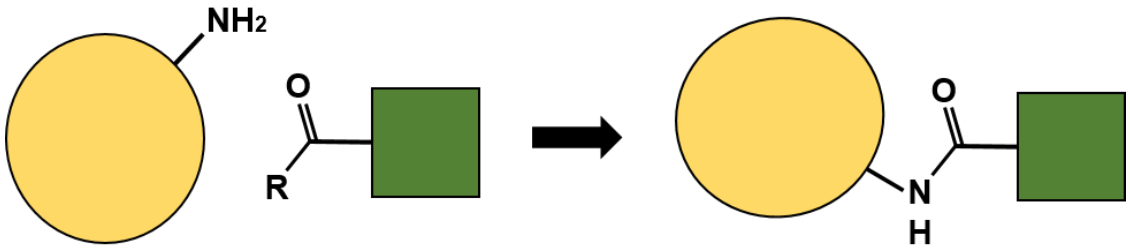


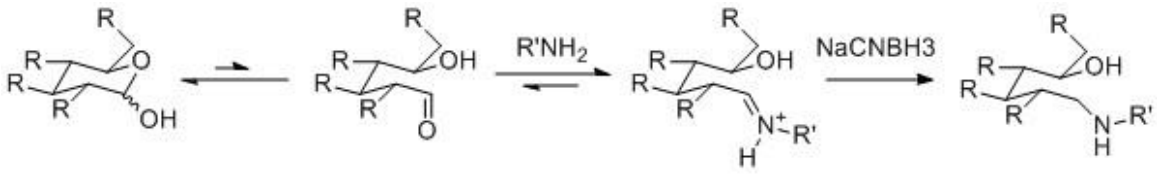
Figure 1.2: Cartoon depiction of protein conjugation strategy via lysine amide formation.

Conjugation of saccharides tends to be more complex due to diverse structural features and require well-defined strategies, however, there are many different techniques

available. Carbohydrates are useful in nature for structural support, such as cellulose found in the cell walls of plants, lubrication such as the highly charged glycosaminoglycans found in the joints[22], and energy such as glucose in the production of ATP. Nature utilizes a multitude of various glycosidic linkages, complicated branching, and a variety of monosaccharide structures to create a code for innumerous protein and cellular interactions, signaling, and processes[23]. These glycan mediated events range from cell signaling[24] to pathogen recognition and genetic disorders[25]. Carbohydrate researchers in a variety of fields aim to better understand life through its sugar code which mediates countless processes. Conjugation offers plentiful techniques for observation of individual events in a controlled manner, isolated from the complex natural environment in which they occur as well as methods for development of biomaterials. Due to the structural complexity and mixture of components in biological samples, isolation, purification, and synthetic modifications are often difficult and expensive. Many lithographic and array technologies have been developed to create a platform for minimal sample usage and high-throughput screening, decreasing the cost and advancing the rate of knowledge-base growth within glycomics.

There are many functional sites found within saccharide structures that can be selectively modified to contain reactive chemistries for attachment such as amines or hydrazides for reaction with activated esters[26, 27], azides for click reactions[28, 29], or aldehydes through oxidation of diols or synthetic terminal alkenes that can be ligated to amines[30, 31]. However, these can present many synthetic challenges and, especially in the case of oxidation, can alter the structure to the extent that the biological recognition and physical properties are lost[32]. It is quite common for carbohydrates, whether

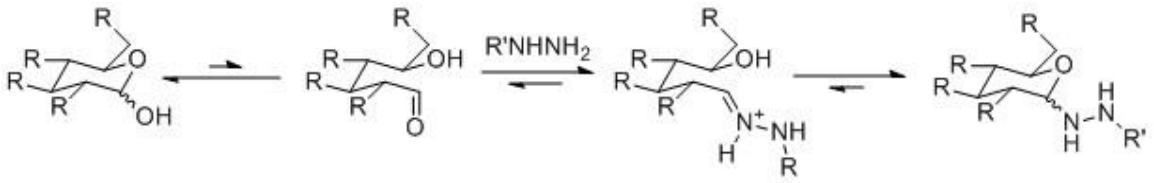
monosaccharide units or large, branched polysaccharides, to naturally carry a reducing terminus with the ability to ring open, revealing an aldehyde group. As an alternative to oxidation, many use this reducing end aldehyde as the handle for conjugation, most commonly by reductive amination[8, 33-35]. When the imine that is formed is reduced, this causes the ring to be permanently opened as shown in Scheme 1. Depending on the importance of the reducing end unit for biological function, this could be problematic.



Scheme 1.1: Strategy utilizing the ring opening of the reducing terminus of a monosaccharide and conjugation via imine formation followed by reductive amination at the aldehyde site.

It is possible to utilize the reactive handle provided by the reducing terminus in the absence of reducing agent, leaving behind an imine-type bond that can reform a ring closed unit. This linkage is not as stable because it is reversible upon hydrolysis, which is a major reason many use reductive amination. Another challenge associated with reducing end conjugation is the slow rate of ring opening in solution. At a given time, less than 0.01% of sugar molecules (depending on its structure) in solution are present in their ring open forms[36, 37]. To better combat rate and stability issues, alternative nucleophiles to amines have been demonstrated useful in addition to aniline-based catalysts. Hydrazone and oxime

ligations are shown in the literature to be efficient due to increased nucleophilicity relative to amines and display increased complex stability at neutral pH[38, 39]. Hydrazone formation is a reversible process, which can have its advantages for various applications involving reversible crosslinking or conjugation as it is stable at neutral pH and can be dissociated at low pH[40]. Aniline-based compounds have been demonstrated by Dirksen et al to have catalytic efficiency in the formation of hydrazones and oximes described for use in dynamic covalent chemistry[41, 42]. The rate of aldehyde conjugation is increased drastically by addition of 10 equivalents of aniline due to its ability to form an unstable complex with the aldehyde, making a highly reactive electrophile for attack by hydrazine. An additional benefit of the large excess of aniline in solution along with saccharides which are rarely ring open is the ability of aniline to capture the aldehyde before it reforms the hemiacetal, allowing the conjugation to hydrazine to be more likely. After hydrazone formation occurs, the sugar unit can return to its native, stable pyranose form as apparent in Scheme 2, which will likely preserve the biological function.



Scheme 1.2: Conjugation strategy involving aniline catalyzed hydrazone formation via the reducing end aldehyde of a saccharide and its subsequent ring closing to form a stable complex.

Glycopolymers

It has been argued in the literature that there is great importance to the three-dimensional presentation, concentration, and multi-valency of natural ligands found on cell surfaces and proteins for the occurrence of binding events[43-45]. In addition, there is argument that polymers are of use in mimicking these natural presentations and multi-valent structures[46]. This is because synthetic materials can provide control over the orientation, ratio, density, and statistical dilution of functionality along the polymer chain. In designing surfaces such as microarrays, a surface coated or grafted with polymer decorated by glycans offers the potential for increased biological recognition and binding affinity over direct surface attachment of the small molecule ligands. These type of hybrid natural synthetic materials are commonly called glycopolymers. Hsieh-Wilson's group has synthesized many GAG-mimetic glycopolymers by ring-opening metathesis polymerization and observed the 100-fold increase in binding affinity to antithrombin III over natural heparin, demonstrating the potential of these types of materials to improve upon natural properties for pharmaceutical applications[47]. There are various routes to obtain a polymer composed of carbohydrates either through polymerization of glycomonomers[24, 45, 48, 49] or post-polymerization modification[50, 51]. Some examples of this include the surface-initiated atom transfer radical polymerization of monosaccharide based monomers by Kizhakkedathu and the copper click of azide-modified sugars to an alkyne acrylate based polymer made via living radical polymerization by Haddleton[45, 52].

A simple route to glycopolymer synthesis is post-polymerization conjugation of sugars to a hydrazine-type nucleophile built into the polymer structure. There have been several reports of synthetic schemes to obtain an acrylate polymer carrying hydrazide

functional moieties along the backbone, called poly(acryloyl hydrazide) (PAH)[39, 53-55]. This material has been demonstrated to have many applications in dynamic covalent chemistry and glycopolymer synthesis such as stimuli responsive hydrogels[32, 53], drug delivery systems[54, 55], ion exchange resins[53], and glycopolymers[39]. The synthetic routes to obtaining PAH are often multistep and performed using solution polymer. Kumar et al showed the synthesis of PAH by modification of polymethyl acrylate with hydrazine which required stirring for 12 hours at 60°C and then demonstrated its use for a variety of materials applications[53]. Bertozzi's group achieved the desired polymer by reversible addition-fragmentation chain transfer polymerization of acetoxime acrylate with a biotinylated chain transfer agent then modification with hydrazine and showed its utility in glycopolymer synthesis for microarrays printed on streptavidin[39]. Both of these routes require long reaction times for the post-polymerization hydrazine modification and are not covalently grafted to a surface.

Activated Ester Polymer Thin Films

Activated esters have been widely used in bioconjugation due to their ability to rapidly form amide linkages, considered a click-like reaction, and are commercially available as a coating on many different devices and substrates for biological research. These types of reactive groups can be cleaved by any amine-containing compound, whether biomolecules like proteins, or dyes. N-hydroxysuccinimide (NHS) esters are one of those most commonly employed, and have been used to create reactive polymers since 1972, when they were pioneered[56, 57], either by post-polymerization modification or controlled polymerization of NHS-based monomers such as surface initiated atom transfer radical polymerization[58] or nitroxide-mediated polymerization[59]. D'Souza et al showed the utility

of copolymers that have an NHS-derived block modified to bear bisphosphonate groups that bind to bone tissue for linking mesenchymal stem cells to damaged bone for improved healing and regeneration[60]. NHS esters have shown themselves useful in bioconjugation, however, it is somewhat unstable to hydrolytic cleavage and can undergo side reactions causing crosslinking between active ester sites[58, 61]. An alternative activated ester for rapid amine cleavage is known as pentafluorophenyl ester[62-64]. It exhibits greater hydrolytic stability and a wider range of solubility in organic solvents, and Pentafluorophenyl acrylate (PFPA) can be synthesized in one step and can undergo free-radical polymerizations[63].

For the purpose of fabricating carbohydrate decorated surfaces that can be used in various studies related to cell or protein interactions, polymeric systems can serve to mimic some facets of the 3D geometry provided by cell surfaces. As a platform for these studies, thin films comprised of this polymer, pPFPA, can be prepared. There are two commonly used techniques to create covalently attached thin films known as polymer brushes. The first is created by "grafting-from" an interface by surface initiated free-radical polymerization, a technique in which an initiator molecule, such as azobisisobutyronitrile (AIBN) modified with a surface reactive functional group, is first allowed to form a self-assembled monolayer (SAM) on the substrate of interest. Subsequently, the polymerization is initiated in the presence of monomer resulting in densely packed polymer chains grown from the surface. Because of the high grafting densities associated with "grafting-from", the polymer exhibits extended chain conformations normal to the surface, forming an architecture with unique properties known as a polymer brush[65, 66]. The second route to obtain polymer thin films that are covalently attached to surfaces is via "grafting-to", a process by which polymer is coated onto a surface and reacted with surface functionality

to form a less dense and thinner film. “Grafting-to” generally leads to low grafting densities and is not considered to have true “brush” architecture as individual chains are not forced to extend upward and may contain multiple attachment points causing loops and tails[66, 67]. Both techniques, as depicted in Figure 3, are useful for creating easily modifiable pPFPA surfaces for a variety of uses. Due to the high reactivity toward amines, PFPA groups have the potential to be reacted with lysine residues present in protein structures or hydrazine forming a hydrazide that is reactive toward carbohydrate reducing termini. In order to make surface grafted PAH, the esters of pPFPA are modified with hydrazine after surface attachment. As mentioned previously, this carbohydrate conjugation chemistry is much more efficient with aniline catalysis, however there are other reaction conditions that require optimization to achieve a high percentage of conjugated groups relative to the number of reactive sites. After the optimization, surfaces containing many patterns, ratios, and statistical dilutions of an endless number of different reducing sugars can be made, likely with retention of biological function, using small amounts of purified ligand and no additional synthetic modifications.

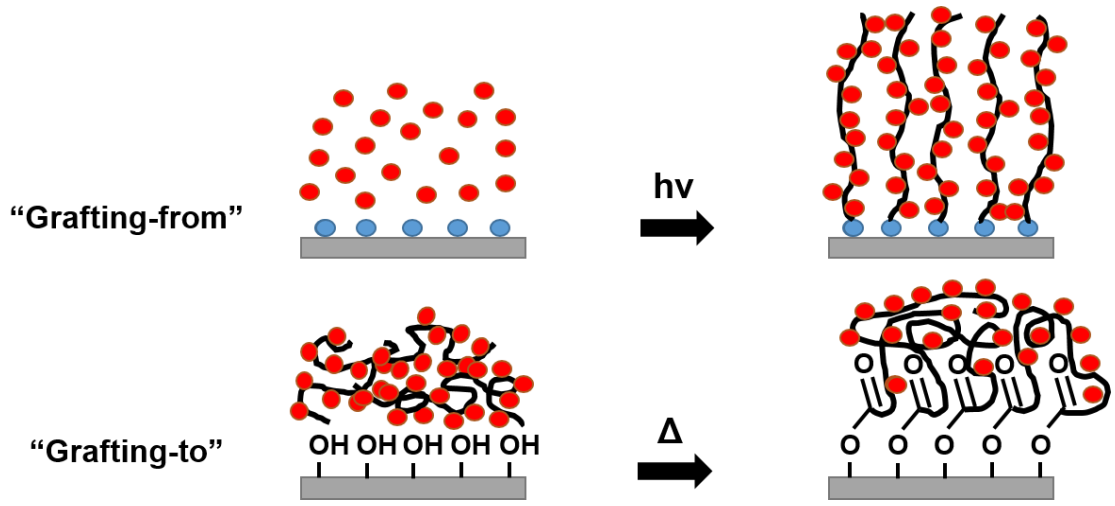


Figure 1.3: Cartoon portraying the processes of "grafting-from" and "grafting-to" for surface modification with pPFPA.

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CHAPTER 2

GLYCOSYLATED SURFACES VIA HYDRAZONE LINKAGE BY POST-POLYMERIZATION MODIFICATION OF SURFACE-GRAFTED POLYMER

Introduction

Nature presents a complex array of interactions between glycans and proteins to mediate function and initiate innumerous physiological, cellular, and chemical events. These range from joint lubrication[1] and cell signaling[2] to pathogen recognition and genetic disorders[3]. In attempts to understand individual interactions, many researchers have developed techniques for attachment and modification of biomolecules for various types of studies as well as biocompatible material development. Naturally occurring glycans are structurally complex, difficult to purify, and present many synthetic challenges in attempts to mimic or maintain functionally significant structural features. Microarray technology and other lithographic methods have provided avenues to engineer surfaces for studying interactions and using high-throughput screening with small amounts of purified material. A commonly used type of covalent ligation chemistry for sugars involves modification of the carbohydrate structure with a clickable or otherwise reactive moiety[4] such as an amine which can cleave an activated ester on a small molecule, surface[5], or polymer. Other types include oxidation to create aldehyde sites for linkage to amines via reductive amination[6, 7] and reducing terminus aldehyde conjugation to an amine group followed by reductive amination[8, 9]. Each of these techniques involves several steps which can lead to difficult purification or changes in the shape and structure of the sugar, potentially affecting the biological activity due to alterations of recognition and binding affinities[10, 11].

It has been argued in the literature that the 3D presentation, concentration and multi-valency of natural ligands are of great importance for binding events[12-14] and that polymer supports have the ability to present glycans in a similar manner to that of a cell surface or protein. The control that a synthetic material allows over orientation, ratio,

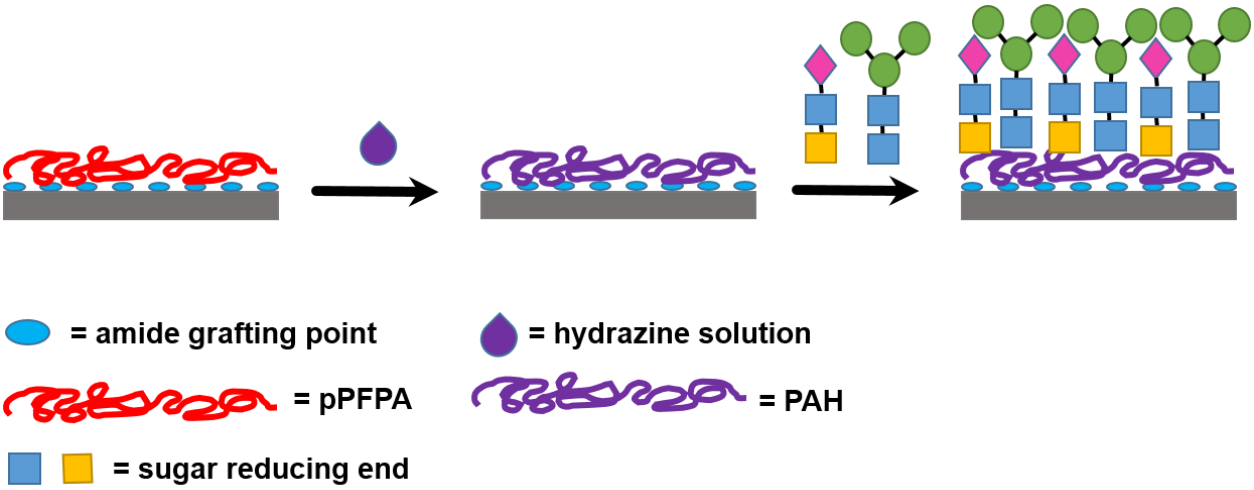
density and statistical dilution of functionality allows for improved biological recognition and activity over direct chemical attachment of residues to a surface[2, 15, 16]. This type of synthetic bioconjugated material, called glycopolymers, can be generated through many different routes and are of interest in development of biocompatible materials. Many have polymerized sugar-based monomers[2, 14, 17] and others use post-polymerization modification steps to reach this goal[18, 19]. Some examples of this include the surface-initiated atom transfer radical polymerization of monosaccharide based monomers by Kizhakkedathu and the copper click of azide-modified sugars to an alkyne acrylate based polymer made via living radical polymerization by Haddleton[14, 20]. Poly(acryloyl hydrazide) (PAH) has recently shown great significance in material science due to its ability to undergo rapid and pH reversible reactions with a variety of functional groups. It has been utilized to create stimuli responsive hydrogels[21], drug delivery systems[22, 23], ion exchange resins[21], and glycopolymers[24]. The hydrazide moiety located along the backbone of the polymer is an effective conjugation site for many types of aldehyde containing molecules including reducing sugars. Hydrazone formation is reversible which can be advantageous in some applications and has increased stability and rate relative to imine formation due to its nucleophilicity[25]. Because of this, hydrazine is commonly used as a bi-functional linker for reversible crosslinking and conjugation. The synthetic routes to obtaining PAH are often multistep and lack an easy method for surface attachment. Kumar et al showed the synthesis of PAH by modification of polymethyl acrylate with hydrazine which required stirring for 12 hours at 60°C and then demonstrated its use for a variety of materials applications[21]. Bertozzi's group achieved the desired polymer by reversible addition-fragmentation chain transfer polymerization of acetoxime acrylate with a biotinylated

chain transfer agent then modification with hydrazine. They then showed its utility in glycopolymer synthesis for microarrays printed on streptavidin[24].

Several techniques have been used to covalently attach carbohydrates to surfaces, although the majority employ surfaces functionalized with small molecule reactive moieties and complementary groups for grafting or binding[24, 26, 27]. Quite commonly these are N-hydroxysuccinimide (NHS) modified surfaces[5]. Pentafluorophenyl acrylate (PFPA) is an activated ester that has been demonstrated to have faster reaction kinetics and greater hydrolytic stability relative to NHS. Additionally, it is easily polymerized by free-radical polymerization in solution or grafted from a surface after a one step monomer synthesis[28]. This polymeric activated ester is easily grafted-to many types of surfaces via cleavage of esters located on occasional units along the backbone by surface hydroxyl groups upon annealing[29]. A one-step post-polymerization modification of surface-grafted or solution pPFPA allows the formation of PAH by reaction with hydrazine. This creates a facile route to aldehyde reactive solution polymer, nanoparticles, surfaces, and scaffolds for use in patterns, microarrays, and gradients of desired functionalities.

This work shows the fabrication of stable grafted-to polymeric surfaces that can be modified in two steps for the presentation of carbohydrates at desired densities and ratios. This direct conjugation technique allows the design of patterns for various applications and does not require any purification steps beyond substrate rinsing. Despite the expense of many purified carbohydrates, small volumes and low concentrations can be used making it cost effective. *Mycoplasma pneumonia* is a human respiratory pathogen, and a major cause of community acquired respiratory disease[30]. *M. pneumoniae* has been shown to recognize a distinct receptor population, sialoglycoproteins containing an alpha 2-3 linked

sialic acid residue[31, 32]. The organism exhibits its distinct gliding motility when bound to this receptor population. Retention of biological activity was demonstrated by observing the motility of *M. pneumoniae* after incubation on a surface containing a conjugated 2,3'-linked sialic acid residue.



Scheme 2.1: General scheme of carbohydrate surface fabrication from grafted-to poly(pentafluorophenol) to poly(acryloyl hydrazide) followed by conjugation of carbohydrate of interest by reducing end hydrazone formation.

Experimental

Materials

Wheat Germ Agglutinin Alexa Fluor® 555 conjugate purchased from Life Technologies. Silicon wafers (orientation <, native oxide) were purchased from University Wafer. Quartz microscope slides were purchased from AdValue Technology. 3'-Sialyllactose sodium salt was purchased from Carbosynth and used as provided. Lactose was purchased from Aqua Solutions. All other chemicals were purchased from either TCI, Alfa Aesar, Oakwood Chemical, or Aldrich and used without further purification.

Amine Monolayer Formation

All substrates (glass, silicon, and quartz) were solvent cleaned using hexane, isopropyl alcohol, acetone, and water by sonication for 5 minutes in each. After drying, the substrates were plasma cleaned for 5 minutes (Harrick Plasma PDC-32G at 0.80 mbar). Monolayers were formed by placing substrates into a solution of 3 mM 3-aminopropyltrimethoxysilane in absolute ethanol overnight, followed by 5 minutes of sonication in fresh ethanol.

PFPA Monomer Synthesis

Dry ether (250 ml) was placed into an oven-dried multi-neck flask with a stir bar and addition funnel. Pentafluorophenol (139 mmol) was added to the flask which was then placed under nitrogen. The flask was lowered into an ice bath and 1.1 equivalents of triethylamine (153 mmol) were added. Acryloyl chloride(1.1 equiv, 153 mmol) was injected into the addition funnel by syringe then added to the solution drop-wise while in ice. White precipitate forms immediately. Let solution come to room temperature and stir for 5 hours. Filtered out the salts then removed ether by rotary evaporation. Pushed product

through a plug of neutral alumina using dichoromethane. Concentrated by rotary evaporation then distilled to obtain the product which was stored in the freezer.

PFPA Free Radical Polymerization

PFPA monomer was dissolved in benzene to obtain a 3 M solution in a flask which then underwent cycles of freeze, pump, thaw to remove dissolved gases. Azodiisobutyronitrile (AIBN) (0.1 mol%) was added under positive nitrogen pressure. The polymerization was performed at 70°C overnight. Remaining benzene was removed by rotary evaporation. Polymer was dissolved in minimal tetrahydrofuran then precipitated in cold methanol and filtered. The precipitation was repeated to further purify polymer.

PFPA Grafting

A solution of 30 mg/ml pPFPA in tetrahydrofuran was prepared. Substrates with freshly prepared amine monolayers were spin coated with PFPA at 1000 rpm for 10 seconds. The films were annealed at 150°C in a glovebox for one hour. The resulting films were rinsed and sonicated in tetrahydrofuran for 30 minutes to remove physisorbed polymer then dried and cut to desired size.

Hydrazine Post-Polymerization Modification

Substrates containing graft-to pPFPA were placed into a flask with a micro stir bar and purged with nitrogen 3 times. Hydrazine monohydrate (0.3 mmol) was dissolved in 3 ml dry dimethylformamide (DMF) along with triethylamine (2 equiv, 0.6 mmol). The solution was injected into the flask containing the substrates and allowed to stir for 1 hour. The substrates were rinsed with DMF, water, then DMF and dried.

p-Nitrobenzaldehyde Conjugation

Dissolved p-nitrobenzaldehyde (0.03 mmol) in 3 ml DMF/water (1:1). Added aniline (0.30 mmol) and drops of 2 M HCl to obtain pH 4.5. Hydrazine modified substrates were placed in the solution and allowed to react for 2 hours. Rinsed substrates with DMF, water, then DMF and dried.

Carbohydrate Conjugation

All carbohydrate conjugations were performed with 10 mM carbohydrate and 100 mM aniline in 100 mM acetate buffer (pH 4.5). Solution was placed onto hydrazine modified substrates and allowed to react in a moisture chamber for 24 hours. The substrates were thoroughly rinsed with water then dried.

Micro-Capillary Printing

PDMS stamps with channels of 250 μm were used to pattern substrates. The stamps were plasma cleaned for one minute to allow wicking of aqueous solution. The stamps were placed on substrates and 3 μL of carbohydrate solution were allowed to wick into channels. The setup was placed into a moisture chamber and allowed to react for 24 hours before stamp removal and rinsing with water.

Wheat Germ Agglutinin Staining

Sialic acid residues were stained using wheat germ agglutinin Alexa Fluor® 555 conjugate (WGA). The lectin was dissolved in phosphate-buffered saline (pH 7.4) at a concentration of 1 mg/ml. Small aliquots were placed onto substrate surfaces to cover patterned regions and allowed to incubate in a dark place at room temperature for 15 minutes. The substrates were then rinsed with PBS and dried.

Mycoplasma pneumoniae Culture Preparation

Wild-type *Mycoplasma pneumoniae* (strain M129) was grown in 30ml of SP4 growth medium[33] for 72 hours at 37°C in flasks. Medium was removed, flask washed with 30ml phosphate buffer saline (PBS), and cells scrapped in to modified SP4 medium (without fetal bovine serum or phenol red). Cells were pelleted by centrifugation (20,000g for 25 minutes at 4°C) and suspended in modified SP4 medium (without fetal bovine serum or phenol red). Cells were syringe passaged through a 22.5 gauge needle ten times and syringe filtered twice using a 0.44μm filter to remove clumps of cells.

M. pneumoniae Gliding Measurement

Cell suspension was incubated on each substrate using a PDMS mold. Incubated for one hour at 37°C and then removed, washed three times with modified SP4 medium (without fetal bovine serum or phenol red), and modified SP4 medium (without fetal bovine serum or phenol red and containing 3% gelatin) was added to the substrate. Substrates were imaged with live *M. pneumoniae* using a Leica DM IL inverted microscope (Leica Micro-systems, Buffalo Grove, IL) with a digital charge-coupled-device (CCD) camera (Hamamastsu Photonics K.K., Hamamatsu City, Japan) and analyzed using Openlab version 5.5.0 (PerkinElmber, MA). A minimum of 20 uninterrupted frames at a constant time interval were analyzed along a collision-free path for cells, paths were tracked using Openlab software.

Characterization of Surface Modifications

The thickness of silicon substrate grafted functionalities were measured after each step using spectroscopic ellipsometry (J.A. Woollam, M-2000V) then the data were fit using a Cauchy model within CompleteEASE software. Static water contact angle

measurements were taken using drop shape analysis (DSA100, Krüss USA, Matthews, NC, DSA3 software) by placing drops of water onto three different spots of each substrate and taking the average angle. Reaction completion was confirmed by utilizing grazing-angle attenuated total reflectance Fourier transform infrared spectroscopy (GATR-FTIR) (Thermo Scientific, Nicolet 6700/Harrick VariGATR). Each of the quartz substrates was analyzed by UV-visible spectrophotometry (Varian, Cary 50Bio UV-visible spectrophotometer). Surface morphologies were examined by atomic force microscopy (AFM) (Bruker, MultiMode, Billerica, MA)

Determination of Surface Functional Density

Post-polymerization modification of surface grafted pPFPA with 1-pyrenemethylamine (AMP) (λmax = 352 nm), a rapid and nearly quantitative reaction that is easily monitored by UV-visible spectroscopy, can be used to determine the total surface functional density. This functional density can be calculated using the absorbance at the attached chromophore's λmax and the following equation:

$$d = \frac{A \times N_A}{\varepsilon \times 1000\ cm^2}$$

where d is functional density (molecules/cm^2), A is absorbance, N_A is Avogadro's number, and ε is the molar absorptivity of the chromophore at λmax[34]. On average, pPFPA films grafted by the methods described were found to have a functional density of 3.23×10^{15} molecules/cm^2 (or on the order of nM) by measurement of functionalization with AMP.

Synthesis of 4-nitrobenzyl hydrazone

A small flask was used to dissolve 91 mg p-Nitrobenzaldehyde (NBA) (0.6 mmol) in six ml tetrahydrofuran (THF). Hydrazine monohydrate was added (19 uL, 0.4 mmol) to

the solution which immediately began product precipitation. The solution pH was reduced to ~4.5 by addition of a few drops of water and 1 M HCl. THF was added (3 ml) then solution was stirred for three hours before filtering. The filtered solid was rinsed with water then THF to remove any starting materials and allowed to dry. Prepared solutions in N,N-dimethylformamide (DMF) at concentrations ranging from 0.7 µg/ml to 3.7 µg/ml to obtain a molar absorptivity coefficient value (19813 $M^{-1}cm^{-1}$) relevant for surface NBA conjugations.

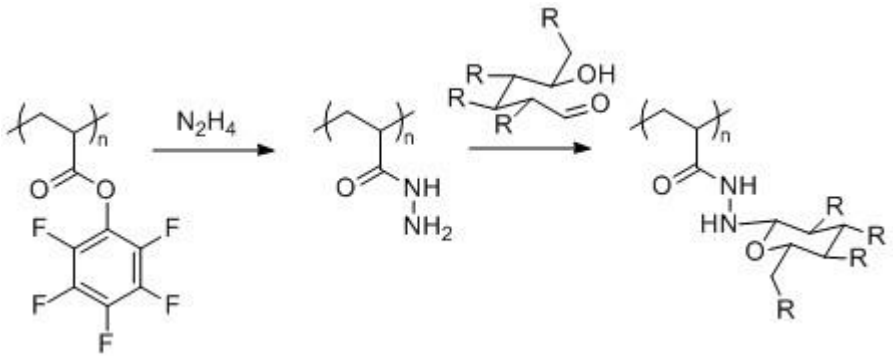
Results and Discussion

PFPA Grafting

The synthesis of PFPA monomer and following polymerization were completed previously by Arnold et al [29] (M_N = 267,062 g/mol, M_W = 364,936 g/mol, Đ = 1.366). The polymer was grafted to surfaces by spincoating and annealing substrates containing monolayers of 3-aminopropyltrimethoxysilane. The preparation of grafted-to pPFPA surfaces does not require an amino- monolayer as the active esters are susceptible to cleavage by surface hydroxyl groups during the annealing process, however the inclusion of the monolayer leads to grafted films with increased thickness and stability arising from the presence of the amide grafting linkage that forms rather than silanol esters. The addition of the amino-silane layer (which is typically 1-2 nm thick) allows for the grafted pPFPA thickness to increase from about 4-5 nm to 10-14 nm. This increase in thickness, however, only slightly increases the concentration of reactive groups based on measurement of functional density using amine containing dyes and remains within the range expected in comparison with reported activated ester polymer surfaces[28, 34].

Hydrazine post-polymerization modification

The quantification method for surface functional density described previously can be used for other surface tethering chemistries that are not entirely quantitative, such as imine and hydrazone attachments. One commonly used pathway to carbohydrate ligation is the use of the reducing end aldehyde as a reactive handle. This occurs in the ring open free aldehyde form which accounts for less than 0.01% of molecules (depending on the sugar) in solution at a given time[35, 36]. Often, sodium periodate oxidation is used to increase the aldehyde concentration which thereby increases the conversion and reaction rate, but also damages the natural structure of the polysaccharide of interest affecting the biological activity[11]. A more mild technique involves simple reductive amination at the reducing end, however reduction forces the terminal sugar unit to be ring opened which may also lead to bio-recognition loss[10, 14]. Recently, Dirksen et al. reported the catalytic efficiency of aniline based compounds for hydrazone and oxime formation which falls under the category of dynamic covalent chemistry[37, 38]. Wang et al. recently demonstrated its catalysis for simple reversible conjugations to polymeric nanoparticles[18]. In the context of carbohydrates, ligation via aniline catalysis to a protein, polymer or surface should lead to retention of biological activity by allowing ring closing to occur after attachment to the reactive site. As described in this work, the general sequence of reactions for aldehyde conjugation can be seen in Scheme 2.2.



Scheme 2.2: Two-step reaction scheme for the post-polymerization modification of either solution or surface-grafted pPFPA to form PAH and its subsequent ligation of carbohydrate (or other aldehyde containing compounds).

Hydrazine and hydrazide containing compounds are common reactive species for conjugation of aldehydes due to increased nucleophilicity relative to primary amines. The quantitative cleavage of reactive esters along the backbone of pPFPA upon addition of hydrazine, forming polyacryloylhydrazide (PAH), occurs rapidly and, despite the low concentration of reactive groups at the surface, there is a statistical likelihood of crosslinking between pairs of ester sites. This occurs due to the bi-functionality of the linker, the proximity of esters to each other and the highly favorable hydrazide formation, which creates the need for a sufficiently high concentration of hydrazine.

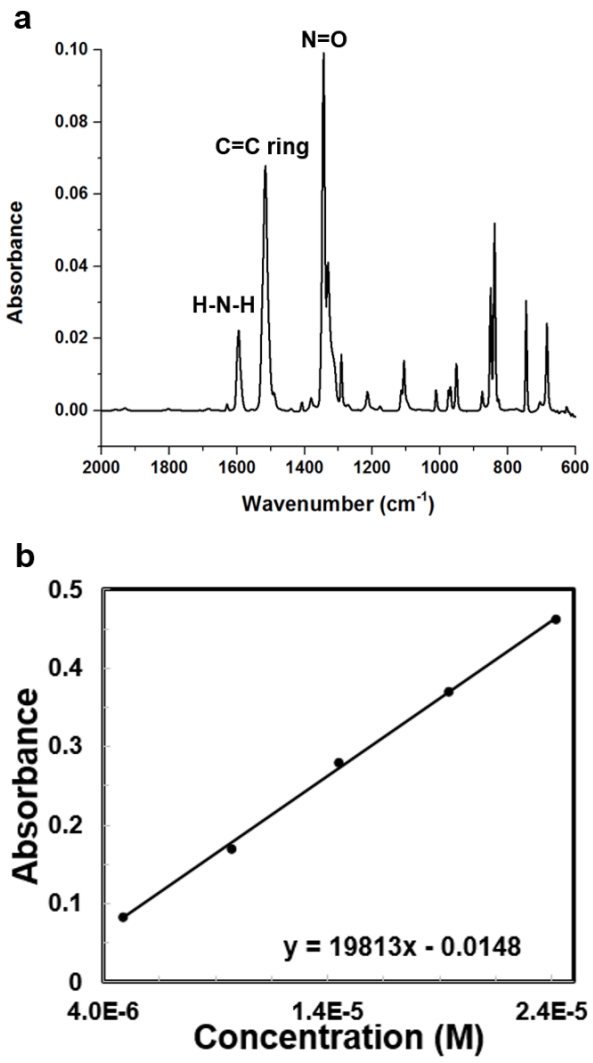


Figure 2.1: a) FTIR spectrum of solid 4-nitrobenzyl hydrazone with major peaks labelled to confirm reaction conversion from aldehyde to hydrazone. b) UV-visible peak absorbance values measured at 333 nm for micromolar concentrations of 4-nitrobenzyl hydrazone. The molar absorptivity coefficient was determined to be 19813 $M^{-1}cm^{-1}$.

The threshold for retaining the full amount of reactive sites after modification was probed by the variation of hydrazine concentration followed by functionalization with an aldehyde moiety containing a chromophore. The aldehyde containing compound chosen

for this work is p-nitrobenzaldehyde (NBA) which is a highly active electrophile to achieve maximized ligation relative to the number of functional sites on the surface. In order to quantify hydrazone formation with NBA, first the small molecule hydrazone, 4-nitrobenzyl hydrazone (NBH), was synthesized. Figure 2.1a contains the grazing-angle attenuated total reflectance Fourier transform infrared spectrum (GATR-FTIR) of NBH revealing free NH_2 functionality near 1600 cm^{-1} as expected due to the presence of the hydrazone. The UV absorbance calibration curve used to determine its molar absorptivity coefficient of NBH (19,813 $M^{-1}cm^{-1}$ at 333 nm) can be seen in Figure 2.1b.

Hydrazine concentration for post-polymerization modification was varied and the resulting PAH surfaces were conjugated with NBA. The UV absorbance at 333 nm for each substrate was compared for determination of the threshold for hydrazine concentration which produces minimal crosslinking. Table 2.1 shows the correlation between increase of functionalization with NBA and concentration of the precursor, hydrazine, revealing the necessity for a large excess of linker in solution to minimize crosslinking. At all concentrations, GATR-FTIR showed complete cleavage of PFPA groups. Upon PFPA side chain cleavage with hydrazine in all samples, the film thickness decreased which is due to the decrease in the molecular weight of the side chain[34]. A large thickness increase upon conjugation with NBA as seen in the substrate reacted with 1 M hydrazine (7.04 nm PAH to 15.38 nm NBA grafted) indicates a large percentage of reactive sites underwent NBA attachment thus increasing side chain molecular weight. At the lowest concentration,1 mM hydrazine, the surface exhibited a static water contact angle of 42.3° and UV absorbance corresponding to $1.52x10^{15}$ NBA molecules/cm^2. This value accounts for approximately 50% of the typical functional density and can be contributed to some significant amount of

crosslinking. At a concentration of 10 mM hydrazine, the water contact angle decreased to 30.8° and the conjugation increased to $5.17x10^{15}$ NBA molecules/cm^2 demonstrating a much higher concentration of reactive hydrazide groups present within the grafted film.. The concentration was increased to 100 mM and 1 M hydrazine to determine the sufficiency of a 10mM concentration. The amount of conjugated NBA slightly increased ($5.47x10^{15}$ and $6.38x10^{15}$ molecules/cm^2 respectively), butconsidering the surface had already reached high coverage, these small increases were not considered significant. It was determined from this data as well as a similar previous experiment on glass that 10-100 mM hydrazine is sufficient due to typical variations in grafting density by this grafting-to technique, however there may still be a very small amount of crosslinking that occurs at the surface due to close proximity of other reactive esters to the unreacted side of the bi-functional linker.

Table 2.1: Effect of hydrazine concentration on reactive group density, thickness, and static water contact angle before and after conjugation of NBA

[hydrazine]	pPFPA (nm)	PAH (nm)[a]	NBA (nm)[b]	pPFPA (°)	PAH (°)	NBA (°)	# molecules/cm^2
1 M	13.08	7.04	15.38	103.1	23.4	67.7	6.38x10^{15}
100 mM	13.08	8.38	14.81	103.1	35.1	61.6	5.47x10^{15}
10 mM	13.08	7.75	13.22	103.1	30.8	69.0	5.17x10^{15}
1 mM	13.08	7.09	8.88	103.1	42.3	68.0	1.52x10^{15}

[a] Hydrazine concentration was varied from 1 mM to 1 M in DMF

[b] NBA conjugation was performed in 1:1 water:DMF at pH 4.5

[c] Percent coverage was density percentage of average AMP calculated surface density

The extent of the NBA functionalization using hydrazine and adipic dihydrazide linkers was determined by comparison with the measured density of functional sites present on grafted pPFPA. Using hydrazine as the linker, the conjugation of NBA led to a high coverage of 3.13x10^{15} molecules/cm^2, whereas adipic dihydrazide consistently only allowed for ~40% coverage under the same conditions (1.19x10^{15} NBA molecules/cm^2). The differences in these two linkers are solubility (adipic dihydrazide is sparingly soluble in dimethylformamide, yet soluble in dimethylsulfoxide), nucleophilicity, hydrophilicity, and size, so it is possible that crosslinking is more favorable due to the length of the spacer or its lower nucleophilicity arising from the initial hydrazide form. Either way, for all future experiments, hydrazine was used because of its linking efficiency and hydrophilicity.

p-Nitrobenzaldehyde Conjugation

Bertozzi carried out many different monosaccharide conjugations to PAH as solution polymer by aniline catalyzed hydrazone formation at pH 5.5 and 50°C for 24 hours which highlights several reaction conditions affecting the conjugation[24]. In the interest of optimizing temperature and pH, NBA was utilized before use of carbohydrate counterparts, as shown in Table 2.2. The conjugation of NBA occurs at a fast rate with and without aniline because of the high reactivity of the electrophilic aldehyde, but trends were observed with variation of pH and temperature. The PAH-grafted substrate that was exposed to an NBA solution of neutral pH only reached about 60% typical coverage (1.98×10^{15} NBA molecules/cm^2) with a thickness increasing from 7.84 nm to 13.21 nm. As expected based on optimal imine formation conditions, decreasing pH leads to increasing reaction conversion as seen by the near complete surface coverage (3.04×10^{15} NBA molecules/cm^2) and thickness change from 7.30 nm to 15.85 nm achieved at pH 4.5 as well as the even higher surface coverage (3.95×10^{15} NBA molecules/cm^2) achieved at pH 3 with a thickness change from 7.20 nm to 17.42 nm. This trend of increasing conversion with decreasing pH extends to pH below that of optimal imine formation (pH 4.5) which can be expected due to the pK_a difference between an amine and a hydrazide. However, for the sake of maintaining relatively mild reaction conditions for use with complex natural structures and due to the fact that it was shown to conjugate near completion, pH 4.5 was chosen for future studies. The conversion can also be driven further by addition of heat, as shown in Table 2.3. The PAH substrate that was heated to 50°C in the presence of NBA at pH 4.5 obtained full coverage (3.50×10^{15} NBA molecules/cm^2) and a thickness change from 8.67 nm to 15.57 nm. At room temperature the coverage only

reached about 70% ($2.43x10^{15}$ NBA molecules/cm^2) with a thickness change from 8.21 nm to 13.16 nm. This may also be affected by the slight variations in surface functional density between substrates. Overall, these trends led to the conclusion that pH 4.5 would produce a sufficient carbohydrate surface concentration without decreasing to more extreme pH conditions and that, when necessary, increased temperatures would drive the reaction to greater completion which may be required by an aldehyde of low reactivity or abundance in solution such as a carbohydrate.

Table 2.2: Effect of pH on surface coverage, thickness, and static water contact angle after conjugation of NBA

pH	pPFPA (nm)	PAH (nm)[a]	NBA (nm)[b]	pPFPA (°)	PAH (°)	NBA (°)	# molecules/cm^2
3	12.97	7.20	17.42	103.9	21.2	67.9	$3.95x10^{15}$
4.5	12.97	7.30	15.85	103.9	28.3	61.4	$3.04x10^{15}$
7	12.97	7.84	13.21	103.9	27.1	65.2	$1.98x10^{15}$
8.5	12.97	7.08	8.27	103.9	22.9	56.3	$0.61x10^{15}$
10	12.97	7.11	8.37	103.9	34.0	64.2	below LOD[c]

[a] Hydrazine modification with 100 mM hydrazine in DMF

[b] NBA conjugation was performed in 1:1 water:DMF at room temperature for the pH variation and pH 4.5 for the temperature variation

[c] Absorbance value below the limit of detection

Table 2.3: Effect of temperature on surface coverage, thickness, and static water contact angle after NBA conjugation

Temp (°C)	pPFPA (nm)	PAH (nm)[a]	NBA (nm)[b]	pPFPA (°)	PAH (°)	NBA (°)	# molecules/cm^2
0	12.73	8.01	11.67	104.3	19.6	67.4	2.46×10^{15}
22	12.73	8.21	13.16	104.3	32.7	70.9	2.43×10^{15}
37	12.73	8.71	15.98	104.3	36.5	70.6	2.89×10^{15}
50	12.73	8.67	15.57	104.3	43.3	72.2	3.50×10^{15}

[a] Hydrazine modification with 100 mM hydrazine in DMF

[b] NBA conjugation was performed in 1:1 water:DMF at room temperature for the pH variation and pH 4.5 for the temperature variation

Carbohydrate Conjugation

After the conditions for attachment of NBA were improved, they were utilized to determine the efficiency of carbohydrate conjugation, namely lactose and 3'-sialyllactose. The sugar solutions were prepared at pH 4.5 in acetate buffer with the same concentration and aniline content then allowed to react with surface grafted PAH for 24 hours. The comparison of the GATR-FTIR spectra for sequential surface modifications can be seen in Figure 2.2. Although the nitro group contained in NBA has strong infrared absorbance as do the carbonyl groups present in pPFPA and PAH, sialyllactose does not contain functional groups that are easily differentiated using GATR-FTIR. There is clear disappearance of the ester carbonyl stretch just below 1800 cm^{-1} and the fluorinated ring alkene stretch near 1500 cm^{-1} upon post-polymerization modification with hydrazine and

formation of amide peaks near 1650 cm^{-1}. After conjugation with NBA, nitro group stretches appear just above 1500 cm^{-1} and around 1350 cm^{-1}, confirming the presence of the nitro containing aldehyde.

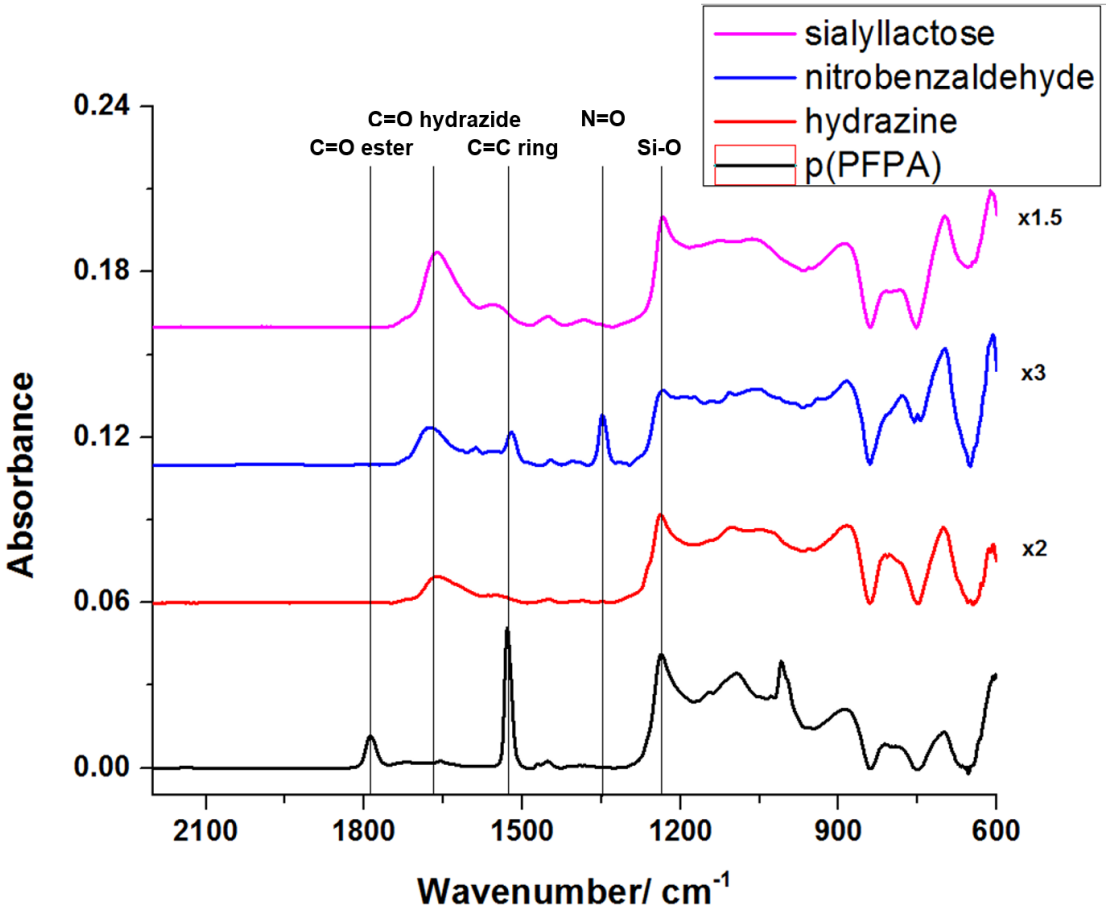


Figure 2.2: FTIR spectra of surface modification steps (scaled to similar SiO_2 peak heights).

For verification of the uniformity of surface functionalization, atomic force microscopy height images were taken for each step beginning with the 3-aminopropyltrimethoxysilane (APTMS) monolayer formed on silicon. Figure 2.3a shows small, but evenly distributed islands of APTMS and has an rms roughness value of 0.970 nm. After grafting-to of pPFPA, the rms roughness value is still only 1.05 nm and appears

mostly uniform as seen in Figure 2.3b. Similar appearance can be noted for PAH (rms roughness = 1.54 nm, Figure 3c), conjugated NBA (rms roughness = 0.841 nm, Figure 3d) and conjugated lactose (rms roughness = 2.02 nm, Figure 3e). The morphologies of each modification contain differences, as would be expected for changes in the surface-grafted polymer structure.

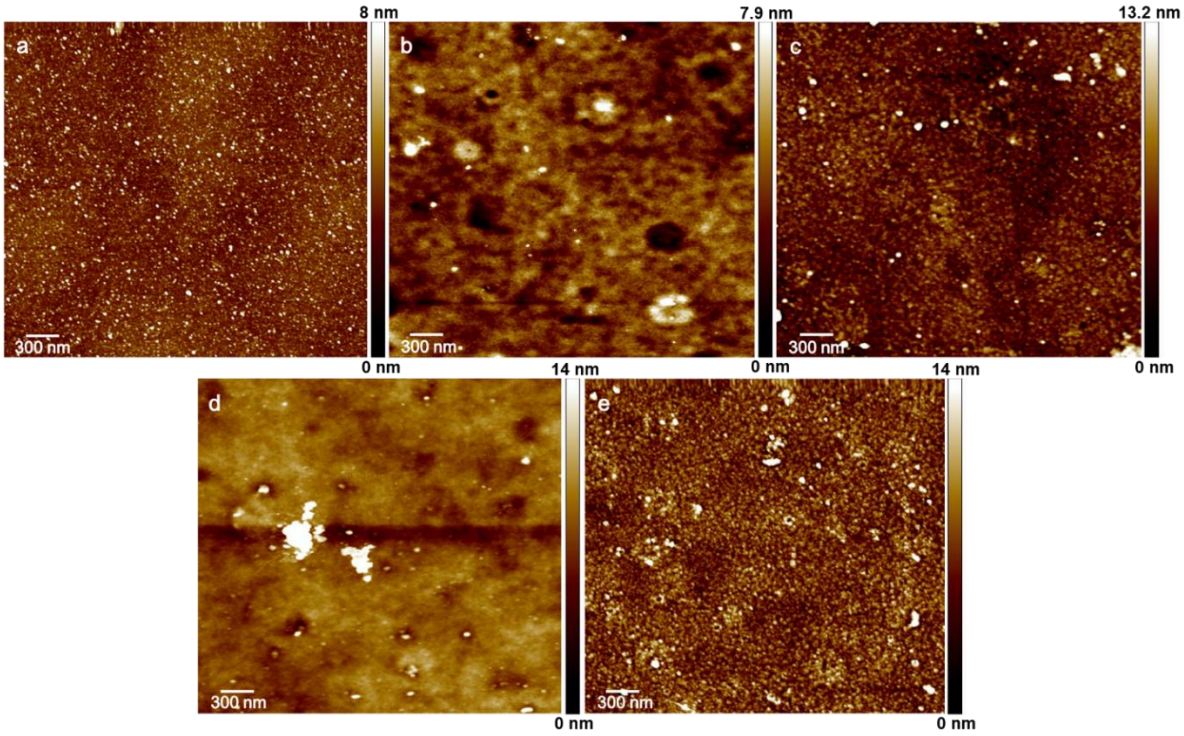


Figure 2.3: Atomic force microscopy height images corresponding to sequential surface modifications. a) APTMS monolayer (rms roughness = 0.970 nm) b) pPFPA grafted-to (rms roughness = 1.05 nm) c) hydrazine modified (rms roughness = 1.54 nm) d) nitrobenzaldehyde conjugated (rms roughness = 0.841 nm) e) lactose conjugated (rms roughness = 2.02 nm)

Carbohydrate Conjugation Efficiency

The method used to determine the carbohydrate surface density was not as direct as that involving NBA. To probe for conjugation efficiency with a sugar, lactose was conjugated to a substrate for 24 hours then placed in a solution of NBA for 15 minutes. The observed UV-visible spectrum revealed the amount of NBA present which attached to hydrazide groups that remained after lactose functionalization. The same was done with a substrate conjugated with lactose at 50°C for 24 hours as it is expected to go to higher conversion. The amounts of NBA present after lactose conjugation were compared to another substrate that was fully conjugated with NBA to obtain carbohydrate coverages. Figure 2.4 shows the greatly reduced absorbance of the lactose surfaces relative to the substrate that was simply reacted with NBA. The percent coverage of lactose was calculated at 74% (9.12×10^{14} NBA molecules/cm^2) for the substrate conjugated at room temperature and 79% (7.29×10^{14} NBA molecules/cm^2) for the 50°C conjugation. This was calculated by dividing the number of NBA molecules/cm^2 of each lactose substrate by the 3.50×10^{15} molecules/cm^2 present on the NBA substrate. The percentages were then subtracted from 100%. These carbohydrate coverage percentages were expected based on the solution polymer equivalents described by Bertozzi[24].

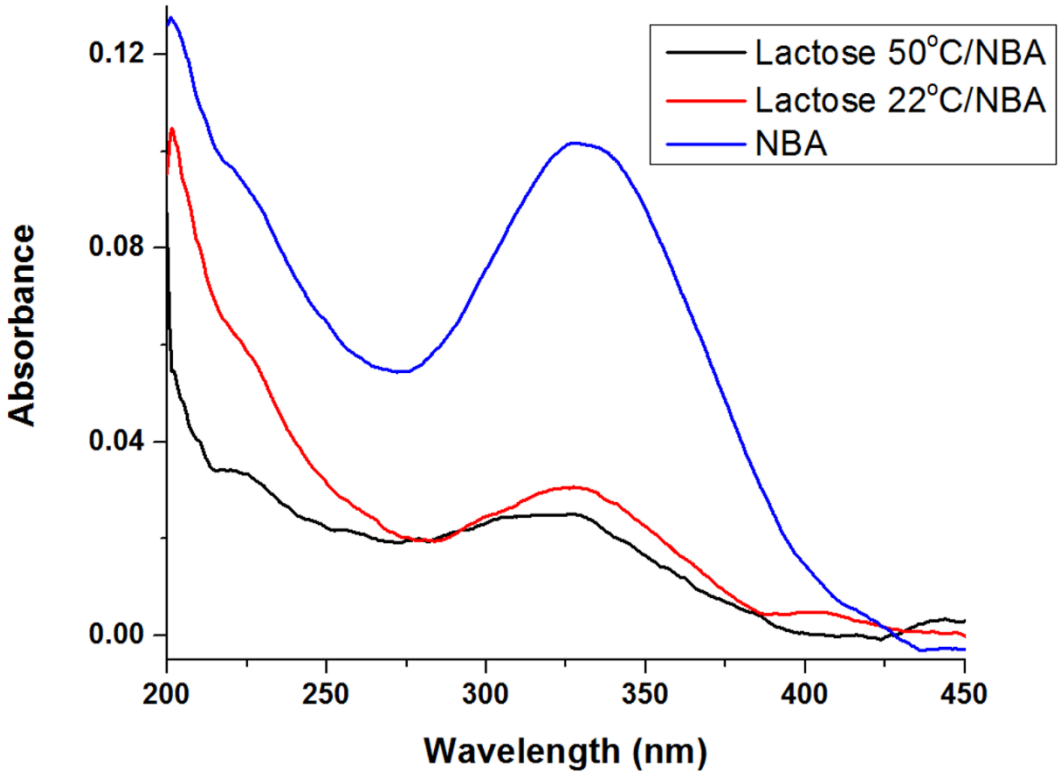


Figure 2.4: Lactose was conjugated at pH 4.5 for 24 hours onto one surface at room temperature and another at 50°C then both were placed in a NBA solution for 15 minutes. NBA was conjugated to a third substrate according to usual conditions for comparison.

Other indications of surface modification were derived from FTIR, water contact angle, and spectroscopic ellipsometry characterization data. Table 2.4 details the characterization data for each substrate in its sequential reaction steps. Substrate 1 corresponds to the fully functionalized NBA sample which was used to compare absorbance with the backfilled substrates and underwent similar surface property changes to the previous NBA conjugation data. Substrate 2 was used to conjugate lactose at room

temperature. Hydrazine modification changed the film thickness from 11.78 nm to 7.94 nm and conjugation of lactose increased thickness to 8.72 nm. After backfilling with NBA, the film thickness only increased 0.32 nm indicating a very small amount of hydrazide groups remained after sugar attachment. A similar sequence of thickness changes is seen for Substrate 3 which corresponds to lactose conjugated at 50°C. As can be expected, there is a larger increase in thickness between PAH and lactose conjugation steps of 4.71 nm due to temperature increased reaction conversion. However, this substrate still exhibits a thickness change of 0.67 nm after exposure to NBA indicating attachment to some remaining hydrazide groups.

Table 2.4: Characterization of substrates for indirect quantification of lactose conjugation by NBA backfill

	Thickness (nm)	Water contact angle (°)	UV absorbance
Substrate 1			
Graft-to pPFPA	11.78	103.7	---
PAH	8.38	21.9	---
NBA	15.39	66.5	0.101
Substrate 2			
Graft-to pPFPA	11.78	103.7	---
PAH	7.94	13.7	---
Lactose 22°C	8.72	44.5	---
Lact 22°C/NBA	9.04	62.7	0.030
Substrate 3			
Graft-to pPFPA	11.78	103.7	---
PAH	6.52	16.8	---
Lactose 50°C	11.23	51.1	---
Lact 50°C/NBA	11.80	47.7	0.024

Wheat Germ Agglutinin Staining

Sialyllactose contains an N-acetylneuraminic acid residue which binds to the lectin, wheat germ agglutinin (WGA), with strong affinity[39]. WGA Alexa Fluor® 555 was used as a fluorescent probe to visualize a sialyllactose conjugated surface. Micro-capillary printing was used to create lines of sialyllactose on a grafted PAH glass substrate. Figure

2.5 contains a fluorescence microscopy image of the patterned and WGA stained substrate (Leica Micro-systems, Buffalo Grove, IL). There is a sharp contrast between the patterned lines of reacted and unreacted hydrazide sites demonstrating the presence of WGA bound sialyllactose and absence within the unconjugated PAH regions of the pattern.

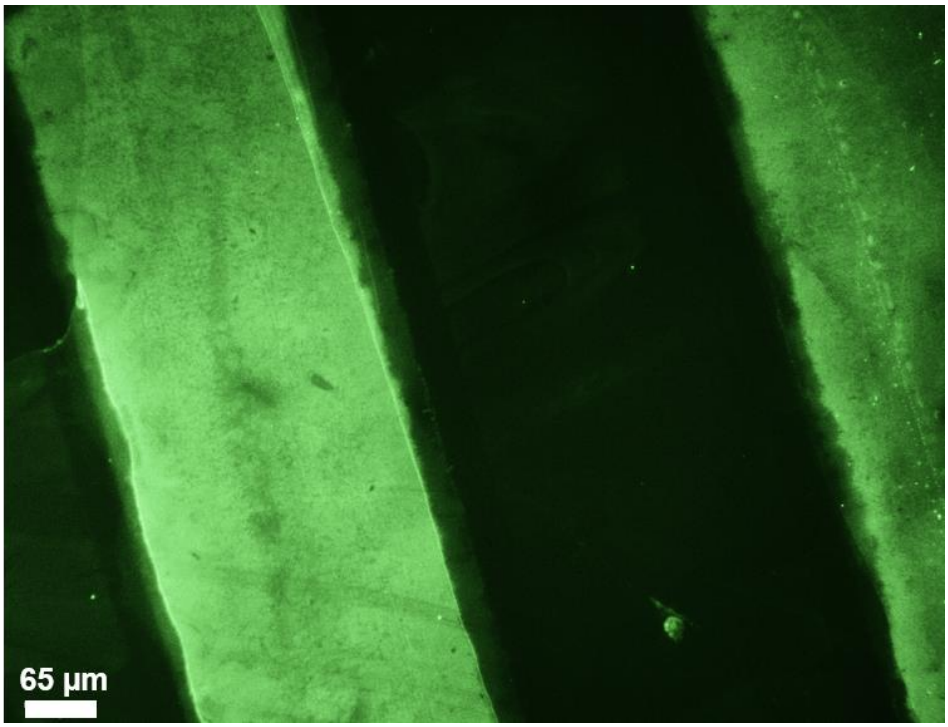


Figure 2.5: Sialyllactose patterned within 250 um lines on a PAH grafted glass substrate which was then stained with dye labelled WGA to show lines of complex and unconjugated PAH between lines.

M. pneumoniae Gliding Measurement

Following the visualization of sialyllactose conjugation, glass coverslips were used to create sialyllactose surfaces on which the behavior of incubated live *Mycoplasma pneumoniae* could be observed. Based upon the knowledge of gliding behavior exhibited

on surfaces coated with physisorbed fetuin or laminin which ceases upon cleavage of the 2,3'-sialic acid residues found on these glycoproteins[31], it was expected that the presence of surface conjugated 3'-sialyllactose (a 2,3'-sialic acid containing residue) would produce the same effect. After incubation with *M. pneumoniae*, the bacteria were observed using a Leica DM IL inverted microscope and were found to exhibit similar percentage of cell movement, micro-colony numbers, and gliding rates as that which is typically seen in the presence of the above mentioned glycoproteins. The gliding motility of several cells was tracked along their paths of movement and can be seen in Figure 2.6 as colored lines. A second experiment was done to confirm the behavior on a substrate prior to conjugation that is covered in unreacted hydrazide groups. The expectation was similar behavior to that which occurs in the presence of L-lysine, adherence of a large number of cells that are static on the surface. Figure 4 shows the live mycoplasma and the differences in number of colonies, cell extension, and motility of several bacteria between PAH and sialyllactose grafted coverslips. The adherence exhibited by mycoplasma to PAH is replaced by "normal" gliding behavior[40] after ligation of sialyllactose demonstrating the retained biorecognition of this surface modification technique.

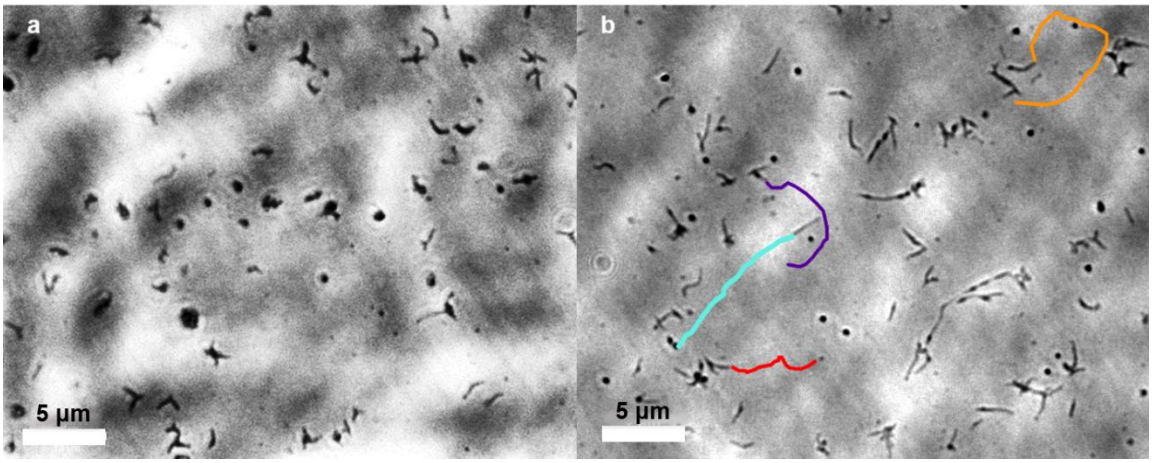


Figure 2.6: a) Live *M. Pneumonaie* imaged adhered to a PAH grafted coverslip and displaying no movement. b) Mobility tracking of live gliding *M. Pneumonaie* on a 3'-sialyllactose conjugated coverslip. Both images were taken at 100x magnification.

Surface characterization data was collected for the sequential surface modifications utilized in the experiment with live mycoplasma and can be seen in Table 2.5. Similar to previously mentioned results, the surface-grafted PAH which was exposed to mycoplasma had a low water contact angle (12.9°) and thickness about half the initial pPFPA thickness indicating high conversion of PFPA reactive sites to hydrazide functionality. Additionally, the substrate which was used for sialyllactose conjugation displayed a thickness increase upon conjugation of 1.07 nm which corresponds to the thickness increases seen previously with lactose conjugation. It can be concluded that, despite small thickness and water contact angle differences, sialyllactose conjugated to the surface in sufficient concentration as to allow gliding motility of the mycoplasma.

Table 2.5: Characterization of silicon substrates to verify hydrazinolysis and sialyllactose conjugation

	Thickness (nm)	Water contact angle (°)
Graft-to pPFPA	16.28	104.4
PAH	7.35	12.9
Graft-to pPFPA	16.28	104.4
PAH	9.79	51.7
3'-Sialyllactose conjugated	10.86	56.5

Conclusions

The simple techniques used for grafting a stable activated ester containing polymer and its one step conversion to PAH allow for facile attachment of reducing sugars for patterning, gradients, and mixtures containing ratios of components depending on the application. The parameters for the post-polymerization modification with p-nitrobenzaldehyde were investigated to show the effect of various conditions upon surface coverage with the desired aldehyde. In the interest of maintaining mild reaction conditions for attachment of natural ligands, slightly acidic (pH 4.5) and room temperature solutions were used for the fabrication of grafted sugar containing polymeric surfaces. We were able to demonstrate surface coverages comparable to conjugation efficiencies of solution polymer analogs described in the literature. In order to provide evidence for the successful retention of structure and bioactivity, conjugated sialyllactose was presented to a culture of mycoplasma which displayed the gliding motility that was expected based on their behavior in the presence of glycoproteins containing the same functional moiety. The

biological recognition was retained due to the absence of oxidative or reductive steps so that the natural structure remained intact and the stable ring closed pyranose form could occur upon linkage to surfaces. This method for formation of surface grafted glycopolymers is cost effective as it uses low amounts of ligand and it allows for synthetic mimics of cell-surface glycan presentation to be designed for innumerous studies of protein-carbohydrate interactions.

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CHAPTER 3

CONCLUSIONS AND OUTLOOK

Conclusions

In this thesis, the design of surface-grafted glycopolymers by post-polymerization modification was described. Chapter 1 provided a literature review of the concepts and methods within bioconjugation as a whole. Emphasis was placed on the potential for glycopolymeric materials to play a large role in mimicking cell surface presentation of ligands for biological interactions. The various methods for obtaining carbohydrate conjugated materials throughout the literature were discussed. Mention was made of techniques for production of glycan surface patterns. Finally, the development of activated esters and grafting techniques were described as they related to the work detailed in this thesis.

In chapter 2, the conditions for hydrazide conjugation of carbohydrates were evaluated for purposes of attachment optimization. The sites for conjugation were located along the backbone of surface-grafted pPFPA after modification with hydrazine. This work was done using p-nitrobenzaldehyde as a probe due to its UV-absorptive structure and highly reactive aldehyde group. Conditions ranging from temperature to pH were tested to show their effects on concentration of hydrazone formation. After determining useful and mild reaction conditions, surfaces were prepared for conjugation with 3'-sialyllactose. The presence of this moiety was verified by staining with wheat germ agglutinin. For the demonstration of biological activity retention after this attachment technique, conjugated 3'-sialyllactose surfaces were incubated with a culture of *Mycoplasma pneumoniae* then washed and imaged. The bacteria displayed the normal gliding motility observed in the presence of the glycoproteins containing 2,3-linked sialic acids. Overall, the techniques and reaction conditions discussed resulted in retention of biological recognition due to the

mild conditions and sufficient conjugation concentrations to obtain gliding of *M. pneumoniae*.

Future Work

The fundamental studies required to prepare glycosylated surfaces from poly(PFPA) have been discussed within this thesis. Current work now focuses on the ability to enhance the sophistication of these carbohydrate surfaces to obtain a more complex design platform. Some of these efforts are occurring in continued collaboration with the Duncan Krause lab and relate to studies of *Mycoplasma pneumoniae* gliding and binding behaviors in the presence of various ligands. The 2,3-linked sialic acids mentioned within this work have been shown to be of significance in this movement, while those with 2,6-linkages show different affinities and gliding velocities. With the ability to pattern multiple residues that are believed to be significant for binding, more can be understood about the complex and unique mechanism used by these pathogens to attach and glide in the presence of certain glycoproteins.

There is also more to be investigated relating to the variations in structure of sugars containing reducing ends and the effects that charge, molecular weight, and solubility may have on the conjugation chemistry. Studies can be done to further analyze the attachment rates and surface concentrations with various structures through use of *in-situ* ellipsometry and dye-labeling. One major hurdle that may be possible to achieve is the reducing-end conjugation of higher molecular weight polysaccharides. One such example is the large variety of glycosaminoglycan (GAG) structures which vary in monosaccharide sequence, charge, and size. These structural changes are very significant as evidenced by large effects

in the mechanical properties as in joint lubrication and mucus as well as neuronal interactions in the nervous system. Any attempts that have been made thus far to attach high molecular weight glycans through this hydrazone formation have resulted only in minute changes in film thickness which leave questions as to the success of conjugation.

Once the technique for conjugation of GAGs and other large polysaccharides is successfully developed, collaboration with the Lohitash Karumbaiah lab is intended for the study of neuron growth. GAGs such as chondroitin sulfate (CS) which contain a large amount of sulfate functionality in varied arrangements have effects on axon regeneration after nervous system injury. Categories of CS structures exist based on overall sulfation patterns and monosaccharide sequence. Studies have looked at inhibition and promotion of neuron regrowth and found correlation to sulfation patterns. The goal is to be able to pattern various CS structures and observe their effects upon neuron growth extent and direction.

In addition to conjugation studies, the stability of these hydrazone bonds require some investigation. At physiological pH, they are believed to be stable for long periods of time. The lower the pH, the more rapidly they are cleaved. Because surface functionality often requires solvation during storage before use to prevent adhesion of extraneous material, the question arises as to how rapidly carbohydrates are cleaved in the presence of water at neutral pH. Studies of the rates of hydrolysis can be done using ellipsometric techniques. To ensure long shelf-life of prepared surfaces, this seems necessary.

Another side to the use of pPFPA graft-to surfaces in bioconjugation is protein attachment. Due to the sensitive nature of native protein structures, this can present a challenge. Organic solvent, higher or lower pH, and heat each cause denaturation of proteins. This leads to the requirement of ambient to physiological temperatures and near-

neutral, aqueous solutions for protein conjugation. However, pPFPA is highly hydrophobic prior to ester cleavage which leads to solvation issues of the polymer chains in aqueous environments. Because of this poor solvation, the rate of PFPA cleavage is extremely slow. Hydrolysis to form the carboxylic acid, though a slow reaction, appears to occur more rapidly than protein conjugation. One way to improve this would be the use of *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC) as a protein coupling reagent for hydrolyzed sites. Since carboxylic acids are reactive to EDC, this strategy would work well for proteins that lack carboxylic acids in their biologically significant regions. However, kinetically speaking, the conjugation would still be extremely slow. Kinetic studies using *in-situ* spectroscopic ellipsometry or UV-visible spectroscopy via dye labeling can be performed in the process of reaction condition optimization for protein conjugation.

Final Remarks

Polymeric materials provide an advantageous direction for surface design to mimic cell surface presentation. They allow for statistical dilution of functionality in a predictable manner, patterning, and ratios of different ligands. In the case of poly(PFPA), simple processes of surface grafting and post-polymerization modification with mild reagents can produce biologically active interfaces. As technology for surface design continues to advance, more complex biological interactions will be able to be investigated. This thesis presents the fundamental aspects of the attachment chemistry that can be used for a wide range of carbohydrates. The ability to achieve a universal platform for surface biological functionalization opens doors for many new areas of research in biological and biochemical sciences.