FUNCTIONALIZATION AND CHARACTERIZATION OF NANOSTRUCTURED MATERIALS FOR SENSING APPLICATIONS

A Dissertation in Chemistry

by

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ABSTRACT

The optimal functionalization of nanoparticle surfaces for sensing applications was investigated in this dissertation. Nanoparticles have emerged as major components in the field of biosensing. For example, metallic nanoparticles have been used as substrates for Surface Enhanced Raman Spectroscopy (SERS) and silicon nanowires have improved the sensitivity in the area of field effect transistors (FETs). Due to the applications of nanoparticles and nanomaterials in sensing, it is vital to have multiple routes to control the surface chemistry of the nanoparticles. Complete characterization of these surfaces is essential for the fabrication and production of reproducible, sensitive, and reliable biosensors. Silica has been used to modify the surfaces of nanoparticles offering advantages such as protection of the metallic core, prevention of aggregation, biocompatibility, and the ability to attach molecules to the surface of the nanowire through well-understood silane chemistry. Modification of these coatings has been accomplished by incorporation of different functional groups for different applications.

Control of surface chemistry is crucial in the performance of nanomaterials and biofunctionalized nanomaterials in applications, such as sensing. In addition to control over the surface chemistry of the nanoparticles, themselves, different routes of biomolecule immobilization must be optimized. Biomolecules can be attached by direct adsorption, covalent attachment, or through cross-linking molecules, and the effect of the immobilization process on the biomolecule function must be characterized to produce reliable sensors. In the following chapters I will address the modification of silica
coatings to control the surface chemistry of nanoparticles, and then I will address methods of biomolecule immobilization.

Chapter 2 of this dissertation describes the coating of metallic nanowires with organically modified silica (ORMOSIL) coatings to incorporate different functionalities into a standard silica coating. The addition of different functional groups, e.g., dihydroimidazole or polyethylene oxide, enabled new chemistry to be incorporated into the coatings, such as metal ion binding or protein resistance, respectively, while still offering the same advantages as standard silane coatings. Metallic nanowires were coated with a variety of different modified silanes, and were then fully characterized with transmission electron microscopy (TEM) and infrared spectroscopy (IR) to confirm the presence of these modified silanes within the coating. Additionally, the metal nanowire cores were dissolved to leave behind silica nanotubes composed of different ORMOSILs. Nanotubes have applications in drug delivery, bioanalysis, and catalysis; the synthesis of nanotubes in this manner enabled control over the chemistry of the nanotubes. Proof-of-concept experiments for applications of these coatings demonstrated the protein resistance and ability to attach DNA using a cross-linking molecule to the polyethylene oxide-containing silica coating, and the metal ion binding of the dihydroimidazole-containing silica coating.

Chapter 3 introduces silica coated, porous Au nanowires for use as Surface Enhanced Raman Spectroscopy (SERS) substrates for the detection of small molecules in biological samples. Au/Ag alloy nanowires were coated, as described in Chapter 2, with SiO₂ or an organically-modified silica containing a protein-resistant polyethylene oxide
functionality. Once coated, the sacrificial silver was etched from the alloy nanowires, leaving behind silica coated, porous Au nanowires, which were characterized using TEM. Due to the nanostructure of these nanowires, enhancement of the inherently weak Raman signal was achieved. A small molecule, 4-mercaptobenzoic acid, was detected, even after the nanowires were exposed to biologically relevant serum concentrations of protein. This demonstrated the potential usefulness of these coated nanowires for detection of small molecules, e.g., metabolites or metabolic products of drugs, in biological fluids using SERS.

The immobilization of HRP onto spherical gold nanoparticles by direct adsorption was investigated in Chapter 4. Direct adsorption of enzymes onto nanoparticles is the most common method of protein immobilization, but it is often not fully characterized. It was determined that there was multilayer formation based on the quantification of the enzymes on the surface of the nanoparticles. Stability of the protein:nanoparticle conjugates was studied using both zeta potential and flocculation assays at different pH values. Additionally, the specific activity of the enzyme was determined. It is generally thought that fluorescent labels do not affect the structure and function of the enzyme. However, comparisons between HRP and fluorescein labeled HRP:Au conjugates determined that the stability of the conjugates differed, particularly at pH 8.0.

Nanoparticle surface modifications for immobilization were further investigated in Chapter 5. The experiments presented in Chapter 5 explored the immobilization of enzymes onto nanoparticle surfaces using barcoded nanowires to detect small molecules in solution. Two different immobilization techniques were studied: encapsulation within
a silica coating or within polyelectrolyte multilayers. These immobilization techniques did not require any covalent attachment to the surface of the nanowires, which may affect the activity of the enzymes, and also enabled encapsulation within a material, preventing reversibility of the immobilization. It was determined that the layer-by-layer (LbL) deposition of polyelectrolytes in the presence of enzymes, rather than encapsulation in silica, enabled the immobilization of a wide variety of enzymes of different molecular weights onto both nanowires and latex beads. Characterization of two different fluorescently labeled enzymes, malate dehydrogenase and citrate synthase, was completed by immobilization onto fluorescent beads. This enabled quantification of the enzyme molecules per bead and calculation of the specific activity, which provided information as to the stability of the immobilized enzymes and their ability to turnover product for biosensor fabrication. Progress towards an enzymatic sensor was accomplished by the enzymatic reaction of horseradish peroxidase (HRP), catalyzing the oxidation of the fluorescein derivative, 2,7′-dichlorodihydrofluorescein, by urea hydrogen peroxide. The production of the fluorescent product was concentrated around the nanowires, but diffusion away from the nanowires was problematic.

The effect of lithographic processes on the ability of the probe DNA oligos to selectively hybridize target sequences was studied in Chapter 6. The use of lithography to fabricate more complex biosensor devices has become very prevalent, and therefore, it is necessary to understand the effect of lithographic processes on the nucleic acids. This was accomplished by exposing nanowires to different photoresists, i.e., Megaposit SP 3012, Shipley 1813, PMGI SF6, that require different pre-exposure bake temperatures
(95°C, 100°C, 190°C, respectively). 3’-Thiolated single-stranded DNA was covalently attached to gold or silica coated wires through thiol-Au or Sulfo-SMCC attachment chemistry, respectively, and these wires were deposited onto a substrate prior to photoresist exposure. It was found that the exposure to standard photoresists, baked at lower temperatures (≤100°C), did not prevent hybridization of target sequences post photoresist removal. Post photoresist exposure, the DNA sequences were able to discriminate a single base mismatch.

The fabrication of bioFETS for the detection of nucleic acids was studied in a collaborative project with Dr. Theresa Mayer’s group in the Department of Electrical Engineering. Previously it was shown that nanowires pre-functionalized with DNA probes were assembled onto a lithographically prepared chip using dielectrophoretic forces. By confirming the lithographic processes did not affect the biomolecules, this assembly method of silicon nanowires can be used for the fabrication of bioFETs. Currently, silicon nanowires are synthesized through vapor-liquid-solid growth off of a surface, but low yield and poor quality control and length homogeneity is often problematic. In collaboration with Xiahua Zhong in the Mayer group, progress towards lithographically prepared axially doped silicon nanowires was accomplished, as also shown in Chapter 6. The biofunctionalization of these particles and dielectric materials was studied.

In conclusion, a variety of different sensing platforms, i.e., SERS substrates, suspension array enzymatic biosensors, and bioFETS, were studied for the development of biosensors to detect small molecules and DNA. The modification of the surface
chemistry of nanoparticles was accomplished through the use of silica coatings and polyelectrolyte multilayers. The understanding of the interaction of biomolecules with nanoparticles, and the ability to control the surface chemistry of the nanoparticles enables the production and fabrication of reproducible and reliable biosensors. In the future, this knowledge can be used to fabricate sensitive bioFETs that will enable multiplexed detection of nucleic acid cancer markers and allow for statistical analysis of the results. Ultimately, this knowledge will permit the fabrication of inexpensive, point-of-care sensors for early detection of diseases based on an electrical readout mechanism.
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<tr>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AcPTMS</td>
<td>3-acyrloxypropyltrimethoxysilane</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>APTMS</td>
<td>3-aminopropyltrimethoxysilane</td>
</tr>
<tr>
<td>AuNP</td>
<td>Gold Nanoparticle</td>
</tr>
<tr>
<td>BioFET</td>
<td>Biological Field Effect Transistor</td>
</tr>
<tr>
<td>BTEB</td>
<td>Bis(trimethoxysilyl)benzene</td>
</tr>
<tr>
<td>BTESE</td>
<td>Bis(triethoxysilyl)ethylene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BTMH</td>
<td>Bis(trimethoxysilyl)hexane</td>
</tr>
<tr>
<td>CHES</td>
<td>N-cyclohexyl-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>CETES</td>
<td>2-cyanoethyltriethoxysilane</td>
</tr>
<tr>
<td>CMOS</td>
<td>Complementary Metal Oxide Semiconductors</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate Synthase</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl Trimethylammonium Bromide</td>
</tr>
<tr>
<td>DAP</td>
<td>2,3-diaminopophenazine</td>
</tr>
<tr>
<td>DCF</td>
<td>2,7’-dichlorodihydrofluorescein</td>
</tr>
<tr>
<td>DI H2O</td>
<td>18.2 MΩ-cm Nanopure water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FE-SEM</td>
<td>Field Emission Scanning Electron Microscopy</td>
</tr>
<tr>
<td>FET</td>
<td>Field Effect Transistor</td>
</tr>
<tr>
<td>F-HRP</td>
<td>Fluorescein Labeled Horseradish Peroxidase</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein 5(6)-isothiocyanate</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>GOx</td>
<td>Glucose Oxidase</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HNO3</td>
<td>Nitric Acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>ICPTES</td>
<td>(3-isocyanatopropyl)triethoxysilane</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>KCN</td>
<td>Potassium Cyanide</td>
</tr>
<tr>
<td>LbL</td>
<td>Layer-by-Layer</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>MBA</td>
<td>4-mercaptobenzoic acid</td>
</tr>
<tr>
<td>MDH</td>
<td>Malate Dehydrogenase</td>
</tr>
<tr>
<td>MESA</td>
<td>Mercaptoethanesulfonic acid</td>
</tr>
<tr>
<td>MPTES</td>
<td>Mercaptopropyltriethoxysilane</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>NW</td>
<td>Nanowire</td>
</tr>
<tr>
<td>NTPDI</td>
<td>N-(3-triethoxysilylpropyl)-4,5-dihydroimidazole</td>
</tr>
<tr>
<td>OPD</td>
<td>o-phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>ORMOSIL</td>
<td>Organically Modified Silane</td>
</tr>
<tr>
<td>PBS</td>
<td>10 mM phosphate buffer, pH 7.4, 300 mM NaCl</td>
</tr>
<tr>
<td>PE</td>
<td>Polyelectrolyte</td>
</tr>
<tr>
<td>PEO</td>
<td>N-(triethoxysilylpropyl)-o-polyethylene-oxide urethane</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric Point</td>
</tr>
<tr>
<td>PMGI</td>
<td>Polymethylglutarimide</td>
</tr>
<tr>
<td>PVP</td>
<td>Poly(vinylpyrrolidone)</td>
</tr>
<tr>
<td>RIE</td>
<td>Reactive Ion Etch</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SBM</td>
<td>Single Base Mismatch</td>
</tr>
<tr>
<td>SERS</td>
<td>Surface Enhanced Raman Spectroscopy</td>
</tr>
<tr>
<td>SiO₂</td>
<td>Silicon Dioxide</td>
</tr>
<tr>
<td>SulfoSMCC</td>
<td>Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TEOS</td>
<td>Tetraethoxysilane</td>
</tr>
<tr>
<td>TMOS</td>
<td>Tetramethoxysilane</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-Visible</td>
</tr>
</tbody>
</table>
VLS  Vapor Liquid Solid
XPS  X-Ray Photoelectron Spectroscopy
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A journey like graduate school cannot be done alone, so I have to take this time to thank all of the people that helped guide me through this process. I thank my committee members, Drs. Tom Mallouk, Carlo Pantano, and Mary Beth Williams, and my advisor, Dr. Chris Keating, for their advice and guidance. I also acknowledge The Pennsylvania Space Grant Consortium and the National Institute of Health for financial support during my graduate years.

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Finally, I thank my parents. I have been thinking for some time now how I could put into words my appreciation and love for my parents. I have concluded it is impossible, but I will make an insufficient effort here. My parents are now, and always will be, my heroes. They taught my sister and I how to work hard, and the importance of giving all you can, which has been indispensable in our development and helping us achieve our goals throughout life. They have never stood in the way of my dreams, but instead, did everything in their power to help me get through each day. Their “one-day-at-a-time” attitude is a mantra that I remind myself of often.

Not only would my car not be running without my dad, but I would not be who I am today without him. He has been such a stable male figure in my life. It is very clear how his daughters are the most important things in his life, and I thank him for demonstrating that. I will never forget the hours he spent with the financial aid counselors at the end of high school and at Wooster to ensure I was able to afford college. His actions were a sign of his love for me, and I hope my success in school is a sign of
my love for him in the same way. Additionally, my mom is the most remarkable woman I know. She has a calming force over everyone, and the way she kept the family going for so many years, with clean clothes, a clean house, good food, all while working, is absolutely remarkable. She has taught me so much, and has turned into my best friend over the years. Although they did not always understand what I was doing with chemistry or why I wanted to be a chemist, their pride in having both their daughters be college graduates has always been evident. My parents have always been my cheerleaders, when I was sitting the bench in volleyball, to acting on stage, to pursuing a career in chemistry. I would not be where I am today without both my mom and dad. My Ph.D. is for them, it always has been.
For my Mom and Dad, always.
Chapter 1

Introduction


This thesis is broadly concerned with nanoparticle-based sensing, with an emphasis on surface functionalization. Three different nanoparticle-based sensing platforms were studied: surface enhanced Raman spectroscopy (SERS) substrates, metal nanowire suspension arrays, and field effect transistors (FETs). Controlling the surface chemistry is essential for all three types of sensors to obtain reproducible, sensitive and statistically relevant results. In this work, silica and polyelectrolyte multilayers were used as nanoparticle coatings, as they provided control over the surface chemistry of the particle and enabled integration of biomolecules.
1.1 Introduction to Sensing Platforms

Sensing platforms are used to detect a wide variety of substances for applications in medicine, security, commercial products, environmental protection, and manufacturing. In particular, early detection of diseases is of the utmost importance in biosensing, e.g., for cancer, where patients could greatly benefit from treatment at the onset of the disease. The most successful commercial biosensors, e.g., glucose detectors for diabetics and the home pregnancy test, are accurate, reproducible, cost-efficient, portable, and easy to use. The incorporation of micro- or nanoparticles into biosensor fabrication can lower the limit of detection and provide a myriad of detection mechanisms. A wide variety of nanoparticle-based biosensors have been developed using electrochemical,\(^1\) colorimetric,\(^2\) electrical,\(^3\) mass-based,\(^4\) fluorescent,\(^5\) or spectroscopic\(^6\) transduction methods; but, these methods are not always amenable to multiplexing for low-cost point-of-care sensing. Here, Raman spectroscopy (SERS) substrates, metal nanowire suspension arrays, and field effect transistors (FETs) were selected to overcome these challenges. These platforms were chosen due to the wide range of molecules that can be detected and/or characterized, e.g., nucleic acids, proteins, small molecules, and the ability to multiplex. We are working on the fabrication of low-cost, multiplexed FETs with integrated complementary metal—oxide—semiconductor (CMOS) technology, in particular, for point-of-care detection of biomarkers through collaborative work with Dr. Theresa Mayer’s group in Electrical Engineering.
1.2 Sensing Platform Fabrication

Three different synthetic procedures were employed to produce nanoparticles or devices for sensing applications in this work: 1.) solution-phase reduction of metal salts, 2.) templated electrodeposition, and 3.) lithographic processes (Figure 1-1). A brief explanation of these will be provided in turn below.

Figure 1-1: Schematic showing examples of three different procedures to produce nanoparticles or devices: (A) solution-phase synthesis of nanoparticles through the reduction of metal salts; (B) templated synthesis of nanowires through electrodeposition; and (C) fabrication of lithographically prepared devices. Adapted and reproduced with permission from reference 7.

Solution-phase reduction of metal salts, i.e., gold(III) chloride hydrate, was used to make colloidal nanoparticles as scaffolds for proteins or as detection mechanisms for
nucleic acids. Solution-phase methods are attractive for their simplicity, relative ease of scale-up, and minimal equipment requirements. The facile synthesis of monodisperse particles over a wide range of sizes has contributed to their appeal in numerous applications, e.g. biodiagnostics and biological imaging.\textsuperscript{8-10} The adsorption and enzymatic activity of proteins on colloidal gold scaffolds was studied in Chapter 4.

The majority of the work presented in this dissertation used metallic nanowires prepared by the electrodeposition of metal salts into porous alumina templates. Templated synthesis produces particles with increased complexity, compared to particles synthesized via solution-phase methods, enabling control over particle size and shape. Chemical reactions or electrodeposition within the pores of template membranes yields arrays of parallel nanowires, where the diameter is controlled by the pore size of the template, and the length by the thickness of the template (for chemical deposition) or the amount of current passed (for electrodeposition).\textsuperscript{11} Electrodeposition is particularly attractive for the preparation of nanowires, because it provides greater overall length control and can be used to prepare more elaborate particles for encoding, i.e., variations in the diameter\textsuperscript{12,13} or composition\textsuperscript{14-16} of the particle along its length. By varying the composition of the nanowires, barcoding patterns can be introduced. By functionalizing different populations of encoded nanowires, i.e., with each population having a different barcoding pattern, nanowires can be mixed for multiplexed suspension array assays,\textsuperscript{14} as discussed in Chapter 5. Additionally, alloy nanowires have also been produced by varying the plating potential during electrodeposition from solutions of mixed metal salts to produce nanoporous particles\textsuperscript{17,18} or multisegment wires\textsuperscript{19-21}. Nanoporous gold SERS
substrates prepared from silica coated Au/Ag alloy nanowires are discussed in Chapter 3 of this dissertation.

The third sensor fabrication method used was lithography, as discussed in Chapter 6. Lithography is the process of imprinting patterns onto a substrate through direct contact onto a treated surface or using a mask to impart a design using light of a specific wavelength or a particle beam. Advances in lithography have allowed for more intricate designs to be patterned onto the substrates with feature sizes ranging from the microscale down to tens of nanometers or below using low wavelength lasers, electron or ion beams.\textsuperscript{22} Due to the emergence of lithography in the fabrication of sensors, and the increasing integration of biomolecules within these devices, these processes will be discussed in some detail here, as a complete understanding of the effects of lithographic processes on biomolecules is necessary to fabricate reliable multiplexable FETs.

Photolithography is a process that uses light to selectively remove or retain different areas of a material to produce devices with controlled geometry, or microparticles that can be released into suspension. Photolithography is used in the work presented here to fabricate field effect transistors, and also to produce axially doped silicon nanowires with controlled length for use in FETs. Within the field of photolithography, photoresists, which are light sensitive polymeric solutions, are commonly used to fabricate devices, structures, or nanoparticles of controlled size and geometry. Photoresists are spun onto a substrate, the spin coat conditions and choice of resist determining the thickness of the material, and then by use of a mask, the substrate can be exposed and developed to leave behind a three-dimensional relief of polymeric materials on the surface of the substrate.
Depending on the application a positive or negative resist may be used. A positive photoresist is a material that becomes soluble in solvent after exposure to a specific wavelength, while a negative resist becomes insoluble.\textsuperscript{23} SU-8, a very common negative resist, is composed of an epoxy resin (Figure 1-2 A) that cross-links to form an insoluble material (Figure 1-3 A).\textsuperscript{24,25} This cross-linking reaction is catalyzed by triarylsulfonium hexafluoroantimonate salts that decompose to form Lewis acids upon UV exposure.\textsuperscript{24,25} Likewise, general use positive resists, e.g., Shipley 1800 series resists, contain a Novolak resin (Figure 1-2 B) and a photoactive compound (Figure 1-3 B) that promote dissolution of the resin material after exposure to UV light.\textsuperscript{23,26} The effect of these compounds — and the necessary processing conditions — on immobilized biomolecules is not fully understood and will be explored in Chapter 6.

![Figure 1-2: (A) Bisphenol A Novolak epoxy oligomer, the main component in SU-8 negative photoresist.\textsuperscript{24,25} (B) Linear novolak resin, the main component in a variety of positive photoresists.\textsuperscript{23,26}](image-url)
1.3 Use of Micro- and Nanoparticles in Sensing

Optical changes of nanoparticle solutions or mass changes resulting from binding of nanoparticles to a resonator device have been used for biological and chemical sensing, but nanoparticle-based biosensor work can also utilize the nanoparticles as scaffolds, e.g., metallic nanowire suspension arrays. Often probe molecules, e.g., probe DNA or an antibody, are attached covalently to the surface of the nanoparticle and the binding event of the target molecule is detected. Here, the focus will be on the use of spherical nanoparticles and anisotropic nanowires as scaffolds for

Figure 1-3: (A) Reaction of epoxy resin, found in SU-8 negative resist, with a reactive diluent, gamma-butyrolactone, catalyzed by a decomposed photoinitiator to form an insoluble cross-linked material (Adapted from Reference 25). (B) Reaction of diazo photoactive species found in positive photoresists that produces a base-soluble species upon irradiation with UV light (Adapted from reference 27).
sensing applications. These particles are introduced in this dissertation for use in SERS detection, as scaffolds for suspension arrays, or integration into FETs.

Nanoparticles have been used for SERS detection because binding of molecules to the surface of metallic nanoparticles increases the inherently weak Raman signal through heightened electromagnetic fields on the surface of the nanoparticles. Gold nanospheres functionalized with dye-labeled oligonucleotide probes have been used to distinguish six different DNA targets in a multiplexed format.\textsuperscript{31} Nanowires, such as porous Au wires prepared by etching the sacrificial Ag from Au/Ag nanowires, have also been used for SERS substrates.\textsuperscript{32} Fabricating substrates using on-wire lithography, Chad Mirkin and George Schatz demonstrated the optimal spacing between gold nanodiscs immobilized using a silica sheath on one side by systematically varying the size of the particles and spacing through experiments and computation.\textsuperscript{33} Control over the surface chemistry of these substrates could enable the selective detection of molecules. In Chapters 2 and 3 of this dissertation, silica coatings for nanowires prepared from organically modified silane precursor molecules were studied for use in sensing applications, providing control over the surface chemistry of the nanowires, while still maintaining attractive qualities of silica, e.g., well-understood surface attachment.

Barcoded nanowires have been used as scaffolds for suspension assays for the detection of nucleic acids\textsuperscript{5} and proteins\textsuperscript{14} using striped nanowires that can be optically distinguished. By immobilizing biomolecules onto the surface of nanowires, the binding of the target molecule, e.g., the hybridization of a target DNA sequence, can be detected through a fluorescence signal, while the barcoding pattern enables identification (Figure 1-4). NBSee computer software has been developed by Nanoplex, Inc. that enables
quantification of the fluorescence signal with corresponding identification of nanowire barcode pattern based on differences in reflectivity between Au and Ag at 430 nm for ease of analysis.\textsuperscript{34}

![Figure 1-4: Schematic of a sandwich assay of DNA on barcoded nanowires. Hybridization of a target sequence to a probe sequence on the nanowire surface is detected by the subsequent hybridization of a fluorescently labeled tag sequence. The target sequence is identified optically based on the barcoding pattern of the nanowire.](image)

Silicon nanowires have also been used for biosensing in field-effect transistors (FETs). An increased sensitivity with nanowire FETs is observed since the accumulation/depletion of carriers happens within the bulk of the semiconductor material, as opposed to just on the surface with planar FETs.\textsuperscript{35} Nanowires immobilized on chip using metal electrodes can be assembled as an array for the detection of biomolecules electrically. When the microenvironment of the nanowire surface changes
– i.e., through a hybridization or binding event – a change in conductance is observed (Figure 1-5). Zheng et al. demonstrated the multiplexed detection of prostate specific antigens down to 0.9 pg/mL in serum samples that were desalted using a microcentrifuge filter. Increases in ionic strength rapidly decreases the Debye length in which binding events can be detected from the nanowire surface, making analysis of biological fluids difficult. To circumvent this, microfluidics can be incorporated into the sensors for purification and neutral peptide nucleic acids (PNA) can be used as probe molecules, which allows for DNA hybridization in low ionic media. Work presented here is currently being conducted in collaboration with Dr. Theresa Mayer’s group in Electrical Engineering on the fabrication of arrays of FETs using nanowires prefunctionalized with probe DNA specific for flu or cancer markers for the multiplexed detection of biomolecules. To fabricate reproducible, sensitive devices, the functionalization of the surface of these nanomaterials and the effect of lithographic processing on the biomolecules must be well-understood, as introduced in Chapter 6.
To study the surface functionalization of dielectric materials and silicon particles for the fabrication of FET devices, the absorbance of gold nanoparticles or DNA:Au conjugates were used. This was done by adsorbing gold nanoparticles onto a functionalized surface or designing a sandwich hybridization assay using a single stranded oligo covalently attached to both the substrate and a nanoparticle for detection of a complementary target sequence. An example of this is seen in Figure 1-6, where gold nanowires were functionalized with primary aptamers specific to S100B protein, a protein marker for melanoma, and 50 nm gold nanoparticles were functionalized with the secondary aptamer. The nanowire on the left was exposed to the 1 µM S100B protein,
while the nanowire on the right was exposed to the same concentration of a control protein. After the capture of the protein using the primary aptamers on the surface of the nanowires, gold nanoparticles functionalized with the secondary aptamer were introduced to act as tags for detection using scanning electron microscopy. Figure 1-6 shows that there is little non-specific binding with the control, while the S100B can be detected by the presence of the bound gold nanoparticles.

![Figure 1-6: 5'-thiol derivatized primary aptamers were coupled to gold nanowires using standard thiol chemistry. Secondary aptamers were coupled to 50 nm AuNPs in the same manner. Purified S100B protein (left) or purified HtrA1 control protein (right) was then bound first to the derivatized nanowires. After thorough rinsing, secondary aptamer-50 nm AuNPs were subsequently bound to the primary-aptamer-nanowire:S100B complexes. After thorough rinsing, bound sandwich complexes were visualized using field-emission scanning electron microscopy. Scale bars = 1 µm. Reprinted with permission from Reference 39.]

1.4 Surface Modification of Nanoparticles

Control over the surface chemistry of nanoparticles is essential for the development of each of the types of sensors introduced above, and is important for nanoparticle function, toxicity in biological applications, interaction with biomolecules or other nanoparticles, and overall stability. The size and shape of solution-phase
synthesized particles are controlled using a variety of molecules, e.g., citrate, CTAB, PVP.\textsuperscript{40} The direct adsorption of molecules onto the surface of nanoparticles not only controls the synthetic route of the particles, but can be used to control function. For example, gold nanowires prepared in solution with a bilayer of CTAB on the surface can bind organic molecules in aqueous solution due to the presence of the hydrophobic CTAB.\textsuperscript{41}

Surface modification of nanoparticles changes the surface properties. This was explored herein by using silica or polymeric coatings. In the literature, spherical metallic particles have been coated with silica in solution by first pre-treating the particles with 3-aminopropyltrimethoxysilane and sodium silicate\textsuperscript{42-45} or PVP\textsuperscript{46} prior to growth of tetraethoxysilane (TEOS) through the Stöber process. Likewise, nanowires have been coated with silica through a solution sol-gel method by the hydrolysis (Figure 1-7) and polycondensation (Figure 1-8) of a silicon alkoxides, e.g., TEOS.\textsuperscript{47} This work was extended here using modified silicon alkoxides, e.g., $N$-(triethoxysilylpropyl)-o-polyethylene-oxide urethane, to incorporate functional groups into the silica coating. Since nanowires are less prone to aggregation than spherical colloid, a prefunctionalization step was unnecessary. The choice of catalyst in sol-gel reactions will change the properties of the resultant materials. The positively charged transition state of the silicon precursor molecule in low pH solutions is stabilized by electron donating silicon alkoxides groups, therefore promoting condensation, resulting in linear structures that then cross-link.\textsuperscript{48,49} Alternatively, electron withdrawing hydroxyl groups stabilize the negatively charged intermediate of base-catalyzed hydrolysis, resulting in further degree of hydrolysis prior to condensation.\textsuperscript{48,49} The resultant structures are
materials composed of polymeric clusters.\textsuperscript{49,50} The silica coating work presented in this dissertation proceeds via base catalysis, which results in a fast hydrolysis step, followed by a slower condensation step, generally resulting in a more porous material.\textsuperscript{48,49,51} Silica coatings are biocompatible; protect the core particle from solution, prevent degradation and aggregation; enable functionalization through well-understood silane chemistry; are heat resistant; and offer mechanical support to the particles.\textsuperscript{52}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{hydrolysis_reactions.png}
\caption{The hydrolysis reactions of alkoxysilanes by (A) acid catalysis or (B) base catalysis (Adapted from references 48, 49, 51).}
\end{figure}
Likewise, coating nanoparticles with polyelectrolyte coatings can change the surface chemistry by the choice of polyelectrolyte, producing a charged surface on the particle. The charge of the nanoparticle surface is controlled by changing the outer layer of polyelectrolyte to either a polycation or polyanion to suit a particular application. The surface charge of the nanoparticles results from the outermost layer due to charge overcompensation, and can be monitored using zeta potential. Through the layer-by-layer deposition of oppositely charged polyelectrolytes, better control over the coating thickness is achieved compared to other polymeric coating chemistries. The thickness can be controlled by the number of layers deposited; a bilayer of PAH and PSS on a gold substrate was found to be 2.9 nm thick by surface plasmon resonance, and this increased to 4.3 nm by the addition of another PAH layer. The choice of ionic species in the solution can also play a role in the deposition of the polyelectrolytes, thus affecting the thickness and roughness of the multilayers at ionic strengths above 0.1 M. In
addition to being able to control the surface charge, thickness, and to some extent roughness, charged species, such as proteins, dyes, and nanoparticles, can be encapsulated within the coating (Figure 1-9).55

Biomolecules can also be used to modify the surface chemistry of a nanoparticle, which is of particular importance for the fabrication of biosensors. Thiolated DNA can be covalently attached to metal nanoparticles. Additionally, proteins and nucleic acids can be attached through a variety of coupling chemistries to surfaces (Figure 1-10).58 The nanoparticles can first be coated with silica and the biomolecule can be covalently attached to the silica using a cross-linking molecule, e.g., sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC). This covalent attachment prevents dissociation of the biomolecule, e.g., DNA, from the surface of the particle with increased temperatures.59
Figure 1-10: Examples of cross-linker molecules to attach biomolecules to surfaces. (A) Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) is used to couple amines, e.g., 3-aminopropyltrimethoxysilane (APTMS), and sulhydryls, e.g., thiolated DNA. Adapted from reference 60. (B) 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride is a zero-length crosslinker that links carboxyls, e.g., carboxylated DNA and primary amines, e.g., APTMS. Adapted from reference 61.
1.5 Objectives

Surface chemistry of nanoparticles can be modified for use in sensing elements using a variety of chemistries, e.g., silica, polymers, and biomolecules, as discussed in this dissertation. The surface chemistry of both spherical and anisotropic wires can be modified and stabilized with silica (Figure 1-11A), providing protection against oxidation, biocompatibility, stabilization of particles against changes in e.g., ionic strength or pH, and structural stability.\textsuperscript{52,62} The metallic core of silica coated nanowires can subsequently be dissolved to produce high yields of silica nanotubes, which have applications in bioanalysis, catalysis and drug delivery (Figure 1-11B).\textsuperscript{63,64} The surface chemistry of nanowires was altered by the incorporation of modified silicon alkoxides, to incorporate additional functionality into the silica coating. Chapter 2 introduces the first reported ORMOSIL (organically modified silica) coatings on metallic nanowires, with characterization by FT-IR spectroscopy, x-ray photoelectron spectroscopy, and transmission electron microscopy. Proof-of-concept experiments showed applications for metal adsorption and protein resistance. Additionally, ORMOSIL nanotubes were produced and characterized.
A major field of sensing is the development of surface enhanced Raman spectroscopy substrates. An enhancement of the signal can be observed by adsorption of molecules onto surfaces, e.g., roughened electrodes, colloidal gold or silver, or nanowires. Furthermore, once Raman molecules have been adsorbed, the particles can be coated in silica to produce core—shell SERS substrates that are stable in a variety of solvents without alterations to the Raman response. Silica also prevents the undesired adsorption of molecular species onto the surface of the metallic core that would complicate the Raman signal. Au/Ag alloy nanowires can be coated with silica, and then the sacrificial Ag can be etched away to leave behind a silica coated porous gold nanowire. The silica coating is porous and small molecules can penetrate through the coating and adsorb to the surface of the gold core, while larger molecules, e.g., proteins, are blocked, allowing detection of small molecules in high concentrations of protein.

Figure 1-11: Transmission electron microscope image of (A) silica coated 13 nm Au nanoparticles and (B) silica nanotubes. Scale bars = (A) 50 nm, (B) 500 nm.
solutions. Porous gold nanowires with a polyethylene oxide silica coating, developed in Chapter 2, were SERS substrates that enabled the repeatable detection of 4-mercaptobenzoic acid (MBA) in biologically relevant solution, demonstrating promise for the detection of metabolic products in serum, as presented in Chapter 3.

Surface modification of nanoparticles can also be accomplished by the physical adsorption of proteins onto the nanoparticle surface through a combination of electrostatic, hydrophobic, van der Waals, and/or hydrogen bonding to the particle and to other protein molecules. Direct adsorption of proteins onto surfaces is the most common and direct method of immobilization, but complete characterization of the complexes is generally lacking. In Chapter 4 we present the characterization of HRP and fluorescein-labeled HRP onto 13 nm Au nanoparticles, including a direct quantification method, determination of the specific activity of the HRP, and an analysis of the complex stability with zeta potential and flocculation assays at different pH values. Adsorption differences between unlabeled and fluorescein-labeled HRP were compared.

Chapter 5 investigates the immobilization of enzymes onto the nanowire surfaces by encapsulation in silica or through the layer-by-layer assembly of polyelectrolytes. Encapsulation of proteins onto the surface of different populations of striped nanowires would enable multiplexed biosensing since the nanowires act as barcodes. Progress towards the development of enzymatic biosensors was accomplished by immobilizing a wide range of enzymes onto nanowire surfaces. Full characterization of these particle complexes is necessary to develop reproducible and sensitive sensors. Characterization of the embedded enzymes is difficult through optical methods, and thus was accomplished by depositing enzymes into polyelectrolyte multilayers on latex beads.
Chapter 6 combines the ability to functionalize and characterize nanomaterials and the fabrication of field-effect transistors (FET) in a collaborative process with Dr. Theresa Mayer’s group in the Department of Electrical Engineering to produce bioFETs for the detection of biomarkers. Multiplexed sensing with FETs has been limited to date due to the inability to control the placement of nanowires with accuracy. It has recently been shown that nanowires pre-functionalized with DNA can be assembled into predefined lithographically prepared wells using dielectrophoresis. Complementary DNA will hybridize to the anchored nanowires on the substrate post assembly, but the extent to which the lithographic processes affects probe DNA stability has not yet been determined. In Chapter 6 the survivability of thiolated DNA covalently attached to gold nanowires and to silica coated nanowires using Sulfo-SMCC chemistry was studied after exposure to a variety of lithographic processes, e.g., deposition and baking of different photoresists. Additionally, the ability of the DNA to selectively hybridize a complementary strand versus a target strand with a single base mismatch was studied. The effect of the lithographic process on nucleic acids will help progress towards the fabrication of multiplexed bioFETs that produce reproducible, sensitive, and quantitatively significant responses.

In conclusion, work has been completed to better understand the role of surface chemistry of nanoparticles for sensing applications. Biofunctionalization of nanoparticles was accomplished through a variety of methods, e.g., direct adsorption, immobilization within polyelectrolyte multilayers, or via cross-linking molecules. Controlling the surface properties of nanoparticles has enabled progress toward the development of SERS sensors for the detection of small molecules in biological fluids, enzymatic
biosensors in a suspension array format, and field effect transistors for the detection of nucleic acid biomarkers.
1.6 References


41. Alkilany, A. M.; Frey, R. L.; Ferry, J. L.; Murphy, C. J. Gold Nanorods as Nanoadmicelles: 1-Naphthol Partitioning into a Nanorod-Bound Surfactant Bilayer.


52. Bumb, A.; Brechbiel, M. W.; Choyke, P. L.; Fugger, L.; Eggeman, A.; Prabhakaran,


2.1 Introduction

Modified silicon alkoxide precursors have been used to control the chemical functionality and structure of bulk ORMOSIL (ORganically MOdified SILanes) materials.\textsuperscript{1-6} ORMOSILs are inorganic-organic hybrid materials, such as RSi(OCH\textsubscript{3})\textsubscript{3}; where the organic moiety, R, is covalently bound to the resulting matrix upon reaction.\textsuperscript{2} This prevents loss of the organic functionalities due to leaching.\textsuperscript{1} The choice of precursor molecule(s) affects the porosity, chemical reactivity, structure, morphology, hydrophobicity and materials properties of the resultant sol-gel, e.g., enabling the encapsulation of proteins, dyes, nanoparticles, or catalysts within the matrix.\textsuperscript{2-4} Mackenzie and Bescher described how using silicon alkoxide precursors modified with methyls, vinyls, polydimethylsiloxanes, or carbazoles resulted in materials that were hydrophobic, able to crosslink with acrylic groups, rubbery, or demonstrated charge transport properties, respectively, compared to sol-gels prepared from TEOS or tetramethoxysilanes (TMOS).\textsuperscript{5} Silicon alkoxide sol-gel precursors with, e.g., anthracene, bridging units that fluoresce when excited with ultraviolet light have produced materials
with the fluorescent molecule covalently attached to the structure, unable to leach away.\cite{7} Monolithic ORMOSILs have also been synthesized with $N$-(3-triethoxysilylpropyl)-4,5-dihydroimidazole (NTPDI) as a sol-gel precursor to introduce a dihydroimidazole functionality into the bulk material for chelating metal ions.\cite{8,9} These advanced materials have proven useful in a variety of applications: adsorbent materials, (bio)chemical sensors, nonlinear optical materials, and coatings, etc.\cite{2,6} In addition to monolithic ORMOSILs, these materials have been prepared as thin films to protect metallic or polymeric surfaces or to provide functionality for sensing or electrical applications.\cite{10-12}

Micro- and nanoparticulate organically modified silicas have also been prepared, for applications including biosensors and in vivo imaging.\cite{13} To the best of our knowledge, ORMOSILs have not previously been reported as coatings for nanowires, nor have they been formed into hollow tubes.

Metallic nanowires are attractive for applications ranging from multiplexed biosensing\cite{14-18} and electronics\cite{19,20} to catalytically driven nanomotors.\cite{21} For most applications, it is desirable to control the nanowire surface chemistry. Previous methods of modifying the surface properties of metallic nanowires have included: direct adsorption of organic molecules onto the metallic surface,\cite{22,23} attachment of DNA through thiol—Au linkages\cite{16} or proteins by first functionalizing the nanowire with an organic moiety,\cite{14,24} formation of a silica sheath into which nanowires were grown,\cite{25} or a modified sol-gel approach in solution.\cite{26-28} Alkilany et al. prepared gold nanowires in solution with a bilayer of cetyltrimethylammonium bromide (CTAB) to act as a hydrophobic layer to bind organic molecules in aqueous solution.\cite{22} Silica is an attractive coating for nanoparticles because it protects the particle core from oxidation and from
molecules in solution, prevents undesired adsorption of molecules onto the metal surface, acts as structural support, stabilizes particles for transfer to different solvents, and provides covalent attachment points for biomolecules through well-understood chemistry. Nanowires have been coated with SiO$_2$ in a variety of ways: (1) forming the silica shell first by depositing SiCl$_4$ into alumina pores and subsequently electrochemically filling the pores with the metal of choice, (2) high temperature reactions, (3) growth of sodium silicate on the surface of the nanowires in suspension, or (4) reaction of a silicon alkoxide, such as tetraethoxysilane (TEOS) in solution. Stabilizing agents, such as 3-mercaptopropyltrimethoxysilane may be used to make surfaces more vitreophilic, but are not always necessary for silica growth.

Removal of the metallic cores after deposition of a SiO$_2$ shell is one route to preparing silica nanotubes, which are attractive in bioanalysis, catalysis, and drug delivery, and are particularly desired due to the hydrophilicity of silica, the ability to functionalize the surface with well-known silane chemistry and an inner void that can be filled. SiO$_2$ nanotubes have also been prepared through a multistep fabrication approach using thermal oxidation of silicon nanowire arrays to form silica tube arrays. Additionally, templated synthesis of inorganic silica nanotubes has been accomplished via negative templating within the pores of alumina or positive templating using biological, carbon nanotube, or nanowire templates. ORMOSIL tubes could offer additional functionality as compared to these inorganic structures.

Literature on silica coatings for nanoparticles generally, and for nanospheres in particular, is more extensive than for nanowires. The procedures to coat nanoparticles with silica have been well investigated; a variety of different processes have resulted in
uniform, well-controlled coatings on particle surfaces with applications in catalysis, diagnostics, SERS, and photothermal therapy. Spherical metal nanoparticles (e.g., Au, Ag, Pt) have been coated with SiO$_2$ through the hydrolysis and polycondensation of TEOS on the particle surface, or by pre-treatment of the particles with 3-aminopropyltrimethoxysilane and then sodium silicate so the particles can be transferred into ethanol without aggregating. Alternately, poly(vinylpyrrolidone) has been adsorbed to the surface of the colloidal particles to prevent aggregation upon transfer of the particles into ethanol for the growth of silica through the Stöber process. Although SiO$_2$ surfaces offer many advantages, it is desirable to tailor surface properties for more advanced applications without an additional functionalization step. Synthesizing hybrid silica coatings using organically modified silicon alkoxide precursors would retain the advantages of SiO$_2$, while enabling control of the surface chemistry via incorporation of different functional groups. Surprisingly, although some reports of more complex, hybrid coatings have appeared, the vast majority of silica coatings on nano- and microparticles have been simple inorganic silicas.

Here, we present the fabrication and characterization of silica coatings incorporating a variety of functional groups onto metal nanowires in suspension. ORMOSIL films on the nanowires were prepared using a modified sol-gel synthesis in which functional group containing silicon alkoxide precursor molecules and TEOS were co-deposited (Figure 2-1). Nanotubes of the various modified silicas were produced by acid dissolution of the nanowire scaffolds (Figure 2-1). The presence of the functional groups in the ORMOSIL shells was confirmed through infrared spectroscopy (FT-IR) and proof-of-concept binding experiments. To our knowledge this is the first example of
ORMOSIL coatings on nanowires or nanoparticles of any kind. Such coatings may prove valuable in a wide range of biomedical and materials applications.

Figure 2-1: Fabrication process for silica coating nanowires and the subsequent dissolution of the nanowire scaffold to produce hollow, silica nanotubes.

2.2 Experimental Materials and Methods

2.2.1 Materials

Tetraethoxysilane, 98% (TEOS), 3-acryloxypropyltrimethoxysilane (AcPTMS), bis(trimethoxysilyl)benzene (BTEB), bis(triethoxysilyl)ethylene (BTESE), 1,6-bis(trimethoxysilyl)hexane (BTMH), 2-cyanoethyltriethoxysilane (CETES), (3-isocyanatopropyl)triethoxysilane (ICPTES), N-(3-triethoxysilylpropyl)-4,5-dihydroimidazole (NTPDI) and N-(triethoxysilylpropyl)-o-polyethylene-oxide urethane (PEO) were obtained from Gelest, Inc. 3-aminopropyltrimethoxysilane (APTM) was acquired from TCI America. Spectrograde KCl was obtained from International Crystal Laboratories. Lactate dehydrogenase from porcine heart was purchased from
Calbiochem. Alexa Fluor® 555 protein labeling kit and Alexa Fluor® 488 goat anti-mouse IgG (H+L) were purchased from Invitrogen. DNA sequences were purchased from Integrated DNA Technologies. Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) was purchased from Pierce Protein Research Products, Thermo Scientific. All water used was 18.2 MΩ-cm Nanopure water from a Barnstead system. All reagents were used as purchased without further purification; the silanes were aliquoted out in a glove box under nitrogen gas to prevent hydrolysis from water vapor in the air.

2.2.2 Nanowire Synthesis

Metallic nanowires were synthesized by galvanostatic electrodeposition into anodized aluminum oxide Anodisc 25 membranes with 0.2 μm pores (I = 1.65 mA) on an in-house system, as has been described previously. Silver (300 nm) was vapor deposited on one side of the membranes to act as the working electrode. Nanowires were electrodeposited in the pores of the membrane, after which the silver backing was dissolved in 33% v/v nitric acid. Nanowires were then released into suspension by vortexing the membrane in 3 M NaOH, and rinsed with 3 M NaOH, followed by DI H₂O, and three times with ethanol (EtOH) before suspension in 1 mL EtOH. One batch of nanowires produced ~10⁹ nanowires.

While most of the work in this manuscript was performed with 6 μm Au nanowires, nanowires composed of segments of both Au and a sacrificial metal (Ag or Ni) were used to facilitate rapid screening of reaction conditions using light microscopy.
For example, if a Au-Ag-Au wire was coated with silica and the Ag segment was subsequently removed by acid etching, the two Au ends would remain associated. If the silica coating was unsuccessful, the two Au ends would not remain associated (Figure 2-2). Once viable reaction conditions were identified, further work was conducted with Au wires, with silica imaging via TEM.

![Image of nanowires](image)

Figure 2-2: Reflectance images of Au/Ni striped nanowires coated with 1TEOS:1AcPTES (left) and 3TEOS:1AcPTES (right) with the Ni segment etched, demonstrating that the silica was not thick enough to keep the Au segments together for the 1:1 sample, but did maintain structure when a 3:1 TEOS:AcPTES ratio was used. Scale bar = 2 μm.

2.2.3 Silica Coating of Nanowires

Nanowires were coated with TEOS silica as described in the literature\textsuperscript{26,27} (Figure 2-1); this procedure was then adjusted to produce different glass chemistries by adding the silicon alkoxide of choice to the reaction mixture at the same time as the TEOS, however acetonitrile (ACN) was used as the solvent, rather than EtOH. Briefly, 300 μL nanowires (batch concentration) were combined with 490 μL EtOH, 160 μL DI H\textsubscript{2}O, 40
μL TEOS, and 10 μL NH₄OH (14.8 M) in a microcentrifuge tube. After one hour of sonication in a Crest Ultrasonicator with a Genesis module operating at a frequency range of 39-41 kHz at 190-200 watts, the coated nanowires were rinsed three times with ~1 mL EtOH by centrifugation (2000 g). Specific reaction conditions to produce the modified silica coatings can be found in Table 2-1.
Table 2-1: Reaction parameters and TEM and IR analysis of silica-coated, 6 µm gold nanowires.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ratio of TEOS to Modified Silane (%)§</th>
<th>Reaction Volumes</th>
<th>Number of Coating s</th>
<th>Reaction Time/Coating (min)</th>
<th>Thickness on Nanowire† (nm)</th>
<th>Observed Characteristic IR Peaks (cm⁻¹)‡⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEOS</td>
<td>n/a</td>
<td>40.0 µL TEOS</td>
<td>1</td>
<td>60</td>
<td>50.8 ± 2.6</td>
<td>2985, 2944, 2933, 2906 (C—H); 1300-996 (Si—O—Si)</td>
</tr>
<tr>
<td>TEOS:AcPTES</td>
<td>75:25</td>
<td>29.9 µL TEOS, 13.2 µL AcPTES</td>
<td>3</td>
<td>60</td>
<td>6.00 ± 1.1</td>
<td>2925, 2954 (C—H); 1726 (C=O); 1334-947 (Si—O—Si)</td>
</tr>
<tr>
<td>TEOS:BTEB</td>
<td>91:9</td>
<td>36.4 µL TEOS, 3.77 µL BTEB</td>
<td>3</td>
<td>60</td>
<td>43.1 ± 4.8</td>
<td>2982, 2945 (C—H); 1314-980 (Si—O—Si)</td>
</tr>
<tr>
<td>TEOS:BTESE</td>
<td>75:25</td>
<td>29.9 µL TEOS, 11.0 µL BTESE</td>
<td>3</td>
<td>60</td>
<td>20.2 ± 3.4</td>
<td>2976, 2932 (C—H); 1284-982 (Si—O—Si)</td>
</tr>
<tr>
<td>TEOS:BTMH</td>
<td>67:33</td>
<td>26.6 µL TEOS, 12.8 µL BTMH</td>
<td>1</td>
<td>60</td>
<td>15.2 ± 1.4</td>
<td>2932, 2864 (C—H); 1325-990 (Si—O—Si)</td>
</tr>
<tr>
<td>TEOS:CETES</td>
<td>75:25</td>
<td>29.9 µL TEOS, 13.2 µL CETES</td>
<td>3</td>
<td>60</td>
<td>8.7 ± 2.6</td>
<td>2930, 2853, 2809 (C—H); 2253 (C≡N); 1309-978 (Si—O—Si)</td>
</tr>
<tr>
<td>TEOS:ICPTES</td>
<td>80:20</td>
<td>31.8 µL TEOS, 11.9 µL ICPTES</td>
<td>3</td>
<td>10</td>
<td>8.3 ± 2.3</td>
<td>2980, 2949, 2887, 2836 (C—H); 1653 (N—C≡O, amide); 1309-978 (Si—O—Si)</td>
</tr>
<tr>
<td>TEOS:NTPDI</td>
<td>25:75</td>
<td>6.65 µL TEOS, 21.7 µL NTPDI</td>
<td>4</td>
<td>10</td>
<td>24.7 ± 3.8</td>
<td>2930, 2888 (C—H); 1651 (C≡N); 1601 (C—N); 1284-897 (Si—O—Si)</td>
</tr>
<tr>
<td>50:50</td>
<td>9.98 µL TEOS, 16.3 µL NTPDI</td>
<td>4</td>
<td>10</td>
<td>24.5 ± 4.7</td>
<td>2930, 2888 (C—H); 1651 (C≡N); 1601 (C—N); 1284-897 (Si—O—Si)</td>
<td></td>
</tr>
<tr>
<td>75:25</td>
<td>15.0 µL TEOS, 8.13 µL NTPDI</td>
<td>4</td>
<td>10</td>
<td>17.5 ± 2.3</td>
<td>2928, 2877 (C—H); 1692 (amide I); 1530 (amide II); 1331-982 (Si—O—Si)</td>
<td></td>
</tr>
<tr>
<td>83:17</td>
<td>16.7 µL TEOS, 5.43 µL NTPDI</td>
<td>4</td>
<td>10</td>
<td>18.5 ± 1.9</td>
<td>2928, 2877 (C—H); 1692 (amide I); 1530 (amide II); 1331-982 (Si—O—Si)</td>
<td></td>
</tr>
<tr>
<td>91:9</td>
<td>18.2 µL TEOS, 2.96 µL NTPDI</td>
<td>4</td>
<td>10</td>
<td>12.7 ± 2.2</td>
<td>2928, 2877 (C—H); 1692 (amide I); 1530 (amide II); 1331-982 (Si—O—Si)</td>
<td></td>
</tr>
<tr>
<td>TEOS:PEO</td>
<td>75:25</td>
<td>30.0 µL TEOS, 24.7 µL PEO</td>
<td>3†</td>
<td>60</td>
<td>16.3 ± 1.8</td>
<td>2928, 2877 (C—H); 1692 (amide I); 1530 (amide II); 1331-982 (Si—O—Si)</td>
</tr>
</tbody>
</table>

§ Ratio of the number of reactive groups of TEOS to the number of reactive groups of the modified silicon alkoxide

† n = 100 measurements made

‡ Nanowires coated 5 times at 60 min each to make nanotubes with thick enough walls to prevent collapse during drying or under the vacuum of the TEM; IR and TEM analysis done using nanowires coated 3 times.
2.2.4 Materials Characterization

Transmission electron microscopy images were obtained using a JEOL JEM 1200 EXII instrument with a high resolution Tietz F224 digital camera at an accelerating voltage of 80 kV. Analysis of the TEM images was done using Image J software to determine glass thickness by averaging over many nanowires and measurements (n=100) (Image J software is available for free at www.NIH.gov).

FT-IR spectra were acquired on a Bruker IFS 66/s spectrometer using a Collector II diffuse reflection accessory equipped with a high temperature/vacuum (HTV) sample chamber. The HTV chamber was employed to reproducibly reduce moisture content and minimize spectral interferences in the water O-H stretching (~3400 cm\(^{-1}\)) and O-H deformation (~1640 cm\(^{-1}\)) regions. Silica coated nanowires (40 µL TEOS-coated nanowires in ethanol, or 200 µL of the modified silica coated nanowires, due to differences in coating thickness, at batch concentration in ethanol) were dispensed into a mortar where residual solvent was evaporated using a heat gun. Nanowires were then ground for 120 s with a pestle in the presence of 60 mg KCl to minimize differences in particle size to keep the scattering coefficient constant. Samples were loaded into the HTV and heated to 75ºC for 5 minutes prior to spectral acquisition to facilitate drying. Preliminary measurements where samples were dried at higher temperatures (115ºC & 150ºC) reveal changes in the spectra of the nanowire coating beyond simple removal of molecular water. Progressed polycondensation of tetraethoxysilane has been observed in the bulk with increasing temperature as water is driven off. All spectra were acquired by averaging 400 scans at 6 cm\(^{-1}\) and were referenced to the spectrum of KCl powder.
prepared in an analogous manner. Spectral acquisition and processing was done with OPUS 6.0 software. All spectra were plotted in absorbance units, thus avoiding baseline offset errors of the Kubelka-Munk transformation.\textsuperscript{52} Vapor phase water features were subtracted from the spectra using the OPUS atmospheric compensation function. The CO\textsubscript{2} mode centered at \(~2350 \text{ cm}^{-1}\) was mathematically removed, a one point baseline offset was applied at \(~3900 \text{ cm}^{-1}\), and the Si—O—Si peak centered at \(~1150 \text{ cm}^{-1}\) was normalized to an absorbance value of one to improve visualization. IR analysis of the silica nanotubes was done in an identical fashion.

X-ray photoelectron spectroscopy (XPS) data was obtained on a Kratos Analytical Axis Ultra with a monochromatic Al K\textalpha{} x-ray source operating at an X-ray power of 240 watts. Spectra were collected at a 90\degree{} photoelectron take off angle with respect to the sample plane with a pass energy of 20 eV and an energy step of 0.1 eV. All spectra were taken with the charge neutralizer on and referenced to the C 1s at 285.0 eV. The samples were analyzed using CasaXPS software (Casa Software Ltd.).

### 2.2.5 Chromate Adsorption

Chromate adsorption onto the nanowire coatings was detected by first coating 300 \(\mu\text{L} \sim 5.5 \ \mu\text{m} \) Au nanowires with TEOS, 10TEOS:1NTPDI, or 1TEOS:1NTPDI, as described previously. Each nanowire sample (~ 3 \(\times\) 10\(^8\) wires) was incubated in 300 \(\mu\text{L} \) 19 \(\mu\text{M} \) K\textsubscript{2}CrO\textsubscript{4} in pH 2.5, 0.57 M acetic acid while vortexing for 4 hrs. A control without nanowires was also made and vortexed to correct for adsorption to the centrifuge tubes. The nanowires were spun down and the supernatant was removed and analyzed on
a Hewlett-Packard 8453 diode-array UV/visible spectrometer with Agilent ChemStation software.

2.2.6 Protein Adsorption

Protein adsorption data on the coated nanowires was collected by first coating 6 µm Au nanowires with TEOS and 3TEOS:1PEO. The nanowires were then rinsed into buffer containing 0.1 µM Alexa 555® LDH or 0.1 µM Alexa 488® goat antimouse IgG. The samples were vortexed for 1 hr and then rinsed extensively with buffer. Nanowire samples were imaged using a Nikon TE-300 inverted microscope equipped with a Photometrics Coolsnap HQ camera, a Plan fluor 60x oil objective (N.A. 1.4), and Image-Pro Plus (v 4.5) software. The source for reflected and fluorescence excitation was a 300 W Xe lamp. Fluorescence from at least 290 wires in each sample was quantified to compare the amount of protein adsorption on the different glass chemistries using NBSSee software (Nanoplex, Inc.).

2.2.7 Covalent Modification with DNA and Hybridization to Complementary Sequence

Two different thiolated single stranded DNA sequences were covalently attached to TEOS and 3TEOS:1PEO coated nanowires through Sulfo-SMCC chemistry, as previously described. Hybridization to fluorescently-labeled complementary DNA sequences from solution was performed by taking 20 µL aliquots of each batch of nanowires, adding 170 µL 10 mM phosphate buffer, pH 7.4, 300 mM NaCl (HB), and 10
uL 20 µM Alexa Fluor® 488 labeled DNA sequence that was complementary to one probe sequence but not the other. After vortexing for 1 hr, samples were rinsed 3x by centrifugation into the HB and imaged as described above.

2.3 Results and Discussion

2.3.1 Synthesis and Characterization of Modified Silica Coatings

Our general approach to incorporating functional groups into the silica coatings was to replace part of the TEOS in our previously reported on-wire sol-gel silica growth protocol with a substituted siloxane. Unlike in our prior work, we chose to use the aprotic solvent ACN for these syntheses; this facilitates the forward reaction by rendering the catalytic hydroxyl ions more nucleophilic, and prevents reverse reactions that can occur when electrophilic protons are available. In combination with the tetrafunctional siloxane molecules (TEOS), alkyl-substituted species (AcPTES, CETES, ICPTES, NTPDI, PEO) and precursors with bridging units (BTEB, BTESE, BTMH) were used to form silica coatings on the nanowires (Figure 2-3).
Reaction conditions (reactant ratios, number of coatings) were adjusted to achieve silica shell formation without excessive free silica sphere generation for each of the substituted siloxanes. In the early stages of this work, coatings were initially formed on striped Au/Ag or Au/Ni metal nanowires. The sacrificial Ag or Ni segments made it possible to more rapidly test synthesis conditions using optical reflectance imaging, rather than collecting TEM data for each set of conditions. A silica coating was considered successful in these preliminary tests if it maintained structural stability after acid etching to remove the sacrificial segments (an example is shown in Figure 2-1). These synthetic conditions were then employed to produce ORMOSIL coatings on 6 µm
Au nanowires for the analysis shown in this manuscript. When necessary, shorter reaction times were used to prevent the formation of free silica spheres, and/or multiple coatings were performed to increase the thickness of the silica deposited. Rinsing by centrifugation and resuspension between subsequent reactions and before analytical characterization was used to remove any free silica that did form in the samples. The large size and high density of the metallic nanowires facilitates their separation from free silica by centrifugation. With the NTPDI silica coating the concentration of the reaction mixture (wires, silanes, water, catalyst) was diluted by half to prevent formation of free silica spheres. When very thin (< 5 nm) silica shells were observed, the thickness of the coating was increased by coating the nanowires multiple times, with rinsing by centrifugation and resuspension of the nanowires into new reagents between each reaction. Table 2-1 lists the ratio of reactive groups between the TEOS precursor molecules and the functionalized silicon alkoxide, the number of coatings, and the coating times that were necessary to produce homogenously thick, silica coatings on the nanowire surface.

TEM imaging of the resulting ORMOSIL coatings on 6 µm Au nanowires was used to determine their mean thickness, which varied from just 6.0 ± 1.1 nm for three consecutive coatings with AcPTES-containing silica to 50.8 ± 2.6 nm for a single coating synthesized from TEOS. Representative images for each of the coating chemistries are shown in Figure 2-4 (left panels), and mean thicknesses are reported in Table 2-1. Low magnification TEM images of TEOS, BTEB, BTMH, PEO, and NTPDI coated nanowires can be found in Figure 2-5, which confirms little to no free silica was present in the sample, and that the nanowires were coated uniformly. ORMOSIL thickness
varied with the length of the nanowires (i.e. available surface area for deposition), with thinner coatings for longer wires and thicker coatings for shorter wires. To allow for direct comparisons, all data presented in Figure 2-4 and Table 2-1 are for 6 μm Au nanowire coatings. TEM analysis also gave qualitative indications as to the homogeneity of the silica coating; some chemistries led to rougher, textured surfaces (and thus larger surface areas) leading to larger standard deviations in the thickness of the glass, for example, BTEB (Figure 2-4C) or NTPDI (Figure 2-4H), (Table 2-1). The differences in coating thickness and structure indicated, qualitatively, the differences in the silica coatings when different silicon alkoxide precursors are incorporated into the nanowire coatings.

![Figure 2-4: TEM images of silica coated nanowires (left panels) and silica tubes (right panels) prepared from the following precursors: (A) TEOS (SiO₂), (B) 3TEOS:1AcPTES (acryloxy group), (C) 10TEOS:1BTEB (ethyl benzene linker), (D) 3TEOS:1BTESE (ethane linker), (E) 2TEOS:1BTMH (hexane linker), (F) 3TEOS:1CETES (cyano group), (G) 4TEOS:1ICPTES (reacted isocyanato group), (H) 1TEOS:1NTPDI (dihydromidizole group), (I) 3TEOS:1PEO (polyethylene oxide group). Scale bars = 100 nm all left panels and 500 nm for right panels.](image-url)
Exposure of the silica coated nanowires to an acid solution resulted in dissolution of the metallic nanowires to form silica nanotubes (Figure 2-1). TEM analysis showed

Figure 2-5: Low magnification TEM images of (A) TEOS, (B) BTEB, (C) BTMH, (D) NTPDI, and (E) PEO coated nanowires with corresponding high magnification images to demonstrate the full coating of each nanowire with minimal to no free silica formation (indicated with a *). Low magnification scale bars = 2 µm; high magnification scale bars = 100 nm.
these nanotubes to be intact, hollow structures replicating the nanowire size and shape (Figure 2-4, right panels). For a 3 µm nanowire, the resulting nanotube would have an estimated interior volume of $\sim2 \times 10^{-16}$ L. Depending on the thickness of the silica walls, nanotubes can be prone to collapse without the structural support of the nanowire scaffold when <10 nm thick; 3TEOS:1AcPTES and 4TEOS:1ICPTES tubes collapsed during drying or under vacuum, even with 5 coatings. Five coatings produced thick enough 3TEOS:1CETES and 3TEOS:1PEO silica to prevent collapse of the majority of tubes.

The different reaction conditions required for formation of the modified silica coatings, as well as their differences in morphology as compared to TEOS silica coating suggested that the substituents were getting incorporated. Differences in the thickness and surface roughness of the various ORMOSILs may be due to changes in the reaction kinetics when different silicon alkoxides were incorporated into the reaction solution, thus changing the way in which the silanes were deposited onto the surface of the nanowires. Spectroscopic analysis of the coated nanowires and nanotubes was done to confirm that the different silicon alkoxide precursor molecules were getting incorporated.

2.3.2 Spectroscopic Analysis of Modified Silicas

To verify the presence of the desired functional groups in the silica shells formed on the nanowires, we performed spectroscopic analysis of the coated nanowires and nanotubes. First, x-ray photoelectron spectroscopy (XPS) was performed on 1TEOS:1NTPDI coated nanowires, which were selected due to the presence of a nitrogen
atom. High resolution XPS indicated a peak at 397.6 eV in this sample, corresponding to the nitrogen 1s photoelectrons of the imidazole functionality (Figure 2-6). No corresponding peak was observed in the TEOS control sample. This confirmed that the addition of the imidazole-containing silicon alkoxide precursor into the reaction solution resulted in its incorporation in the silica coatings on the nanowire surface.

Figure 2-6: High resolution XPS data for the nitrogen 1s region for silica coated nanowires prepared from 1TEOS:1NTPDI (A) and TEOS (B).

To further evaluate the incorporation of functionalized silicon alkoxides, the coated nanowires were analyzed using Fourier transform infrared spectroscopy using a diffuse reflection accessory. Figure 2-7 shows the resultant IR spectrum for each of the samples prepared and Table 1 lists the characteristic peaks. A very strong band at ~1300-960 cm\(^{-1}\) was observed in each spectra; this peak is the Si—O—Si asymmetric stretch,\(^{55}\)\(^{57}\) and was very intense due to the large number of crosslinked SiO\(_2\) in every sample.
Given the intensity of this band, some of the peak distortion here is likely due to specular reflection of the sample. The functional groups were observed using FT-IR due to their characteristic vibrational signatures, confirming the incorporation into the silica coating on the nanowires. The presence of more pronounced CH$_2$ asymmetric and symmetric stretches at 2932 cm$^{-1}$ and 2864 cm$^{-1}$, respectively,$^{57}$ in the BTMH sample with no other peaks observed other than the Si—O—Si stretch indicates the incorporation of this hexane-bridged silicon alkoxide. Likewise, peaks for other functional groups were observed, confirming the incorporation of these silicon alkoxides into the coating material (Table 2-1).$^{57}$ For example, the C=N functionality in the imidazole in the NTPDI sample absorbed at 1651 cm$^{-1}$, while the C—N absorbed at 1601 cm$^{-1}$. $^{57}$ The amide I and II groups of the PEO coating absorbed at 1692 cm$^{-1}$ and 1530 cm$^{-1}$, respectively.$^{57}$

We were unable to unambiguously identify the absorbance due to the C=C functionality in the AcPTES, BTEB, or BTESE materials. This is most likely due to the weak absorption of a carbon-carbon double bond, and the low concentration, particularly with the BTEB sample (only 9% added benzene containing silicon alkoxide). The absence of the weak C=C mode in the spectra of the AcPTES, BTEB, or BTESE coated nanowires does not necessarily mean that these groups were absent from the coating on the nanowires; for example, the carbonyl peak at 1726 cm$^{-1}$ is observed in the AcPTES sample, indicating the presence of the molecule in the coating, even though no C=C vibration was observed. We suspect that BTEB and BTESE may also have been incorporated based on the morphologies of the coatings and necessary modifications to the reaction conditions, but cannot confirm their presence.
It is also interesting to note that no strong peak at 2280 cm\(^{-1}\) was observed from the N=C=O functionality in the ICPTES sample, even after coating the nanowires with three ICPTES coatings.\(^{55}\) Although this still produced a thin coating in comparison to other chemistries studied, 8.3 ± 2.3 nm, the isocyanate group should strongly absorb at 2300-2250 cm\(^{-1}\).\(^{57}\) When compared to the TEOS spectrum, there were peaks present in the ICPTES spectrum not present in the TEOS spectrum. The peak at 1653 cm\(^{-1}\) suggested that the ICPTES was incorporated into the coating, but was converted to another species, possibly containing an amide group, due to the reactivity of isocyanates,

Figure 2-7: (A) FT-IR spectra of TEOS and hybrid silica samples on Au nanowires. Characteristic peaks enlarged to the right: (B) carbonyl stretch of AcPTES; (C) alkyl stretch of BTMH; (D) cyano stretch of CETES; (E) peaks indicative of reacted ICPTES; (F) C=N stretch of NTPDI; (G) amide stretches of PEO.
specifically in the presence of water. It is well known that isocyanates will react with hydroxyl groups to form urethanes.\textsuperscript{58} The peak at 1653 cm\textsuperscript{-1} could be assigned to the carbonyl stretch, while the peaks at 1548 cm\textsuperscript{-1} and 1448 cm\textsuperscript{-1} may be the N—H bend and C—N stretch, respectively, lending evidence to the formation of urethane groups.\textsuperscript{57,59}

IR analysis of the silica nanotubes prepared by etching silica coated Au nanowires with \textit{aqua regia} showed the varying functional groups remained present even after the strong acid etchant (Figure 2-8). IR analysis was done for the same modified silica materials analyzed above as nanowire coatings, except for the silicas containing the C=C functionalities (i.e., BTEB, BTESE), as these peaks were not observed with the initial analysis. New peaks at 3740 cm\textsuperscript{-1} were observed, corresponding to isolated silanols on the surface.\textsuperscript{60} However, vibrational signatures of the functional groups remained, indicating that acid etch conditions did not result in loss of the organic groups.
We then varied the ratio of TEOS to NTPDI to test whether this resulted in a change in the amount of modified silicon alkoxide incorporated into the nanowire coating. Figure 2-9 shows the TEM images of samples prepared with 10:1, 5:1, 3:1, 1:1, and 2:1 TEOS:NTPDI. When more NTPDI was added into the reaction mixture, thicker coatings were observed (Table 2-1). IR spectra were collected to determine if the concentration of NTPDI in the coating could be controlled, as indicated by the signature peak intensity. We were able to vary the amount incorporated qualitatively (Figure 2-10), however, a strong quantitative correlation between ratio of precursors added and the

Figure 2-8: (A) FT-IR spectra of nanotubes composed of TEOS and each hybrid silica post acid etching. Identifying peaks enlarged to the right indicating presence of the functional peaks post etching: (B) carbonyl stretch of AcPTES; (C) alkyl stretch of BTMH; (D) cyano stretch of CETES; (E) peaks indicative of reacted ICPTES; (F) C=N stretch of NTPDI; (G) amide stretches of PEO.
amount of functional group in the resulting glass was not observed due to day-to-day variability in the on-wire sol-gel reactions. It is clear from IR spectra of coated wires prepared and analyzed on separate days that the organically modified silane molecules were incorporated into the silica coating, but the exact amount varies due to a number of factors, e.g., the number of wires (loss of wires can occur during rinsing and due to adsorption to centrifuge tube walls), how well the wires are sonicated, the humidity, etc., thus contributing to the larger error bars observed in Figure 2-11.

Figure 2-9: TEM images of organically modified silica coatings with different amounts of dihydroimidazole added during synthesis: (A) 10:1, (B) 5:1, (C) 3:1, (D) 1:1, and (E) 1:2 TEOS:NTPDI coated nanowires. Scale bar = 100 nm.
Figure 2-10: FT-IR spectra for silica coatings prepared using different amounts of imidazole-containing precursor: 1:1 TEOS: NTPDI (a), 10:1 TEOS: NTPDI (b), and TEOS alone (c).

Figure 2-11: Quantification of NTPDI incorporation. The IR spectra of the TEOS:NTPDI silica coated nanowires with varying amounts of NTPDI added to the reaction mixture were then integrated to determine the area under the C=N peak (1720-1620 cm\(^{-1}\)) and the Si—O—Si peak (1280-850 cm\(^{-1}\)). This showed qualitative control over the amount of imidazole functionality incorporated into the coating by changing the reagent concentrations. Error bars are the standard deviation between three sets of data from three sets of coated nanowires, fabricated and analyzed on separate days.
2.3.3 Chromate adsorption on imidazole-containing silica shells.

In a proof-of-concept experiment, NTPDI silica coatings were studied to further validate presence and retained function of the modified silica. Bulk imidazole-containing sol-gel materials have been shown to chelate metal ions, such as chromate, and remove them from solution.\textsuperscript{8,9} To demonstrate that our ORMOSIL coatings retained the properties conferred by their functional groups in bulk ORMOSILs, we tested the ability of NTPDI-containing silica coated nanowires to remove chromate from solution, as compared to TEOS-silica coated nanowires. The nanowires were vortexed in chromate solution for a given amount of time, spun down, and the supernatant was analyzed. Figure 2-12 shows UV-VIS spectra of chromate in solution before and after contact with the coated nanowires. The chromate absorbed at \( \sim 350 \) nm; a loss of 8.1% of the initially 19 \( \mu \text{M} \) chromate was observed upon introduction of the TEOS coated nanowires, presumably due to nonspecific binding. Nanowires with NTPDI incorporated into the silica coating removed a substantially larger amount of chromate from solution (62% and 80%, for 10:1 and 1:1 TEOS:NTPDI coatings, respectively), as evident from the decreased chromate absorbance. Approximately an order of magnitude more chromate ions were adsorbed onto the 1TEOS:1NTPDI coated nanowires (1.5 \( \times 10^{14}/\text{cm}^2 \)) compared to the TEOS coated nanowires (1.3 \( \times 10^{13}/\text{cm}^2 \)), while \( \sim 1.3 \times 10^{14} \) chromate/cm\(^2\) was adsorbed onto the 10TEOS:1NTPDI coated nanowires. Integrated IR data for the coatings prepared with different ratios of TEOS:NTPDI, twice as much NTPDI was incorporated into the silica coating when a 1:1 ratio of TEOS:NTPDI is used in the reaction mixture compared to 10:1 (Figure 2-11). These data suggest that not every
imidazole group was accessible for chromate binding, potentially due to the inaccessibility of a greater percentage of groups in the 10:1 sample or charge repulsion leading to a saturation in chromate binding at higher surface densities.

Figure 2-12: UV-VIS absorbance for $K_2CrO_4$ supernatant before (a) and after incubation with silica coated nanowires prepared from: TEOS alone (b), 10:1 TEOS:NTPDI (c) and 1:1 TEOS:NTPDI (d).

2.3.4 Resistance of protein binding by PEO-functionalized silica shells.

Although there are many advantages to SiO$_2$ surfaces, such as, well-understood attachment chemistry, structural support, relatively inert surface, etc., proteins are known to adsorb to the surfaces of materials, such as silica.$^{61,62}$ This undesired adsorption of proteins onto the surface of silica materials needs to be minimized for specific applications, for example, biosensors or implantable devices.$^{61}$ The PEO moiety is attractive as a surface coating to resist protein adsorption and prevent biofouling.$^{63}$ We
compared protein adsorption on PEO modified- and standard SiO$_2$ coated nanowires by suspending TEOS and TEOS:PEO coated gold nanowires in solutions of fluorescently labeled proteins. After incubation in a solution of either Alexa-Fluor® 555-labeled lactate dehydrogenase (LDH) or Alexa-Fluor® 488-labeled goat anti-mouse IgG, the nanowires were rinsed well with buffer and imaged on a fluorescence microscope (Figure 2-13 A). Figure 2-13 B shows nanowires coated with the standard TEOS silica showed strong fluorescence after exposure to the fluorescent proteins, however very little fluorescence was observed on the TEOS:PEO ORMOSIL coated nanowires, confirming their greater resistance to protein adsorption.

2.3.5 Surface reaction of ORMOSIL coatings.

The organically modified silica coatings prepared here could also be surface functionalized analogous to standard inorganic silicas in order to attach molecules of interest such as DNA oligonucleotides. We verified this by attaching 5’ thiolated DNA oligonucleotides to the surface of silica and ORMOSIL coatings on metal nanowires and then incubating with fluorescent complementary or noncomplementary DNA oligonucleotides. Figure 2-14 compares fluorescence intensity data for these experiments on inorganic silica and TEOS:PEO coated nanowires. Differences in intensity for the complementary strand on the two samples can be understood in light of both the greater number of reactive groups and the greater separation between the dye molecule and the metal surface for the thicker TEOS coating as compared with TEOS:PEO. Nonetheless,
both the standard TEOS silica and the TEOS:PEO exhibited good selectivity between the complementary and noncomplementary sequences.

Figure 2-13: (A) Reflectance and fluorescence microscopy images of Alexa Fluor® 488 anti-IgG (a,c) and Alexa Fluor® 555 LDH (b,d) adsorbed onto TEOS coated Au nanowires (top images) and 3TEOS:1PEO coated Au nanowires (bottom images). (B) Mean intensity data for the adsorbed Alexa Fluor® 555 LDH and Alexa Fluor® 488 goat anti-mouse IgG (H+L) on the TEOS (grey) and 3TEOS:1PEO coated Au nanowires (white). Scale bar = 5µm.
2.4 Conclusion

We have demonstrated the incorporation of organically modified precursor molecules with TEOS during sol-gel synthesis of silica shells on metallic nanowires. Most of the organically modified precursors evaluated here were successfully incorporated with minor modifications of the protocol used for standard TEOS glass shell formation, which suggests that additional functional groups not tested here should also be able to be incorporated without difficulty. A wide variety of modified silicon alkoxides with different functionalities and properties are commercially available and could potentially be used to synthesize ORMOSIL surface coatings on nanowires or other nano-
or microparticulate scaffolds. Such coatings offer functionalities beyond those possible for traditional inorganic silica shells or the surface silanization approaches commonly used to vary the chemistry of these silica shells. Examples include the ability to incorporate desired organic groups covalently throughout the silica shell rather than just on the surface, to control porosity, provide permselectivity, and enhanced protection against protein adsorption, biofouling, and/or corrosion.
2.5 References


48. Use caution when working with aqua regia, as it will cause severe burns. Dispose of strong acid properly.

49. All spectra were acquired using: a mid-band MCT detector, 3.0 mm internal aperture, and a 40.0 kHz scanning velocity. The interferograms were processed using a Mertz phase correction, Norton-Beer medium apodization, and a zero-filling factor of 8.


Silica-Coated, Porous Gold Nanowires as SERS Substrates

3.1 Introduction

A number of approaches have been reported in the literature to enhance the inherently weak Raman signal. Complex structures, such as nanostructured arrays, on-wire lithography, nanowire pillars, and nanoshells, have all been shown to enhance the Raman signal. Aggregated colloidal silver particles are among the simplest scaffolds for molecules of interest, and have been shown to produce $10^{14}$ – $10^{15}$ signal enhancement. Interaction of light with these nanostructured metallic materials produces collective oscillation of electrons, i.e., surface plasmons, that generate intense electromagnetic fields at the surface of the particle, which enhances the Raman signal. Surface enhanced Raman spectroscopy (SERS) has been used as a characterization tool and a detection mechanism for small molecules, biological species, warfare agents, and drug molecules. Since water generally does not interfere with species of interest in a Raman spectrum, this technique is an advantageous tool compared to its counterpart, infrared spectroscopy, for samples in aqueous solution. Herein, we report the use of silica coated porous gold nanowires for the detection of a small molecule, 4-mercaptobenzoic acid (MBA), in a biologically relevant protein solution.
Nanostructured gold and silver have been shown to provide shape- and size-dependent enhancement of the Raman signal. Aggregated nanoparticles have been shown to dramatically enhance Raman signal, but only one in every 100-1000 particles demonstrate optical hot spots, thus making reproducibility a problem. In the presence of high concentrations of proteins, e.g., serum concentrations, 1 mM, the adsorption of the proteins onto the metal surface may also prevent the detection of small molecules, e.g., metabolites, drug molecules, from being detected. It is therefore desirable to design SERS substrates that will produce good enhancement of the inherently weak Raman signal, while controlling the surface chemistry of the substrates.

Nanowire materials are beneficial for use as sensing platforms because their size and composition can be readily controlled. Mirkin’s group introduced the technique of on-wire lithography (OWL), which used template-assisted electrodeposition to synthesize striped metal nanowires. These nanowires were then drop-cast onto a surface and a thin (50 nm) layer of SiO$_2$ was deposited on the surface through plasma-enhanced chemical vapor deposition. After removal from the surface, the sacrificial metal could be selectively etched, leaving behind well controlled nanoparticle assemblies, held in place by the silica support. Qin et al. were able to quantitatively show the optimal spacing between gold nanoparticle dimers and between the sets of particles to produce the greatest signal enhancement. Although OWL has been shown to be a very useful technique in the field of Raman spectroscopy, the silica support being present on only one side of the nanowires is a limiting factor, as it does not prevent solution-phase molecules from adsorbing to the surface of the gold particles. By coating the entire surface of the nanowires with silica, adsorption of larger molecules onto the surface of
the gold can be prevented. This can be accomplished by coating nanowires in solution with silica by the hydrolysis and condensation of tetraethoxysilane (TEOS).\textsuperscript{20,21}

Control over the surface chemistry of nanoparticles, in general, is important for the design and function of sensing platforms. This was demonstrated using roughened electrodes functionalized with positively charged thiol groups for the detection of anions using SERS, with selectively of anion based on the structure of the thiol molecule.\textsuperscript{22} Van Duyne has demonstrated that self-assembled monolayers (SAMs) on SERS substrates limit fouling and improve signal by aiding in the partitioning of a small molecule, e.g., glucose, and localizing it near the metal surface for enhancement.\textsuperscript{11,23-27} By functionalizing a SERS substrate with an alkanethiolate tri(ethylene glycol) monolayer, the protein resistant nature of ethylene glycols enabled detection of glucose even after exposure of the substrates to bovine serum albumin (BSA).\textsuperscript{24} Likewise, environmental pollutants, e.g., polycyclic aromatic hydrocarbons\textsuperscript{28} and polychlorinated biphenyls,\textsuperscript{29} were detected by partitioning into a 1-decanethiol monolayer on a SERS substrate. Rather than a self-assembled monolayer, compounds that can adsorb to both a metal surface and the analyte of interest have been used to detect molecular species that do not display affinity for metals. For example, a viologen compound, which is known to bind strongly to a metal surface, was used to functionalize Ag nanoparticles to complex with polycyclic aromatic hydrocarbons for detection.\textsuperscript{30,31} The direct modification of the metallic, nanostructured surface did produce complications of the Raman spectra, as signal from the SAM was observed,\textsuperscript{10,23} which could lead to difficulties in identification of an unknown molecule.
Silver nanoparticles functionalized with thiolated SAMs were stabilized for transfer into buffer solutions using the layer-by-layer deposition of polyelectrolytes. These complexes can then be transferred into 2-propanol and silanized, rendering stable particles that are biocompatible with consistent surface chemistry that can be functionalized with well-understood chemistry. Likewise, SERS tags have been made by attaching Raman active molecules onto single Au or Ag nanoparticles, Au/Ag shell particles, aggregated Au particles, or clusters of Ag nanoparticles reduced onto the surface of a non-metallic particle with subsequent glass coating. The addition of a silica coating prevents the unwanted adsorption of solution molecules. It is thus desirable to have the beneficial aspects of silica incorporated into a SERS substrate for the label-free detection and identification of small molecules in solution.

We report a simple route to produce porous Au nanowires for use as SERS substrates by the synthesis of Au/Ag alloy wires, which are coated with silica in solution, and then etched to remove silver, leaving behind a nanostructured gold wire encased in silica (Figure 3-1). Searson et al. previously reported the synthesis of Au/Ag alloy nanowires through the electrodeposition of a single plating solution containing both Au and Ag that could then be etched to produce porous nanowires. The resultant porous Au nanowires have a surface area that is approximately 10× greater (6.9 m²/g) than a solid gold nanowire (~0.6-0.7 m²/g). The porosity of these nanowires is on the nanoscale, and can be controlled by the concentrations of each component in the plating solution. Lee et al. has previously shown that porous gold nanowires prepared similarly can be used as SERS substrates with enhanced signal compared to the unetched wires due to the increased surface area.
In this chapter, we describe the use of porous gold nanowires as SERS substrates that were coated with silica to provide structural strength and prevent breakage. The addition of a silica coating produced a controlled, biocompatible surface to the nanowires, without interfering with the Raman signal. The nanowires were also coated with a polyethylene oxide-containing silica, which prevented proteins from adsorbing to the outside of the nanowire surface, blocking the entry sites to the nanostructured gold, which could potentially cause protein aggregation in vivo. Additionally, it was demonstrated that the porous nanowires were encoded by incorporating different striping
patterns, similar to what has been done with noble metal nanowires,\textsuperscript{44} for multiplexing capabilities. These nanowires were encoded by plating segments of Ag or Au, in addition to Au/Ag alloy; after the nanowires were coated with silica, the Ag was etched to leave behind regions of porous Au, solid Au, and empty regions.

3.2 Experimental Materials and Methods

3.2.1 Materials

Silver potassium cyanide, 99.9\% and gold(I) potassium cyanide, 99.99\% were obtained from Alfa Aesar. Sodium carbonate used to pH adjust the plating solution was purchased from EM Science. Silver Cyless R RTU (3 troy oz/gallon) and Orotemp 24 RTU Rack (0.25 troy oz/quart) were acquired from Technic, Inc. Tetraethoxysilane (TEOS) and $N$-(triethoxysilylpropyl)-o-polyethyleneoxide urethane (PEO) were purchased from Gelest, Inc. 4-Mercaptobenzoic acid was obtained from Aldrich. Bovine Serum Albumin (BSA) (IgG-free, Protease-free) was purchased from Jackson ImmunoResearch Laboratories, Inc. and Aldrich. All water used was either 18.2 MΩ-cm Nanopure water from a Barnstead system or BDH Aristar Plus water (HPLC, Low TOC). All reagents were used as purchased without further purification; the silanes were opened and aliquoted out in a glove box under nitrogen gas to prevent hydrolysis from water vapor in the air.
3.2.2 Nanowire Synthesis

Au/Ag alloy nanowires were synthesized by electrodeposition into anodized aluminum oxide (AAO) Anodisc 25 membranes with 0.2 µm pores (E = -1.3V) on an in-house system using a plating solution of 100 mM KAg(CN)$_2$, 20 mM KAu(CN)$_2$, and 0.25 M Na$_2$CO$_3$, as adapted from reference 42. A cyclic voltammogram was taken to assure a high enough potential was used to electroplate both silver and gold (Figure 3-2). Silver (300 nm) was vapor deposited on one side of the membranes to act as the working electrode. Nanowires were electrodeposited in the pores of the membrane for 10 min, after which the silver backing was dissolved in 33% (v/v) nitric acid. Nanowires were then released into suspension by dissolving the membrane in 3 M NaOH to dissolve the alumina, and rinsed with 3 M NaOH, followed by DI H$_2$O, and three times with ethanol (EtOH) before suspension in 1 mL EtOH. One batch of nanowires produced $\sim 10^9$ nanowires, $\sim 5.7 \pm 1.5$ µm in length.

Encoded nanowires were synthesized as described above by alternating the plating of Au/Ag with the galvanostatic electrodeposition of silver and/or gold (I = -1.65 mA) using Silver Cyless or Orotemp 24 plating solutions, respectively. The length of each segment was controlled by the plating time.
3.2.3 Silica Coating

Nanowires were coated with TEOS silica\textsuperscript{20} or PEO:TEOS silica,\textsuperscript{21} as described in the literature. Briefly, 300 µL alloy nanowires (batch concentration, $1 \times 10^9$ wires/mL) were TEOS coated by combining 490 µL ethanol (EtOH), 160 µL DI H$_2$O, 40 µL TEOS, and 10 µL NH$_4$OH (14.8 M) in a microcentrifuge tube. After one hour of sonication, the coated nanowires were rinsed three times with ~1 mL EtOH by centrifugation (2000 g). The nanowires were coated with a thin TEOS coating by sonication for 12 min. The procedure was adjusted to coat nanowires with PEO:TEOS silica by combining 300 µL alloy nanowires (batch concentration), 490 µL acetonitrile (ACN), 160 µL H$_2$O, 30 µL

![Cyclic voltammogram of aqueous solution containing 100 mM KAg(CN)$_2$, 20 mM KAu(CN)$_2$, and 0.25 M Na$_2$CO$_3$ versus a Ag/AgCl reference electrode, obtained using a 2.01 cm$^2$ area silver working electrode at a potential scan rate of 20 mV/sec.](image-url)
TEOS, 24.7 µL PEO, and 10 µL NH₄OH (14.8 M) in a microcentrifuge tube, coating 3× by sonication for 1 hr with rinsing by centrifugation 3× ACN between each coating.

The alloy wires were etched to form porous Au wires by first rinsing the wires into water, and then resuspending in ~ 500 µL 33% HNO₃. The nanowires were spun to prevent them from settling in the tube for 0.75-1 hr. The alloy wires turned black in color upon etching. The etched wires were rinsed 2× H₂O and 3× EtOH by centrifugation.

3.2.4 Characterization

Transmission electron microscopy images were obtained using a JEOL JEM 1200 EXII instrument with a high resolution Tietz F224 digital camera at an accelerating voltage of 80 kV. Ultraviolet-visible spectra of the alloy nanowires before and after etching were obtained of 5 µL porous Au nanowires in 195 µL EtOH and 1 µL alloy nanowires in 199 µL EtOH using a Hewlett-Packard 8453 diode-array UV/visible spectrometer with Agilent ChemStation software (different concentrations used due to loss during handling). Optical images of the nanowires were obtained using a Nikon TE-300 inverted microscope equipped with a Photometrics CoolSnap HQ camera, a Plan fluor 60× oil objective (N.A. 1.4), and Image-Pro Plus (v 7.0) software. The source for reflected excitation was a 300 W Xe lamp.
3.2.5 Raman Spectra

Raman spectra of the nanowires were collected after vortexing 10 µL nanowires (~1×10⁷ nanowires) in a 5 mM MBA solution in 10 mM phosphate buffer, pH 7.4, 150 mM KCl in a non-stick microcentrifuge tube for 1 hr. To demonstrate the use of these wires for detection of small molecules in biological solutions, a set of the wires were first vortexed in a 1 mM BSA solution for 1 hr, followed by rinsing of the wires 3× with buffer by centrifugation. After 1 hr exposure to the MBA solution, the wires were rinsed 3× with buffer and 3× H₂O. The wires were resuspended in 200 µL H₂O, and a 50 µL aliquot of each sample was dried onto a microscope slide. The etched samples were dried onto TEM finder grids to correlate each nanowire to the individual Raman spectrum. To confirm the BSA was not desorbing from the surface of the silica coated wires when rinsed into the MBA solution, 12.5 µL TEOS coated porous Au nanowires were rinsed 3× buffer and then resuspended in a solution composed of 20 mM MBA and 1 mM BSA in 10 mM phosphate buffer, 150 mM KCl, pH adjusted to 9.5 to facilitate MBA dissolution. A control sample was resuspended in 20 mM MBA solution in the same buffer. After vortexing overnight at 4°C, the samples were rinsed 1× buffer, 3× H₂O. Samples were resuspended in 200 µL H₂O, and 20 µL of each sample was dried on a glass microscope slide for analysis on the Raman microscope.

Raman spectra were collected using a Renishaw Invia MicroRaman Spectrometer using 633 nm light from a HeNe laser source. A 100× air objective (N.A. = 0.9) was used to focus the laser light directly onto a single nanowire, with a spot size of ~430 nm. The laser intensity was set to ~50 µW, with an exposure time of 100 seconds. The
samples were scanned from 800-1800 cm\(^{-1}\). For each sample, >30 spectra were collected on separate, individual nanowires for quantitative work. Data was collected using Wires 2.0 Renishaw Raman software.

### 3.3 Results and Discussion

#### 3.3.1 Production and Characterization of SERS Substrates

Alloy nanowires were coated with TEOS, as previously described\(^{20}\), and the sacrificial silver was etched in nitric acid. The extinction spectra of micrometer sized metallic nanowires show significant scattering and reflectance\(^{20}\). Figure 3-3 shows the optical characterization of the alloy nanowires and TEOS coated porous gold nanowires. The alloy nanowires showed absorbance of the Ag at ~460 nm, which disappeared upon etching. A prominent peak at ~540 nm appears upon etching TEOS coated nanowires, similar to what has been seen in the literature\(^{45}\).
TEM analysis of the nanowires prior to etching and after etching can be seen in Figure 3-4. Figure 3-4 A shows the surfaces of the alloy wires pre- and post- etching without the silica coating present. The nanowires were coated with silica through the hydrolysis and condensation of TEOS on the surface of the nanowires,\textsuperscript{21} as seen in Figure 3-4 B,C. Significant differences in the nanostructure of the porous gold nanowires were not observed from wire to wire; the nanowires have ~30-40 nm Au ligaments with ~10 nm spacing between\textsuperscript{42,43} (Figure 3-5).

Figure 3-3: Extinction plot of the (grey) Au/Ag alloy nanowires and (black) TEOS coated porous Au wires.
Figure 3-4: TEM images of (left) alloy nanowires and (right) porous, gold nanowires that are (A) uncoated or coated with (B) 37 ± 2 nm TEOS or (C) 24 ± 2 nm TEOS. Scale bar = 200 nm
In addition to controlling the surface properties of the porous nanowires, the silica shell provides structural support. Figure 3-6 demonstrates the brittleness of the uncoated, etched nanowires; handling the wires, e.g., rinsing, sonicating, centrifugation, caused breakage and heterogeneity in length. By silica coating the nanowires prior to etching, the nanowires are significantly less likely to break and/or aggregate upon etching, in addition to providing control over the surface chemistry of the nanowires, in comparison to the etched, uncoated wires (Figure 3-5). Liu and Searson have noted that the resultant porous nanowires are brittle upon etching, and have circumvented problems with breakage by etching the nanowires while still within the alumina template\textsuperscript{46} and/or by annealing the nanowires at elevated temperatures.\textsuperscript{42} Annealing (>50°C) does decrease
the surface area by 71% due to coarsening of the nanowires, which would be detrimental to for use as SERS substrates.\textsuperscript{42}

3.3.3 Raman Spectroscopy with TEOS Coated Nanowires

Raman spectra were collected using a Raman microscope, which allowed individual nanowires to be analyzed. Inconsistencies in signal were observed from wire to wire using TEOS coated wires with a shell thickness of 37 nm. Therefore, the 25 nm TEOS coated nanowires were analyzed. Figure 3-8 A shows the Raman spectra of MBA

Figure 3-6: Low magnification TEM images of uncoated, etched nanowires showing brittle qualities of the nanoparticles and aggregation upon etching. The higher magnification image demonstrates how the nanowires crack upon handling. Scale bars = 1 \( \mu \text{m} \) (low magnification images), 200 nm (higher magnification image on right)
obtained by irradiating nanowires that were exposed to a 5mM MBA solution. No signal was observed for the TEOS coated alloy nanowires. A \(16.5 \times\) increase in signal based on the area under the peak at 1589 cm\(^{-1}\) is observed when using the coated porous Au nanowires (Figure 3-7) compared to the uncoated alloy wires (Figure 3-8) due to the enhancement of the Raman signal, primarily due to the \(10 \times\) increase in the surface area upon etching. On average, the signal intensity of the coated, etched samples were higher than the uncoated counterparts, most likely due to breakage of the uncoated, etched nanowires resulting in smaller areas being analyzed by the Raman microscope (spot size = 430 nm).

The Raman spectra are dominated by peaks at 1078 and 1589 cm\(^{-1}\), which correspond to the \(\nu_{12}\) and \(\nu_{8a}\) ring breathing modes, respectively.\(^{47,48}\) The \(\delta(C=H)\) mode was observed at 1184 cm\(^{-1}\).\(^{49}\) Peaks at 844, 1422, 1710 cm\(^{-1}\) correspond to the \(\delta(COO^-)\), \(\nu_s(COO^-)\), and \(\nu(C=O)\), respectively.\(^{47}\) This agrees with analysis of MBA monolayers in the literature, which have reported a mixture of protonated and unprotonated forms of MBA.\(^{50}\) In further experiments, the nanowires were initially exposed to a 1 mM BSA buffered solution, followed by a 1 mM MBA solution to challenge the detection of the small molecule with a protein (Figure 3-7 B). Significant differences in intensity were not observed, indicating that the small molecule could be detected even after the nanowires were vortexed in a solution containing a biologically relevant protein concentration.
Figure 3-7: Raman spectra of TEOS coated porous gold nanowires, (black) etched and (grey) unetched, exposed to (A) 5 mM MBA in buffer and (B) 1 mM BSA in buffer followed by 5 mM MBA in buffer, and the TEM images of the etched nanowire SERS substrates from which the Raman spectra were collected. Scale bars = (left) 1 µm, (right) 200 nm
To confirm the BSA is not desorbing from the surface of the silica coated nanowires upon resuspension in the MBA solution not containing BSA, TEOS coated porous Au nanowires were exposed to a solution of 20 mM MBA in a 1 mM BSA in buffer (Figure 3-9 A). Signal was still observed for the MBA, as evident by the peaks at 1078 and 1589 cm\(^{-1}\). Signal was also observed for the control samples exposed to only MBA (Figure 3-9 B), demonstrating diffusion of the MBA onto the surface of the porous Au regardless of if BSA was in solution.\(^{51}\)

Figure 3-8: Uncoated etched (black) and unetched (grey) nanowires exposed to (A) 5 mM MBA in buffer and (B) 1 mM BSA and then 5 mM MBA in buffer.
3.3.4 PEO-Modified Silica Coated Porous Au Nanowires

We have previously shown that the surface chemistry of nanowires can be controlled using organically modified silica coatings.\textsuperscript{21} Incorporation of organically modified silicon alkoxides into bulk sol-gel materials have been shown to change the surface properties and porosity of the bulk materials.\textsuperscript{52,53} Alloy nanowires were coated with PEO modified silica (Figure 3-10 A), followed by etching away the Ag in nitric acid to leave porous Au encased in a PEO silica shell (Figure 3-10 B). The alloy nanowires were coated 3× with the PEO silica, resulting in a 24 ± 2 nm coating, which is of similar thickness to the TEOS coated wires for comparison. A decreased number of nanowires with signal was observed for the thicker TEOS coated nanowires, demonstrating decreased diffusion of the MBA through the silica to the Au surface. Thus it is important

Figure 3-9: TEOS coated porous Au nanowires exposed to (A) 20 mM MBA and 1 mM BSA simultaneously and (B) 20 mM MBA in buffer.
to compare the TEOS and PEO coated nanowires with similar coating thicknesses. Coating the nanowires with PEO also produced structurally stable nanowires upon etching, with minimal breakage of the brittle nanowires (Figure 3-11). Additionally, the optical properties of these wires remain the same, as compared to the TEOS coated porous gold nanowires, with a peak at ~540 nm (Figure 3-12).

![TEM images of PEO coated (A) alloy nanowire and (B) porous Au nanowire. Scale bar = 200 nm](image)

Figure 3-10: TEM images of PEO coated (A) alloy nanowire and (B) porous Au nanowire. Scale bar = 200 nm
Figure 3-11: Low magnification TEM images of PEO coated nanowires with corresponding higher magnification TEM images of the nanowires on the right. Scale bars = 1 µm (left) and 200 nm (right).

Figure 3-12: Extinction spectrum of PEO coated porous Au nanowires in solution.
Similar SERS results were observed for the PEO coated nanowires compared to the TEOS coated porous Au (Figure 3-13 A). No significant change is observed in the signal when the nanowires are first exposed to 1 mM BSA (Figure 3-13 B), indicating that these nanowires could be used as SERS substrates to study biological samples. Although TEOS coated nanowires also showed signal after exposure to BSA solutions, biofouling of the surfaces can occur over time, as proteins are known to adsorb to surfaces such as silica.\textsuperscript{54,55} The buildup of proteins on the surface of the nanowires would decrease the amount of analyte that could diffuse to the porous gold nanowire surface and be detected. Coating porous Au nanowires with PEO modified silica is a viable way to protect the surface without requiring a post-synthesis functionalization step, and would provide protection against an immune response in whole blood.\textsuperscript{56}
3.3.5 Statistical Analysis of Raman Data

When the 25 nm TEOS or PEO coated nanowires were compared to the 37 nm TEOS coated nanowires, a significant increase in the number of substrates with a detectable signal (S/N>3) was observed, presumably due to improved diffusion of the
MBA through the SiO$_2$ (Table 3-1). For each type of sample over 30 nanowires were analyzed. Exposure of the nanowires to BSA did not cause a significant decrease in the signal with the PEO coated nanowires, demonstrating their use for the detection of the MBA in a biologically relevant solution.

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>37 nm TEOS</th>
<th>25 nm TEOS</th>
<th>24 nm PEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM MBA</td>
<td>38%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>1 mM BSA and then 5 mM MBA</td>
<td>48%</td>
<td>97%</td>
<td>98%</td>
</tr>
</tbody>
</table>

Table 3-1: Percentage of silica coated nanowires with detectable Raman signal.$^i$

$^i$ Measured for $>$30 nanowires per sample.

Differences in intensity between nanowires from the same batch, analyzed under identical conditions, were observed (Figure 3-14). This is in most part due to instrumental resolution limitations in focusing the laser directly on a nanowire (spot size of the laser, 430 nm, similar to diameter of the nanowires, 320 nm), but may also be due to slight differences in the nanostructure of the nanowires, as observed in the TEM images. Although differences in intensity were observed for the etched nanowires, all of the porous Au nanowires demonstrated significantly higher signal than the unetched alloy nanowires. The peaks at 1000 and 1022 cm$^{-1}$ (Figure 3-14) have been observed previously to appear over time for MBA; this has been attributed to a surface reaction that leads to monosubstituted benzene derivatives.$^{47}$
Figure 3-14: Further examples of (A) Raman spectra and (B) the corresponding TEM images of the PEO coated porous gold nanowires exposed to 1 mM BSA in buffer and then 5 mM MBA in buffer. Debris observed in the TEM images is due to the BSA present in solution. Scale bars = (left) 1 µm, (right) 200 nm
3.3.6 Encoded Substrates

To demonstrate the possibility of multiplexed sensing using porous gold nanowires as SERS substrates, different striping patterns were deposited, in addition to the Au/Ag alloy. This produced encoded nanowires that, once silica coated and etched, contain segments of porous gold, solid Au, and/or hollow regions. Figure 3-15 shows TEM images of TEOS coated and etched wires that can be used as encoded nanowires prepared from (A) Au/Ag alloy nanowires, (B) Au-Ag-Au-Ag-Au/Ag alloy nanowires, and (C) Au-Ag-Au/Ag alloy nanowires. Upon etching away the sacrificial segments with nitric acid, gold stripes and porous Au segments remain, as evident in the TEM images. Movement of these segments within the silica shell was not observed. Other barcode designs with smaller segments have had this issue, which has been solved by the initial functionalization of the wires with 3-mercaptopropyltriethoxysilane to produce vitreophilic surfaces prior to coating with the TEOS to minimize this shifting.20 The encoded wires were mixed and imaged under white light on an optical microscope, as well, with the Au stripes being more reflective than the porous Au segments, and the SiO$_2$ tube segments being non-reflective (Figure 3-16). The nanowires were imaged using white light compared to the typical blue light at 430 nm since the porous nature of the nanowires makes the nanowires less reflective (Figure 3-17). This demonstrates the potential ability to use striped alloy nanowires for multiplexed Raman assays using a MicroRaman instrument. Encoded SERS substrates can be used to image mixed nanowires that have been exposed to different target solutions for multiplexed assays. Additionally, these nanowires could be used as tags, similar to glass-coated, analyze-
tagged spherical particles,\textsuperscript{34-36} by introducing a Raman active molecule to different populations of encoded nanowires and then functionalizing the silica coating with biomolecules, e.g., antibodies, for localized identification of molecular species on a surface. This would provide collective encoding by the physical pattern of the nanowire and the spectroscopic analysis of the pre-adsorbed analyte, similar to Mirkin’s “Nanodisk Codes”\textsuperscript{3}. 
Figure 3-15: TEM and optical images of silica-coated, encoded, porous nanowires. SiO$_2$-coated (A) porous gold nanowire, (B) Au/gap/Au/gap/porous gold nanowire, and (C) Au/gap/porous gold nanowire. Left images show entire length of nanowire (Scale bar = 1 µm) and right images show a high magnification image of wire shown on the left (Scale bar = 200 nm).
Figure 3-16: Optical image under white light of the three different encoded nanowires (Scale bar = 5 µm) with associated line scans.
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3.4 Conclusion

We have demonstrated that silica coated Au/Ag nanowires can be etched to produce porous Au nanowires encased in a silica sheath, which prevents breakage of the brittle nanowires. These porous nanowires provided enhancement of the Raman signal to detect a small molecule, 4-mercaptopentanoic acid. The addition of a polyethylene oxide functionality into the silica coating provided a protein-resistant surface on the nanowire, which enabled the detection of the MBA in solution containing serum protein and biologically relevant salt concentrations. As a proof-of-concept, encoded nanowires containing a porous Au segment were also synthesized and imaged using TEM and

Figure 3-17: Reflectance images of alloy nanowires and porous Au nanowires illuminated under white light and 430 nm. Contrast of the white light images adjusted the same; contrast of the 430 nm image set adjusted 2× that of the white light images for visual clarity. Scale bar = 5 µm
optical microscopy to show the possibilities of multiplexing. Additional metals, e.g., Ni, could be incorporated to add in a magnetic component, or to increase the number of different striping patterns; addition of a third metal into a 6.5 µm wire with 500 nm stripes increases the number of possible barcodes from 4160 to over $10^5$.44

By optimizing the synthetic methods to produce the alloy nanowires, improvements in the reproducibility of the intensity of the signal may be obtained. In the future, changes in the coating properties could enable a higher concentration of analyte to diffuse through to the surface of the Au by decreasing the thickness or by increasing the porosity of the silica. The thickness of the coating is controlled by reaction time and/or the concentration of silicon alkoxides as compared to the available surface area of nanowires. Increased porosity of the coatings could be accomplished by incorporating in a bridged silicon alkoxide, which have been shown to increase porosity in the bulk.53 Additionally, by adjusting the silicon alkoxides used, permselective silica coatings could be synthesized for the detection of different molecules based on, e.g., size or charge. By controlling the amount/type of analyte to be detected, and with the protein resistant properties of the PEO modified silica coating, detection of small molecules, e.g., metabolic products, could be accomplished in biologically relevant samples, such as serum.
3.5 References


51. Swelling of the TEOS coating was observed during this experiment, possibly leading to an increased porosity, but this was not controllable. The two samples analyzed here were from the same batch of coated nanowires, and thus can be directly compared. Swelling of the silica was not observed when the samples were prepared fresh and vortexed for only a few hours.


Chapter 4

Synthesis and Characterization of Enzyme—Au Bioconjugates: HRP and Fluorescein-Labeled HRP

“The activity assays and surface coverage work presented in this chapter, along with the writing of the manuscript, was done in collaboration with Dr. Ann-Sofie Cans of the Keating group. Dr. Cans prepared conjugate samples and collected fluorescence data for analysis. The author of this dissertation was responsible for the colloid synthesis, zeta potential measurements, flocculation assays, and working alongside Dr. Cans on the preparation of the samples and UV-Vis data collection for the activity assays and surface coverage measurements. Writing of the manuscript was done in collaboration with all authors presented.

4.1 Introduction

Enzymes conjugated with Au or other nanoparticulate materials are increasingly important in bioanalysis and biocatalysis. Characterization of both the bioconjugation process and the properties of the resulting enzyme:Au assemblies is critical for the maturation of this field. The simplest method for enzyme assembly onto Au nanoparticles is direct adsorption, where an enzyme solution typically at low ionic strength is mixed with Au nanoparticles and allowed to react for some time, followed by centrifugation and resuspension to remove unbound enzyme. This approach has been used, for example, by Sastry and coworkers for pepsin, fungal protease, and endoglucanase Au bioconjugates, and by ourselves and others for horseradish peroxidase (HRP) Au nanoparticle conjugates. Although direct adsorption is simple,
some loss of enzyme activity is often observed.\textsuperscript{13,14} More elaborate routes to enzyme:Au conjugation have been reported, which should lead to greater retention of enzyme activity. Schiffrin and coworkers functionalized Au nanoparticles with nitrilotriacetic acid–Co(II), which then bound histidine-tagged enzymes; excellent retention of activity was observed for HRP:Au and ferredoxin-NADP+ reductase:Au bioconjugates.\textsuperscript{15} This method is appealing for its excellent retention of activity and applicability to any histagged enzyme, however enzymes to be conjugated must be genetically engineered for expression with histidine tags. “Click” chemistry has also been used to immobilize lipase onto azide-modified Au nanospheres via reaction with the enzyme’s free amine residues, e.g., lysines.\textsuperscript{16}

Regardless of the method of enzyme coupling to the nanoparticle surface, characterization of the resulting conjugates with respect to the number of enzyme molecules bound and the extent to which they retain their bioactivity is crucial. This is because, although the high curvature surfaces of nanospheres are more biocompatible than planar surfaces,\textsuperscript{17,18} enzyme function is often compromised by surface attachment. Reduced bioactivity can result from denaturation and/or physical blockage of the active site by the particle surface or adjacent enzymes. Packing on the surface can influence enzyme orientation,\textsuperscript{19} conformation,\textsuperscript{20,21} and consequently activity; thus, surface density is an important variable in enzyme bioconjugation. Protein coverage on nanoparticle surfaces can be difficult to determine directly, and is often estimated based on protection of the particles from salt-induced aggregation, or by quantifying the unbound protein remaining in a supernatant after pelleting the protein:Au bioconjugates. Direct
determination of protein: Au nanoparticle stoichiometry requires labeling of the protein, which may alter adsorption behavior.

In this manuscript, we report investigations of HRP and fluorescein-labeled HRP (F-HRP) assembly on 13-nm Au nanospheres as a function of solution pH. The assembly process is characterized by resistance of protein-coated Au to salt induced flocculation and by in-situ zeta potential measurements. The F-HRP: Au nanoparticle binding stoichiometry is determined via fluorescence after dissolution of the Au nanoparticles to avoid quenching by the metal particles. Our results indicate HRP multilayer formation on the Au nanoparticles, and suggest that low surface density HRP molecules have higher specific activity than high surface density molecules. Enzymatic activity per conjugate is essentially constant over a large range of HRP: Au ratios, due to the lower specific activity of HRP at high surface densities.

4.2 Experimental Materials and methods

4.2.1 Materials

Gold (III) chloride hydrate, fluorescein 5(6)-isothiocyanate (FITC), peroxidase type VI from horseradish (HRP), dimethyl sulfoxide, SigmaFast o-phenylenediamine dihydrochloride tablets, as well as buffers, salts, and general chemicals were purchased from Sigma-Aldrich (St Louis, MO). Centri-spin-20 size exclusion columns were purchased from Princeton Separations (Adelphia, NJ). BCA protein assay kit was purchased from Pierce Biotechnology Inc (Rockford, IL). Deionized water with a
resistivity of $\geq 18.2$ M$\Omega$ from a Barnstead NANOpure Diamond water purification system (Van Nuys, CA) was used in all experiments.

### 4.2.2 Au nanoparticles

Colloidal Au nanoparticle suspensions were prepared by the citrate reduction of HAuCl$_4$; the experimental procedure has been described elsewhere.$^{22-26}$ The Au nanoparticles were $13 \pm 1$ nm in diameter, as analyzed using a JEOL JEM 1200 EXII transmission electron microscope (TEM). A total of 8869 particles were measured using Image J software to determine the average particle size. The concentration of the colloid solution was determined to be 18.2 nM, based on the amount of Au used for synthesis and the measured size of the resulting nanoparticles. The absorbance at 520 nm was found using a Hewlett-Packard 8453 diode-array UV/visible spectrometer with Agilent ChemStation software.

### 4.2.3 F-HRP labeling

A protocol from Molecular Probes was used to prepare fluorescein-labeled HRP (F-HRP). Briefly, 100 µL of freshly made FITC solution (0.1 mg/mL) in DMSO was added to a solution of 16.5 mg/mL HRP dissolved in freshly made 0.1 M carbonate buffer pH = 9.0. The reaction mixture was incubated while stirring for 1 h at room temperature. The conjugated F-HRP was purified from unreacted FITC by size exclusion chromatography using centri-spin 20 columns and labeled protein was eluted in 25 mM
phosphate buffer pH = 7.4. Aliquots of the purified F-HRP solution were rapidly frozen in liquid nitrogen and were lyophilized for storage until use. The F-HRP concentration was determined by measuring the absorbance of the heme group at 403 nm and compared to a standard curve of HRP using the extinction coefficient $\varepsilon = 100 \text{ mM}^{-1} \text{ cm}^{-1}$. The degree of labeling of the protein was 1.4 dyes per HRP as determined from fluorescein absorbance ($\varepsilon_{\text{FITC, 494 nm}} = 68 000 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 8.0).

### 4.2.4 Flocculation assay

Flocculation assays were performed to determine the amount of HRP or F-HRP necessary to prevent Au nanoparticle aggregation when subjected to a high ionic strength media. The buffers used for conjugation experiments were: 5 mM acetate pH = 4.0, 5 mM citrate pH = 6.0, 1 mM Tris pH = 8.0 and 5 mM carbonate pH = 10.0. Buffer concentrations were kept low enough that bare Au-nanoparticles did not spontaneously aggregate in the buffers. Samples with mole ratios of HRP versus Au-nanoparticles ranged from 5:1 to 400:1 and were prepared by first dissolving HRP in the buffer used to study the conjugation. The conjugation process was initiated by the addition of $2.5 \times 10^{-12}$ mol Au nanoparticles in a total volume of 500 µL, and followed by incubation for 20 min at room temperature. 180 µL of each of the conjugate solutions was then added to 20 µL of 1.5 M NaCl solution, to induce aggregation of unprotected nanoparticles. After 1 h incubation, samples were analyzed by absorbance spectroscopy using a Hewlett-Packard 8453 diode-array UV/visible spectrometer with Agilent ChemStation software.
4.2.5 HRP conjugation and purification

The amount of HRP or F-HRP needed in solution at conjugation to form a stable conjugate was determined based on the flocculation results. To make sure HRP was present in excess to fully cover the Au-colloid surface, a mole ratio higher than that needed to prevent aggregation was used during the conjugation process. HRP was first dissolved in buffer and the conjugation was initiated by addition of $1.0 \times 10^{-11}$ mol of 13-nm Au in a total volume of 1 mL, followed by 20-min incubation at room temperature. HRP:Au conjugates were then purified by centrifugation at 10 000 g for 40 min using a Biofuge Pico centrifuge from Kendro Laboratory Products. The supernatant, containing unbound F-HRP, was removed and the pellet was re-dissolved in 5 mM Tris buffer pH = 8.0. The purification was repeated twice and the pellet was re-suspended in 200 µL of 5 mM Tris buffer pH = 8.0.

In a separate experiment, we tested the effect of excess bovine serum albumin, BSA, added after conjugation but before purification of the HRP:Au conjugates. Here, 200 µL 0.15 mM BSA was added, to give a final concentration of 25 mM, after the HRP conjugation to the gold particles. The conjugates were incubated for 20 min before purification as described above.

4.2.6 Zeta Potential

Zeta potential of the conjugates was done using a ZetaPALS Zeta Potential Analyzer from Brookhaven Instruments Corporation. The 13-nm Au particles used here scatter light very weakly, and therefore, alterations to the standard instrumental
parameters were necessary to increase the sample count rate. The reference beam was manually adjusted to produce a reference count rate between 30-140 kcps prior to each sample set by setting the collimator appropriately. Voltage was set to 10 V to produce an electric field of ~26-28 V/cm. The temperature was maintained at 25°C.

The zeta potential of the particles was analyzed by placing 528 µL (7.5 x 10^{12} mol) of Au nanoparticle suspension into a buffered solution of protein. The molar amount of protein compared to Au was varied for each sample. The suspension was sonicated for 1 min prior to preparation of each sample. Samples were then allowed to equilibrate for 15 min before analysis. The zeta potentials reported are averaged values from between two and six identical samples for each ratio of protein to particle. The zeta potential was taken after 25 scans for 5 runs per sample, and the mean zeta potential calculated based on the multiple measurements for identical samples. The zeta potential for “bare”, citrate-reduced Au nanoparticles in water was -12± 2 mV.

### 4.2.7 Quantification of enzyme:particle stoichiometry

To determine the number of enzyme molecules adsorbed per Au nanoparticle, F-HRP was first adsorbed to Au nanoparticles in the buffer used at conjugation, and conjugates were purified from unbound enzyme as described above. To avoid interference from fluorescence quenching, scattering, and absorbance due to the metallic Au, we then dissolved the Au nanoparticles as reported previously for DNA:Au bioconjugates by Demers et al.\textsuperscript{28,29} KCN was added to a concentration of 16 mM (480,000 CN\textsuperscript{−}:1 Au mole ratio), and samples were stored in the dark until Au dissolution
was complete and samples were colorless (approximately 10-20 minutes). The concentration of F-HRP was then determined by fluorimetry using a Fluorolog 3 Horiba Jobin Yvon fluorimeter (Edison, NJ) measuring the emission at 518 nm with 492 nm excitation. The fluorescence was compared to a standard curve for F-HRP generated in a matrix of appropriate Au and cyanide concentration; background fluorescence was corrected by comparison to a sample containing no F-HRP. The quantified amount of F-HRP was correlated to the Au particle concentration in the conjugate samples as determined by visible absorbance spectroscopy.

We evaluated how effectively unbound F-HRP was removed from F-HRP:Au conjugates by the centrifuge purification process in order to determine whether any of our fluorescence signal arose from unbound protein. In this experiment, F-HRP was followed through all steps in the conjugation, washing and analysis protocols described above, except that Au nanoparticles were omitted (an equal volume of buffer was used in place of the Au nanoparticle suspension). After each centrifugation step, a few microliters of solution corresponding to the same size as a pellet was left in the bottom of the tube (no actual pellet was present in these control experiments due to the lack of Au nanoparticles). New buffer was added and the purification was repeated twice. Samples were resuspended in 200 µL 5 mM Tris buffer pH = 8.0. After purification and before the fluorescence analysis of the sample, Au nanoparticles were added and dissolved using KCN as described above, in order to accurately mimic assay conditions for HRP:Au conjugates. We found that F-HRP corresponding to only 0.3% of the added F-HRP persisted through the purification steps, which corresponded to < 0.1 molecule of F-HRP.
for each Au nanoparticle normally present in these experiment. Thus, free F-HRP does not contribute significantly to our reported F-HRP:Au ratios.

Quantification of non-labeled HRP was performed by centrifugation of conjugates at 16 000 g for 40 min using a Biofuge Pico centrifuge from Kendro Laboratory Products. 500 µL of the supernatant was collected and the concentration was determined using a Micro BCA protein assay kit. Subtraction analysis of initial amount added HRP vs amount HRP in supernatant was made to determine the number of HRP bound per conjugate.

4.2.8 Enzyme activity assay

A protocol from Sigma was used to study the enzyme activity of HRP bound to Au nanoparticles as compared to free enzyme in solution. SigmaFast OPD was used as the substrate. This consisted of one o-phenylenediamine dihydrochloride (OPD) tablet and one tablet of urea hydrogen peroxide that was dissolved in 20 mL of water immediately before use. The final concentration was 0.4 mg/mL OPD, 0.4 mg/mL urea hydrogen peroxide in 0.05 M phosphate-citrate buffer pH = 5. The conjugates were added to a microtitre plate by adding 50 µL of conjugate solution to 50 µL of water in serial dilutions with a final total volume of 100 µL. The reaction was started by the addition of 200 µL of substrate solution for a timed period. As the reduction of OPD proceeded to form the product 2,3-diaminophenazine (DAP) the samples turned orange in color. The reaction was stopped by the addition of 50 µL of 3 M HCl and the final reaction time recorded. Product formation was determined by measuring its absorbance
at 492 nm ($\varepsilon_{DAP} = 67,143 \, \text{M}^{-1} \text{cm}^{-1}$, determined from a standard curve of DAP). One unit of enzyme is the amount necessary to form 1 $\mu$mol of product in 1 min. The retained specific activity of enzyme adsorbed to the particles was compared to the enzyme activity of free enzyme that had been in solution for the same amount of time.

4.3 Results and Discussion

4.3.1 Enzyme adsorption to Au nanoparticles

Conventionally, protein:Au bioconjugate formation is characterized by flocculation analysis.$^{26,30}$ This approach is based on the sensitivity of the Au surface plasmon absorbance to the aggregation state: isolated nanoparticles are red, exhibiting a maximum absorbance at $\sim$520 nm, however aggregation leads to a redshift and broadening of absorbance features, turning the suspension purple or blueish.$^{30,31}$ Au nanoparticles are initially stabilized by electrostatic repulsion due to negative surface charge from adsorbed anions (Cl$^-$ and citrate for the Au particles used here);$^{26}$ addition of electrolyte to the initially low ionic strength suspension compresses the electrical double layer and leads to aggregation due to van der Waals attraction among the highly polarizable Au nanoparticles. This process can be prevented by adsorption of macromolecules such as proteins to the nanoparticle surface, which provides steric stabilization against flocculation. Thus, the minimum concentration of protein necessary to fully coat the surface of colloidal Au nanoparticles can be readily determined by addition of a salt such as NaCl to solutions of Au nanoparticles that have been incubated
with increasing concentrations of protein. The lowest concentration at which aggregation is prevented should correlate with monolayer formation on the particles; this concentration or a slight excess beyond it can then be used in future preparations of stable protein:Au nanoparticle conjugates. Flocculation analysis is simple to perform, and since the color change upon aggregation is readily apparent by eye, it is possible to perform this assay without any instrumentation.

Unfortunately the aggregation properties of colloidal Au suspensions can lead to some confusion in flocculation analysis. Both the shape and position of the absorbance feature(s) after aggregation depends on the properties of the aggregates (i.e. how many particles per aggregate, shape of aggregate, inclusion of proteins, etc.), and will vary from assay to assay in response to variables such as the identity of the protein used, the amount of time after NaCl has been added, etc. The fact that adsorption of molecules (e.g., proteins) to Au nanoparticles causes a slight redshift in the 520 nm absorbance due to the refractive index change even in the absence of any aggregation,32-34 can cause further uncertainty in determining the minimum protein concentration at which flocculation is prevented. Furthermore, if the protein molecules to be adsorbed are dissolved in a buffer solution as is common for biomolecules, the buffer alone may destabilize the Au nanoparticles by screening the interparticle electrostatic repulsions, which can greatly complicate experimental flocculation assay results.

Despite these complications, flocculation analysis is a common and useful means of evaluating protein adsorption to colloidal Au suspensions. We compared fluorescein-labeled and unlabeled HRP adsorption to 13-nm nanoparticles using this method. Care was taken in adjusting the pH: we tested 25, 5, and 1 mM buffer concentrations, and
found that addition of 25 mM buffer alone often led to immediate or eventual nanoparticle aggregation. In general, 5 mM buffers did not cause aggregation at pH 4.0, 6.0, or 10.0, and thus 5 mM was used for those experiments. At pH 8.0, 1 mM Tris buffer was used to avoid aggregation (which had still occurred at 5 mM with this buffer). Flocculation spectra for F–HRP and HRP at pH 8.0 are shown in Figure 4-1. In both cases, a large excess of enzyme over that predicted for monolayer coverage was necessary to prevent salt-induced aggregation. Expected monolayer coverage for HRP was determined based on the surface area of the 13-nm Au spheres and the dimensions of HRP (6.0 × 4.4 × 4.0 nm), which were estimated from the crystal structure (PDB ID: 1HCH).35 Thus, monolayer coverages in the range of 19 to 37 HRP per Au nanoparticle were anticipated, depending on whether HRP adsorbs via its long or short axis. Figure 1 shows that full protection from flocculation occurs at protein:Au ratios corresponding to much higher than monolayer coverage. These data can be interpreted as: a) HRP adsorption requires a relatively high solution HRP concentration (i.e. low $K_{ads}$), b) adsorbed HRP are readily displaced upon NaCl addition and/or c) nanoparticle flocculation may occur despite a full coating of HRP on the Au. Since intervening HRP molecules will decrease plasmon coupling between the Au particles, alternative a) and/or b) are most likely.
The shapes of the aggregate curves for HRP vs. F-HRP adsorption in Figure 4-1 are somewhat different; for example, the 50:1 HRP:Au ratio spectrum for HRP suggests a
greater degree of aggregation than the 50:1 spectrum for the fluorescein-labeled HRP. These differences may indicate weaker adsorption of the unlabeled as compared to the labeled HRP. In an effort to compare the F-HRP with HRP, we plotted flocculation results in terms of the absorbance at 520 nm vs. enzyme:Au ratio added. Results for pH 4.0, 8.0, and 10.0 are plotted in Figure 4-2 and summarized in Table 4-1. At pH 4.0, absorbance at 520 nm increases rapidly between 0 and 20 enzyme:Au for both labeled and unlabeled HRP. After this, the unlabeled enzyme increases at a slower rate and labeled HRP levels off; the two converge after ~200:1 enzyme:Au ratio. We report the stabilization ratio as 20:1 for both enzymes in Table 4-1 based on this initial–nearly complete–protection from flocculation. Additional protection does occur beyond this, and differs for the unlabeled vs. labeled HRP. These differences may indicate a somewhat lower adsorption affinity for the fluorescein-labeled HRP at this pH, and highlight the difficulties in selection of stabilization ratio from flocculation analysis.
Figure 4-2: Flocculation results plotted as the absorbance at 520 nm vs. enzyme:Au ratio added prior to addition of NaCl. Open symbols are the F-HRP and filled symbols are HRP. Insets show a magnified view of the low enzyme:Au ratio region. Each data point is the average from three separate flocculation experiments at that pH; error bars shown are the standard deviation in the measurements.
At pH 8.0, the flocculation data for F-HRP and HRP are quite different, with F-HRP absorbance at 520 nm saturating at ~50 per Au particle, and unlabeled HRP saturating at much higher enzyme concentrations. We also observed greater sample-to-sample variability for the HRP binding at pH 8.0, which is apparent in the larger standard deviations for this data set. Additional repetitions did not decrease the variability. These data suggest weaker binding of HRP vs. F-HRP at pH 8.0. At pH 10.0, flocculation results for F-HRP and HRP were almost identical, with both enzymes saturating by ~20 per Au particle. The flocculation results at pH 4 and pH 10 is suggestive of a high binding affinity and monolayer coverage, with the HRP molecules adsorbing mainly via their long axis rather than end-on.

In some cases electrostatic interactions appear to dominate protein adsorption to surfaces, for example where highly charged surfaces and proteins were used, and where proteins had regions of high charge density.\textsuperscript{36-40} However, for adsorption to Au nanoparticles, protein size has been shown to be in general a better predictor of binding affinity than isoelectric point, suggesting the importance of hydrophobic interactions.\textsuperscript{37} While the maximum number of protein molecules bound has been shown to increase at lower pH, this change is independent of protein pI.\textsuperscript{37} In the case of HRP vs. F-HRP, few

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<th>pH</th>
<th>HRP:Au from flocculation</th>
<th>HRP:Au from zeta potential</th>
<th>F-HRP:Au from flocculation</th>
<th>F-HRP:Au from zeta potential</th>
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<tr>
<td>4.0</td>
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differences between the proteins exist to account for the observed changes in adsorption behavior. Dye conjugation not only adds fluorescein, which may modulate adsorption via, e.g., hydrophobic interactions with the surface, but also changes the charge distribution on the protein. The HRP used here is a mix of isoforms with isoelectric points ranging from 5.5 to 9.0. Thus, at pH 4.0 the unlabeled protein has a positive overall charge. Labeling occurs at amine residues on the protein surface, such that for each dye molecule added one positive charge is removed. Fluorescein can exist in $-2$, $-1$, neutral, and $+1$ forms, with pKa values of 6.4, 5.0, and 2.1, respectively. Thus, at pH 4.0, when HRP has a net positive charge, F-HRP should be less positively charged, and at all other pH values tested F-HRP is more negatively charged than HRP.

The flocculation data in Figure 4-2 and Table 4-1 indicate that replacing on average one to two (positively charged) lysine or arginine residues with (negatively charged) fluorescein dye molecules does affect the surface adsorption behavior of these molecules onto colloidal Au nanoparticles. Differences between HRP and F-HRP are most significant at pH 8.0. The Au nanoparticles are negatively charged at all pH values investigated here. Thus, while higher affinity for unlabeled protein at pH 4.0 can be rationalized via electrostatics, the greater apparent affinity of labeled protein at pH 8.0 argues against a simple electrostatic interpretation, as F-HRP should be more negatively charged than HRP and thus repelled from the Au surface more effectively. Reduced stability of the protein in solutions of pH near the isoelectric point may contribute to the higher enzyme:Au ratios needed to prevent flocculation at pH 6.0 and 8.0. At pH = 8.0, the pH may be closer to the isoelectric point of unlabeled HRP than F-HRP.
To more fully understand the adsorption of F-HRP and HRP at the Au nanoparticle surface, we also performed zeta potential measurements as a function of enzyme concentration for several pH values. These measurements provide information on the surface potential of the particles and can indicate changes in surface charge upon biomolecule binding. Zeta potential measurements are simple to perform and report on changes in the surface of the nanoparticles in situ, and do not require addition of reagents such as NaCl to the sample, which might alter adsorption for weak-binding proteins. We interpret the HRP:Au mol ratio necessary to produce constant zeta potentials as indicative of the minimum amount of protein needed to coat the nanoparticle. Unfortunately, once near-monolayer coverage is achieved it is unlikely that binding of additional enzyme would alter the surface potential, and therefore any further binding would go undetected. Nonetheless, zeta potential measurements are a second means of probing the enzyme:Au binding interaction and should provide complementary information to flocculation analysis. We performed zeta potential measurements for F-HRP and HRP at several pH values; the results are summarized in Table 4-1. Figure 4-3 shows the effect of F-HRP adsorption on the surface potential of colloidal Au nanoparticles. The initial zeta potential for “bare” citrate-stabilized Au particles in all of the buffers is approximately –25 mV. Exposure to F-HRP leads to enzyme adsorption, eventually saturating at zeta potentials near neutral (~ –5 mV) for all of the samples except pH 10.0, which levels off around –10 mV. The more negative surface potential for the F-HRP:Au conjugates at this pH is presumably due to deprotonation of amino acid residues on the enzyme surface (cysteine, tyrosine, and lysine have pKa 8.3, 10.1, and 10.8, respectively).
Very similar results were obtained for HRP and F–HRP via zeta potential, suggesting that the adsorption behavior of this enzyme is not significantly altered by...
addition of between one and two fluorescein groups per enzyme molecule. We infer that zeta potential is less sensitive to differences in HRP binding due to fluorescein labeling than flocculation analysis. Enzyme:Au ratios obtained from zeta potential are similar to those from flocculation at pH 4.0 and 10.0, but much lower at pH 6.0 and 8.0. This difference suggests either that the high enzyme:Au ratios needed for protection against flocculation were due not to poor adsorption, but rather instability upon NaCl addition or that the change in nanoparticle charge saturates before HRP monolayer formation is complete.

4.3.2 Quantification of bound HRP and F-HRP

While both flocculation analysis and zeta potential measurements provide information on enzyme binding to colloidal Au nanoparticles, neither method directly reports the number of enzyme molecules bound per particle. In either approach, a percentage of the added HRP remains unbound, the magnitude of which is dependent upon the equilibrium constant for adsorption. Additionally, neither method provides information on the possibility of multilayer formation. For applications where enzyme:Au conjugates will be prepared and used as reagents in later experiments, it is desirable to quantify both the number of molecules bound per particle, and their activity relative to free enzymes.

Several methods exist for determining the protein:Au ratio in bioconjugates. The simplest method is to measure the unbound protein in the supernatant after pelleting conjugates by centrifugation, and estimate the number of molecules bound by
This method is most accurate when the amount of protein in the supernatant is similar to the amount of protein bound, and is high enough for facile determination by standard absorbance assays. Indirect methods in which the supernatant concentration is used to determine protein adsorption are subject to overestimating the protein:Au ratio in the event of protein adsorption to container walls during conjugate preparation and analysis. Direct determination of the number of protein molecules bound per particle has been performed using proteins labeled with radioactive or fluorescent probes. Of these approaches, radioactivity is more straightforward, however fluorescence is attractive as it avoids the safety concerns associated with radioisotopes.

Unfortunately, emission from fluorescently-tagged, nanoparticle bound enzymes is complicated due to quenching by the metallic particles. Franzen, Feldheim and coworkers recently reported both static and time-correlated single photon counting spectroscopy (TCSPC) for quantification of protein binding to Au nanoparticles. In the static measurement, fluorescence was used to quantify the unbound, labeled protein in the supernatant after BSA:Au conjugates were pelleted, and coverage was determined by subtraction. TCSPC required more complex instrumentation than traditional steady state fluorescence, but differences in lifetimes for tags on Au-bound vs. free proteins provide a means of differentiating these two species without prior separation. Finally, Colvin and coworkers have shown that the separation technique of analytical centrifugation can provide information on unlabeled biomolecule binding to Au nanoparticles based on changes in sedimentation due to adsorption.

To determine the number of enzyme molecules bound to each Au nanoparticle directly using traditional fluorescence instrumentation, we adapted an assay developed by
In this approach, complications due to quenching at the Au surface are avoided by dissolving the Au particles in KCN(aq) prior to fluorescence measurements. Figure 4-4 gives the average number of F-HRP bound per particle as a function of the amount of F-HRP added to solution and the pH during conjugation, as determined by Au dissolution after washing of HRP:Au nanoparticle conjugates. At pH 8.0, incubation with 50 F-HRP for each Au particle leads to immobilization of approximately 20 enzyme molecules per particle. To reach ~60 F-HRP:Au, samples must be incubated in a solution with 200× higher concentration F-HRP than Au nanoparticles. Additional F-HRP can be bound to the Au nanoparticles well beyond monolayer coverage, indicating multilayer formation at the higher enzyme:Au ratios. HRP adsorption continued beyond monolayer coverage for pH 4.0 and 10.0 as well.
HRP multilayers on Au nanoparticles are unexpected based on some previous literature accounts for protein:Au bioconjugation. Baudhuin and coworkers reported HRP binding saturation on 15 nm Au particles at 61 HRP per Au using radioactivity measurements.\textsuperscript{37} We note that calculated monolayer adsorption for this size particle would give 27 to 53 HRP per Au depending on orientation. These authors determined a $K_d$ of 79 nM and a molecular area of 11.8 nm$^2$ per HRP molecule for adsorption performed at the native pH of the citrate-reduced colloidal Au suspension, which was ~5.2. A difference between experimental conditions used here and in de Roe et al.\textsuperscript{37}
includes the addition of a large excess of BSA (24 nM) prior to sedimentation by Baudhuin and coworkers. We tested higher centrifugation speeds and addition of 25 µM BSA followed by 20 min incubation prior to centrifugation, as well as different pH during conjugation. Our data continue to show HRP multilayer formation at high HRP concentrations under any of these conditions.

Several other experimental differences may explain the apparent discrepancy. Our experiments employed higher maximum enzyme concentrations (up to 16 µM as compared to 1 µM) as well as higher Au nanoparticle concentrations (140 µg/mL vs 10-60 µg/mL) as compared to de Roe et al. Baudhuin and coworkers mention substantially increased “nonspecific adsorption” at higher nanoparticle concentrations. We note that Feldheim, Franzen and coworkers found that dye-labeled BSA was exchanged from the nanoparticle surface by unlabeled BSA at a rate of 2 molecules per 12 minutes, when the concentration of unlabeled BSA in solution was 1.2 nM. Also, in a report from Goodman et al up to 40% of radiolabeled concavalin A and Ig antibody adsorbed to gold nanoparticles was released in a time period of 3 hours by subjecting the conjugates to a competing protein. BSA incubation time in Baudhuin and coworkers was not given, although a binding constant for BSA twice that for HRP was reported. It is therefore likely that bound HRP was replaced with BSA at a higher rate than 2 per 12 minutes; incubation with 24 nM BSA, possibly for long times, could have resulted in replacement of a significant percentage of the bound HRP with BSA.

To determine whether the observed high F-HRP:Au ratios were due to the fluorescent labeling of the enzyme, we repeated the adsorption quantification experiments using unlabeled HRP. These data, plotted in Figure 4-5, also show
increasing amounts of bound enzyme well beyond monolayer coverage. Because it was necessary to quantify the bound, unlabeled HRP indirectly from the amount of free HRP remaining in the supernatant, we anticipate that these results somewhat overestimate the HRP:Au ratio. Nonetheless, the bound HRP:Au ratios observed for unlabeled HRP are higher than for fluorescein-labeled HRP, indicating that the fluorescent label is not mediating multilayer formation. Rather, we interpret the lower F-HRP:Au ratios for the labeled enzyme as possibly resulting from increased electrostatic repulsion due to the addition of the negatively-charged fluorescein.
The literature contains many examples where protein binding does not saturate once monolayer coverage has been achieved. In 1984, Horisberger and Vauthey noted from an examination of the cytochemical marker literature that, “in many instances, the number of molecules adsorbed per particle was vastly superior to that a particle can accommodate as a monolayer...” Indeed, several radiolabeling studies including of protein adsorption to colloidal Au point to multilayer formation.\textsuperscript{36,48-52} For example, multilayer formation onto gold nanoparticles has been reported for protein A,\textsuperscript{36} human immunoglobulin,\textsuperscript{36,49} concavalin A,\textsuperscript{36} $\alpha_2$-macroglobulin,\textsuperscript{48} and BSA.\textsuperscript{50} Franzen, Feldheim and coworkers also found that adsorption of a peptide-modified BSA does not saturate with increasing protein concentration, which indicates that more than a monolayer of

\begin{figure}[h]
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\caption{Number of unlabeled enzyme molecules adsorbed per Au nanoparticle as determined by supernatant analysis, as a function of the number added to solution at pH 8.0. Filled and open circles correspond to duplicate experiments performed on different days. Error bars are standard deviations of three independent samples and are in most cases smaller than the data points.}
\end{figure}
protein is adsorbing to the nanoparticles. Extensive protein multilayers have been observed previously for cytochrome c on Ag nanoparticles, although these structures were observed under special circumstances including aerogel formation, which may have stabilized their formation.

4.3.3 Comparison of free vs. Au-bound enzyme activity

When enzymes adsorb to solid surfaces such as colloidal Au nanoparticles, their native enzymatic activity can be altered. Often, losses in activity are observed due to conformational changes (e.g., partial or full denaturation) and/or steric effects (e.g., physical blocking of the active site by the surface or adjacent enzyme molecules). In some cases, improved enzyme function over time or under adverse conditions (e.g., heating) is observed for particle bound molecules. Since enzyme activity is generally changed, and often substantially so, by surface adsorption, direct comparison of the activity for bound and free enzymes is of interest. Knowledge of the enzyme:Au stoichiometry can be used to enable quantification of specific activity for bound vs. free enzymes.

The percent retained specific activity for HRP:Au conjugates as compared with the same concentration of free HRP is shown in Figure 4-6. At ~20 HRP:Au, more than 50% activity is retained for the nanoparticle-bound enzyme. As the HRP:Au ratio is increased, the specific activity as compared to the same number of free enzyme molecules in solution decreases (to only ~5% for 140 HRP:Au), however the total activity per particle (Figure 4-6, inset) remains essentially unchanged. This most likely
result from poor accessibility of enzyme active sites not located in the outermost layer, and may also reflect decreased accessibility as the lateral packing of enzymes increases within a layer. Dordick and coworkers have reported a similar decrease in activity with increasing surface density for lysozyme adsorbed to silica nanoparticles, which correlated with a loss of α-helicity, and may have been due to self-association of the adsorbed lysozyme.\textsuperscript{17}

Figure 4-6: Percent retained specific activity for HRP:Au conjugates as compared with free HRP as a function of the HRP:Au ratio. (inset) Activity per HRP:Au conjugate as a function of HRP:Au ratio.
4.4 Conclusions

We have presented a simple method for direct measurement of the number of fluorescein-labeled enzymes conjugated per Au nanoparticle by fluorescence quantification after dissolution of the bioconjugate gold core. Fluorescent labeling of proteins is standard in many analyses, and is generally thought not to alter enzyme structure, function, or interaction with surfaces. We have compared HRP and F-HRP adsorption to colloidal Au nanoparticles. Flocculation analysis indicates some differences in adsorption behavior for the labeled and unlabeled enzymes, particularly at pH 8.0 and to a lesser extent also at pH 4.0. The higher concentrations of HRP necessary to prevent flocculation at intermediate pH values (6.0 and 8.0) may be related to reduced stability of the enzyme near its isoelectric point. Lower enzyme:Au ratios were observed for both labeled and unlabeled HRP via zeta potential. Since zeta potential measurements are performed in situ and require no addition of electrolyte, comparison of these values with those from flocculation may give insight into the effect of added electrolyte on conjugate stability.

Quantification of the number of HRP molecules bound per Au nanoparticle indicated multilayer formation when high HRP concentrations were present in solution. Multilayers had not been reported previously for directly adsorbed HRP on Au nanoparticles to our knowledge, however previous studies had observed multilayer formation for a range of other proteins directly adsorbed to colloidal Au nanoparticles. High protein concentrations such as were used here and in some previous studies may increase the propensity for protein dimerization and/or multilayer adsorption. We note
that common methods such as flocculation analysis and zeta potential measurements cannot directly detect multilayer formation.

Knowledge of the HRP:Au stoichiometry made it possible to determine the specific activity of bound enzyme. The specific activity of Au-bound HRP was in all cases reduced as compared to free enzyme (≤ 50%), and was inversely proportional to the number of HRP molecules bound per particle. Indeed, the total activity per conjugate was fairly constant over a range of HRP:Au ratios, suggesting that only enzymes located in the outer layer of the HRP:Au conjugates contributed significantly to activity.
4.5 References


24. Sutherland, W. S.; Winefordner, J. D. Colloid Filtration – A Novel Substrate


47. Control experiments in which high F-HRP concentrations were used in the absence of Au nanoparticles demonstrated that the high F-HRP:Au ratios observed at high F-HRP solution concentrations were due to bound F-HRP and not incomplete washing.


Chapter 5

Immobilization of Enzymes on Nanoparticle Surfaces

The layer-by-layer work on latex beads presented in this chapter, in addition to activity assays and the work on immobilized enzymes for biosensing, were done in conjunction with Dr. Jacqueline Keighron of the Keating group. Dr. Keighron also provided a majority of the labeled enzymes for this work. The initial glucose oxidase immobilization studies were done by Gregory Hild, a summer undergraduate student, mentored by the author in the Keating lab. The author’s focus was immobilization and characterization of enzymes onto nanowire surfaces for use in biosensing applications.

5.1 Introduction

Interfacing biology and materials science has produced great advances in the field of biosensing. Immobilized biomolecules provide control over reactions for biosensing applications. By immobilizing enzymes or nucleic acids onto a nanoparticle surface, biosensors can be fabricated with probe molecules accessible to target molecules of interest for a number of sensing scaffolds, e.g., field effect transistors, suspension arrays, or surface enhanced Raman spectroscopy substrates. Here, we looked at two methods of protein immobilization onto nanoparticles surfaces – encapsulated in a silica matrix and immobilized within polyelectrolyte multilayers – that does not require modification of the protein molecule.

Loss of enzymatic activity upon immobilization due to denaturation or blockage of the active sites is a concern. In Chapter 4 of this dissertation it was shown that 50% of the HRP enzymatic activity was retained with low surface coverages of HRP on Au
particles, compared to the native enzyme, while a significant decrease in activity was observed with increasing concentration of enzyme on the surface.\textsuperscript{7} Although an initial loss of enzymatic activity is generally observed for immobilized enzymes on surfaces, studies have found that once immobilized, these enzymes resist further activity loss, even with changes in pH or temperature.\textsuperscript{8} Thus, it is necessary to characterize enzyme:particle conjugates to understand the effect of immobilization on the enzymatic activity, which is important for the development of commercial and reproducible biosensors.

We explored the immobilization of enzymes on the surface of striped metallic nanowires for the production of multiplexed biosensors for enzymatic substrates and metabolites. Nanowires provide an excellent scaffold for the immobilization of biomolecules, and can be used for multiplexing by incorporation of barcoding patterns. If different enzymes were immobilized onto different barcode populations, then the production of fluorescent products could be followed and attributed back to what metabolites are present in complex solutions based on what enzymes were involved in the reaction. Metabolites are associated with a variety of diseases and could be used as biomarkers. Immunoassays have been performed on barcoded wires,\textsuperscript{3,9} but direct detection of metabolites in solution has not yet been achieved.

5.1.1 Encapsulation of Enzymes in Silica

In the bulk, enzymes have been physically entrapped within a silica matrix, which isolates the biomolecule while maintaining a controlled aqueous microenvironment.\textsuperscript{10} The bioactivity of enzymes immobilized into sol-gel materials can be retained.
Yamanaka et al. synthesized sol-gels with glucose oxidase and peroxidase incorporated to produce optically based glucose samples with enzymatic activity comparable to that in solution.\textsuperscript{11}

Although there have been reports of protein/nanoparticle complexes being immobilized into a sol-gel material to produce a glassy carbon electrode,\textsuperscript{12} or nanoparticles covalently attached to a sol-gel material with HRP subsequently immobilized,\textsuperscript{13} to the best of the author’s knowledge, there have been no reports of encapsulating enzymes within silica coatings on nanoparticles. Nanoparticles have been coated with silica.\textsuperscript{14-19} For example, Sioss and Keating demonstrated that metallic nanowires can be efficiently coated with SiO$_2$ in solution using TEOS,\textsuperscript{14} but this method could not be used to encapsulate enzymes without denaturing the proteins due to the high concentration of ethanol and high pH necessary. The nanowires need to be coated in a process that initiates nucleation of the sol-gel on the surface on the nanowire with minimal free silica formation using a biocompatible solvent and pH. Due to the potential challenges associated with this, an alternative route of enzyme immobilization – the layer-by-layer assembly of polyelectrolytes – was also explored.

### 5.1.2 Layer-by-Layer (LbL) Assembly of Enzyme/Polyelectrolyte Multilayers

Polyelectrolyte (PE) multilayers are well-studied surfaces.\textsuperscript{20-23} LbL assembly of PEs occurs due to the alternate deposition of surfaces into solutions containing oppositely charged molecules with the deposition of each layer occurring through electrostatic interactions. This methodology allows control over the thickness of the multilayers by
the number of bilayers assembled, and the thickness of the PE layers can be adjusted with Angstrom precision by addition of salts, as an increase in ionic strength screens the charges between the highly charged polyelectrolyte molecules.\textsuperscript{23}

PE multilayers were quickly adapted from planar surfaces to colloidal surfaces. Sukhorukov et al. showed the formation of PE multilayer films onto the surface of polystyrene latex particles.\textsuperscript{24} Biomolecules can be embedded within these multilayers by addition into the oppositely charged layer or as its own layer, alternating with the oppositely charged polymer,\textsuperscript{20} or into either the polyanion or polycation layer\textsuperscript{25}. Caruso and Möhwald first introduced the methodology for coating colloidal particles with protein multilayers onto colloidal particles.\textsuperscript{22} This work was later extended to study the immobilization of glucose oxidase (GOx) and horseradish peroxidase (HRP) onto polystyrene microparticles.\textsuperscript{20} Caruso and Schüler showed that as the number of enzyme containing bilayers increased, the observed activity of the overall assembly increased.\textsuperscript{20} In addition, Caruso and Schüler also demonstrated sequential enzymatic catalysis on particle by immobilizing alternating layers of GOx and HRP and following the reaction through a colorimetric oxidation reaction.\textsuperscript{20}

A variety of biosensors have already been introduced in the literature by embedding enzymes into multilayer films through the LbL method. Yang et al. developed a cholesterol biosensor by the immobilization of cholesterol oxidase onto the surface of a multilayer film modified electrode that detected the presence of cholesterol electrochemically with high sensitivity.\textsuperscript{26} Likewise, hydrogen peroxide can be detected by immobilization of horseradish peroxidase onto gold electrodes using hydroquinone as an electrochemical mediator.\textsuperscript{27} Many examples of glucose sensors have been presented
in the literature. The oxidization of glucose is detected by immobilization of glucose oxidase onto substrates with a wide linear response on the second timescale.

In this chapter, we report initial work to develop multiplexed enzymatic biosensors. Enzymes, e.g., glucose oxidase (GOx), horseradish peroxidase (HRP), malate dehydrogenase (MDH), and citrate synthase (CS), were immobilized on nanoparticles by mixing into solutions of polyelectrolytes (e.g., poly(allylamine hydrochloride) [PAH] and poly(sodium 4-styrenesulfonate) [PSS]) during the LbL assembly of the multilayers on the nanowires. Figure 5-1 shows a schematic of a striped, metallic nanowire with polyelectrolyte (PE) multilayers on the surface with enzymes, indicated by a white circle, embedded within one of the two polyelectrolyte layers. This experimental design allows for detection of low concentration of metabolites as fluorescent product will be turned over and concentrated on wire. We have shown that fluorescently labeled enzymes can be immobilized on nanowires, and that these enzymes continue to turn over product. We have also shown that unlabeled HRP immobilized on wire will catalyze the oxidation of 2,7′-dichlorodihydrofluorescein to produce a fluorescent product localized around the wire. In the future, this can be further explored to produce multiplexed, enzymatic biosensors.

Although barcoded nanowires will be used for the final biosensor assembly, the size and density of the metallic wires can make characterization by non-optical methods challenging. Therefore, LbL assemblies were characterized using latex beads, which due to their smaller size and density enable characterization using techniques like fluorescence and UV-Vis absorbance spectroscopy. Using micron sized beads we
characterized the enzyme activity to improve the design and use of our nanowire based biosensors.

![Figure 5-1: Schematic showing the process of immobilization of enzymes (depicted by white circles) by alternating negatively and positively charged polyelectrolytes onto the surface of striped, metallic nanowires. Procedure adapted from reference 20.](image)

### 5.2 Experimental Materials and Methods

#### 5.2.1 Materials

Poly(allylamine hydrochloride) (PAH) (average molecular weight = 56 000 g/mol), poly(sodium 4-styrene sulfonate) (PSS) (average molecular weight = 70 000 g/mol), 2-mercaptoethanesulfonylic acid (MESA), L-malic acid, β-nicotinamide adenine dinucleotide hydrate (NADH), β-nicotinamide adenine dinucleotide (NAD+), oxaloacetate, acetyl-coenzyme A, dithionitrobenzoic acid, Sigma FAST o-
phenylenediamine dihydrochloride tablet sets, β-D-glucose, alkaline phosphatase from *Escherichia coli*, citrate synthase from porcine heart, glucose oxidase Type X-S from *Aspergillus niger*, peroxidase Type VI from horseradish, lactate dehydrogenase from bovine heart, malate dehydrogenase from porcine heart (cytoplasmic), and urease from Jack Bean were purchased from Sigma-Aldrich. Silver Cyleess and Orotemp 24 plating solutions were acquired from Technic, Inc. 3-Aminopropyltrimethoxysilane (APTMS), mercaptopropyltriethoxysilane (MPTES), tetramethoxysilane (TMOS), *N-* (triethoxysilylpropyl)-o-polyethyleneoxide urethane (PEO), and tetraethoxysilane (TEOS) were purchased from Gelest, Inc. Fluoresbrite YG Carboxylate Microspheres (2.5% solides-latex), diameter = 0.398 µm, were purchased from Bangs Laboratories. 2,7′-Dichlorodihydrofluorescein diacetate (DCF) and Alexa Fluor™ 488, 555, 633, 647 protein labeling kits were obtained from Invitrogen Molecular Probes. All water used was 18.2 MΩ-cm Nanopure water from a Barnstead system. All reagents were used as purchased without further purification; the silanes were opened and aliquoted in a glove box under nitrogen gas to prevent hydrolysis from water vapor in the air.

5.2.2 Nanowire Synthesis

Nanowires were made by the galvanostatic electrodeposition of silver succimide or potassium aurocyanide into porous, alumina templates on an in-house system, as previously described.3, 30-32 A ~300 nm layer of silver was evaporated onto one side of the template to act as the working electrode. A sacrificial silver layer was then deposited prior to alternating silver and gold to produce striped nanowires for barcoding purposes.
Wires were released into solution by dissolution of the silver backing using ~33% HNO₃, following dissolution of the alumina template in 3M NaOH. The wires were then rinsed 1× 3M NaOH, 1× H₂O, and 3× EtOH. Once in solution, the barcoding pattern can be identified by illumination at 430 nm light using light microscopy, as the reflectance of silver is brighter than gold at this wavelength.

5.2.3 Silica Coating Nanowire

Nanowires were coated with SiO₂ through the hydrolysis and condensation of tetraethoxysilane (TEOS), as described in the literature. Briefly, 300 μL of batch concentration wires (~1×10⁹ wires/mL) were sonicated in 490 μL ethanol, 160 μL H₂O, 40 μL TEOS, and 10 μL NH₄OH (28%) for 45-60 min, followed by rinsing 3× EtOH. Adjustments to make a more biocompatible silica coating procedure were necessary for enzyme encapsulation. Tetramethoxysilane (TMOS) was used in place of TEOS, and a pre-hydrolysis step was done prior to addition of the enzymes. The hydrolysis step was catalyzed with HCl, and was then neutralized with buffer to prevent denaturation of the enzymes, following a separate condensation step. The TEOS:PEO coated nanowires were prepared following the procedure outlined in Chapter 2. Briefly, 300 μL of batch concentration wires (~1×10⁹ wires/mL) were rinsed into acetonitrile (ACN) and were sonicated in 790 μL ACN, 160 μL H₂O, 30 μL TEOS, 24.7 μL PEO and 10 μL NH₄OH (28%) for 60 min, 3×, with rinsing with ACN between each coating step.

Once coated, 50 μL batch concentration wires were rinsed into pH 9.5 CHES buffer to deprotonate the surface hydroxyls, and then the nanowires were resuspended in
a solution volume of 180 µL with 0.5 M TMOS + 3.7 mM HCl in water. After the pre-
hydrolysis step, 117 µL pH 7.5 10 mM phosphate buffer was added to bring the pH to 6.
An additional 50 µL water was added, in addition to 50 µL 1.2 mg/mL Alexa Fluor 488-
HRP. The samples were spun for 2 hrs to keep the nanowires suspended, followed by
imaging on the inverted microscope using the appropriate filters.

5.2.4 Layer-by-Layer Assembly of Polyelectrolytes/Enzyme Complexes on Particle
Surfaces

Polyelectrolytes were deposited onto metallic nanowires by alternating anionic
and cationic polyelectrolytes in buffered solution while vortexing to keep the nanowires
suspected. Figure 5-2 depicts the structures of the (A) anionic poly(styrene sulfonate)
(PSS) and (B) cationic poly(allylamine hydrochloride) (PAH) used in the work presented
here. This pair of polyelectrolytes is well studied, and have been used to immobilize
enzymes onto nanoparticle surfaces previously.²⁰ Silica coated or bare, metallic
nanowires were suspended in buffer in which 0.05 mg/mL PAH (56 000 g/mol) or 0.2
mg/mL PSS (70 000 g/mol) in 10 mM buffer, 0.5 M NaCl was added. Samples were
vortexed for 15 – 60 min per layer. Wires were rinsed 3× with 10 mM buffer, 0.5 M
NaCl in between each PE layer by centrifugation. PE concentrations were increased to 1
mg/mL for both PSS and PAH when coating latex beads due to the larger surface area.
Three to six bilayers of PE were generally deposited. Enzymes were deposited within the
oppositely charged PE layer, depending on the isoelectric point of the enzyme. Table 5-1
provides the immobilization conditions for each enzyme deposited within PE multilayers
on nanoparticle surfaces; this includes the multilayer formation, e.g., PSS/PAH-GOx, which indicates that a PSS layer is deposited, the nanowires were rinsed, and then a solution containing PAH and glucose oxidase was deposited to immobilize the glucose oxidase within the PAH layer. The multilayers were then built up by alternating between the two solutions.

Figure 5-2: Structures of the polyelectrolytes used to immobilize enzymes on the surface of nanoparticles by alternating negatively charged (A) PSS and the positively charged (B) PAH.
In some situations, the nanowires were first silica coated to bring the fluorescently labeled enzymes off the surface of the metallic nanowire to prevent quenching. This could also be accomplished by initially depositing sacrificial layers of PE not containing enzyme. When the nanowires were silica coated, the silica surface was first prefunctionalized with 3-aminopropyltrimethoxysilane (APTMS) by vortexing the nanowires in a ~0.2 M solution of APTMS in EtOH for 30 min (followed by rinsing 3× EtOH) to produce a positively charged surface for PSS deposition. Likewise, when the nanowires were not coated with silica, the nanowire surfaces were first functionalized with 2-mercaptoethanesulfonic acid (MESA) in a 43.5 mM solution of MESA in H$_2$O for 1-2 hr (followed by rinsing 3× H$_2$O) to produce a negatively charged surface for PAH.
deposition. The latex beads were carboxylated, as received, and thus, PAH was first deposited onto the negatively charged beads.

MDH and CS were immobilized in PE multilayers on 8 µL Fluoresbrite YG Carboxylate Microspheres (diameter = 0.398 µm) in Safe Seal Microcentrifuge tubes (Soreson Bioscience, Inc.). Polymer solutions, 500 µL, 1 mg/mL, were added sequentially for 30 min depositions. The particles were rinsed 3× 500 µL 50 mM Tris, 0.5 M NaCl, pH 8.1 (16 000 x g, 12 min, 4°C). Supernatants of rinse samples were saved to determine particle loss. Enzymes were immobilized as part of a PE layer (i.e., PAH/PSS-MDH and PSS/PAH-CS) or as an individual layer for comparison (i.e., PSS/MDH and PAH).

5.2.5 Fluorescence and Transmission Electron Microscopy of Coated Particles

Transmission electron microscopy images of PE coated nanowires and beads were obtained using a JEOL JEM 1200 EXII instrument with a high resolution Tietz F224 digital camera at an accelerating voltage of 80 kV or 120 kV. Fluorescence microscopy analysis of the immobilized labeled enzymes within PE multilayers on nanowires was done using a Nikon TE-300 inverted microscope equipped with a Photometrics Coolsnap HQ camera, a Plan fluor 60× or 100× oil objective (N.A. 1.4), and Image-Pro Plus (v 4.5) software. The source for reflected excitation was a 300 W Xe lamp.
5.2.6 Characterization of PE/Enzyme Complexes on Latex Beads

Enzymes were characterized through kinetic assays and fluorescence measurements of all the samples and supernatants, taken to quantify the amount of AlexaFluor 555-MDH (ex. 550 nm, em. 560-580 nm), AlexaFluor 633-CS (ex. 621 nm, em. 633-670 nm), and fluorescent latex beads (ex. 470 nm, em. 490-520 nm). Fluorescence measurements of polyelectrolyte coated beads were carried out on a Horiba Jobin Yvon Fluorolog 3-21 using right angle detection. All measurements were taken using a 0.5 sec integration time, 5 nm excitation and emission slits and using the excitation/emission wavelengths noted above. From the kinetic and fluorimetry data, the number of particles per milliliter, the number of MDH per particle, the activity (U/particle), and the specific activity (U/mg) were calculated.

5.2.7 Activity Assays

Activity assays were conducted colorimetrically using a Hewlett-Packard 8453 UV/Vis spectrophotometer, or fluorescently using a Horiba Jobin Yvon Fluorolog 3-21. Immobilized GOx activity was monitored through a coupled reaction of HRP, where GOx catalyzed the production of H$_2$O$_2$ from glucose and O$_2$. A two enzyme reaction resulted in the production of hydrogen peroxide, which oxidizes o-phenylenediamine dihydrochloride (OPD) to produce the colored product 2,3-diaminophenazine (DAP) (Figure 5-3).$^{41}$ Samples were prepared in triplicate with GOx immobilized on nanowires, 0.6 M glucose, 1 mg/mL HRP, and 2.2 mM OPD in 5mM acetate buffer. Colorimetric assays of embedded GOx were conducted using a discontinuous method in which 10 µL
of embedded wires were allowed to carry out an enzymatic reaction which was stopped by addition of 100 µL 3 M HCl. Reactions occurred in a total volume of 500 µL in which the wires were kept in solution by use of a tumbler. HRP activity was monitored through the absorbance of the product of OPD and hydrogen peroxide at 492 nm.

![Figure 5-3: Schematic showing the two enzyme reaction to produce the oxidized form of OPD that can be detected at 492 nm (when reaction is stopped by HCl). (Adapted from References 41, 42)](image)

The activity of MDH and CS was studied by following the production of a colorimetric product using UV-visible spectroscopy. The forward reaction of MDH was monitored by the consumption of NADH in the presence of MDH immobilized on nanowires (20 µL) through the reaction of malic acid (1 mM) to produce oxaloacetate (Figure 5-4). The nanowires were spun down by centriguation, and the supernatant was analyzed. Similarly, for the MDH and CS immobilized in PE multilayers on latex beads, full characterization of the samples was completed by following the reverse reaction, the more favorable reaction, of the conversion of oxaloacetate to malate (Figure 5-4). This was accomplished by resuspending 150 µL beads in 2.8 mL buffer and substrates with a
final concentration of 0.5 mM oxaloacetate and 0.1 mM NADH in a total reaction volume of 3 mL. The production or consumption of NADH was monitored at 340 nm for 300 sec for both the forward and reverse reactions. The CS reaction was monitored by mixing 150 µL latex bead samples with the substrates for a final concentration of 0.5 mM oxaloacetate, 0.15 mM dithionitrobenzoic acid, and 0.1 mM acetyl CoA by bringing the volume up to 3 mL with buffer (Figure 5-4). The interaction of CoA and dithionitrobenzoic acid was monitored at 412 nm.

![Reaction schemes](image)

**Figure 5-4:** Reaction schemes for the production of (top) oxaloacetate by the reduction of NAD⁺ to NADH in the presence of malate dehydrogenase and (bottom) citrate by the reaction of oxaloacetate with water in the presence of citrate synthase. (Adapted from Reference 8)

### 5.2.8 Production of Fluorescent Products on Wire Surface

Striped nanowires were synthesized and coated either with silica/PE multilayers or just PE multilayers, immobilizing unlabeled HRP onto the nanowire, as described previously. Microscope samples were prepared by diluting 10 µL (~ 1 × 10⁷) nanowires with 90 µL buffer or water onto a 25 × 60 mm coverslip with a silicone spacer attached to
contain the liquid sample. The samples were imaged using 430 nm light, and then 10 µL urea hydrogen peroxide (0.4 mg/mL in water) and 10 µL of DCF (0.5 mM in 30 mM NaOH) were added in sequence. The DCF must be prepared in a basic solution to cleave the diacetate functionality to make the molecule soluble in aqueous solutions. Fluorescence images were obtained over time using the appropriate filter (ex. 480 ± 20 nm; em. 540 ± 20 nm) to monitor the production of the fluorescent fluorescein derivative due to the oxidation of the DCF.

5.3 Results and Discussion

Herein, attempts to coat nanoparticles with silica in a biocompatible manner will be discussed. Additionally, the immobilization of a variety of enzymes by the layer-by-layer adsorption of polyelectrolytes onto the surface of nanoparticles will be studied and characterized.

5.3.1 Immobilization of Enzymes in Sol-Gels on Nanowire Surfaces

Typically, sol-gel materials are made by the acid or base catalysis of tetraethoxysilane (TEOS) or tetramethoxysilane (TMOS) in an organic solvent. When enzymes are introduced into the system, TMOS is preferred over TEOS since the byproduct is methanol compared to ethanol, which has a polarity closer to water. Ellerby et al. developed a biocompatible sol-gel synthetic method that had an initial pre-hydrolysis step of the TMOS, followed by neutralization with buffer prior to introduction
of the enzyme into solution. To translate this onto the nanoscale, this protocol was adapted to attempt to coat gold nanowires with SiO$_2$ in a biomolecule-friendly manner, as discussed below. Initially, no enzyme was added to the reaction mixture to determine if the nanowires could be coated with silica in non-extreme conditions, without wasting the enzymes. The same molar ratio of TMOS to water to HCl was used, but in smaller volumes, in the presence of 150 uL batch concentration gold nanowires. Ellerby et al. used approximately 2 moles of water for every mole of TMOS, so a secondary sample with 2x the amount of water was prepared to assure complete TMOS hydrolysis. An initial pre-hydrolysis step was done with TMOS, H$_2$O, and HCl for either 30 min, 1 hr, or 2 hr for the 1× and 2× water samples. After this time period, 10 mM phosphate buffer, pH 7.4 was added to each sample to increase the pH to 6. This resulted in complete gelation of the TMOS, resulting in nanowires within the sol-gel matrix. To avoid this in the future reactions, the catalyst was omitted to slow down the reaction. This resulted in no silica deposition on the nanowires (Figure 5-5).
Figure 5-5: TEM image of nanowire after suspension in a reaction mixture of TMOS and water showing no formation of silica on the surface. No catalyst was used to prevent immediate gelation of the TMOS. Scale bar = 100 nm

Since the catalyst was necessary to promote the hydrolysis and condensation reactions of the TMOS, the concentration of the TMOS, water, and HCl was decreased by an order of magnitude, while keeping the number of wires, and thus the total surface area, constant. A pre-hydrolysis step was again done by sonicating the reaction mixture and wires for either 30 min, 1 hr, or 2 hr, for both the 1× and 2× water, following the addition of 10 mM phosphate buffer, pH 7.4 to increase the solution pH. After addition of the buffer, the samples were spun for 1 hr. All six samples showed minimal to no silica growth on the surface of the nanowires. The decreased concentration of TMOS did prevent the complete gelation of the material, however, large chunks of free silica were observed in each sample, indicating that the reaction was not nucleating on the surface of the nanowires.
To promote reaction on the surface of the nanowire, the wire surfaces were first functionalized with mercaptopropyltriethoxysilane (MPTES) in ethanol. The wires were resuspended in reaction solutions containing either 1× TMOS or 2× TMOS, water, and a catalyst, either HCl or NH₄OH. Figure 5-6 shows silica growth on the surface of the nanowire, but it is very thin and heterogeneous. The wires were not well coated for any of the samples.

![Silica coated Au nanowires prepared by the pre-hydrolysis of (A) 1× TMOS, catalyzed by 3.70 mM HCl, (B) 2× TMOS, catalyzed by 6.35 mM HCl, (C) 1× TMOS, catalyzed by 27.4 mM NH₄OH, and (D) 1× TMOS, catalyzed by 5.80 mM NH₄OH, followed by addition of buffer to neutralize the solution pH. Scale bars = 100 nm](image)

Although the MPTES did provide a nucleation site for the growth of the silica on the nanowire surface, the coating was much too thin. Thus, to increase adhesion, the nanowires were first silica coated using the standard procedure with TEOS in solution, followed by the biocompatible reaction of TMOS with a pre-hydrolysis step,
neutralization of the solution, and the addition of water and 50 µL 1.2 mg/mL Alexa Fluor 488-HRP. After rinsing the samples, the nanowires were imaged on the microscope. Figure 5-7 shows that a significantly different amount of labeled HRP was immobilized on the nanowires compared to the control not containing the enzyme. TEM analysis of the coated wires showed the thickness of the coating was not significantly different after the second silica coating step with TMOS.

![Figure 5-7: Fluorescence of TEOS coated wires before and after immobilization of AlexaFluor 488-HRP within a biocompatible TMOS coating.](image)

These results were promising, but proteins are known to adsorb onto the surface of SiO$_2$. It was unclear if the AlexaFluor 488-HRP was encapsulated within a silica coating produced by the hydrolysis and condensation of the TMOS on the surface of the TEOS coated nanowires, or if the protein just adsorbed to the surface of the silica coated nanowires. To test this, the experiment was repeated using TEOS:PEO coated nanowires, which were shown in Chapter 2 to be a protein-resistant coating due to the
presence of a polyethylene oxide functionality. These were compared to the standard TEOS coated nanowires. AlexaFluor 488-HRP was added to a solution of TEOS and TEOS:PEO coated nanowires (grey) and compared to the samples where a pre-hydrolysis of TMOS prior to the addition of the enzyme took place (white) (Figure 5-8). A significant decrease in protein adsorption was observed for the TEOS:PEO samples. Figure 5-8 does show that the amount of protein observed immobilized onto the nanowire in Figure 5-7 was most likely due to physical adsorption of the protein onto the silica surface and not encapsulation into a secondary TMOS coating. Enzymes were not being encapsulated at detectable concentrations within silica; therefore, to move forward with this project, layer-by-layer assembly of polyelectrolytes was explored for controlled immobilization of proteins on nanowire surfaces.

![Graph](image_url)

**Figure 5-8:** Fluorescence intensity of AlexaFluor 488-HRP added to TEOS or TEOS:PEO coated nanowires (grey) or added to nanowires in solution with pre-hydrolyzed TMOS (white).
5.3.2 Immobilization of Enzymes onto Nanowire Surfaces with PE Multilayers

Layer-by-layer assembly of polyelectrolytes onto the surface of the nanowires was accomplished by suspension of nanowires that were either silica coated or functionalized with a small molecule in alternating solutions of the polycation or polyanion in buffer. Once assembled onto the nanowires, the PE multilayers were imaged using transmission electron microscopy (TEM) to differentiate PE multilayers from the silica coated nanowires based on differences in electron density between the SiO$_2$ and the less electron dense PAH/PSS multilayers (Figure 5-9). In both experiments, 1 µM HRP was added to the PSS layer. The PE multilayers in Figure 5-9 were (A) 14.3 ± 1.7 nm for 6 bilayers of PAH/PSS-HRP and (B) 9.7 ± 1.7 nm for 3 bilayers of PAH/PSS-HRP with an additional 3 layers of PE deposited initially to bring the fluorescently labeled dye off the surface of the metal nanowire. These are in accordance with the literature values. Caruso and Schüler determined that three bilayers of PAH/PSS-HRP on beads to be ~12 nm thick. The decreased values observed here compared to by Caruso and Schüler are most likely due to drying of the particles for TEM analysis compared to analyzing the PE multilayer thickness using light scattering methods in solution. The PE multilayers can be deposited onto either silica coated nanowires or bare wires efficiently.
Studies were completed to determine the optimal conditions for protein immobilization on nanowire surfaces. It was determined that 30 min of deposition of polyelectrolyte per layer was more than enough to immobilize nano- to micromolar concentrations of enzyme on the surface of the nanowires. Figure 5-10 A shows the immobilization of AlexaFluor 555-GOx immobilized onto silica coated Au/Ag striped nanowires. The striping pattern can be distinguished under 430 nm light, as silver is more reflective than gold at this wavelength. Figure 5-10 B shows the quantification of the fluorescence on the nanowires, indicating that there is no inherent PE fluorescence since the control containing no enzyme is not fluorescent, and there is sufficient immobilization of the labeled enzyme onto the nanowire surface. Fluorescently labeled horseradish peroxidase (HRP), malate dehydrogenase (MDH), alkaline phosphatase (AP), citrate synthase (CS), lactate dehydrogenase (LDH), glucose oxidase (GOx) and urease
have all been embedded in PE multilayers (Table 5-1). This demonstrates that enzymes of a wide range of molecular weights can be immobilized onto the nanowire surfaces using LbL assembly methods.

Figure 5-10: (A) Optical reflectance at 430 nm and corresponding false-colored fluorescence microscopy images of striped 0101010 nanowires with Alexa 555-GOx embedded on wire. Scale bar = 5 µm (B) Quantification of the fluorescence intensity on nanowire surface. (Data collected in collaboration with Gregory Hild).

5.3.3 Characterization of Immobilized Enzymes in PE Multilayers

To further characterize the immobilization process of enzymes within PE multilayers, activity assays were used to determine if the immobilized enzyme could still turn over product. Nanowires with GOx immobilized onto the surface were exposed to glucose, OPD, and HRP, and the production of a colored product, DAP, was monitored as a function of time at 492 nm (Figure 5-11).\textsuperscript{41} The increase in OPD concentration in the solution containing the nanowires with GOx immobilized within PE multilayers indicates that product is being turned over. The activity of the GOx was calculated to be
4.3 U/µL wires. Similarly, Dr. Jacqueline Keighron of the Keating group calculated the activity of MDH immobilized per wire to be 0.0005 U/µL wires, compared to 632 U/mg protein for the free MDH in solution. These values cannot be compared to activity reported for GOx or MDH in PE multilayers in the literature because the exact number of wires and immobilized enzyme cannot be determined with accuracy.

A significant loss of activity is expected upon immobilization. Depending on the enzyme and conditions, loss of up to 90% enzymatic activity has been observed for enzymes immobilized within PE multilayers on a particle surface. Determination of the specific activity of the enzymes within PE multilayers would be valuable information. To do this, enzymes were immobilized within PE multilayers on fluorescent latex beads.
that could be quantified using fluorimetry to determine the concentration of particles, even after loss due to rinsing. Significant differences in the properties of immobilized enzymes within PE multilayers on a latex bead surface and a metallic nanowire are not anticipated, so the characterization on the bead should be comparable to the nanowire samples.

MDH and CS were immobilized within PE multilayers on separate populations of fluorescent latex beads. Separate samples were prepared with the enzyme either in the first, second, or third bilayer of polyelectrolytes (away from the latex bead) to understand the effect of the position of the enzyme (Figure 5-12). Additionally, enzymes were immobilized within the oppositely charged layer (e.g., PSS/PAH-CS) or as the, e.g., polyanion layer itself (e.g., PAH/CS).

![Figure 5-12: Schematic showing the immobilization of enzyme in the (A) first, (B) second, or (C) third layer of polyelectrolyte.](image)

The amount of enzyme immobilized and the thickness of the polyelectrolyte multilayers vary based on how the enzyme is incorporated. This was demonstrated by the immobilization of MDH and CS onto the latex particles. Particles with the enzyme
immobilized in the third bilayer were analyzed via TEM and 30 particles of each sample were measured by hand and averaged (Figure 5-13). The polyelectrolytes are similar electron density compared to the latex beads, so the actual layers could not be distinguished, but the overall size of the particles did change, as compared to bare latex particles. As expected, the particles with bilayers prepared with PSS and PAH were significantly larger at a 99.9% confidence in diameter than the particles with bilayers of one polymer and one enzyme. The deposition of a second polyelectrolyte adds additional material to the surface of the particle which increases the size of the overall enzyme:particle conjugate. Additionally, particularly for the MDH, an increased number of enzyme per particle was observed when the enzyme was immobilized as part of a PE layer, which also increases the size of the conjugate. The number of particles/mL, the number of enzyme molecules per particle, the activity, and the specific activity were determined for MDH (Figures 5-14) and CS (Figure 5-15) by characterization of these particles.
Figure 5-13: Size analysis by TEM of MDH or CS immobilized into the third bilayer on latex beads. The enzymes were either added in the oppositely charged layer or as the oppositely charged layer. Error bars are the standard deviation from measurement of 30 separate beads per sample.

Figure 5-14: Characterization of MDH immobilized on latex particles with PSS/MDH bilayers (blue) and PAH/PSS-MDH bilayers (red) to determine (A) the number of MDH molecules per particle, (B) the activity per particle, and (C) the specific activity of the MDH immobilized on the particles. (Data collected in conjunction with Dr. Keighron; Dr. Keighron collected fluorescence data).
The characterization of enzymes on the latex beads showed the amount of CS:particle remained fairly constant for both deposition types with enzyme in each layer. The amount of MDH:particle was also constant as the enzyme was incorporated into different layers, but significantly more MDH was incorporated in PAH/PSS-MDH than PSS/MDH. The further away from the interior of the particle (and closer to the surface), the more active the enzymes were due to the ease of the substrate to reach the enzyme. Higher activity per particle was observed for both the CS and MDH when it was near the surface in the outer layer compared to being in the first bilayer, as also seen with the specific activity. As expected, a significant loss of activity was observed upon immobilization to the particle surface. Keighron and Keating reported the MDH catalyzed conversion of oxaloacetate to malate to have a specific activity of 182 ± 3 U/mg and 73.6 ± 0.6 U/mg for the reaction of CS. The specific activity decreased substantially to the order of 10^{-4} U/mg for both enzymes on the particle surface within the

Figure 5-15: Characterization of CS immobilized on latex particles with PAH/CS bilayers (green) and PSS/PAH-CS bilayers (purple) to determine (A) the number of CS molecules per particle, (B) the activity per particle, and (C) the specific activity of the CS immobilized on the particles. (Data collected in conjunction with Dr. Keighron; Dr. Keighron collected the fluorescence data).
PE multilayers. Despite this loss, the enzymes still turn over product, and improved specific activity upon immobilization might be accomplished using more robust enzymes, e.g., HRP and/or GOx.

5.3.4 Production of a Fluorescent Product on a Wire Surface

To progress towards the development of multiplexed biosensors, unlabeled HRP was immobilized onto the surface of 011110 nanowires (3 bilayers of HRP-PSS/PAH). HRP was chosen because it is a well-studied, stable enzyme. These PE coated nanowires were imaged at 430 nm (Figure 5-16, right), and then fluorescent images were acquired over time immediately upon the addition of urea hydrogen peroxide and 2,7′-dichlorodihydrofluorescein (DCF). The oxidation of the DCF by the urea hydrogen peroxide is catalyzed by HRP. Figure 5-16 shows that the fluorescent product is concentrated around the surface of the nanowires. The product diffuses away from the surface of the nanowires, and in future experiments this could be slowed by immobilization of the nanowires within an, e.g., agarose gel.
5.4 Conclusions and Future Directions

5.4.1 Conclusions

Despite the advantages of encapsulating enzymes within sol-gel materials, it proved to be difficult to initiate reaction on the surface of nanowires in a biocompatible manner. Thus, polyelectrolytes were used to immobilize enzymes onto the surface of striped, metallic nanowires. This methodology of protein immobilization is an inexpensive and fast method, compared to other methods, e.g., cross-linker molecules, while simultaneously controlling the surface chemistry of the particles, which is beneficial for sensor design. These materials are well studied, and can be used to immobilize enzymes of a wide range of molecular weights on nanowires of varying sizes. Characterization of the enzymes on the nanowire surfaces and immobilized onto latex
beads showed that the enzymes still turn over product, although a significant percentage of enzymatic activity is loss, which agrees with literature findings. The ability to immobilize enzymes onto nanoparticles of any size or shape will aid in the production of biosensors using nanoparticles with controlled surface chemistry. Progress towards the production of an enzymatic biosensor for small molecules, e.g., H$_2$O$_2$, was made by immobilizing HRP onto the surface of nanowires and detecting a fluorescent product, DCF, when the substrates are present in solution. Competitive diffusion of the substrates to the wires and the product away from the surface of the wire, remains a problem, thus making identification difficult in a multiplexed format, but this may be solved by encapsulating the nanowires in a gel material to slow diffusion. Due to the simplicity of immobilizing enzymes onto particle surfaces using polyelectrolyte multilayers, the production of biosensors is still a feasible goal.

### 5.4.2 Future Applications

The process of immobilizing molecular species onto nanoparticles with polyelectrolyte multilayers can be utilized for alternative applications. Other species, e.g., fluorescent dyes, can quickly and efficiently be immobilized onto the surface of nanowires without the need for covalent attachment, while also having control over the surface charge of the nanowires using the PEs (Figure 5-17).
By adding in free dye into the PE multilayers, striped wires can also have a fluorescent signal associated with them. This will be used for identification purposes in assembling different populations of nanowires. By having fluorescent dye immobilized in PE multilayers, the surfaces of the nanowires are charged, and the attractive van der Waals forces between the nanowire cores is compensated. The fluorescent tag allows nanoparticle tracking to better understand the diffusion of single nanowires within a separate population of nanowires (labeled with a second dye). This would provide a better understanding of interparticle interactions.

Figure 5-17: Reflectance (left) and false-colored fluorescence (right) microscopy images showing the immobilization of free AlexaFluor 633 dye within 3 polyelectrolyte bilayers of PSS/PAH-AlexaFluor 633 on the surface of uncoated ~6 µm Au nanowires. Scale bar = 5µm.
5.5 References


39. Literature reports that the isoelectric point for malate dehydrogenase is 5.0 for cytoplasmic MDH and 10 for mitochondrial MDH. The manufacturer (Sigma) reports a pI of 10 for their cytoplasmic product. Using 50 mM Tris buffer, pH 8.1, MDH was immobilized within PSS/PAH-MDH layers on nanowires and PAH/PSS-MDH on latex beads with MDH successfully getting incorporated in both situations.


Chapter 6

Field Effect Transistor Characterization for Nucleic Acid Sensing

The work done towards the fabrication of multiplexed field effect transistors to be integrated with CMOS technology is a highly collaborative process. The authors’ role was to study the effects of lithographic processing on DNA hybridization and to confirm photoresist deposition did not cause damage to the probe oligos. Additionally, the author worked on the biofunctionalization of dielectric materials and silicon nanowires. Work done in collaboration is noted within the text. The microarray experiment was done by Dr. Gary Clawson’s lab at the Hershey Medical Center with Dr. Mingwei Li from Dr. Theresa Mayer’s group in the Department of Electrical Engineering. The device fabrication was led by the Mayer group, specifically Xiahua Zhong and Kaige Sun. Dr. Wenchong Hu studied device stability in terms of the dielectric material. Xiahua Zhong also fabricated silicon nanowires lithographically. Dr. Thomas Morrow developed the assembly process for the controlled placement of nanowires to predetermined locations through dielectrophoretic forces. Kristi Liddell is working on the functionalization of dielectrics.

6.1 Introduction

There is a steady rise in the use of various forms of lithography to fabricate biosensors.\textsuperscript{1,2} Herein, three components of lithographically prepared devices were studied: 1.) the effect of lithographic processes on biomolecules, 2.) the functionalization of lithographically prepared silicon nanoparticles, and 3.) the functionalization of dielectric materials, e.g., SiO\textsubscript{2}, Al\textsubscript{2}O\textsubscript{3}, and HfO\textsubscript{2}. 
6.1.1 Biomolecule Damage Upon Exposure to Photoresists

Biofunctionalized nanoparticles are incorporated into sensing platforms, such as nanoelectromechanical sensors (Figure 6-1) or field effect transistors (Figure 6-2), to improve sensitivity for the detection of biomolecules by, e.g., the accumulation/depletion of charge carriers throughout the bulk of the nanowire in FETs. These devices are fabricated by assembling pre-functionalized nanowires onto a lithographically prepared chip, using multilevel photolithography to have control over the registry, i.e., positional accuracy, between layers of photoresist. Multilevel lithography results in multiple steps typically including deposition of numerous layers of photoresist and inter-level alignment steps during exposure. These processes introduce nanomaterials to a variety of conditions, which could be detrimental to nanowires prefunctionalized with biomolecules for the fabrication of biosensors. For fabrication of these devices, photoresists are spun onto the substrate, followed by UV exposure, and removal of photoresists with solvents; the photoresists and removers contact the biofunctionalized nanowires directly. Destruction of the biomolecules by the UV light exposure is prevented by covering the DNA functionalized nanowires with a photoresist during these steps; the photoresist absorbs the UV light, preventing the DNA from being damaged.
A variety of different photoresists exist for use in lithographic processes. These polymeric solutions vary in composition, viscosity, and cross-linking properties, allowing different photoresists to be used for different applications with control over the thickness of the material deposited, the light absorption properties, and therefore, the resultant...
Herein, the compatibility of three types of commonly used positive photoresists with biomolecules was studied. DNA immobilized on nanowires was exposed to Shipley 1813 and 1805, PMGI SF6, and Megaposit SPR 3012, which were chosen due to their use in fabrication of nanoelectromechanical and field effect transistor sensors. Additionally, these photoresists require different prebake temperatures, i.e., 100°C, 190°C, and 95°C for the Shipley, PMGI, and SPR 3012, respectively (Table 6-1).

The Shipley 1800 and SPR 3000 series photoresists are general use resists composed of a novolak resin (Figure 6-3 A) and a diazo photoactive compound (Figure 6-4). The PMGI series of resists are composed of a polyaliphatic imide copolymer (Figure 6-3 B) and are of particular interest due to the high thermal stability, adhesion to Si, and ability to undercut in a controlled manner.\(^8\)

<table>
<thead>
<tr>
<th>Photoresist</th>
<th>Curing Temperature Used (°C)</th>
<th>Curing Time Used (min)</th>
<th>Composition(^9)</th>
</tr>
</thead>
</table>
| Microposit Shipley 1813 | 100                         | 1                      | 71-76% propylene glycol monomethyl ether acetate  
10-20% mixed cresol novolak resin  
1-10% diazo photoactive compound  
0.01-0.99% cresol                                                             |
| PMGI SF6          | 190                         | 9                      | 65-85% cyclopentanone  
10-15% tetrahydrofurfuryl alcohol  
1-25% polyaliphatic imide copolymer                                             |
| Megaposit SPR 3012 | 95                          | 1                      | 55-65% ethyl lactate  
20-30% cresol novolak resin  
7-12% anisole  
<10% organic siloxane surfactant  
1-10% diazo photoactive compound  
1-5% 2-methyl butyl acetate  
1-5% n-amyl acetate  
<1% cresol                                                                   |
For the assembly of prefunctionalized nanowires for the top-down fabrication of FETs, not only are the particles being exposed to lithographic conditions, the nucleic acids bound to the surface of the nanoparticles are also being exposed. Morrow et al. demonstrated that nanowires functionalized with different probe sequences could be assembled to predetermined locations on a chip, and this process did not affect the ability of the DNA to hybridize a perfect complementary sequence. Although these results were promising towards the fabrication of chips with a high density of FET devices for
multiplexed sensing, damage to the nucleic acid structure could lead to false positives and/or negatives.

Much work has been done to detect damage to DNA in vivo and understand how damaged DNA leads to disease, e.g., cancer.\textsuperscript{14-16} DNA can undergo a variety of base modifications, such as deamination, oxidation, and alkylation.\textsuperscript{14-16} Particularly, DNA is susceptible to oxidative damage, resulting in the formation of 8-oxo-guanine most prevalently, due to its low ionization potential.\textsuperscript{17} Although significant work has been done to understand how endogenous species damage DNA sequences, leading to disease,\textsuperscript{15} significantly less work has been done to study the effect of processing conditions on DNA in vitro. With the increase in biosensor technology, this information will be essential as the degree of multiplexing is increased to prevent false positive/negative signal output from occurring due to, e.g., target DNA binding to modified probe sequences. Herein, probe oligos attached to silica coated nanowires were exposed to different lithographic conditions to determine if the DNA can discriminate a perfect match target sequence versus a single base mismatch. Silica coated nanowires were chosen to mimic the surface chemistry of silicon nanowires, as they are more readily available. This will provide insight to the extent, if any, of DNA damage upon exposure to photoresist materials.

6.1.2 Functionalization of Lithographically Prepared Nanoparticles and Dielectric Materials

Silicon nanowires used in FET devices are typically grown from catalyst particles by vapor-liquid-solid techniques.\textsuperscript{18,19} This synthetic procedure, although commonly used,
produces nanowires that are heterogeneous in length due to breakage of the nanowires and a low yield of the particles (Figure 6-5). Due to these disadvantages, our collaborators in Dr. Theresa Mayer’s group in Electrical Engineering are working on the production of lithographically prepared silicon nanoparticles. By the careful design of lithographic masks and through the use of electron beam lithography, rectangular nanoparticles have been prepared from a ultrahigh purity silicon wafer that are axially doped through a multi-step process. These lithographically prepared particles were etched to produce the size and final geometry desired. Since these nanoparticles were prepared lithographically, the yield, size, and quality are better controlled. Preliminary studies on the functionalization of the native oxide of these particles using nucleic acid sequences were completed to determine the feasibility of using these in biosensor devices.

Figure 6-5: FE-SEM image of silicon nanowires grown from gold nanoparticles (Image taken by Xiahua Zhong). Scale bar = 2 µm
Further characterization of FET devices has enabled a deeper understanding of the transfer characteristics of FETs in aqueous solutions.\textsuperscript{20} With devices becoming increasingly smaller, higher dielectric constant materials are desirable for passivation of devices.\textsuperscript{21} Due to the enhanced properties of these high \( k \) materials, the biofunctionalization of surfaces coated with \( \text{Al}_2\text{O}_3 \) or \( \text{HfO}_2 \) was studied. The amination process of these materials was monitored by the adsorption of gold nanoparticles, since a primary amine on the surface can be reacted to couple thiolated or carboxylated species through Sulfo succinimidyl-4-(\( \text{N}\)-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) or 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) chemistry, respectively.

6.2 Experimental Methods and Materials

6.2.1 Materials

Silver Cyless R RTU (3 troy oz/gallon) and Orotemp 24 RTU Rack (0.25 troy oz/quart) were acquired from Technic, Inc. Tetraethoxysilane, 98\% (TEOS) and 3-aminopropyltrimethoxysilane (APTMS) were acquired from TCI America. DNA sequences were purchased form Integrated DNA Technologies. Photoresists and remover were acquired from Micro-Chem. Sulfo succinimidyl-4-(\( \text{N}\)-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) was purchased from Pierce Protein Research Products, Thermo Scientific. Gold nanoparticles, 50 nm, were purchased from Ted Pella. Gold (III) chloride hydrate was acquired from Sigma-Aldrich. All water used was 18.2
MΩ-cm Nanopure water from a Barnstead system. All reagents were used as purchased without further purification; the silanes were aliquoted out in a glove box under nitrogen gas to prevent hydrolysis from water vapor in the air.

### 6.2.2 Nanowire Synthesis

Metallic nanowires were synthesized by galvanostatic electrodeposition into anodized aluminum oxide Whatman Anodisc 25 membranes with 0.2 µm pores (I = 1.65 mA) on an in-house system, as has been described previously. Silver (300 nm) was vapor deposited on one side of the membranes to act as the working electrode. A sacrificial silver layer was initially electrodeposited for 1 hr, followed by the deposition of the metal(s) of choice. After electrodeposition, the silver backing and sacrificial silver were dissolved in 33% (v/v) nitric acid. Nanowires were then released into suspension by vortexing the membrane in 3 M NaOH, and rinsed with 3 M NaOH, followed by DI H₂O, and three times with ethanol (EtOH) before suspension in 1 mL EtOH. One batch of nanowires produced ~10⁹ nanowires.

Nanowires were coated with silica in solution by the hydrolysis and condensation of tetraethoxysilane (TEOS), as previously described. Briefly, 490 µL EtOH, 300 µL nanowires at batch concentration, 160 µL H₂O, 40 µL TEOS, and 10 µL NH₄OH were combined in a microcentrifuge tube, sonicated 1 hr, and then rinsed 3× EtOH.
6.2.3 Microscopy

Nanowires were imaged using a Nikon TE-300 inverted microscope equipped with a Photometrics Coolsnap HQ camera, a Plan fluor 60× oil objective (N.A. 1.4), and Image-Pro Plus (v 7.0) software. FET devices were imaged using a Nikon Eclipse E800 upright microscope with Image-Pro Plus (v 7.0) software. Transmission electron microscopy experiments were performed using a JEOL JEM 1200 EXII transmission electron microscope at 80 keV accelerating voltage. Images were captured with a Tietz F224 digital camera. Field-emission scanning electron microscope (FE-SEM) images of the particle-nanowire conjugates were acquired using a Leo 1530 Field Emission Scanning Electron Microscope with a Schottky field-emission electron source and In-Lens secondary electron detector. FE-SEM images were collected with an accelerating voltage of 5.00 kV.

6.2.4 Preparation of DNA Sequences

DNA was received as a lyophilized pellet from Integrated DNA Technologies, Inc. The DNA used in this work can be found in Table 6-2; the flu sequences were designed from the Influenza virus sequences, identified by the CDC approved primer and probe sets. A seven base T AAC ATT spacer or ten T spacer was added to the functionalized end of the probe sequences to bring the oligos off the surface of the nanowire once immobilized. Thiolated DNA was cleaved by resuspension in a 100 µL 100 mM dithiothreitol (DTT) solution in 0.1 M sodium phosphate buffer, pH 8.3. After 1 hr the solution was purified using a Centrispin 10 desalting column (Princeton
Separations). The fluorescently labeled target sequences were rehydrated in water. The concentrations of all sequences were calculated based on absorbance values taken at 260 nm using a Hewlett-Packard 8453 diode-array UV/visible spectrometer with Agilent ChemStation software. Sequences were diluted to 20 µM in water and stored at -80°C. Sequences used as tags for nanoparticle conjugates were diluted to 100 µM in water.

6.2.5 Single Base Mismatch Assay

To determine if damage was occurring during lithographic processing, a single base mismatch assay was developed using a mismatch at position 6 from the 5’ end of the flu B target. Silica coated nanowires with flu B probe covalently attached via Sulfo-SMCC chemistry were rinsed into 35 µL 5× SCC buffer (20× SSC = 3 M NaCl, 0.3 M sodium citrate, pH 7.0) and 5 µL 20 µM flu B target DNA (perfect match or mismatch) was added (Table 6-2). The nanowires were tumbled or vortexed to keep them in suspension for 1 hr or overnight. The wires were rinsed with 5× SSC and then were imaged.
Table 6-2: DNA sequences used in this work.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Name</th>
<th>Sequence 5’→3’</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanowires</td>
<td>Flu B Probe</td>
<td>TTTTCAGTTCTTTTACAAT-Thiol</td>
<td>Probe DNA with 3’ T AAC ATT spacer with first 12 NT on 5’ end complementary to Flu B Target</td>
</tr>
<tr>
<td></td>
<td>Flu A Probe</td>
<td>GACCAATCCTGTTTACAAT-Thiol</td>
<td>Probe DNA with 3’ T AAC ATT spacer to act as a non-complementary sequence to Flu B target DNA</td>
</tr>
<tr>
<td></td>
<td>Flu B Target</td>
<td>AAGAACTGAAAA-Alexa Fluor 647</td>
<td>12 NT sequence perfectly complementary to flu B probe</td>
</tr>
<tr>
<td></td>
<td>Flu B Mismatch Target</td>
<td>AAGAAAATGAAAA-Alexa Fluor 647</td>
<td>12 NT sequence with single base mismatch at position 6 from 5’ end</td>
</tr>
<tr>
<td>Microarray</td>
<td>ASO&lt;sub&gt;683&lt;/sub&gt;</td>
<td>TGCCATCAAGATTTTCTCGTC</td>
<td>ASO targeting DD3 at the region immediately 3’ to nucleotide 683</td>
</tr>
<tr>
<td></td>
<td>ASO&lt;sub&gt;683&lt;/sub&gt;</td>
<td>TTTTTTTTTTTGCCATCAAGATTTTCTCGTC</td>
<td>ASO&lt;sub&gt;683&lt;/sub&gt; with 10 Ts added at the 5’ end as noted</td>
</tr>
<tr>
<td></td>
<td>ASO&lt;sub&gt;683&lt;/sub&gt;MM</td>
<td>GCCATCAAGACCTTTTCTCGTC</td>
<td>Single nucleotide mismatch of ASO&lt;sub&gt;683&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>ASO&lt;sub&gt;735&lt;/sub&gt;</td>
<td>GCT GCC TCA TGT CAT CAC AG</td>
<td>ASO targeting DD3 at the region immediately 3’ to nucleotide 735</td>
</tr>
<tr>
<td>Silicon Nanotile</td>
<td>DD3 Probe</td>
<td>GCT GCC TCA TGT CAT CAC AGT TTT TTT TTT-thiol</td>
<td>Probe sequence attached to silicon nanowires in fluorescence experiment</td>
</tr>
<tr>
<td></td>
<td>Noncomp Probe</td>
<td>CTC GTA TCT CAA CTC GTA TCT TTT TTT TTT-thiol</td>
<td>Probe sequence that is non-complementary to target DD3 DNA mimic</td>
</tr>
<tr>
<td></td>
<td>DD3 mimic</td>
<td>CTG TGA TGA CAT GAG GCA GCT TTT TGA CGA GAA AAT CTT GAT GGC-(Alexa 647)</td>
<td>Target sequence complementary to DD3 probe with Alexa label for fluorescence experiments</td>
</tr>
<tr>
<td></td>
<td>DD3 Tag</td>
<td>Thiol-TTT TTT TTT TGC CAT CAA GAT TTT CTC GTC</td>
<td>Thiolated tag sequence for nanoparticles conjugates</td>
</tr>
</tbody>
</table>
6.2.6 Effect of Photoresist Deposition on DNA Hybridization

The effect of the deposition and removal of photoresists on the ability of probe DNA to hybridize target DNA was studied by first attaching DNA to nanowires through thiol/Au chemistry or Sulfo-SMCC chemistry. Probe DNA with a 3’ thiol (10 µL, 20 µM) was added to 20 µL 6 µm Au nanowires (~2 × 10^7 nanowires) rinsed into 490 µL PBS buffer. After vortexing for 30 min, the nanowires were rinsed by centrifugation 3x PBS. The same thiolated probe DNA was attached to silica coated nanowires via Sulfo-SMCC chemistry, as previously described (Table 6-2). Nanowires, 10 µL (~ 1 × 10^7 nanowires), were rinsed into water, and were dried onto a glass microscope slide. Photoresist (Shipley 1813, PMGI SF6, or SPR 3012) was spun onto the glass slides statically (45 sec, 4000 rpm). The samples were baked as outlined in Table 6-1.

After the photoresist was deposited, the glass slides were cut into ~ 1 × 3 cm pieces and placed into 1165 photoresist remover (95% 1-methylpyrrolidinone), 50ºC, 30 min. After removal of the photoresist, the pieces were placed into microcentrifuge tubes filled with water and were sonicated 1 hr, followed by 1 hr vortexing, to knock the nanowires off of the surface of the glass slides. Nanowires were spun down, rinsed 1x buffer, and were resuspended in either 190 µL PBS and 10 µL 20 µM flu B target DNA for the direct hybridization studies or 35 µL 5x SSC buffer and 5 µL 20 µM flu B target or mismatch DNA (Table 6-2). The samples were vortexed overnight and then rinsed 4-8× buffer. The nanowires were imaged on the inverted microscope using appropriate filter cubes. Fluorescence intensity on the nanowires was quantified using NBSee software from Nanoplex, Inc.
In parallel, work was completed through a collaboration with Dr. Gary Clawson’s lab at the Hershey Medical Center using probe DNA specific for the prostate cancer marker DD3 that was immobilized in a microarray format using Codelink Activated Slides and a GeneMachines OmniGrid Microarraying robot (Table 6-2). After immobilization, Shipley 1805 was deposited and baked at 100°C for 1 min. Photoresist was removed using 1165 remover at either 55°C or 100°C for 10 or 5 min, respectively. Following photoresist removal, in vitro transcribed DD3 RNA (500 ng) that was prehybridized with Alexa Fluor-labeled ASO735 (at a ratio of 1:40, 1 hr, in Nexterion hybridization buffer) was hybridized to the spotted arrays for 1 h at 47°C in ArrayIt 1 × 16 Hybridization cassettes. The ability to distinguish mismatches was completed using a probe sequence with a single nucleotide mismatch (Table 6-2). Following hybridization of the DD3 to the probe sequence on the surface, the arrays were rinsed with 2× SSC + 0.2% SDS, 2× SSC, and then 0.2× SSC, for 30 sec each at room temperature. Once dried, the arrays were imaged using a GenePix 4000B Microarray Scanner with GenePix Pro or GeneSpring 7 software.

6.2.7 Biofunctionalization of Silicon Particles

The ability to covalently attach DNA to silicon particles was confirmed using nanoparticle conjugates and fluorescence studies using DNA mimic sequences for the prostate cancer marker DD3 (Table 6-2). Nanoparticle conjugates were prepared by adding 50 μL, 100 μM 5’ thiolated DNA tag sequence to 1 mL 50 nm Au nanoparticles in a non-stick microcentrifuge tube at 37°C, 1 hr. A salting procedure has been developed
to increase the coverage of the tag molecule on the surface of the nanoparticles: 1M NaCl, 10 mM phosphate buffer, pH 7.4 added every 30 min in 25 µL, 25 µL, 100 µL, 150 µL, and 128 µL aliquots.\textsuperscript{7,31,32} The conjugates were left at 37°C overnight. The surface of the nanotiles was aminated by suspension of the particles in 275 µL EtOH and 10 µL APTMS. After vortexing for 30 min the nanotiles were spun down (16 000 g, 1 min) and rinsed 3× EtOH, 3× 10 mM CHES buffer, pH 9.5. The nanotiles were resuspended in 150 µL CHES buffer, to which 1.0-1.5 mg Sulfo-SMCC cross-linker was added. The sample was vortexed 1 hr, followed by rinsing 3× CHES buffer and 3× PBS buffer. The sample was resuspended in 300 µL PBS and was aliquotted into two 150 µL samples. The DD3 probe sequence or the non-complementary probe was added (50 µL, 20 µM) to the centrifuge tubes. After vortexing 2 hrs the samples were rinsed 3× PBS and were resuspended in 180 µL PBS and 20 µL DD3 mimic (target sequence). The samples were vortexed 1 hr. During this time the conjugates were rinsed (8100 g, 10 min) 3× PBS and were resuspended in 100 µL buffer. The nanotiles were rinsed 3x PBS, the supernatant was removed, and 100 µL nanoparticle conjugates were added directly to the nanotile pellet. After vortexing 1 hr the samples were rinsed with PBS and resuspended in buffer.

Samples for a fluorescence assay were prepared similarly by first aminating the surface of the nanotiles and then functionalizing with Sulfo-SMCC, which linked the thiolated probe sequences to the aminated surface. The Sulfo-SMCC functionalized nanotiles were resuspended in 50 µL PBS buffer and 16.7 µL 20 µM DNA was added, and the samples were vortexed 2 hrs, followed by rinsing. Once the probe DNA was
attached, 16.7 µL 20 µM Alexa Fluor 647 labeled target DNA was added to 433 µL PBS (Table 6-2). The samples were vortexed overnight and then rinsed 3× PBS, followed by analysis on the inverted microscope.

6.2.8 Biofunctionalization of Dielectric Materials

To determine if alternative dielectric materials could be functionalized with biomolecules using well-understood chemistry, e.g., Sulfo-SMCC chemistry to cross-link thiolated nucleic acids to an amine, the reaction between 3-aminopropyltrimethoxysilane (APTMS) and the dielectric was studied. This silane reaction was characterized by the adsorption of negatively charged, citrate reduced ~13 nm gold nanoparticles (~17 nM). Al₂O₃ or HfO₂ (10 nm) was deposited onto the surface of glass slides using atomic layer deposition. These coated slides were acquired, cut into ~1 × 3 cm pieces, rinsed with EtOH, and dried with N₂. The samples were placed into microcentrifuge tubes containing 1425 µL EtOH and 75 µL APTMS, and were left to react for 30 min at room temperature or were placed on a 90°C heat block for 5 hrs. Once cooled, the glass slide samples were rinsed with EtOH, 30 sec each side, and then water, 15 sec each side. The glass slides were placed in 3 mL 12.6 ± 0.1 nm gold colloid for 30 min, followed by rinsing in water, 15 sec each side. The samples were stored in water until analysis. The nanoparticle coverage on the surfaces was analyzed via visible spectroscopy (λ = 524 nm; εsurf = 2.8 × 10⁸).³³

In a separate experiment, functionalization experiments on Al₂O₃ were completed by taking ~1 × 1 cm pieces of 9 nm of oxide deposited onto silicon wafers, rinsing with
EtOH, and placing into centrifuge tubes containing 950 µL EtOH and 50 µL APTMS. After 30 min at room temperature, the pieces were removed and rinsed with EtOH and water. The samples were heated to 120°C for 1 hr to facilitate the reaction between the Al₂O₃ and the APTMS. After heating, the samples were rinsed with water and placed in 2 mL 14 ± 2.5 nm gold colloid for varying amounts of time. The samples were rinsed, allowed to dry, and imaged on the FE-SEM. Surface coverage was determined by counting the number of nanoparticles by hand and averaging over different locations of the sample.

6.3 Results and Discussion

Nanowires have been assembled to pre-determined locations using dielectrophoretic forces. This assembly process was integrated with a multi-step lithographic procedure, optimized primarily by Dr. Thomas Morrow of the Keating group, to prepare devices that could be used as field effect transistors (Figure 6-2). Briefly, gold electrodes were evaporated onto lithographically patterned areas on a silicon wafer. Well structures fabricated by undercutting PMGI photoresist were then produced to guide nanowires to predetermined locations during assembly. Following nanowire assembly, anchor windows were lithographically patterned over the ends of the nanowires to enable the electrodeposition of gold working electrodes for FET measurements. The final step of fabrication was lithographically isolating each device with a cut layer. This resulted in arrays of nanowire FETs, as shown in Figure 6-6.
6.3.1 Effect of Photoresist on DNA Hybridization

Fabrication of bioFETs for the detection of nucleic acid biomarkers will be accomplished by assembling nanowires prefunctionalized with single stranded DNA probes. The effect of lithographic processes was studied by drop casting functionalized nanowires onto a glass slide. Once dry, photoresist was deposited, baked, and later removed. Here, metallic nanowires (Au) were used due to their availability for the proof-of-concept of DNA survival. The mean fluorescence from hybridization of a fluorescently labeled target sequence to a complementary or non-complementary probe DNA on nanowires was used to evaluate the effects of the photoresist deposition/removal process on the biomolecules. The DNA was attached to the surface of the nanowires through thiol/Au chemistry (Figure 6-7) or by attaching thiolated DNA to silica coated nanowires through Sulfo-SMCC chemistry (which is comparable to the native oxide on silicon nanowires in terms of the attachment chemistry) (Figure 6-9).
For the thiolated DNA on gold nanowires, fluorescence values were low due to quenching of the fluorophore on the target DNA by the metallic nanowire surface (Figure 6-7). Minor decreases in fluorescence were observed after photoresist exposure and removal, presumably due to the bake temperatures being ≥ 95°C, causing thiolated DNA to desorb from the surface of the gold (Table 6-1). A significant decrease in fluorescence was observed for the DNA exposed to PMGI SF6 and baked at 190°C for 9 min, indicating very little hybridization of the target DNA to the probe sequence. The observed decrease in fluorescence could be the result of desorption of DNA from the nanowire surface and/or damage to the probe sequence, preventing hybridization of the target. Lindahl has reported that at temperatures greater than 100°C, DNA is prone to hydrolytic damage, which would inhibit hybridization to the target sequence. The large error and minor signal observed in the non-complementary control samples was the result of significant background fluorescence from target DNA adsorbed to the surface of broken glass in the sample (from vortexing the glass slides) (Figure 6-8). We had difficulty with powdered glass fragments particularly in this sample. The nanowires showed little to no signal.
Figure 6-7: Fluorescence intensity of hybridized flu B target DNA to complementary (dark grey) or non-complementary (light grey) probe DNA attached to the nanowire surface via thiol/Au bonds after exposure to and removal of photoresists on a surface.
Decreases in fluorescence intensity, indicative of decreased hybridization, were not observed for the DNA attached to silica coated nanowires through Sulfo-SMCC chemistry after exposure to Shipley 1813 and SPR 3012 (Figure 6-9). Although there was a slight decrease in hybridization with the DNA exposed to PMGI SF6, potentially due to the high bake temperature, as noted above, the probe molecules are still capable of binding target DNA. The ratio of fluorescence of the probe post PMGI exposure in the presence of the complementary sequence versus the non-complementary is 81:1 compared to 88:1 for the control. These samples show excellent discrimination against the non-complementary probe, as well. Higher signal overall is observed in comparison to the DNA bound to Au nanowires through thiol/Au chemistry due to the silica coating.

Figure 6-8: Reflectance and false-colored fluorescence images of hybridization of a fluorescently labeled target sequence to probe oligos attached to the nanowire surface via Au/thiol chemistry. Scale bar = 5 µm
bringing the fluorophore off of the surface of the nanowire. Additionally, by use of a Sulfo-SMCC crosslinker, the thiolated probe DNA was covalently attached to the nanowire which minimized desorption upon heating, as observed with the thermally labile thiol/Au chemistry. The literature reports a loss of 47-53% loss of probe DNA bound via Sulfo-SMCC chemistry to a microscope slide, while a loss of only 16% was observed for DNA attached similarly to silica coated nanowires, upon thermocycling to a maximum temperature of 94°C. It is unclear why the SPR 3012 enhanced the fluorescence of the DNA, producing 511× greater fluorescence when exposed to the complementary sequence versus the non-complementary. It was initially hypothesized that it was not fully removed in the 1165, but the photoresist exhibited no inherent fluorescence when tested on the fluorimeter. The components of the dissolved photoresist may be interacting with the labeled DNA in a way that enhances fluorescence.

Figure 6-9: Fluorescence intensity of hybridized flu B target DNA to complementary (dark grey) or non-complementary (light grey) probe DNA attached to the silica coated nanowire surface via Sulfo-SMCC chemistry after exposure to and removal of photoresists on a surface.
Due to the multiple lithographic steps DNA functionalized nanowires are exposed to during FET device fabrication to produce the structures in Figure 6-6, the effects of these materials on the biomolecule function must be analyzed. The effect of photoresist deposition on DNA in a microarray has been studied by Dr. Gary Clawson’s lab at Hershey Medical Center in collaboration with Drs. Keating and Mayer at Penn State University. Microarrays are commonly used for multiplexed detection of nucleic acids by spotting probe DNA in predetermined locations, and can be used to determine optimal conditions in a high throughput manner.

Here, the DNA, specific for prostate cancer marker, was immobilized onto a Codelink microarray slide. After immobilization, Shipley 1805, which is a similar photoresist to Shipley 1813, also containing a mixed cresol novolak resin, a diazo photoactive compound, cresol, and fluoroaliphatic polymer esters, was applied to the microarray and baked for 1 min at 100°C. The photoresist was removed using 1165 remover, and the microarray was exposed to the prostate specific target oligo that was prehybridized to a fluorescent tag sequence. In addition to the complementary target, the probe molecule was tested against a single base mismatch. Figure 6-10 demonstrates that the probe DNA was able to hybridize the complementary target sequence effectively and discriminate a single base mismatch. Although both removal conditions produced high signal for the complementary sequence compared to the non-complementary, higher fluorescence was observed when the photoresist was removed for 10 min at 55°C, presumably due to a more complete removal of the photoresist. The addition of a ten T spacer also improved hybridization by bringing the DNA off of the surface of the nanowires, minimizing stercics.
The ability of the DNA on a microarray to discriminate a mismatch provided evidence that the photoresist deposition was not damaging the DNA. We wanted to confirm this to be true for DNA covalently attached to nanowires after exposure to exact photoresists, removers, and bake temperatures used for device fabrication. The additional photoresists studied above in the direct hybridization assays were evaluated using a single base mismatch assay to confirm deposition did not cause damage to the DNA immobilized on the nanowires, specifically oxidative damage. DNA covalently attached to silica coated nanowires were drop cast onto a surface. Photoresist was spun on,
heated, and then removed with 1165 photoresist remover, 50°C, 30 min (Table 6-1). A single base mismatch target sequence was developed substituting an adenine nucleobase in place of the cysteine. The single base mismatch experiments should be more sensitive to subtle change in DNA structure due to damage of individual bases, e.g., oxidation, hydrolysis, alkylation. Additionally, this particular substitution was chosen since the presence of 8-oxo-dG in vivo results in mutagenesis due to G:C to T:A transversions during synthesis.36

Single base mismatch assays are more challenging than the direct hybridization assays presented above. Hybridization conditions, e.g., buffer selection, temperature, sequence concentration, rinsing, must be optimized to discriminate a perfect match from a mismatch.37,38 The rinsing protocol and hybridization conditions are essential for a sequence to discriminate a single base mismatch, as high salt buffers facilitate DNA binding. Optimization of this assay was performed by attaching flu B probe DNA to silica coated nanowires, as previously described, and modifying hybridization conditions used in the microarray experiments. Hybridization of fluorescently labeled target molecules, i.e., complementary and containing a single base mismatch, was done at room temperature in 5× SSC buffer. Figure 6-11 demonstrates these conditions provided discrimination of the probe to a single base mismatch with 9.9 times more signal for the perfect match compared to the mismatch.
The DNA exposed to photoresist was also able to discriminate a single base mismatch (Figure 6-12). A slight increase in the fluorescence intensity from the hybridization of the probe molecule to the single base mismatch target in the control sample, compared to the initial experiments done to optimize the binding conditions, is most likely due to changes in the number of wires, i.e., probe concentration, due to less control of the number being sonicated off the surface of the glass slides. Very similar fluorescence intensities were observed for the DNA exposed to Shipley 1813 and SPR 3012 as to the control not exposed to photoresist. The DNA exposed to PMGI SF6 did not demonstrate discrimination against a single base mismatch, indicating damage after this process. Decreased fluorescence was observed after exposure to this photoresist with the direct hybridization assay, but hybridization of a target sequence still was clearly occurring (Figure 6-12). This further supports damage occurs upon exposure to the
PGMI resist with a 9 min bake at 190°C. This is not problematic for the assembly process to fabricate multiplexed FETs, as outlined in reference 7, as the PMGI photoresists are never deposited on top of functionalized nanowires, but it is valuable information for the development of processing conditions for new device fabrication.

**Figure 6-12:** Fluorescence intensity of hybridized perfect match target DNA (dark grey) or target DNA with a single base mismatch (light grey) to flu B probe covalently attached to silica coated nanowires via Sulfo-SMCC chemistry on a surface post deposition and removal of photoresist.

### 6.3.2 Biofunctionalization of Lithographically Prepared Silicon Nanoparticles

In addition to optimizing photoresist processes to ensure DNA survivability, the choice of particle is essential for the development of reproducible, multiplexed biosensors. Currently, silicon nanowires are used for the development of nanoFETs. NanoFETs have enhanced sensitivity compared to planar FETs due to the accumulation/depletion of charge carriers throughout the bulk of the material, as opposed...
to just the surface.\textsuperscript{39} Silicon wires as prepared by VLS methods often are heterogeneous in length, which is problematic in assembly applications. Wires need to be accurately placed in predefined locations for proper registry in device fabrication. Thus, there is a need for alternate synthetic methods for silicon wires. Xiahua Zhong in the Mayer group has developed a lithographic process to fabricate axially doped silicon nanotiles (Figure 6-13 A).

The nanotiles have a surface oxide; therefore, functionalization of the particles was expected to be straightforward and identical to silica coated nanowires. Using Sulfo-SMCC cross-linking chemistry, probe DNA was attached to a batch of nanotiles that was functionalized with APTMS in ethanol. Nanoparticle conjugates were initially used as a detection mechanism through a sandwich hybridization assay. Due to the geometry of these nanotiles (300 nm thick × 5 \(\mu\)m long × 1.5 \(\mu\)m wide), it was necessary to spin these particles down very fast (16 000 g) in order to pellet the particles. Multiple rinse steps were necessary to isolate the functionalized nanotiles from the excess nanoparticle conjugates. Figure 6-13 B shows a FE-SEM image of a functionalized nanotile. The high density of nanoparticles on the surface indicates good surface coverage of the DNA probe. Unfortunately, non-complementary samples could not be isolated. We hypothesize that the added mass from the nanoparticles on the complementary sample enabled better pelleting during rinsing. Although this data does demonstrate functionalization of the nanotiles, a fluorescence assay was done to validate the successful functionalization. Direct hybridization of a fluorescently labeled target sequence to complementary and non-complementary probe DNA confirmed the nanotiles
were biofunctionalized, and that the bound probe DNA retained selectivity for the complementary strand (Figure 6-14).

Figure 6-13: (A) TEM of a silicon nanotile prepared lithographically (scale bar = 2 µm). (B) FE-SEM image of a biofunctionalized silicon nanotile with gold nanoparticle conjugates used for detection through a sandwich hybridization (scale bar = 1 µm). Silicon nanotiles fabricated by Xiahua Zhong.

Figure 6-14: (A) Reflectance and corresponding false-colored fluorescence images and the (B) fluorescence quantification of silicon nanotiles functionalized with probe DNA with a fluorescently labeled target directly hybridized to the surface; 58-60 nanotiles analyzed for each sample for statistical relevance. Silicon nanotiles fabricated by Xiahua Zhong. Scale bar = 5 µm
6.3.3 Biofunctionalization of Dielectric Materials

A third important characteristic of FET devices is the choice of dielectric material, which is particularly important for the stability of electrical measurements in aqueous solution. Generally, high dielectric constant, $k$, materials are desired for devices since capacitance is proportional to $k/d$, where $d$ is the gate dielectric thickness.\textsuperscript{21} The device current is enhanced between a source and drain when the capacitance of the dielectric is increased.\textsuperscript{21} SiO\textsubscript{2} has a $k=3.9$, with tunneling current increasing exponentially with decreasing thickness below 2 nm.\textsuperscript{21} The ion sensitive FET literature has reported decreased drift in the surface potential for Al\textsubscript{2}O\textsubscript{3} passivated FETs compared to SiO\textsubscript{2} passivated.\textsuperscript{20} Because of these disadvantages, passivation of FETs with Al\textsubscript{2}O\textsubscript{3} ($k=8$) was attempted.\textsuperscript{21} The stability of the electrical measurements of FETs was monitored over time in solution by applying a constant $V_{DS}$ and maintaining a constant $I_{DS}$ by adjusting the $V_{GS}$ (Figure 6-15).

![Figure 6-15: Schematic of solution-phase electrical measurements using a FET device.](image)
Figure 6-16 shows the results of the stability study on SiO$_2$ and Al$_2$O$_3$ passivated FETs. The drift in the voltage was significantly higher for the SiO$_2$ passivated device (80 mV) compared to the Al$_2$O$_3$ (8 mV) over 12 hrs. Additionally, the Al$_2$O$_3$ demonstrated stabilization after 2 hrs. This demonstrated Al$_2$O$_3$ passivated devices had significantly less leakage current.

![Figure 6-16](image_url)

**Figure 6-16:** Long term stability tests for FETs with (A) 4 nm SiO$_2$ and (B) 4 nm SiO$_2$ / 9 nm Al$_2$O$_3$ gate dielectrics ($V_{DS} = 0.1$ V, $I_{DS} = 10$ nA). Data collected by Dr. Wenchong Hu, Electrical Engineering; reproduced from reference 20.

We therefore turned our attention to defining conditions where Al$_2$O$_3$ could be reproducibly functionalized. Since thiolated DNA can be reacted with Sulfo-SMCC, the amination reaction between APTMS and Al$_2$O$_3$ was studied. This can be studied systematically by the detection of citrate reduced gold nanoparticles adsorbed to the surface of ATPMS functionalized surfaces, as has been demonstrated on SiO$_2$ surfaces (Figure 6-17).$^{33,34}$
Functionalization of HfO$_2$ ($k=22$-25) was also studied. Figure 6-18 shows the absorbance spectra for the gold nanoparticles adsorbed onto (A) SiO$_2$, (B) Al$_2$O$_3$, and (C) HfO$_2$ surfaces treated with APTMS. The different spectra for each plot represents replicate samples prepared and analyzed to provide information to the reproducibility of the reaction between the APTMS and the dielectric surface. As expected, the SiO$_2$ coated surface could be reproducibly aminated, but this was not observed for the other dielectric materials. Evidence of aggregation of the nanoparticles was even observed for the Al$_2$O$_3$ surfaces, presumably due to APTMS not covalently bound to the Al$_2$O$_3$ surface desorbing off and into the colloidal solution.
Szczepanski et al. compared the stability of different silane molecules on alumina membranes and speculated based on the immobilization of a dye molecule to the amine groups on the surface that the increase in local pH from the amines is detrimental to the highly polarized Al—O—Si bond. Immobilization of a silane molecule with two amine groups was even less stable. Szczepanski et al. suggested the silane molecule initially physiosorbs to the alumina surface, and covalent attachment only occurs with increased temperature. The amination step was completed at 90ºC to facilitate the covalent modification of the dielectric surfaces (Figure 6-19). Heating did improve the reproducibility of the amination process.

Figure 6-18: Absorbance of 13 nm Au nanoparticles on the surface of aminated (A) SiO₂, (B) Al₂O₃, and (C) HfO₂, materials commonly used as gate dielectrics in FET devices. The different spectra correspond to replicate samples.
A second set of conditions that was studied was the immersion of Al₂O₃ on Si wafers in APTMS solution at room temperature, followed by 2 hr at 120°C to promote the reaction of the physically sorbed silane molecules. The samples were then left in gold colloid solution for varying amounts of time. Upon drying and analysis on the FE-SEM, the surface coverage of nanoparticles could be determined. The surface coverage versus time can be found in Figure 6-20. The diffusion of spherical particles to a planar surface is proportional to \( t^{1/2} \), as seen in Equation [1];\(^{33,34,41,42}\) the data on Al₂O₃ showed minor deviation from this trend. By taking the coefficient, \( k \), from the \( t^{1/2} \) plot, the sticking probability, \( p \), can be calculated for Al₂O₃ using Equations [1] and [2]:

\[
\Gamma = kt^{1/2} \tag{1}
\]

\[
k = pC\left(\frac{D}{2}\right)^{1/2} \tag{2}
\]
where $\Gamma$ is the surface coverage of Au nanoparticles on the surface (#/cm$^2$), $C$ is the concentration of particles (#/cm$^3$), $t$ is the time (sec), and $D$ is the diffusion constant of the particles (cm/sec).\textsuperscript{33,34,41,42} The diffusion coefficient is defined in Equation [3],

$$D = \frac{kT}{6\pi\gamma r}$$

[3]

where $k$ is the Boltzmann constant ($1.38 \times 10^{-16}$ g cm$^2$ s$^{-1}$K$^{-1}$), $T$ is the temperature (K), $\gamma$ is the viscosity of the solution (for water, 0.010 g cm$^{-1}$ s$^{-1}$), and $r$ is the radius of the particle (cm).\textsuperscript{33,41,42} The sticking probability is related to the kinetics of the interaction between the gold nanoparticles and the surface, and is a ratio of the number of particles that are actually bound to the surface compared to the number that reach the surface.\textsuperscript{34,41} This value was calculated to be 0.4 for APTMS functionalized Al$_2$O$_3$, which is considerably lower than the 1.2 ± 0.4 reported for a mercaptosilane-functionalized SiO$_2$ microscope slide, reported in the literature,\textsuperscript{34} but is similar to the sticking probability reported for 12 nm Au colloid adsorbed onto an APTMS functionalized glass surface, $p=0.52$ at pH 5.5.\textsuperscript{42}

The difference between the two reported values is attributed to different mechanisms of adsorption onto thiol versus amine groups.\textsuperscript{42} These data demonstrate that although these dielectric materials can be functionalized similarly to SiO$_2$, the process is not as reproducible. Also, desorption of the silicon alkoxide over time from the surface of the Al$_2$O$_3$ has been observed in the literature.\textsuperscript{40} New attachment chemistries will need to be explored.
6.4 Conclusions

The work presented in this chapter investigated three main characteristics of field effect transistors for optimized device fabrication. Through direct hybridization and single base mismatch studies, it was concluded that DNA can be exposed to photoresists that do not require a high temperature bake step (<100°C), but exposure to PMGI SF6 that is heated to 190°C for 9 min is detrimental to the probe DNA. It can not be said with certainty if it is just the high temperature bake step that is detrimental to the DNA, or if an interaction with some component of the PMGI photoresist is causing damage. Regardless, having the knowledge of what photoresists are biocompatible will help in the design of fabrication processes. The study of the effect of different photoresists, e.g., negative resists, or further processing steps, e.g., plasma etch, electrical assembly, on
nanowires functionalized with probe oligos can provide further information about how lithographic processes effect DNA binding and/or if oxidative damage is occurring.

In separate experiments, the biofunctionalization of lithographically prepared nanoparticles and dielectric materials was studied. Lithographically prepared nanoparticles can be made in high yield with excellent control over the size, geometry, and doping profile, and will enable the replacement of VLS silicon nanowires. The native oxide on these particles could be functionalized with Sulfo-SMCC cross-linking chemistry to couple thiolated nucleic acids and primary amines, in the same way as silica coated nanowires. Further optimization of the fabrication of these nanoparticles is currently being studied by Xiahua Zhong in the Mayer group.

Due to the improved properties of higher dielectric constant materials, e.g., Al$_2$O$_3$, on the electrical properties of passivated devices, the biofunctionalization of these materials was studied. The Al—O—Si bond is less stable than Si—O—Si, producing decreased functionalization with the APTMS. By heating the samples during/after the amination step, the extent of covalent attachment of physiosorbed APTMS can be improved. These data indicate that Al$_2$O$_3$, and to a greater extent HfO$_2$, can be aminated, and therefore, biofunctionalized, but the control over surface coverage and reproducibility is not as great as with SiO$_2$. Different attachment chemistries, e.g., phosphonic acids in place of APTMS, are currently being explored.
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Chapter 7

Conclusions and Future Directions

7.1 Conclusions

The work presented in this dissertation investigated the interaction of biomolecules and nanoparticles. By understanding the surface chemistry of nanoparticles, and different methods of immobilization or attachment of biomolecules onto nanoparticles, advancements in biosensor technology can be realized. Surface chemistry modifications with silica coatings and polymers were explored, with the immobilization of biomolecules through direct adsorption, cross-linker molecules, and through the layer-by-layer assembly of proteins in polyelectrolyte multilayers.

The work presented in Chapter 2 introduced organically modified silica coatings for metallic nanowires. Silica coatings for nanoparticles offer advantages, including biocompatibility, protection of the core particle, and attachment of biomolecules through well-understood silane chemistry. Organically modified silicon alkoxides were included in the reaction mixture, in addition to tetraethoxysilane (TEOS), which enabled incorporation of different functional groups, such as dihydroimidazole and polyethylene oxide. The incorporation of these functional groups was characterized using transmission electron microscopy (TEM) and infrared spectroscopy. Proof-of-concept experiments
demonstrated application for these coatings by the binding of chromate ions from solution using the dihydroimidazole-containing silica coated nanowires and protein resistance with the polyethylene oxide coatings. Additionally, the benefits of SiO$_2$ were retained, as demonstrated by the attachment of DNA sequences using Sulfo-SMCC cross-linking chemistry. These results have been published in *Langmuir*.$^1$

Chapter 3 expanded upon the chemistry presented in Chapter 2 by producing Surface Enhanced Raman Spectroscopy substrates by coating Au/Ag alloy nanowires with the polyethylene oxide-containing silica. Once coated, the nanowires were resuspended in ~ 33% HNO$_3$ to etch the sacrificial silver, resulting in silica coated, porous gold nanowires. The nanostructure of these wires enhanced the inherently weak Raman signal. Detection of a small molecule, 4-mercaptobenzoic acid, was accomplished, even after the nanowires were exposed to serum concentrations of protein (1 mM BSA), showing promise towards the detection of metabolic products in biologically relevant solutions. Additionally, the silica coating provided structural strength to the nanowires, which enabled vortexing, sonication, and handling of the nanowires without breakage of the brittle porous nanowires. This work is being prepared for submission to *Chemistry of Materials*.$^2$

The work in Chapter 4 investigated a simple immobilization strategy of an enzyme onto a metallic nanoparticle surface through direct adsorption. Direct adsorption is one of the most common methods of biomolecule attachment to nanoparticles, but it is often not well characterized. The work presented in this chapter, in collaboration with Dr. Ann-Sofie Cans, investigated the adsorption of horseradish peroxidase (HRP) and fluorescein-labeled HRP (FITC-HRP) onto 13 nm Au colloid. Quantification of the HRP
on the nanoparticle surfaces revealed multilayer formation. The stability of the enzyme:nanoparticles complexes were studied using both the traditional flocculation assays and also zeta potential measurements, in addition to the specific activity of the enzyme. Comparisons between HRP and FITC-HRP:Au complexes indicated differences in stability, particularly at pH 8.0. This work was published in *Nanobiotechnology*.

The work presented in Chapter 5 expanded upon the immobilization of proteins onto the surface of nanoparticles, presented in Chapter 4, for the development of enzymatic suspension array biosensors for the detection of small molecules. Two immobilization strategies were explored, including encapsulation within a silica shell and within polyelectrolyte multilayers. The layer-by-layer assembly of polyelectrolytes proved to be a more successful immobilization approach for detectable concentrations of enzymes on the nanowire surface. By the alternate assembly of poly(styrene sulfonate) and poly(allylamine hydrochloride) onto the surface of nanowires in the presence of the enzyme of interest a variety of enzymes with a range of molecular weights were immobilized onto different populations of nanowires. Characterization of the enzymes once immobilized was essential for the development of reproducible and reliable biosensors; this was accomplished by the immobilization of malate dehydrogenase and citrate synthase onto latex beads. It was concluded that a significant loss in activity was observed upon immobilization, but was less severe when the enzyme was within the layer closest to the surface of the particle, presumably due to ease of diffusion of the substrates. Progress towards an enzymatic biosensor for H$_2$O$_2$ was accomplished using immobilized horseradish peroxidase on nanowires, but diffusion of the fluorescent product away from
the nanowire remained a problem. Optimization of this system will help realize the goal of producing enzymatic biosensors.

The final chapter of this dissertation explored the effects of lithographic processes on biomolecules. Lithography has been used throughout the field of biosensing to fabricate more complex structures and devices, such as field effect transistors, for the electrical detection of molecules. Our group previously showed that nanowires can be pre-functionalized with DNA and assembled into predetermined locations on chip through dielectrophoretic forces. Once assembled, source and drain electrodes can be electroplated using lithography to fabricate isolated FET devices. Although direct hybridization of the perfectly complementary sequence was accomplished post lithography, the effects of the lithographic process needed to be further explored to enable multiplexed sensing of DNA. DNA was attached to nanowires through thiol-Au chemistry or using a cross-linking molecule onto a silica coating. The nanowires were exposed to different lithographic conditions. If the cure temperature was <100ºC, hybridization of the target molecule to the probe was observed on the surface of the nanowire. Additionally, it was demonstrated that the DNA could still distinguish a single base mismatch, indicating minimal to no oxidative damage to the DNA. The DNA survivability work presented here is being compiled for publication.

7.2 Future Directions

The knowledge acquired about surface properties of nanoparticles and different attachment chemistries for biomolecules will be used to fabricate biosensors for the early
detection of diseases. The early detection of disease, e.g., cancer, could significantly improve the survivability rate by providing treatment at the onset of the disease prior to it spreading throughout the body. We believe the future of biosensing is in the electrical detection of biomolecules using field-effect transistors to overcome a lot of the current limitations in the field. For example, chip-based sensors can be integrated with CMOS technology to enable point-of-care detection in a timely fashion, inexpensively, and without excessively trained personnel. Additionally, the ability to multiplex and sensitivity are improved using FET based sensors. By incorporating in multiple nanowires with the same probe sequence immobilized, statistically relevant results can be obtained without false positives or negatives.

Further optimization of the sensor needs to be accomplished prior to actual sensing. It has already been shown that FET stability can be enhanced using Al$_2$O$_3$ as the dielectric, compared to just SiO$_2$. Different attachment chemistries are being explored for the covalent immobilization of probe DNA on the surface of the dielectric coated nanowire, i.e., phosphonic acid functionalized Al$_2$O$_3$ and EDC coupling chemistry. It is desirable to have controllable surface coverages of probe DNA on the surface of the nanowires for reproducible analysis. Once this is accomplished, the survivability of the DNA must again be explored to ensure the lithographic processes are not affecting the attachment chemistry.

Furthermore, an alternate for VLS grown silicon nanowires is currently being explored by our collaborators in the Theresa Mayer group in Electrical Engineering. VLS grown silicon nanowires are produced in low yield and are often heterogeneous in length due to breakage. As introduced in Chapter 6, Xiahua Zhong is making great
progress in the fabrication of lithographically prepared, axially doped silicon nanowires. Access to well controlled, doped silicon nanowires will move this project forward quickly, enabling the pre-functionalization of the nanowires prior to FET fabrication.

Finally, once the FET devices have been optimized through new stable attachment chemistries to the Al₂O₃, biomolecules can be detected in a multiplexed fashion. To enable detection in biologically relevant solutions, i.e., high salt concentrations, a rinse step or an initial purification step may be necessary. Additionally, microfluidics could be used to produce lab-on-a-chip devices. In the future, it would be desirable to incorporate aptamers as the probe molecule, in addition to DNA, to enable the detection of nucleic acids, proteins, and small molecules all on the same device for a complete detection tool for use in the field or in physician offices for diagnostics.
7.3 References


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