Novel Biosensing Platforms with Advanced Nanomaterials for Electrochemical and Optical Detection of Proteins and Viruses

A Dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Chemistry

by

Bryce William Davis

Dissertation Committee:
Dr. Quan Cheng, Chairperson
Dr. Yinsheng Wang
Dr. Cynthia Larive
The Dissertation of Bryce William Davis is approved:

______________________________________

______________________________________

______________________________________

Committee Chairperson

University of California, Riverside
ACKNOWLEDGEMENTS

My path into graduate school has been untraditional, but as a chemist I have learned that things don’t always go the way you think they should. When I graduated high school, I decided to join the Navy and spent 6 years in service. When I left the US Navy in 2001, I did not know that I would end up studying chemistry, let alone pursue a doctoral degree. Although my career in the Navy as an Explosive Ordnance Disposal (EOD) technician was very intense, little did I know that the attention to detail required to dispose of bombs safely would be the perfect preparation I would need to study science. Aldous Huxley said, “Experience is not what happens to you; it's what you do with what happens to you.” This quote has served as tremendous motivation because the variety of experiences in my life have inspired me to go into a field where through trial and error one can makes steps towards a scientific discovery.

First and foremost, I would like to thank my advisor Prof. Quan (Jason) Cheng who always offered me his encouragement, unrelenting support and, most of all, patience. Dr. Cheng was always available to discuss the complexities of research, but most importantly his mentorship developed my ability to solve problems independently. I feel fortunate and proud to have had the opportunity to carry out research at UCR, alongside professors who excel in both the lecture and laboratory areas of academia. I am also extremely grateful to my committee members, Prof. Yinsheng Wang and Prof. Cynthia Larive, for their oversight, guidance, expertise and for taking precious time from their demanding schedules to help me during this process.
I would also like to thank all of my labmates – Dr. Thomas Wilkop, Dr. Jong Ho Han, Dr. Scott Phillips, Dr. Thomas Owen, Dr. Zhuangzhi “Max” Wang, Dr. Guangyu Ma, Dr. Yi Dong, Dr. Na Zhang, Dr. Matt Linman, Ying Liu, Chih-Yuan (Derek) Chen, Junqing (Stuart) Xu, Andrew Burris and Fatma Buhajer – thank you for your valuable help and the wonderful time we spent together at the Cheng Lab. I also had the opportunity to work with three undergraduate students Harry Lee, Kamara Linley and Chris Hare and I would like to thank them for their contributions.

The nature of science itself would be greatly hindered without the concept of collaboration. I would like to thank those at UCR and elsewhere who empowered and enriched my research by sharing their wisdom and knowledge with me. First, Prof. Marylynn Yates and Dr. Jason Cantera (UCR, Environmental Microbiology) who were instrumental in the research project involving the enzyme-amplified electrochemical detection of poliovirus type 1 by providing the expertise and training in the cultivation of the concentrated stock virus solution. Prof. Mario Leclerc and Dr. Patricia Harding (University Laval, QC, Canada) also helped on my first project by providing a ferrocene-functionalized cationic polythiophene, a redox-active polymer, to incorporate into the nanoarchitecture of the electrochemical virus sensor. Next, Prof. Mongkol Sukwattanasinitt (Chulalongkorn University, Thailand) and his visiting graduate student Nakorn (Na) Niamnont played a critical role in the research and development of both optical nanofibers projects by synthesizing all the dendritic fluorophores and Polydiacetylene (PDA) compounds used. Lastly, Dr. Christopher Bardeen and Robert
Dillon were instrumental in my research by providing fluorescence lifetime analysis of the dendritic fluorophores used in the electrospun nanofiber pattern recognition project.

Last but not least, I would like to thank those in my family who were always supportive of my drive to gain a higher education. Thanks to my parents for all they have been through to raise me and for their sincere and unreserved love. Most importantly, I thank my best friend and beautiful wife, Laura, for being my support through this long and tumultuous path. I truly appreciate you and thank God you are in my life. Thank you all for your unconditional love and for never giving up on me.
COPYRIGHT ACKNOWLEDGEMENTS

The text and figures in Chapter 2 are reprinted from *Electrochimica Acta* 55 (2010) 4468–4474, with permission from Elsevier. The co-author Quan Cheng was the principle investigator of the research that forms the basis for this. The co-author Matthew J. Linman assisted with the atomic force microscopy experiments and interpretation. Co-authors Kamra S. Linley and Christopher D. Hare carried out some of the experiments.

The text and figures used in chapter 4 are reprinted from *ACS Appl. Mater. Interfaces*, 2010, 2 (7), pp 1798–1803. Copyright 2010 American Chemical Society. The co-author, Quan Cheng, directed and supervised the research that forms the basis for this chapter. Co-author Nakorn Niammont assisted with synthesis of the fluorescent dendrimers and their interpretation when incorporated into electrospun nanofibers, under guidance of co-author and Mongkol Sukwattanasinitt. Co-author, Christopher D. Hare, carried out some of the experiments.

The text and figures used in chapter 5 are reprinted from *Langmuir*, 2011, 27 (10), pp 6401–6408. Copyright 2011 American Chemical Society. The co-author, Quan Cheng, directed and supervised the research that forms the basis for this chapter. Co-author Nakorn Niammont assisted with synthesis of the fluorescent dendrimers and their interpretation when incorporated into an array of electrospun nanofiber surfaces, under guidance of co-author and Mongkol Sukwattanasinitt. Co-author, Robert Dillon assisted with the fluorescence lifetime experiments and interpretation, under the guidance of co-author Christopher Bardeen.
This dissertation is dedicated
To my Father, Richard W. Davis & Mother, Knoxie A. Mathis

and

In memory of my friend, Michael Marco
ABSTRACT OF THE DISSERTATION

Novel Biosensing Platforms with Advanced Nanomaterials for Electrochemical and Optical Detection of Proteins and Viruses

by

Bryce William Davis

Doctor of Philosophy, Graduate Program in Chemistry
University of California, Riverside, August 2011
Dr. Quan Cheng, Chairperson

Novel nanomaterials are actively sought by chemists, biologists and engineers for a variety of applications. Biosensor platforms used for diagnostic purposes would benefit greatly from the development of new electrochemical or optical transducer substrates. This dissertation is a detailed account of the research effort to develop novel nanotechnology platforms for advanced electrochemical and optical detection of biological molecules. Specifically, my goal was to design and fabricate operationally simple yet inexpensive new electrochemical and optical biosensors that are highly selective and sensitive.

The first section will cover the early work in the development of a porous polyelectrolyte interfaces with well-defined structure and electrochemical behavior using electrochemical surface plasmon resonance (ESPR) spectroscopy. This early work will also cover the application of the unobstructed electron transfer on porous polyelectrolyte nanostructures for the detection of poliovirus type 1 (PV1) using electrochemical enzymatic amplification. The electrochemical behavior of this sensor for whole viral particles is characterized using an enzymatic sandwich based immunoassay, with the final
antibodies tagged with an ultra sensitive electrochemically active enzyme. Two important factors that make this sensor design innovative is the high loading capacity within a 3-D nano-assembly and the unhindered fast electron transfer through the nanofilm to the electrode surface. The enzymatic amplification scheme is an alkaline phosphatase (ALP) system, which has shown superb properties to enhance the redox current of electroactive species in the presence of aminophenyl phosphate (APP).

The majority of the thesis will focus on the electrospinning technique and how electrospun nanofibers can be used to create novel optical transducer platforms. Electrospinning is a polymer processing technique used to create continuous fibers with diameters ranging from a few nanometers to micrometers, and is ideal for encapsulating functional units, especially fluorescent and optical receptors, for biosensing applications. This section of the thesis will be divided into three specific parts. The first part will focus on a solid-state reusable and porous nanofiber-based optical (fluorescent) transducer for detecting proteins using an array of fluorescent dendrimers. The second part will cover the details of an optical (colorimetric and fluorescent) transducer using electrospun nanofibers doped with the conjugated polymer polydiacetylene (PDA) for the detection of volatile organic compounds (VOCs) and proteins. Lastly, the third part will describe the development of 2D-silica nanofiber thin films doped with gold nanoparticles, their enhanced optical properties, and the potential use in future sensor technology.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><strong>CHAPTER ONE: Introduction</strong></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A. Biosensors</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1) Introduction</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2) Electrochemical Biosensors</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>3) Optical Biosensors</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>B. Design and fabrication of analytically significant nanomaterials</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>1) Self-Assembled Monolayers</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>2) Layer-by-Layer Assembly</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>3) Electrospun Nanofibers</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>C. References</td>
<td>48</td>
</tr>
<tr>
<td>2.</td>
<td><strong>CHAPTER TWO: Unobstructed Electron Transfer on Porous Polyelectrolyte Nanostructures and its Characterization by Electrochemical Surface Plasmon Resonance</strong></td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>A. Abstract</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>B. Introduction</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>C. Experimental Section</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>1) Materials</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>2) Preparation of Redox Active Self Assemble Monolayer Electrodes</td>
<td>63</td>
</tr>
</tbody>
</table>
3) Cyclic Voltammetry .............................................................. 64
4) Surface Plasmon Resonance ............................................... 64
5) Atomic Force Microscopy (AFM) ........................................ 65
6) Ellipsometry Measurements .............................................. 65

D. Results and Discussion ....................................................... 66
  1) Electrochemical Behavior of FUT/MUA Interface ................. 66
  2) Characterization of Electrostatic Self-assembly by SPR .......... 71
  3) Electrochemical Behavior of the Nanostructured Multiple Layers .......... 75
  4) AFM and Ellipsometry Characterization .............................. 82

E. Conclusions ........................................................................ 85

F. References ........................................................................ 86

3. **CHAPTER THREE: Enzyme-Amplified Electrochemical Detection of Viral Particles** ......................................................... 91

A. Abstract ............................................................................. 91

B. Introduction ........................................................................ 92

C. Experimental Section ........................................................ 95
  1) Chemical and Materials .................................................. 95
  2) Poliovirus type 1 (PV1) ...................................................... 96
  3) Electrochemical and SPR experiments ............................. 98

D. Results and Discussion ....................................................... 99
1) Design and fabrication of the electrochemical PV1 sensor 99
2) Enzyme activity and loading capacity. 102
3) Signal amplification and detection of PV-1 108

E. Conclusions 114
F. Reference 115

4. CHAPTER FOUR: Nanofibers Doped With Dendritic Fluorophores for Protein Detection 117
   A. Abstract 117
   B. Introduction 118
   C. Experimental Section 120
   D. Results and Discussion 126
   E. Conclusions 136
   F. References 137

5. CHAPTER FIVE: FRET detection of proteins using fluorescently doped electrospun nanofibers and pattern recognition 140
   A. Abstract 140
   B. Introduction 141
   C. Experimental Section 145
   1) Materials 145
2) Preparation of Polymer Solutions. 145
3) Electrospinning of Dendritic Fluorophore-doped CA fibers. 145
4) Preparation of Sensor Interface. 146
5) Lifetime Fluorescence Measurements. 147
6) Characterization of Protein Sensing. 147

D. Results and Discussion 148
   1) Electrospun Nanofibers with Fluorescent Dendrimers. 148
   2) Protein-Dependent Fluorescence Quenching. 156
   3) Protein-Specific Response Pattern by PCA. 166

E. CONCLUSIONS 170

F. References 172

6. CHAPTER SIX: Colorimetric and Fluorescence Detection with Electrospun Polydiacetylene (PDA)-Embedded Nanofibers 176

A. Abstract 176

B. Introduction 177

C. Experimental Section 182
   1) Materials 182
   2) Preparation of PDA-Embedded Electrospun Fibers 182
   3) PDA Fiber Fluorescence Measurements 183
   4) Colorimetric Experiments 184
   5) SEM and TEM studies. 184
### D. Results and Discussion

1) Organic Solvent Vapor Tests ................................. 184
2) Colorimetric Detection of STA .................................. 189

### E. Conclusions .......................................................... 195

### F. References ............................................................ 196

#### 7. CHAPTER SEVEN: Electrospun Silica Nanofiber Thin Films and Functionalization with Gold Nanoparticles .......................... 198

### A. Abstract ............................................................... 198

### B. Introduction .......................................................... 199

### C. Experimental Section .............................................. 202

1) Materials ............................................................... 202
2) Preparation of Fused Mesoporous Silica Nanofibers .......... 202
3) Characterization ...................................................... 204

### D. Results and Discussion ............................................ 205

1) Effects of Humidity and Electrostatic Discharge ............... 205
2) Morphology and Durability of Silica Nanofibers ............... 207
3) Fusing Silica Nanofibers onto a Glass Substrate ............... 211
4) Silica Nanofibers Functionalized with AuNPs .................. 215

### E. Conclusions .......................................................... 218

### F. References ............................................................ 219
8. CHAPTER EIGHT: Conclusion and Outlook

A. References
LIST OF FIGURES

Figure 1.1 Schematic of a biosensor that includes the bioreceptor (1), and the transducer (2). The physical change accompanying the reaction is converted into an electrical signal, amplified (3), processed (4) and displayed (5). ........................................ 4

Figure 1.2 A schematic illustration of direct (A) and indirect (B) electron transfer from the active site of the redox center of the enzyme to the electrode surface.20 ....................... 12

Figure 1.3 A schematic illustration of a standard Kretschman SPR biosensor setup and how a sensor chip is used to detect target analyte in a flowing solution (A). Also a schematic representation of a typical SPR sensorgram and how the interactions at the sensor interface corresponds to different responses in the sensorgram (B).47 ...................... 19

Figure 1.4 A schematic representation of the spectral overlap integral illustrating Förster resonance energy transfer (FRET).64 ................................................................. 25

Figure 1.5 (A) Topochemical photopolymerization of diacetylene monomers to form PDA CPs. (B) A schematic illustration of the molecular orbital in the π-conjugated PDA backbone and how the energy levels are affected by the overlap of the orbitals, which is caused by rotation of the C-C bonds in the backbones.76 .................................................... 29

Figure 1.6 Examples of typical absorption and fluorescence spectral changes upon environmental perturbation to PDA CPs.77 ................................................................. 30

Figure 1.7 (A) Schematic illustration of an ideal, single-crystalline SAM of alkanethiolates supported on a gold interface and its characteristics are highlighted. Schematic diagram of (B) a mixed SAM and (C) a patterned SAM. Both illustrations (B
and C) demonstrate how SAMs are used in current biosensor technology and biological studies using SAM architectures. 89

Figure 1.8 Schematic depiction of the electrostatic Layer-by-Layer nanoassembly of cation/anion multilayers onto flat and spherical surfaces (A). Schematic illustration of different types of cations and anions used (B); polyallylamine hydrochloride (PAH), polysodium 4-styrenesulfonate (PSS), and polyglutamic acid (PGA). 38

Figure 1.9 Relationship of the surface area versus fiber fineness (diameter). 41

Figure 1.10 Schematic diagram showing the three main components of a typical electrospinning set-up. 43

Figure 1.11 Potential applications of electrospun polymer nanofibers. 107 46

Figure 2.1 A schematic of LbL buildup of polymeric thin films on a mixed SAM with ESPR configuration and structure of polyelectrolytes and alkylthiol derivatives for self-assembly. 62

Figure 2.2 Cyclic voltammograms for MUA (curve 1) and 1:1 FUT/MUA (curve 2) in 0.1 M NaClO₄ (pH 6.6). The scan rate is 100 mV·s⁻¹. 67

Figure 2.3 Cyclic voltammograms for 1mM FUT in 0.1 M NaClO₄ (pH 6.6) at a scan rate of 100 mV/s. 69

Figure 2.4 Cyclic voltammograms for 1:10 (green), 1:5 (red), and 1:1 (black) FUT to MUA ratios in 0.1 M NaClO₄ (pH 6.6) at a scan rate of 100 mV/s. 70

Figure 2.5 SPR sensogram of a 10-layer nanostructure build up by alternating injection of oppositely charged polyelectrolytes in 0.1 M NaClO₄ on a 1:1 FUT/MUA SAM surface. Inset is an SPR sensogram of a 5-layer build up on a MUA surface. The circle illustrates
the specific electrostatic interaction of PAH with surface carboxylate (red curve) while PSS (black curve) shows no interaction with the negatively charged surface. Arrows indicate first (1) and second (2) injection of the polyelectrolyte demonstrating complete adsorption of the polymer in the first injection.

**Figure 2.6** SPR angular scan curves of reflectivity versus incident angle recorded after each layer of polyelectrolyte (top) and linear relationship between experimental and theoretical SPR minimum angle shifts for polymer films on SPR gold substrates (bottom).

**Figure 2.7** Overlay of the cyclic voltammograms taken after each layer of polyelectrolyte was adsorbed on a 1:1 FUT/MUA surface in 0.1 M NaClO$_4$. The scan rate is 100 mV·s$^{-1}$. Inset: The dependence of the $i_{pa}$ on the scan rate after each layer. ($n=11$)

**Figure 2.8** The average peak separation versus the scan rate up to 800 mV/s for all layers ($n=11$).

**Figure 2.9** (a) Zoomed-in SPR sensogram of a 10-layer nanostructure on a 1:1 FUT/MUA SAM surface in a 0.1 M NaClO$_4$ solution. The spikes indicate SPR response to CV cycles. Inset is the overlay of cyclic voltammograms at different scan rates (100-800mV). (b) Zoomed-in SPR sensogram of a 10-layer nanostructure on a MUA SAM surface in a 0.1 M NaClO$_4$ solution. Inset is the overlay of cyclic voltammograms at different scan rates (100-800mV).

**Figure 2.10** Atomic force microscopy (AFM) images of (a) bare Au, (b) 1:1 FUT/MUA SAMs, (c) 5 layer build up, and (d) 10 layer build up.
**Figure 3.1** Photographic image of the plague assay for poliovirus type 1 (PV-1) using Buffalo Green Monkey kidney (BGMK) cell lines. The stock solution was determined to be $5.3 \times 10^7$ pfu mL$^{-1}$. ............................................................ 97

**Figure 3.2** Schematic representation of the preparation of the amperometric ALP enzyme PV1 sensor based on the mixed SAM (FUT/MUA) and polymer bilayer (PAH/PGA) film. ............................................................ 101

**Figure 3.3** Enzymatic signal enhancement using an alkaline phosphatase (ALP) labeled anti-PV1. The ALP label converts p-aminophenyl phosphate (APP) into the electroactive compound p-aminophenol (AP). This product is electrooxidized to quinoneimine (QI) by the reduction of ferrocenium back to ferrocene, producing an electrocatalytic anodic current. ............................................................ 104

**Figure 3.4** SPR sensorgrams showing the difference in loading capacity of anti-PV-1 (100 µg mL$^{-1}$) on a SAM monolayer surface (left) and a polyelectrolyte 3D multilayer surface (right). ............................................................ 107

**Figure 3.5** Cyclic voltammograms of the enzyme-amplified electrochemical detection of poliovirus type 1 (PV-1) with (B-C) normal ($5.3 \times 10^6$ pfu mL$^{-1}$ PV-1) and (D) negative (10 µg mL$^{-1}$ rabbit IgG) control samples. A is the cyclic voltammograms of 5 bilayers (PAH/PGA) on a mixed SAM (FUT/MUA) modified Au electrode. In (B-C), Cyclic voltammograms of the (ALP) enzyme-amplified sandwich type detection (B) before and (C-D) after the incubation of 1mM APP for 10 min. In (D), rabbit IgG was used instead of PV-1. All Cyclic voltammograms were obtained using EB solution and at a scan rate of 50 mV s$^{-1}$. ............................................................ 111
**Figure 3.6** Cyclic voltammograms of a sandwich-type electrochemical virus sensor for poliovirus type 1 (PV-1) at various concentrations of PV-1: (A) 0 pfu mL\(^{-1}\), (B) 5.3×10\(^{1}\) pfu mL\(^{-1}\), (C) 5.3×10\(^{2}\) pfu mL\(^{-1}\), (D) 5.3×10\(^{3}\) pfu mL\(^{-1}\), (E) 5.3×10\(^{4}\) pfu mL\(^{-1}\), (F) 5.3×10\(^{5}\) pfu mL\(^{-1}\), (G) 5.3×10\(^{6}\) pfu mL\(^{-1}\), and (H) 5.3×10\(^{7}\) pfu mL\(^{-1}\). Cyclic voltammograms were obtained 10 min after incubation in EB containing 1 mM APP and at a scan rate of 50 mV s\(^{-1}\).

**Figure 3.7** Calibration plot showing the correlation between the anodic peak current at 0.2 V and the concentration of target poliovirus type 1 (PV-1) from 5.3×10\(^{1}\) pfu mL\(^{-1}\) to 5.3×10\(^{7}\) pfu mL\(^{-1}\) after incubation in EB containing 1 mM APP and at a scan rate of 50 mV s\(^{-1}\).

**Figure 4.1** A schematic illustration of the electrospinning setup, encapsulation of the fluorescent dendrimer, and deacetylation process used in this study are shown. The 5 water-soluble fluorescent dendritic compounds (AFD-1, AFD-2, AFD-3, AFD-4 and AFD-5) composing of phenylene-ethynylene repeating units are illustrated. The circled AFD-3 was the fluorescent dye used to dope the CA nanofibers for detection of metalloproteins.

**Figure 4.2** Photographic image of the fluorophore solutions (10 µM) in phosphate buffer saline (10 mM, pH 7.4) under black light (360 nm).

**Figure 4.3** FT-IR spectra of cellulose acetate (CA) and cellulose fibrous materials (top, before deacetylation; bottom, after deacetylation). The characteristic adsorption peaks attributed to the vibrations of the acetate group at 1745(\(\nu_{C=O}\)), 1375(\(\nu_{C-CH_{3}}\)), and 1235 cm\(^{-1}\)(\(\nu_{C-O-C}\)) disappeared after deacetylation of CA. An adsorption peak at 3500 cm\(^{-1}\)(\(\nu_{O-H}\)....
The FT-IR spectrum obtained after deacetylation agrees with that of pure cellulose fibers.

**Figure 4.4** SEM image of electrospun AFD-doped deacetylated cellulose fibers (17% CA/0.1% AFD dissolved in 8:1 acetone/water, 10,000× magnification).

**Figure 4.5** Fluorescence emission spectra of the AFD-functionalized nanofibers in response to varied concentrations of (A) cyt c, (B) Hgb, and (C) BSA ($\lambda_{\text{Ex}}/\lambda_{\text{Em}} = 370/475$ nm).

**Figure 4.6** Stern-Volmer plots of the nanofibers for cyt c (■) and Hgb (●). Inset: Analyte-dependent pattern for 200 nM of bovine metalloproteins (cyt c, Hgb) and non-metalloprotein (BSA) in PBS buffer solution (pH 7.4).

**Figure 4.7** Confocal fluorescence images of the electrospun nanofibers before (left) and after (right) incubation in a 10 μM cyt c solution for 15 minutes ($\lambda_{\text{Ex}} = 364$ nm).

**Figure 4.8** Repeated switching of normalized fluorescence emission of the nanofibers for 5 cycles of 25 μM cyt c of quenching/regenerating process. Quenching time: 15 min, regenerated by immersing into 50 mM NaOH ethanol solution for 5 min, PBS buffered solution (pH 7.4) for 5 min and dried with N2 gas, $\lambda_{\text{Ex}}/\lambda_{\text{Em}} = 370/475$ nm.

**Figure 5.1** Structures of anionic and cationic fluorescent dendritic molecules AFD-3, AFD-6, CFD-3 and CFD-6.

**Figure 5.2** SEM images of pure dendritic fluorophore-doped cellulose sensing materials produced from (a, b, c) 15% and (d, e, f) 17% CA solutions in various solvents of acetone/DMAc: (a, d) 5:1, (b, e) 8:1, and (c, f) 10:1 (10,000× magnification).
Figure 5.3 Pure electrospun dendritic fluorophore-doped cellulose fiber sensing interface: (a) digital image of as-spun electrospun fibers as a free standing paper under a black light; (b) SEM images of electrospun dendritic fluorophore-doped cellulose fibers (17% CA/0.1% AFD-6 dissolved in 10:1 acetone/DMAc, 10,000× magnification)...... 152

Figure 5.4 Fluorescence emission spectra of the different electrospun dendritic fluorophore-doped cellulose fibers in PBS buffer solution (pH 7.4): (a) AFD-3 (solid line) and AFD-6 (dashed line) doped cellulose sensor interface; (b) CFD-3 (solid line) and CFD-6 (dashed line) doped cellulose sensor interface (λ<sub>Ex</sub> = 370 nm).................. 155

Figure 5.5 (a) Fluorescence lifetime decay curves (λ<sub>Ex</sub> = 400 nm) of the AFD-3 dendrimer fibers under four different conditions [(solid line) dry fiber, (dashed line) wet fiber, (dotted line) wet fiber with 3.2 μM of Mb, and (dashed-dotted line) 10 μM AFD-3 only in PBS buffer solution.] (b) Plots of the average AFD-3 fluorescence lifetime τ<sub>avg</sub> as a function of Mb concentration.................................................. 160

Figure 5.6 Plots of I<sub>0</sub>/I of four different electrospun dendritic fluorophore-doped cellulose fibers (AFD-3, AFD-6, CFD-3 and CFD-6) against Mb concentration. Inset: Stern-Volmer plots of fluorophore-doped sensing membranes for Mb. (λ<sub>Ex</sub>/λ<sub>Em</sub> = 370/460 nm). ................................................................. 162

Figure 5.7 Histogram plot of the Stern-Volmer constants (K<sub>sv</sub>) of the arrangement of electrospun dendritic fluorophore-doped cellulose fibers against five target proteins (Hgb, Mb, cyt c, BSA, avidin) in PBS buffer solution (pH 7.4). .................. 165

Figure 5.8 Change-in-fluorescence (ΔI) response patterns at 460 nm (λ<sub>Ex</sub> = 370 nm) of the fluorophore-doped fiber collection against five target proteins (Hgb, Mb, cyt c, BSA,
avidin) at 400 nM in PBS buffer solution (pH 7.4). Each value is an average of three independent measurements. .............................. 167

**Figure 5.9** 3D Plot of the first three principal components of the change-in-fluorescence (ΔI) response patterns obtained through the fluorophore-doped fiber array against five target proteins (Hgb, Mb, cyt c, BSA, avidin) at 400 nM in PBS buffer solution (pH 7.4). Note that the first three principal components account for 97.875% of the total data variance................................................................. 169

**Figure 6.1** Structures of diacylene monomers investigated for electrospun fibrous colorimetric sensors................................................................. 181

**Figure 6.2** Fluorescence emission profiles of the polymerized PCDA (a), ECDA (b), and PCDA-EDEA (c) embedded electrospun fibers after exposure of the organic solvent vapor at 25 °C for 1 h. (λ<sub>Ex</sub> = 490 nm) Organic solvents; THF (black solid), Chloroform (red dash), Methanol (blue dot), and Hexane (green dash-dot)....................... 187

**Figure 6.3** Fluorescence emission profiles at 640 nm (λ<sub>Ex</sub> = 490 nm) for the polymerized PCDA, ECDA, and PCDA-EDEA embedded electrospun fibers against four organic solvent vapors (THF, Chloroform, Methanol, Hexane) at 25 °C for 1 h. Each value is an average of three independent measurements......................................................... 188

**Figure 6.4** Photographs of electrospun fiber mat embedded with PCDA-biotin before (a) and after (b) 254 nm UV-irradiation (1 mW cm<sup>-1</sup>) for 5 min; SEM image of the microfibers containing polymerized PCDA-biotin (c)........................................ 191

**Figure 6.5** TEM image of PDA-biotin electrospun nanofiber before (a) and after (b) UV-irradiation.......................................................... 192
Figure 6.6 Optical microscope image showing color change of polymerized PCDA-biotin embedded electrospun fiber mat upon addition of 500 ng (top), 250 ng (middle), and 0 ng (bottom) of streptavidin (a). Fluorescence emission profile of the polymerized PCDA-biotin embedded electrospun fibers after a 5 sec emersion into a 250 μg mL⁻¹ streptavidin solution (b).......................... 194

Figure 7.1 Illustration of 3D silica nanofiber system that is transformed into a 2D silica nanofiber network and fused onto a transducer substrate....................................................... 201

Figure 7.2 SEM images of precursor silica nanofibers electrospun with a relative humidity (A) greater than 50% and (B) less than 50%. SEM images of calcinated nanofibers (C) without a protective foil cover and (D) with a protective foil cover. ..... 206

Figure 7.3 EDX energy spectra of electrospun silica nanofibers (A) before and (B) after calcination................................................................................................................. 209

Figure 7.4 SEM images of the electrospun silica nanofibers (A) before and (B) after calcination. The insets are SEM images with higher magnifications......................... 210

Figure 7.5 AFM image (lower) and line profile (upper) of electrospun silica nanofibers after calcination.......................................................... 213

Figure 7.6 Photographic images of electrospun silica nanofibers after calcination on an aluminum substrate (A) and a silica substrate (B). C is the SEM image of the calcinated silica nanofibers fused onto a silica substrate........................................... 214

Figure 7.7 TEM micrograph (A) and confocal fluorescence image (B) of electrospun AuNP embedded silica nanofibers after thermal treatment (λₑₓ = 543 nm)............... 217
LIST OF TABLES

Table 1.1 Types of electrochemical transducer for classified types of measurements. ........................................ 12

Table 2.1 Voltammetric results for peak current ($i_p$), formal potential ($E^\circ$), and peak separation ($\Delta E_p$) obtained before and after each layer. ......................................................... 76

Table 5.1 Fluorescence lifetime decay data (biexponential) of fibrous and nonfibrous AFD-3 dendrimers under four different conditions. ......................................................... 159
1. CHAPTER ONE: Introduction

The overall objective of the research presented in this dissertation is to develop novel biosensor interfaces using new functional nanomaterials and advanced signal transduction schemes. The building blocks of these biosensor interfaces include known chemical structures such as self-assembled monolayers (SAMs), conjugated polymers (CPs) and electrospun nanofibers. However, it’s the combination of these structures and functions that has led to unconventional results. Specifically this research introduces new bioanalytical methods for detection of biological molecules and agents (i.e., viruses and proteins) using advanced electrochemical and optical techniques. The dissertation is divided into four main sections. First, an introduction (Chapter One) is provided on the principles and types of electrochemical and optical sensors covered in the thesis and the new nanomaterials (i.e., SAMs, polyelectrolytes, dendrimers, and nanofibers) used for fabrication of the sensing interface. This is followed by the construction of a structurally controlled electroactive nanoarchitecture (Chapter Two), and its use in the detection of whole viral particles (Chapter Three). In the third section advanced optical sensors using electrospun polymer nanofibers are presented. This includes 1) the fluorescence pattern recognition and detection of proteins using fluorescent dendrimers doped into electrospun nanofibers (Chapters Four and Five), 2) the fluorescent and colorimetric detection of proteins and volatile organic compounds (VOCs) using silica-reinforced polydiacetylene(PDA)-embedded nanofibers (Chapters Six), and 3) the development of 2D-silica nanofiber thin films doped with gold nanoparticles, their enhanced optical
properties, and the potential use in future sensor technology (Chapter Seven). Finally the current state and future outlook of the topics presented in the dissertation are discussed (Chapter Eight).

A. Biosensors

1) Introduction

What is a biosensor? The International Union of Pure and Applied Chemistry (IUPAC) defines “a chemical sensor as a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal”.¹ In the context of this thesis, ‘biosensors’ are defined as analytical tools used to understand the biological composition, structure and function by converting a biological response into a detectable analytical signal. The ideal biosensor should be small in size, sensitive, selective, easy to use, portable, economical, and acquire data quickly. These bio-analytical devices are composed of a biological recognition element directly attached (or confined to a transducer substrate) which together relates the concentration of an analyte (or group of related analytes) to a measurable response.² Therefore, many sensors used for biological purposes are not true ‘biosensors’ including those for temperature, pH, Ca²⁺, and pressure. Biological sensing elements have the ability to interact with extremely low concentrations of analyte and it is this sensitivity makes the usage of biosensors ideal for a variety of applications. Biosensors can play a key role in monitoring the level of harmful pollutants in the
environment and detecting medically important molecules like proteins, sugars, and peptides found in body fluids.

Biosensors are typically divided up into five general components: 1) a bioreceptor, 2) transducer, 3) amplifier, 4) processor and 5) display (Fig. 1.1). The bioreceptor is the part of a biosensor where the biochemical information (substrate) is transformed into a form of energy (product) which is then measured by the transducer. Some examples of common biological receptors include antibodies, aptamers, protein receptors, and DNA. The transducer is the component that transforms the sample’s biochemical information into a useful analytical signal and does not show any selectivity. The signal from the transducer is often low and superimposed upon a relatively high and noisy baseline. Thus the resultant signal should be amplified and then processed by subtracting the ‘reference’ baseline signal and electronically filtering out the unwanted signal noise. The analogue signal generated is then converted to a digital signal and correlated to concentration units, via a microprocessor. Finally the digital signal is transferred to the display device, or data storage unit, for analysis. These five components are essential in developing a successful biosensor that is accurate, precise, reproducible, and is linear over a useful analytical range.
Figure 1.1 Schematic of a biosensor that includes the bioreceptor (1), and the transducer (2). The physical change accompanying the reaction is converted into an electrical signal, amplified (3), processed (4) and displayed (5).
What makes a biosensor distinctive is the operating principle of the transducer. Some examples include electrochemical, electrical, optical, mass sensitive, and magnetic, just to name a few. The focal point of this dissertation is to detect biological molecules using advanced electrochemical and optical detection methods. Therefore other transducer operating principles will not be discussed. An electrochemical biosensor contains a transducer that transforms a biological interaction of the analyte at the electrode, or receptor, into an electrical signal. This electrochemical interaction may either be stimulated electrically or may result in a spontaneous interaction at the zero-current condition. An optical biosensor contains a transducer that transforms changes in optical phenomena caused by a biological interaction of the analyte at the receptor into a signal. Optical transducers are further subdivided according to the type of optical property being detected including absorbance, reflectance, luminescence, fluorescence, and refractive index. A brief review covering the principles and applications of both electrochemical and optical biosensors is presented in the following sections.

The analyte is the substance or chemical constituent that defines what type of receptor/transducer component should be used in the biosensor. There are two main bioanalytes of significance within this research: viruses and proteins. Effective detection of viruses will and has played a critical role in medicine and public health, in bio-industry, and in bio-defense worldwide. Currently two of the six CDC category A bioterrorism agents/diseases are viruses and the presence of human enteric viruses in water have accounted for more than half of the world’s outbreaks and epidemics. Thus, a
rapid and reliable viral sensor which could swiftly improve response time and treatment, and thus prevent the spread of disease is presented in Chapter Two and Three.

A significant portion of the thesis research centers on the detection of proteins using an optical biosensor. Protein detection is a powerful tool for diagnosis, prognosis, and monitoring of cancers and other medical conditions, such as HIV, Alzheimer’s disease, prostatitis, and hypoalbuminemia. However, a key requirement in the development of methods for use in the early diagnosis of cancer or other disease states is the ability to rapidly “detect” and “identify” the presence of certain biomarker proteins and/or irregular protein concentrations found in biofluids (e.g., serum, blood, and urine). While substantial efforts have been devoted to develop precise and efficient protein detection arrays, they still remain underdeveloped due to the lack of highly selective and specific binding agents. Currently, antibodies, antibody fragments, nucleic acid aptamers, and peptoid protein ligands are used as ligands that provide the needed protein surface complementary interaction. The application of these lock and key binding interactions, however, is limited by convoluted preparation procedures, chemical instability, and the need to label protein analytes with fluorescent tags. Therefore, the focus of the protein detection research has been in developing straightforward optical transducers that could detect multiple biological molecules within a complex mixture of biofluid. This sensing operation has been accomplished by using functional polymers and new nanomaterials for creating a “smart” interface for quick label-free detection.
2) Electrochemical Biosensors

Electrochemical detection of glucose in the blood was the first and is still the most widely used biosensing application today.\(^8\) The idea of analyzing glucose oxidase electrochemically was first introduced in 1962 by Leland C. Clark at the New York Academy of Sciences Symposium, and the first commercial electrochemical glucose biosensor appeared on the market in 1975.\(^9\) Currently, there are many electrochemical biosensors for pathogens and toxins based on the similar biosensing principles as those first generation glucose sensors.\(^10\) In addition, new electrochemical devices and techniques have evolved including a variety of electrode surface architectures, immobilization chemistries, genetically engineered recognition molecules, microarray of capture molecules, and fluidics for continuous monitoring. For example, Cai and coworkers have recently developed an ultrasensitive and selective electrochemical method for identifying hepatitis C virus genotype 1b in real clinical samples based on specific endonuclease combined with gold nanoparticle signal amplification.\(^10\) Also, a Magnetic Integrated Microfluidic Electrochemical Detector (MIMED) that integrates sample preparation and electrochemical sensors in a monolithic disposable device to detect influenza H1N1 viruses directly from throat swab samples has been created by Soh and coworkers.\(^11\)

An electrochemical biosensor contains either a potentiometric, impedimetric, amperometric, or field effect transducer (Table 1.1).\(^12\) The transducer surface is typically chemically modified as the chemical modification actually allows for better signal transduction. For biological recognition the electrode surface architecture (e.g., inorganic
films, monolayers, polymers) must be further modified with a biologically sensitive
receptor (e.g., an enzyme, DNA, antibody) which can interact with the target analyte and
convert the biological event into an electrochemical signal. This biologically created
electrochemical signal is transduced to the electrode surface via a carefully designed
“relay” interface, creating a selective and sensitive readout. Electrochemical sensors are
attractive for the detection of biological materials due to their inherent capability of being
fast, sensitive, simple, inexpensive, and portable.
Table 1.1 Types of electrochemical transducer for classified types of measurements.\textsuperscript{12}

<table>
<thead>
<tr>
<th>Measurement type</th>
<th>Transducer</th>
<th>Transducer Analyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Potentiometric</td>
<td>Ion-selective electrode (ISE); Glass electrode;</td>
<td>K\textsuperscript{+}, Cl\textsuperscript{-}, Ca\textsuperscript{2+}, F\textsuperscript{-}; H\textsuperscript{+}, Na\textsuperscript{+}…; CO\textsubscript{2}, NH\textsubscript{3}; Redox species</td>
</tr>
<tr>
<td></td>
<td>Gas electrode; Metal electrode</td>
<td></td>
</tr>
<tr>
<td>2. Amperometric</td>
<td>Metal or carbon electrode; Chemically modified</td>
<td>O\textsubscript{2}, sugars, alcohols…; Sugars, alcohols, phenols, oligonucleotides…</td>
</tr>
<tr>
<td></td>
<td>electrodes (CME)</td>
<td></td>
</tr>
<tr>
<td>3. Conductometric, impedimetric</td>
<td>Interdigitated electrodes; Metal electrode</td>
<td>Urea, charged species, oligonucleotides…</td>
</tr>
<tr>
<td>4. Ion charge or field effect</td>
<td>Ion-sensitive field-effect transistor (ISFET);</td>
<td>H\textsuperscript{+}, K\textsuperscript{+}…</td>
</tr>
<tr>
<td></td>
<td>Enzyme FET (ENFET)</td>
<td></td>
</tr>
</tbody>
</table>
Typically in biologically based electrochemistry, the reaction under investigation either generates a measurable potential or charge accumulation (potentiometric), a measurable current (amperometric), or both electrical resistance and reactance are altered that change the conductive properties of the medium (conductometric) between electrodes.\textsuperscript{13-16} Therefore the type of electrochemical transducer used depends on the biological signal created in the detection process. A potentiometric transducer measures the electrical potential (equal to or near zero) on an electrode when ions accumulate at an ion-selective membrane, such as silicon nitride (Si\textsubscript{3}N\textsubscript{4}), alumina (Al\textsubscript{2}O\textsubscript{3}), zirconium oxide (ZrO\textsubscript{2}) and tantalum oxide (Ta\textsubscript{2}O\textsubscript{5}). Ion-selective electrodes (ISE) and ion-sensitive field effect transistors (ISFET) are also based on the same operating principle as a potentiometric sensor. An impedimetric transducer measures either the impedance (Z) or its components resistance (R) and capacitance (C) based on:

\[ Z^2 = R^2 + \frac{1}{(2\pi f C)^2} \]

where \( f \) is the frequency of the electrical signal. The most commonly used electrochemical transducer is amperometric because it is highly sensitive, simple and suited for mass production. An amperometric transducer measures the production of current when a potential waveform is applied to the working electrode. Amperometric based transducers are used in the thesis research and thus will be the theme of the rest of the electrochemical introduction discussion.

An essential prerequisite for development of ultrasensitive and rapid amperometric biosensors is to establish unhindered fast electron transfer from the biological component to the electrode. In general it is best to have the bio-electrochemical reaction take place as
close to the electrode as possible. This is because the measurable products of the bioreaction diffuse in all directions and this may lead to a decreasing signal with increasing distance from the surface. The catalytic enzyme-electrochemical method is one of the most ideally suited approaches to generate a highly sensitive, long-range electron transfer, which is needed for most biological detection schemes. The function of amperometric biosensors is related to the electron-transfer process between an immobilized enzyme and the electrode surface which is set to the appropriate working potential for activation.\textsuperscript{17}

Catalytic enzyme-electrochemical biosensors can either be direct or indirect, depending on whether a mediator is used or not. For direct electron transduction the redox enzyme is immobilized directly onto the surface and acts as an electrocatalyst. The electrons are directly transferred from the substrate to the electrode as shown in Fig. 1.2A and no mediator is required to transfer the electrons. For indirect transduction an electron transfer mediator shuttles the electrons between the redox center of the enzyme and the working electrode.\textsuperscript{18} Specifically mediator molecules are oxidized (or reduced) at the electrode and reduced (or oxidized) at the reaction site of the enzyme.

Electron transfer mediators are typically small, facile redox-active molecules with low oxidation potential, and are used to react rapidly with the enzyme. The most commonly used mediators are ferrocene, ferrocyanide, methylene blue, and benzoquinone.\textsuperscript{19} Mediators can either be directly attached to the surface or freely diffuse in solution as shown in Fig. 1.2 B.
Figure 1.2 A schematic illustration of direct (A) and indirect (B) electron transfer from the active site of the redox center of the enzyme to the electrode surface.\textsuperscript{20}
Amperometric sensors involving enzymes have shown utility and versatility for low level detection of biological material. The near “real-time” detection is unrivaled among the wealth of bio-analytical techniques. After the determination of glucose in 1962, the amperometric sensing of DNA, antibodies and proteins has been established using the specific adsorption or complexing reactions of guest molecules tagged with reactive enzymes. Miniaturized biosensors have been developed on the basis of these enzymatic redox properties. For example a horseradish peroxidase (HRP) microsensor for the local detection of photoelectrochemically produced hydrogen peroxide has been reported, as well as a method for pathogenic bacteria, where the detection of the bacteria is with HRP labeled antibodies. Although these sensors show substrate dependent currents there is a lot of work remaining for improvement, particularly in areas of sensor miniaturization, preparation of multi-sensor arrays and achievement of high level of sensitivity that is required for viral detection.

Another aspect is enzymes with highly desirable ET properties. Compared to many other enzyme systems, alkaline phosphatase (ALP) and p-aminophenyl phosphate (p-APP) have proven to be an efficient electrochemical scheme for low concentration detection. This enzyme pair has been successfully demonstrated for direct and particle-tolerant detection in unpurified liquids. It remains linear for long incubation times and yields high sensitivity. Yang and coworkers have shown impressive results with a 1 fg/mL detection limit of prostate specific antigen (PSA) and a 100 fg/mL detection limit of mouse immunoglobulin G (IgG) using ferrocene dendrimers as the electron mediator on the working electrode.
3) Optical Biosensors

One of the main goals in biosensor development is to have the ability to study biomolecular interactions in their natural environment, in real time and without the need to label the target analyte. Labeling the target analyte alters the natural function and in some cases can interfere with the desired molecular interaction by occluding a binding site, which leads to false negatives. While electrochemical biosensors offer many advantages (i.e. size, cost, and ease-of-use) recent developments in label-free optical instrumentation and experimental design have led to the increasing use of optical biosensors in biomedical research, homeland security, and drug discovery. Most notably, optical biosensors are immune to electromagnetic interference, capable of performing remote sensing, and can provide multiplexed label-free detection within a single device. Optical transducers are subdivided according to the type of optical property being detected; they include absorbance, reflectance, luminescence, fluorescence, and refractive index. While various types of optical biosensors have been investigated for sensitive label-free detection, the next sections will only review the principles and current applications of SPR, fluorescence, and “litmus” type colorimetric biosensors.

These optical methods are the focus of the thesis research because of their inherent sensitivity and ability for direct label-free detection. Moreover, these optical sensing techniques enable miniaturization and high throughput multiplexed analysis. All of which are competitive with current state-of-the-art biosensing techniques which usually involve the use of labeled compounds. Thus, besides development of new
transduction platforms and innovations in label-free optical techniques, the potential application of such optical techniques can be applied to a variety of bioanalytical problems.

\[\textit{a) Surface Plasmon Resonance}\]

Since the first report in 1983, surface plasmon resonance (SPR) biosensing has been extensively investigated and has gradually become an incredibly powerful label-free tool to study the interaction between target analyte and biorecognition molecules. SPR biosensors monitor molecular interactions taking place in the close vicinity (<200 nm) of the transducer surface (i.e. gold or silver). It offers high sensitivity (<10^{-9}) and fast time resolution (1s) for changes in refractive index of a surface coating or solution near the SPR active surface.\(^{36}\) SPR biosensors have proven to be an important biosensing technology in the areas of biology, chemistry, and material science owing to its continuous, real-time, label-free, and multitarget nature.\(^{37-38}\) The principle, development, and applications of SPR biosensors have been well described in several recent review papers.\(^{39-40}\)

The surface plasmon resonance phenomena was first observed in 1902.\(^{41}\) But it was not until Otto, Kretschmann and Raether established a better understanding of SPR theory that SPR became a common surface analysis tool. First in 1968, Otto demonstrated that the drop in the reflectivity in the attenuated total reflection method is due to the excitation of surface plasmons.\(^{42}\) And later that same year, Kretschmann and Raether reported the excitation of surface plasmons in the attenuated total reflection method configuration.\(^{43}\) SPR can be physically described using electromagnetic theory
A complete understanding of electromagnetic theory requires a strong background in physics. The basic principles and applications of SPR are briefly described below.

SPR is closely related to total internal reflection (TIR) which occurs at an interface of two dielectric media. TIR occurs when a p-polarized light beam propagating from the side of a higher refractive index medium (\(n_1\)) into a lower refractive index medium (\(n_2\)) is partially reflected and refracted. During TIR a small electrical field intensity, called evanescent waves, penetrates a short (tens of nanometers) distance into the low refractive index medium creating an exponentially attenuating evanescent wave. The penetration depth of the evanescent field wave is defined as the distance over which the wave decays to 1/e, or about 37%, of its maximum intensity. When a nonmagnetic conducting material (i.e., gold, silver, conducting oxide, or amorphous carbon) with suitable thickness (~ 50 nm) is positioned at the low refractive index side of the interface; the p-polarized component of the evanescent wave, created in TIR, will excite the delocalized electrons or plasmons of the gold interface, causing an enhanced evanescent wave. The incident angle of the light with the greatest loss and minimum reflectivity is called the surface plasmon angle (\(\theta_{\text{SPR}}\)).

For the surface plasmons to be excited, the wave vector of the light and plasmon must be equal in direction and magnitude for the same frequency of the waves. The direction of the wave vector is the same direction as the transmission of incident light. Because the surface plasmon is surface bound, only the component of incident light that is parallel to the surface (p-polarized) can be equal to the wave vector of the surface
The magnitude of the parallel wave vector of the evanescent wave \( (k_{evan,\parallel}) \) is expressed as:

\[
k_{evan,\parallel} = \frac{2\pi}{\lambda} n_1 \sin(\theta)
\]

where \( \lambda \) is the wavelength of the incident light, \( n_1 \) is the refractive index of the higher refractive index medium, and \( \theta \) is the incident angle. The magnitude of the wave vector of the surface plasmon \( (k_{sp}) \) is related to the dielectric constants of both the solution medium and the gold film. For nonabsorbing media, the dielectric constant equals the square of the refractive index \( (\varepsilon = n^2) \), where \( \varepsilon \) is the dielectric constant and \( n \) is the refractive index. Therefore, \( k_{sp} \) is determined by \( n_2 \) and \( n_g \) according to:

\[
k_{sp} = \frac{2\pi}{\lambda} \sqrt{\frac{n_2^2 n_g^2}{n_2^2 + n_g^2}}
\]

where \( n_2 \) is the refractive index of medium at the vicinity of the interface and \( n_g \) is the refractive index of the gold layer.

When a surface plasmon is excited by an evanescent wave a phenomenon called surface plasmon resonance (SPR) occurs. The one requirement for SPR is that \( k_{sp} \) equals to \( k_{evan,\parallel} \). Since the \( k_{sp} \) is fixed for any given substrate the surface plasmon resonance condition \( (k_{sp}=k_{evan,\parallel}) \) can be satisfied by tuning either the incident angle \( (\theta) \), or the wavelength \( (\lambda) \) of the incident light wave vector \( (k_{evan,\parallel}) \) where:

\[
\theta_{SPR} = \sin^{-1}\left(\frac{1}{n_1} \sqrt{\frac{n_2^2 n_g^2}{n_2^2 + n_g^2}}\right)
\]
The angle required for the resonance of the $\theta_{SPR}$ is related to $n_2$ when $n_1$ and $n_g$ are fixed. Since there are two tunable components ($\theta$ or $\lambda$), SPR measurements are collected in either scanning angle SPR or SPR wavelength shift. Scanning angle SPR is the most common method used because it utilizes a single-wavelength light source for excitation and measures the refractive index changes from a prism/gold assembly as a function of incident angle, making the setup easier.

Both the Kretschmann geometry and Otto geometry require a prism, gold film, and a dielectric medium. The Otto geometry is best suited for analysis of solids of low refractive indices because of the space between the metal layer and prism, while the Kretschman geometry is best suited for analysis of liquids and gasses because of the close metal-prism configuration and thus is the most commonly used. Fig. 1.3 (A) is a schematic representation of a standard Kretschman SPR biosensor setup.\textsuperscript{47}
Figure 1.3  A schematic illustration of a standard Kretschman SPR biosensor setup and how a sensor chip is used to detect target analyte in a flowing solution (A). Also a schematic representation of a typical SPR sensorgram and how the interactions at the sensor interface corresponds to different responses in the sensorgram (B).
Biomolecular interaction taking place at the SPR interface will change the surface mass; as a result the lower refractive index ($n_2$) will change. When $n_2$ changes the $\theta_{\text{SPR}}$ will shift and this shift is measured as a response signal. These changes caused by biological events on the surface are monitored in real-time and in a continuous manner and these results form a sensogram as shown in Fig. 1.3 (B). Fig. 1.3 (A and B) illustrates how a sensor chip detects target analyte molecules (green spheres) in the flow solution, which passes by the bioreceptor molecules (pink diamonds) immobilized to the surface. The blue SPR angle defines the position of the reduced-intensity beam.\(^{47}\) Time points T1 and T2, shown in the schematic sensorgram correspond to the two red SPR angles, which shift as the target analyte binds to the bioreceptor molecules over time. As the concentration of bound analyte increases (arrow), the resonance units (RU) response approaches saturation, where 1000 RU correspond to an angle change of $\sim 0.1^\circ$. When analyte molecules in the test solution dissociate the mass decreases and the response will decrease. The sensorgram provides quantitative information on specificity, activity, affinity and kinetics of biomolecular interactions in real-time.

The use of SPR to monitor sensor nanofabrication is advantageous because it allows one to promptly observe film thickness, screen for new building blocks, and identify possible defects during the initial stages of biosensor design and development. Limitations of current commercial SPR devices (e.g. Biacore, Horiba, and Bio-Rad) are the cost of the instrument, requirement of proprietary sensor chips that fit certain specifications, and a trained technician to operate them.\(^{48}\) This is the reason why more efforts need to be made on instrumental design, principally to take advantage of different
experimental conditions including the use of different excitation wavelengths, materials, geometry, and assay environment.

Another important but less explored area of SPR innovation is the coupling of SPR with other analytical techniques, such as surface enhanced Raman spectroscopy (SERS), high-performance liquid chromatography (HPLC), mass spectrometry (MS), nuclear magnetic resonance (NMR), and electrochemistry, and has been the focus of multiple projects by Cheng and others. Coupling SPR with other analytical tools would improve and expand the capabilities of traditional analytical technology and workflow by enabling multiple quantitative and/or qualitative experiments in parallel. The coupling of SPR can be divided into two different approaches depending on research emphasis: 1) combining different physical phenomena in the same instrument in order to achieve a higher sensitivity and/or resolution; and 2) achieving serial use of different instruments for the same sample analysis. The combination of electrochemistry and SPR is of particular interest to this thesis research because of its ability to simultaneously probe the interface properties for two different physical quantities (refractive index and electron movement), at the same surface, from inside an electrochemical cell.

One of the first SPR studies of electrochemical reaction was based on detecting protein binding to supported lipid membranes using impedance spectroscopy and SPR simultaneously. Since then there has been a tremendous amount of research using electrochemical-SPR (ESPR). Investigations include surface-bound redox species, redox induced conformational changes in surface bound proteins, potential controlled DNA melting, electrochemical polymerization, and anodic stripping for the detection of metal
ions. In this thesis (Chapters Two and Three) electrochemistry is combined with SPR in efforts to fabricate and develop an electroactive surface with high loading capacities, both of which are required for an electrochemical whole viral particle sensor.

\textbf{b) Fluorescence Spectroscopy Involving Energy Transfer}

A fluorescent biosensor is a molecular system for which the physicochemical properties of the fluorescent transducer change upon interaction with a chemical species so that a change in fluorescence is produced. Fluorescent sensors have been employed to detect a wide variety of analytes, including metal ions, volatile agents, aromatic amines, amino acids, carbohydrates, and proteins. The major advantages of using fluorescence based biosensors are its inherent high sensitivity and large linear concentration range, which are both significantly greater than those encountered in absorption based biosensors.

The principles of supramolecular chemistry have been explored with a number of signaling mechanisms for the optical detection of various biological compounds. Examples of signaling mechanisms include photoinduced electron transfer (PET), intramolecular charge transfer (ICT), metal–ligand charge transfer (MLCT), twisted intramolecular charge transfer (TICT), electronic energy transfer (EET), and fluorescence resonance energy transfer (FRET). These fluorescence sensing principles have been well reviewed. This section will briefly discuss the fluorescence biosensors using the energy transfer mechanisms.

The signal mechanisms of current fluorescence biosensors can be best described with three classifications; (1) electron transfer, (2) charge transfer, or (3) energy transfer.
The first classification, electron transfer (ET), primarily happens *via* photo-induced electron transfer (PET) and is typically used for cation and anion sensors. Charge transfer occurs primarily when an analyte, usually heavy-metal complexes, perturb the energy levels of the fluorophore’s excited states inducing inter-conversion among the different charge transfer states, thereby producing a change in the fluorescent signal of the fluorophore. Energy transfer is based on Förster resonance energy transfer (FRET), also commonly referred to as fluorescence resonance energy transfer, which is dependent on a change in the energy transfer between the energy donor and acceptor after binding with analyte. The distance between the donor and acceptor should be from 1 to 10 nm for efficient FRET to occur. Consequently, biosensors based on energy transfer process are very much distance dependent. The energy transfer dipolar interaction is best described by the Förster rate equation:63

\[
E_{\text{FRET}} = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6, \quad R_0^6 = 8.785 \times 10^{-5} \frac{K_2 \phi_D I}{n^4}
\]

where the FRET efficiency \(E_{\text{FRET}}\) depends on the donor-to-acceptor separation distance \(r\) with an inverse 6\(^{th}\) power law due to the dipole-dipole coupling mechanism, donor lifetime \(\tau_D\), and Förster, or critical transfer distance, \(R_0\) at which the energy transfer rate is equal to the decay rate and 50\% efficient. The Förster distance depends on the overlap integral of the donor emission spectrum with the acceptor absorption spectrum as shown in Fig. 1.4. The Förster distance \(R_0\) is related to the dipole orientation factor \(K^2\), the refractive index \(n\) of the medium, the quantum yield \(\phi_D\) of the donor in the absence of the acceptor, and the degree of overlap integral \(I\) between the donor and acceptor.
Fluorescent biosensing methods can be divided into two classes, direct and indirect. Direct methods are based on the specific binding of the target analyte with a fluorescently tagged recognition molecule (e.g., antibody, DNA, or aptamer) which together forms a fluorescent complex. Indirect methods are based on the selective binding of the target analyte with a fluorescent tethered-ligand (e.g., porphyrins, dendrimers, and polymers) which together will cause either a decrease in fluorescence, also known as quenching (turn-off approach), or in some cases an increase in fluorescence (turn-on approach). In the chemical nose-based sensor approach an array of selective fluorescent receptors bind to the antigen and the displacement of the fluorophores generates a stimulus response pattern that can be statistically analyzed. While the direct method offers excellent affinity and specificity, it is limited by production cost, cumbersome preparation procedures, chemical instability and a need to label analytes with a fluorescently tagged recognition molecule that may interfere with the function of a biomolecule. However, the indirect method (chemical nose) has not only eliminated the need for a direct label but has also proven to be able to identify individual target analytes with in complex mixtures with the same degree of sensitivity found in the direct method.
Figure 1.4 A schematic representation of the spectral overlap integral illustrating Förster resonance energy transfer (FRET).
Recently, a number of indirect detection methods have been proposed, including the chemical nose approach that may have potential for analyzing a complete protein expression profile with the speed and sensitivity required. Bajaj et al. has used the chemical nose approach to not only indicate the presence of cancer cells (cervical, liver, testis and breast) in human serum samples but also to distinguish between primary cancer and metastatic disease. Miranda and others have developed an enzyme-nanoparticle sensor array for the indirect detection of several proteins in biofluids through the amplified enzymatic catalysis of β-galactosidase bound to AuNPs. It should be mentioned that the current indirect chemical nose techniques are still limited by poor selectivity, low sensitivity and complex sample preparation. For these reasons, one of the objectives behind this thesis research was to use novel “smart” nanomaterials developed in our lab for a new type of protein sensor array utilizing the indirect method.

The pattern of fluorescent data created by the nanofiber array can be very complex. As a result, the analysis of data involves the selection of a pattern recognition method that will reduce the dimensionality of the data set so that it can be examined in a straightforward two- or three-dimensional plot. The linear methods principal component analysis (PCA), linear discriminate analysis (LDA), and partial least squares (PLS) are the best suited for sensor arrays that respond linearly. The PCA method has been a popular statistical method used for protein array data analysis and has been utilized in this thesis research.
c) **CP-Based Colorimetric Detection**

The colorimetric sensing technique involves the conversion of a chromogen substrate (transducer) into a colorful compound as a result of the attachment of the target analyte to the sensing element (receptor).\(^6\) The single greatest advantage to using colorimetric biosensors over other types of biosensor is the biorecognition signal can be distinguished with the naked eye. In the design of colorimetric detection techniques, conjugated polymers have gained considerable attention due to their attractive optical and electrical properties.\(^7\) Some examples of conjugated polymers that have been used in sensing matrices include polyaniline, polyphenylene, polypyrrole, polythiophene, and polydiacetylene (PDA).\(^7\) In fact, multiple colorimetric biosensors using PDA polymers have been reported for *E. coli*, influenza virus, avidin, and phospholipase.\(^7\) One of the focal points of this thesis research has been with PDA polymers and for that reason will be the subject of this discussion.

The molecular assembly of PDA forms a conjugated backbone with the side chains of the diacetylene monomers through a 1,4-photopolymerization and is shown in Fig. 1.5A. The intriguing optical properties found in PDA CPs are primarily due to the extensive delocalized π-system and the intrinsic conformational restrictions within the polymer chain backbone as shown in Fig. 1.5B. PDA molecules can report environmental changes by chromic transitions detected photometrically or visually in response to specific biomolecular recognition events.\(^7\) The typical absorption change that takes place upon environmental perturbation in PDAs is from blue-to-red (Fig. 1.6). The absorption spectra of blue PDA materials are associated with maxima at *ca.* 650 nm, while the red
forms of PDA are generally at absorption maxima of *ca.* 550 and 500 nm. It is generally believed, the visible color change takes place when the backbone of these conjugated polymer chains are perturbed, causing the delocalized π-network to induce changes in electronic absorption and emission properties. While the blue form of PDA is non-fluorescent, the red form of PDA exhibits strong fluorescent emission maxima at 560 and 640 nm when excited above 450 nm as shown in Fig. 1.6. The intensity of the emission can vary considerably. The ratio of the emission spectra at 560 and 640 nm is dependent on the extent of PDA polymerization which is dependent on the general properties of material used to contain the PDA (*i.e.*, thin films, bulk samples, or solutions). Another great aspect about using PDA CPs is that no chemical initiators or catalysts are needed for the polymerization process. Since the backbone of these conjugated polymers can easily be modified, a variety of different surface ligands and substrate functionalities can be applied to design a chemo/biosensor for specific analyte detection.
Figure 1.5  (A) Topochemical photopolymerization of diacetylene monomers to form PDA CPs. (B) A schematic illustration of the molecular orbital in the π-conjugated PDA backbone and how the energy levels are affected by the overlap of the orbitals, which is caused by rotation of the C-C bonds in the backbones.⁷⁶
Figure 1.6  Examples of typical absorption and fluorescence spectral changes upon environmental perturbation to PDA CPs. The top absorption spectra of blue PDA materials are associated with maxima at ca. 650 nm, while the red forms of PDA are generally at absorption maxima of ca. 550 and 500 nm. The blue form of PDA is non-fluorescent, the red form of PDA exhibits strong fluorescent emission maxima at 560 and 640 nm when excited above 450 nm as shown in the bottom fluorescence spectra.
Recently Kim and coworkers reported a new approach for constructing silica-enforced nanostructured PDA supramolecules in electrospun micro/nanofibers.\textsuperscript{77-79} This work demonstrated that PDA conjugated polymers embedded into electrospun fibers could easily be used as a new colorimetric sensor based on a combinatorial approach. Similarly, Tong \textit{et al.} used PDA monomers embedded into a template melt of anodized aluminum oxide to create a more stable platform for the electrospun PDA nanofibers.\textsuperscript{80} More recently a study by Schmuck and coworkers demonstrated an intriguing approach to colorimetrically detect \textit{E. coli} bacteria using mixed PDA liposomes.\textsuperscript{81} Although all these works established a qualitative approach for the blue-to-red colorimetric transition, no effort was made to differentiate the change using fluorescence properties of the red polymer, which would give a better quantitative analysis of the PDA sensor system. Therefore the experimental work in Chapter Six primarily focuses on two approaches for analytical measurements: 1) a combinatorial quantitative fluorescent approach for the detection of VOCs using various electrospun PDA nanofibers; and 2) a biotin-based solid-state nanofiber PDA fluorescent and colorimetric sensor for protein-protein interactions.
B. **Design and fabrication of analytically significant nanomaterials**

Nanomaterials are the building blocks of nanotechnology and they play a critical role in the area of sensor technology. In particular, the electrochemical and optical research of this thesis is only possible because of the unique physical properties that nanomaterials provide. Specifically, nanomaterials can be used to immobilize enzymes and antigens onto transducer platforms, to promote direct or indirect electron and optical transfer reactions, and to amplify and orient the analytical signal of the biorecognition events. There are many forms of nanomaterials; some examples include nanofibers, nanowires, nanoparticles, surfaces and thin films, two-dimensional arrays of nanoparticles, and three-dimensional structures (superlattices), just to name a few. Moreover, nanomaterials have demonstrated exceptional physical properties (e.g., chemical, photoemission, particle aggregation, and electrical and heat conductivity) to improve the size, specificity, sensitivity, resolution, and/or signal-to-noise ratio of current state-or-the-art sensor technology. In addition, the use of these different types of nanomaterials allow for the introduction of many new strategies to build biosensing platforms (e.g., electrospinning, the LbL method, and self-assembly of monolayers).

While there are many types of nanomaterials being used in the development biosensor technology, only the key nanomaterials used in this thesis research will be presented. The explicit details of the synthesis and processing, physical properties, characterization, and sensor fabrication using these different nanomaterials and nanostructures will be discussed in greater detail in the following chapters.
1) Self-Assembled Monolayers

The spontaneous chemisorption of organic molecules that form a single uni-directional layer onto the surface of a substrate from the liquid or gas phase are known as self-assembled monolayers (SAMs). Nuzzo and Allara first demonstrated the concept of spontaneous bond formation between sulfur-containing molecules and various metals in the early 1980’s.\textsuperscript{84-86} More notably, Ullman extensively reviewed the assembly of alkyl thiols onto gold, propelling forward the mainstream use of SAMs into the field of nanoscience.\textsuperscript{87} In 2005, Whitesides et al. comprehensively outlined the involvement and contribution of SAMs in nanotechnology.\textsuperscript{88} The thickness of most SAMs is only a few nanometers and current patterning techniques allow for dimensional control, perpendicular along the plane of the substrate on which the SAMs are formed (Fig. 1.7A).\textsuperscript{89} The main reasons SAMs are well-suited for biosensor research and development is that they are easy to prepare and can form and add function on objects of all sizes and shapes. Because SAMs can couple the external environment to the electronic and optical properties found in metallic structures they can link biomolecular structures to the interfacial properties of sensors providing the needed biological recognition mechanism.

A major limiting factor for electrochemical and optical biosensors is the lack of surface architectures that allow high detection sensitivity for the desired biochemical event. The initial sublayer on the substrate is important for the functionality of the nanostructure and a versatile technique to achieve this first layer is SAMs. Moreover, SAMs are a well established and a proven surface design technique that can provide a convenient, flexible, and straightforward system with which to tailor the interfacial
properties of organic functional groups and metal oxide surfaces.\textsuperscript{87,90, 88} A mixture of various SAMs on the surface can provide the required multiple functionality, such as electron communication and linkage for bioreceptors (Fig. 1.7 B and C). For example, a ferrocene tethered SAM on an electrode surface can supply this desirable fast electron mediation and thus increase the sensor’s sensitivity and/or resolution.\textsuperscript{91} For direct immobilization of biomolecules, an appropriate functionalized SAM could be used with the electroactive SAM. The same is also true when attaching ligands required for biomolecule immobilization above the electrode surface. There are many functional groups that can be used depending on the desired targets, such as an amino group or carboxylic acid group for attachment. However, the SAM surface alone will not provide the needed loading capacity to generate high sensitivity for enzymatic relays to an electroactive surface. A slightly thicker layer, with thickness around 10-20 nm, is ideal and more desirable. One nanofabrication technique that combines well with SAMs is the layer-by-layer technique.

Some recent examples of how SAMs are being used to build functional interfaces include the work by Luo \textit{et al.}, where they used an array of SAMs to probe the chemical effects on human mesenchymal stem cells for applications in biomaterials and tissue engineering,\textsuperscript{92} and by Molhotra and coworkers, who created an electrochemical genosensor based on modified octadecanethiol SAMs for the detection of the pathogen \textit{Escherichia coli}.\textsuperscript{93}
Figure 1.7 (A) Schematic illustration of an ideal, single-crystalline SAM of alkanethiolates supported on a gold interface and its characteristics are highlighted. Schematic diagram of (B) a mixed SAM and (C) a patterned SAM. Both illustrations (B and C) demonstrate how SAMs are used in current biosensor technology and biological studies using SAM architectures.\(^ {89} \)
2) **Layer-by-Layer Assembly**

A useful technique for building a 3D multi-layer molecular nanostructure is the “layer-by-layer” (LbL) method, which works by alternating physiosorption of oppositely charged molecules. Figure 1.8A is a schematic depiction of the electrostatic LbL nanoassembly of cation/anion multilayers onto flat and spherical surfaces. This is a favorable method for biosensor technology because it is simple, straightforward and works well in a broad range of environmental conditions. Many types of charged molecules and nano-objects are suitable for deposition by the LbL method, such as DNA, enzymes, viruses, and polyelectrolytes. Figure 1.8B is a schematic illustration of different types of cations and anions used in this thesis research, polyallylamine hydrochloride (PAH), polysodium 4-styrenesulfonate (PSS), and polyglutamic acid (PGA). One advantage of the LbL method is that the amount of material deposited on each cycle approaches a constant and reproducible value, allowing a large number of controllable layers and specific properties to be incorporated into the thin films. Additionally, the thin films it generates have a higher loading capacity than traditional surface design methods, which can only produce a single layer of biomolecule immobilization. The 10-20 nm thick films made during this work allow for more binding sites for the analyte of interest, an increase in signal per event, and thus, enhanced sensitivity.

The combination of LbL and SAMs is an ideal bottom-up approach for creating a functionally active nanostructure. While the capacitance of a SAM based biosensor can be tuned by a variety of thiol functionalized molecules for creating a multitude of design
schemes, SAMs alone are limited to a 2D plane. LbL method lends itself well to building above the SAM interface because of its straightforward chemistry, robustness and potential 3D nanoarchitecture that is created. Until recently non-electroactive polymer structures above a redox active surface have created a larger electronic shield, which would hinder the electronic mediation. This would have to be improved if thicker films are used for ET-based, mediation-oriented detection schemes.

In Chapter Two the research demonstrates a new 3-dimensional nanoassembly interface with the desirable surface properties of facile electron transfer, free counter ion movement, and controlled multilayer build-up using the LbL method. This work opens avenues for a potential multitude of new applications in the science of depositing thin polymer films over the surface of electron active electrodes such as, studying the mass transport of molecules across these films, thin-film electrochemical detection, and creation of electroactive sites for biological detection. With a significantly increased capacity for hosting capture molecules on the polymer layer while continuing to have facile electron transfer properties, Chapter Three demonstrates how the LbL method can be used in biosensor technology to detect whole viral particles.
**Figure 1.8** Schematic depiction of the electrostatic Layer-by-Layer nanoassembly of cation/anion multilayers onto flat and spherical surfaces (A). Schematic illustration of different types of cations and anions used (B): polyallylamine hydrochloride (PAH), polysodium 4-styrenesulfonate (PSS), and polyglutamic acid (PGA).
3) **Electrospun Nanofibers**

Within the connotation of nanotechnology and nanostructured materials, a nanofiber generally refers to a fiber having a diameter less than 100 nm. In the past two decades research and development of polymer nanofibers has undergone significant progress. Applications of these nanofibers can now be found in filtration systems, scaffolds for tissue engineering, protective clothing, reinforcement in composite materials and sensors, just to name a few. The fundamental feature that makes polymer nanofibers attractive is the high specific surface area. A much larger specific surface area is created as the diameter of electrospun fibers are reduce in size from micrometers (e.g., 10–1000 μm) to submicrometers or nanometers. Consequently, reducing the fiber diameter proportionally increases the ratio of the exposed polymer chains together with its functional groups. This relationship between specific surface area and fiber diameter is shown in Figure 1.9. Moreover, this larger surface area provides an opportunity to more effectively modify the fiber surface with specific functions such as enhanced energy density per weight, biocompatibility, and bio-recognition.

Numerous nanofiber techniques such as melt-blowing, drawing, template synthesis, phase separation, self-assembly and electrospinning have been employed to generate suitable polymer nanofibers for different functions. Amongst the techniques used, electrospinning is the most popular and preferred technique because electrospun nanofibers offer several advantages. The term “electrospinning”, derived from “electrostatic spinning”, was created around 1994 by Reneker and colleagues. However, the first patent (US Patent Number: 2116942) on electrostatic spinning was
issued in 1934 for the fabrication of cellulose based textile yarns by Antonin Formhals. Even though this non-woven fiber manufacturing technique has been around for over 75 years it is only recently that science has been able to exploit this technology in order to produce nanoscale fibers. Over 1000 patents have been issued on the topic of electrospinning.
Figure 1.9 Relationship of the surface area versus fiber fineness (diameter).
The electrospinning process works when a flowing polymer solution is subject to a high electric field, thereby inducing free charges into the polymer solution. The charged ions move in response to the applied electric field towards the electrode of opposite polarity, thus transferring tensile forces to the polymer solution. When the repulsive electrostatic force overcomes the surface tension of the polymer solution a pendent hemispherical polymer drop takes a cone-like shape and a jet of liquid is ejected from the tip of the cone. As the jet of liquid travels through the atmosphere, the solvent evaporates, and a dry membrane-like web of small fibers is emitted onto a ground electrode. This cone effect has been investigated in detail by Taylor and others, which highlights the existence of a critical angle for the droplet tip, called the “Taylor cone”. Electrospinning is typically conducted at room temperature with atmosphere conditions. The polymers and dopants must be completely dissolved in solvent before introduced into the spinneret for electrospinning. A schematic diagram of a typical electrospinning set-up and its three main components are shown in Fig. 1.10: 1) a feeding unit which contains the polymer solution, capillary tube and spinneret (e.g., a pipette tip); 2) a high voltage power supply in the range of several tens of kVs; and 3) a grounded collecting plate (usually a metal screen, plate, or rotating mandrel).
Figure 1.10  Schematic diagram showing the three main components of a typical electrospinning set-up.
Electrospinning method offers continuous fibers with an extremely high surface-to-volume ratio, tunable porosity, malleability to conform to a wide variety of sizes and shapes and the ability to control the nanofiber composition to achieve the desired results.\textsuperscript{112} It is a relatively robust and simple top-down fabrication technique to produce nanofibers. A wide variety of organic (e.g. polyethyleneoxide, polyaniline, polystyrene, etc.), inorganic (e.g. TiO\textsubscript{2}, Al\textsubscript{2}O\textsubscript{3}, SiO\textsubscript{2}, etc.), metallic (e.g. Pt, Cu, Mn, etc.), and biopolymer (e.g. cellulose, collagen, polylactic acid, etc.) materials have been used.\textsuperscript{113} Because of their immense advantages, electrospun nanofibers have been extensively investigated in the past several years. Several current and potential applications using electrospun polymer nanofibers are shown in Fig. 1.11.

Recently, electrospun nanofibers have received great attention for biological and chemical sensor applications because of their uniquely large surface area. This is the most desirable property for improving the sensitivity of any type of sensor because of the increase in percentage of exposed receptors on the nanofiber interface. Russell and coworkers recently created a high surface area bioelectrode, based on nanofibers of electrospun gold with immobilized glucose isomerase for the detection of glucose and demonstrated an 8-fold higher enzyme loading capacity than conventional gold electrode biosensors.\textsuperscript{114} Also, Alocilja et al. recently showed that electrospun nitrocellulose nanofibrous membranes functionalized with antibodies can be utilized for the high sensitivity detection of the pathogens \textit{E. coli} (61 CFU mL\textsuperscript{-1}) and bovine viral diarrhea virus (10\textsuperscript{3} CCID mL\textsuperscript{-1}).\textsuperscript{115} Both of which further demonstrate the versatility and significance of using electrospun nanofibers in biosensor technology by significantly
increasing the surface area and mass transfer rate, which further improves the biochemical binding effect and sensor signal to noise ratio.
**Figure 1.11** Potential applications of electrospun polymer nanofibers.\textsuperscript{107}
In summary, there are a number of aspects that need to be considered for biosensor development when using electrospun nanofibers: 1) the signal transduction generation either through the increase in signal or decrease of noise; 2) the fluidics design, such as sample injection and drainage, reduction of sample consumption, increase of analyte transport, and/or reduction in detection time; 3) the type of surface immobilization chemistry and how one can increase analyte capture efficiency and eliminate non-specific binding; 4) the detection format, some examples include direct or indirect binding, sandwich-type binding, and competitive binding; and finally, 5) the analysis of the data, i.e., concentration or binding kinetics. In this dissertation, the focus is primarily placed on the transduction component (electrochemical and optical) with the emphasis on novel nanomaterial structures and their interactions with the various analyte. One of the strategies of this thesis research was to create novel platforms using electrospun nanofibers for label-free detection of proteins. The first strategy was done by using the unique indirect chemical nose approach for the fluorescent detection of analyte proteins (Chapter four and five). The second strategy was done by using a direct lock-and-key approach for the colorimetric/fluorescent detection of protein (Chapter six). Finally, the thesis research examines the early development of 2D-silica nanofiber thin films doped with gold nanoparticles, their enhanced optical properties, and the potential use in future sensor technology (Chapter seven).
C. References


(40) Linman, M. J.; Abbas, A.; Cheng, Q. A. Analyst 2010, 135, 2759.
(42) Otto, A. Zeits Phys 1968.


(64) Held, P. *An Introduction to Fluorescence Resonance Energy Transfer (FRET) Technology and its Application in Bioscience*, BioTek Instruments, Inc., 2005.


(72) Tieke, B. Advances in Polymer Science 1985, 71, 79.


(91) Owen, T. W., University of California, Riverside, 2007.


(107) Huang, Z. M.; Zhang, Y. Z.; Kotaki, M.; Ramakrishna, S. Compos. Sci. Technol. 2003, 63, 2223.


2. CHAPTER TWO: Unobstructed Electron Transfer on Porous Polyelectrolyte Nanostructures and its Characterization by Electrochemical Surface Plasmon Resonance

A. Abstract

Thin organic films with desirable redox properties have long been sought in biosensor research. We report here the development of a polymer thin film interface with well-defined hierarchical nanostructure and electrochemical behavior, and its characterization by electrochemical surface plasmon resonance (ESPR) spectroscopy. The nano-architecture build-up is monitored in real time with SPR, while the redox response is characterized by cyclic voltammetry in the same flow cell. The multilayer assembly is built on a self-assembled monolayer (SAM) of 1:1 (molar ratio) ferrocenylundecanethiolate (FUT) and mercaptoundecanoic acid (MUA), and constructed using a layer-by-layer deposition of cationic poly(allylamine hydrochloride) (PAH) and anionic poly(sodium 4-styrenesulfonate) (PSS). Electron transfer (ET) on the mixed surface and the effect of the layer structures on ET are systematically studied. Under careful control, multiple layers can be deposited onto the 1:1 FUT/MUA SAM that presents unobstructed redox chemistry, indicating a highly ordered, extensively porous structure obtained under this condition. The use of SPR to trace the minute change during the electrochemical process offers neat characterization of local environment at the interface, in particular double layer region, allowing for better control over the redox functionality of the multilayers. The 1:1 SAM has a surface coverage of $4.1 \pm 0.3 \times 10^{-10}$
mole∙cm-2 for ferrocene molecules and demonstrates unperturbed electrochemistry activity even in the presence of a 13 nm polymer film adhered to the electrode surface. This thin layer possesses some desirable properties similar to those on a SAM while presenting ~15 nm exceedingly porous structure for high loading capacity. The high porosity allows perchlorate to freely partition into the film, leading to high current density that is useful for sensitive electrochemical measurements.

B. Introduction

Electroactive thin organic films have attracted much interest because of their wide range of applications as electro-optics, semi-conductor devices, molecular memory electronics, electrochromic displays, and sensors.1-9 A number of methods have been developed to create these ultrathin multilayer films, such as the Langmuir-Blodgett (LB) assembly, dip-coating, spin-coating, and vapor-deposition, among others.10-15 Although the LB assembly technique offers a versatile way to build a functional surface at a liquid/gas interface, LB films must be formed in a very condensed manner to obtain proper film stability due to the weak nature of the non-covalent forces.16 Dip-coating and spin coating are commonly adopted techniques for achieving thin multilayer films because of simplicity, low cost, and efficiency of coverage. However, these approaches usually yield molecularly disordered structures, and are difficult to control and monitor at nanometer scale. Vapor-deposition method is a useful technique for coating substrates with high purity and high performance metal oxides and semiconductors. Its use for depositing organic molecules is limited due to the required operational conditions of high temperature, vacuum and volatile precursors and the formation of volatile by-products is
a common result.\textsuperscript{17} There is a need to explore alternatives to creating nanoscale 3-D architecture that provides a more ordered surface with high quality and desirable features such as controlled porosity.

One attractive technique for building a 3-D multi-layer molecular nanostructure is the “layer-by-layer” (LbL) method, which works by alternating physiosorption of oppositely charged molecules.\textsuperscript{18} Many types of charged molecules and nano-objects are suitable for deposition by the LbL method, such as DNA, enzymes, viruses, and polyelectrolytes.\textsuperscript{19-22} One advantage of the LbL method is that the amount of material deposited on each cycle approaches a constant and reproducible value, allowing a large number of controllable layers and specific properties to be incorporated into the thin films. The initial layer on the substrate is important for the functionality of nanostructure and a versatile technique to achieve this first layer is self-assembled monolayers (SAMs). SAMs provide a convenient, flexible, and straightforward system with which to tailor the interfacial properties of organic functional groups and metal oxides.\textsuperscript{23-25} The combination of LbL and SAMs is a great bottom-up approach to creating a functionally active nanostructure.

Many methods have been used to analyze the build-up of these molecular structures on a solid substrate, such as ellipsometry, atomic force microscopy (AFM), cyclic voltammetry (CV), and surface plasmon resonance (SPR) spectroscopy. The combination of electrochemistry and SPR is of particular interest to us due to its ability to probe the interface properties simultaneously on two different physical quantities at the same surface inside an electrochemical cell.\textsuperscript{26-28} Cyclic voltammetry in particular offers
kinetic measurement of heterogeneous electron–transfer reactions and coupled chemical reactions.\textsuperscript{29-30} SPR, a direct optical-sensing technique, measures the refractive index change due to specific interactions occurring at a metal dielectric interface.\textsuperscript{31-32} The use of SPR to monitor the creation of multilayer films is advantageous because one could promptly observe film thickness change and screen for new polymers in real time with no external/internal labels using only small volumes of sample. Application of electrochemical surface plasmon resonance (ESPR) for monitoring the development of polymeric film buildup while simultaneously examining redox characteristics has appeared in a few recent publications.\textsuperscript{33-35}

In this work, we report the development of a polymer thin film interface with well-defined hierarchical nanostructure and electrochemical behavior, and its characterization by ESPR spectroscopy. Our focus is to build nano-architectures less than 20 nm in thickness that offer higher loading capacity while still exhibiting attractive physiochemical (redox in this case) properties as those observed on a 2 nm alkylthiol homogeneous monolayer. Thin films of these characteristics are very useful for creation of energy-efficient minituarized devices and biosensors, but have not been widely studied largely due to lack of proper fabrication methods. Figure 2.1 illustrates the scheme of the design and the structure of the SAMs and polyelectrolytes utilized in this work. A mixed SAM surface of 11-ferrocenyl-1-undecanethiol (FUT) and mercaptoundecanoic acid (MUA) is used, which lends itself to two different primary functions. The ferrocene surface provides electrochemical signal while the carboxylic acid will allow for a more desirable electrostatic attachment for the polyelectrolytes. Specifically we plan to seek to
achieve 3-D nano-architecture with properties matching those on 2-D surfaces in terms of efficient ET with a bottom-up approach. A facile redox communication is sought to take place after organic layers are coated to the surface. We will also take advantage of the high sensitivity of SPR to explore the doping and dedoping process on the thin films, in an attempt to understand and optimize the ion mobility property across the membrane.
Figure 2.1 A schematic of LbL buildup of polymeric thin films on a mixed SAM with ESPR configuration and structure of polyelectrolytes and alkylthiol derivatives for self-assembly.
C. Experimental Section

1) Materials

Poly(sodium 4-styrenesulfonate) (PSS), poly(allylamine hydrochloride) (PAH), mercaptoundecanoic acid (MUA) and NaClO₄ were purchased from Aldrich (Milwaukee, WI). Dojindo Molecular Technologies (Japan) supplied the 11-ferrocenyl-1-undecanethiol (FUT). Microscope slides were purchased from Fisher (Pittsburgh, PA) and the platinum wire (0.5 mm, 99.997 %) was purchased from Alfa Aesar (Ward Hill, MA). All chemicals were of the highest analytical grade, and were used without further purification. Milli-Q (>18 MΩ) water was used in the preparation of all electrolyte solutions, and absolute ethanol was used to prepare all thiol solutions.

2) Preparation of Redox Active Self Assemble Monolayer Electrodes

The Au electrodes were prepared by e-beam evaporation of a thin film of Au (ca. 46 nm) onto microscope glass slides with 2-nm Cr as the adhesive layer. Prior to modification, the Au substrates were cleaned with piranha solution (Caution!), then extensively rinsed in Milli-Q water, absolute ethanol, and dried with a stream of N₂ gas. The electrodes were then immersed in a 1 mM (1:1) FUT/MUA for 12-24 h and rinsed with ethanol, DI water and dried in a N₂ stream. The same procedure was repeated for all other SAM surfaces used in this work.
3) Cyclic Voltammetry

The electrochemical measurements were carried out with a three-electrode system using a CHI 650 electrochemical work station (CH Instruments, Austin, TX). The modified Au electrode served as the working electrode, and an Ag/AgCl electrode was used as the reference electrode which was placed at the outlet of the nanoSPR flowcell. The platinum counter electrode was machined into the top of the SPR flow cell. The 0.1 M NaClO₄ (pH 6.6) solution was used as the supporting electrolyte. The CV data fitting was performed using Origin software (Microcal, Inc.). The quantity of electro generated ferrocenium (Γ_{Fc+}) was calculated using the following equation (Equation 1),

\[ \Gamma_{Fc^+} = \frac{Q_{Fc^+}}{nF} \]

where \( Q_{Fc^+} \) is the charge associated with the ferrocene oxidation determined through the integration of the voltammetric anodic peak corrected for the charging current, \( n \) is the number of electrons involved in the electron-transfer process (\( n = 1 \) for the ferrocene redox couple), \( F \) is the Faraday constant, and \( A \) is the geometric surface area of the exposed FUT/MUA substrate electrode, which is defined by a silicon rubber cast.

4) Surface Plasmon Resonance

The surface plasmon resonance (SPR) spectrometer employed in the procedure is a dual channel nanoSPR in the Kretschmann configuration (Morton Grove, IL) with a semiconductor laser (\( \lambda = 670 \) nm) as the excitation source. Surface interaction and modification were monitored and characterized using the tracking mode of SPR angular scanning at the minimum angle. The SAM modified working electrodes were clamped.
down to a flow cell on a high-refractive index prism (Figure 2.1). Once the electrolyte solution had established a stable baseline, 1 mg·mL$^{-1}$ of PAH and PSS (in 0.1 mM NaClO$_4$) were injected in sequential order for layer build up.

5) **Atomic Force Microscopy (AFM)**

AFM images were obtained using a Veeco Dimension 5000 atomic force microscope (Santa Barbara, CA) with manufacturer provided software. All images were obtained under the tapping mode, and root-mean-square (rms) surface roughness values were obtained by averaging multiple 25 µm$^2$ areas across the entire surface area at a scan rate of 1.5 Hz.

6) **Ellipsometry Measurements**

Spectroscopic ellipsometric (SE) measurements were conducted using a Jobin Yvon UVISEL (Edison, NJ) Spectroscopic Phase Modulated Ellipsometer (SPME) Version M200 in the spectral range of 300-800 nm. For all SE measurements, the chip was placed on a sample stage situated on a goniometer with light from a 75W Xenon arc source at 70° angle of incidence, and reflected light was detected by a PMT. Three spots on each substrate surface were analyzed, and the results were averaged. The thickness of the FUT/MUA monolayer and the subsequent polymer layers was determined from using the Levenberg-Marquardt nonlinear optimization algorithm within the vendor’s DeltaPsi2 software. The thickness of the chromium and gold were fixed to the measured thickness during the calibrated quartz crystal monitor during e-beam evaporation (2 nm and 46 nm, respectively). The n ($\lambda$) and k ($\lambda$) values provided in the vendor’s materials database for

65
gold, polycrystalline chromium, and BK7 glass were used in the fitting process. For the FUT/MUA monolayer, the refractive index \( n \) used were \( n = 1.464^{26,37} \) and \( n = 1.45^{38} \), respectively. The \( n \) value used for the polyelectrolytes was \( n = 1.54^{39-40} \). The extinction coefficient \( (k) \) for all polymers in the calculations was set to be \( k = 0^{41} \).

D. Results and Discussion

1) Electrochemical Behavior of FUT/MUA Interface

Ferrocenylalkanethiolate SAMs are a much studied electroactive system, and their Faradic electrochemistry is extensively documented.\(^{26,42-46}\) Figure 2.2 shows a cyclic voltammogram of a well-defined reversible redox wave for 1:1 mixture of FUT/MUA (curve 1) and a flat wave for MUA (curve 2) in 0.1 M NaClO\(_4\). Symmetrical redox peaks were obtained with a slight peak separation at a scan rate of 100 mV/s. The oxidation, reduction and formal potential (\( E^\circ \)) of the terminal ferrocene group in the monolayer was found to be 412 mV, 382 mV and 397 mV, respectively. A peak separation (\( \Delta E_p \)) of 30 mV is a strong indicator that a facile electrochemical process occurs on the electrode surface. The coverage of FUT, from the charge of the anodic peak (45 \( \mu \)C\( \cdot \)cm\(^{-2} \)), is calculated to be \( 4.7 \times 10^{-10} \) mole\( \cdot \)cm\(^{-2} \). The CV shown in Figure 2.2 and the data reported compare well to values previously reported in the literature.\(^{26,42,45}\) To screen for the most suited electrolyte for this work, solutions of 0.1 M HClO\(_4\), LiClO\(_4\), and NaClO\(_4\) were tested. NaClO\(_4\) offers the most consistent signal among the three, especially with polyelectrolytes, and has thus been used throughout the work.
Figure 2.2  Cyclic voltammograms for MUA (curve 1) and 1:1 FUT/MUA (curve 2) in 0.1 M NaClO₄ (pH 6.6). The scan rate is 100 mV·s⁻¹.
It should be noted that a small shoulder peak is located on the negative potential side of the FUT/MUA response. In fact, this is a common trait found in ferrocene containing SAMs, and it is believed that the shoulder is due to the inhomogeneous spatial distribution of ferrocene in the SAM.\textsuperscript{26} The shoulder peak was further examined by comparing the FUT/MUA SAM mixture with a pure FUT surface (Figure 2.3). As expected, the pure FUT SAM surface shows a larger current due to more ferrocene attached to the surface. However, the shoulder peak for the pure FUT SAM surface is also larger, indicating that mixed FUT/MUA SAMs can improve spatial distribution within the layer.

In order to perform an effective LbL buildup, the ratio of FUT to MUA was varied and optimized as it affects the negative surface charge density and thus the attachment of the polycations and redox current density. We compared the ratios of 1:10, 1:5, and 1:1 of FUT to MUA to screen for the optimal surface conditions and found that the 1:1 FUT/MUA surface gave the strongest and most reproducible redox communication at the same time achieving a high charge density (Figure 2.4). This surface was chosen for further study and building of a 3-D nanostructure with multiple layers.
Figure 2.3  Cyclic voltammograms for 1mM FUT in 0.1 M NaClO₄ (pH 6.6) at a scan rate of 100 mV/s.
**Figure 2.4** Cyclic voltammograms for 1:10 (green), 1:5 (red), and 1:1 (black) FUT to MUA ratios in 0.1 M NaClO₄ (pH 6.6) at a scan rate of 100 mV/s.
2) Characterization of Electrostatic Self-assembly by SPR

Real time monitoring of assembly of a polycation-polyanion molecular film with bottom-up construction was carried out with SPR. Figure 2.5 shows the sensorgrams for the process. The result clearly reveals the buildup of the film by alternating the injection of polyelectrolytes with the corresponding charge. Since the MUA film contains carboxylic acid headgroup, polycationic PAH solution was first injected. The inset of Figure 2.5 illustrates the specificity of the PAH onto the surface while polyanionic PSS does not bind on FUT/MUA. The first layer of polycation adhesion produced a signal increase of 95 millidegrees. Once the baseline was stabilized, PSS was injected, giving an SPR angular increase of 200 millidegrees. PSS is a considerably larger molecule than PAH, therefore leading to a larger refractive index change and response in the SPR sensorgram. The electrostatic interaction between PAH and PSS is very effective and complete adsorption of the layers did not require any incubation time. For proof of principle all layers were injected twice. The second injection shows little signal as compared to the first injection, clearly demonstrating the completion of the electrostatic interaction right after the initial injection (Figure 2.5). Flow rate was optimized at 138 µL·min⁻¹, yielding a 10-layer nanoassembly in less than 100 min.
Figure 2.5 SPR sensogram of a 10-layer nanostructure build up by alternating injection of oppositely charged polyelectrolytes in 0.1 M NaClO$_4$ on a 1:1 FUT/MUA SAM surface. Inset is an SPR sensogram of a 5-layer build up on a MUA surface. The circle illustrates the specific electrostatic interaction of PAH with surface carboxylate (red curve) while PSS (black curve) shows no interaction with the negatively charged surface. Arrows indicate first (1) and second (2) injection of the polyelectrolyte demonstrating complete adsorption of the polymer in the first injection.
The pH-dependent thickness behavior of the sequentially adsorbed polyions layers must be considered when creating multilayer thin films. Rubner et al. has studied the pH dependence properties extensively, demonstrating that control over the bulk and surface composition of the resulting multilayer films is readily achievable via simple pH adjustments.\textsuperscript{47-48} Furthermore, pH controls the linear charge density of an adsorbing polymer as well as the charge density of the previously adsorbed polymer layer. In this work we focus on optimizing deposition solution at a relatively neutral pH for all polyion adsorption for better control over the surface charge density and film thickness while at the same time offering high-quality voltamograms. We used 0.1 M NaClO\textsubscript{4} because it is a nonreactive electrolyte with a pH of \textasciitilde6.6, which in turn allowed for us to obtain controllable adsorption layers within the film.

A more careful study on the quantitative layer adding up has been performed with SPR and Fresnel simulation to understand the correlation of layer deposition and thickness increase. Figure 2.6 shows a series of SPR angular scan curves of reflectivity versus incident angle recorded after each layer of polyelectrolyte was deposited. The left-most curve corresponds to the monolayer of FUT/MUA on the Au surface. A total of 10 layers of organic polyelectrolyte were added to the SAM surface, leading the reflection curves shifting from left to right. SPR angular shifts correlate very well with theoretical predictions from the Winspall program ($R^2 = 1$) developed by the Knoll group (Figure 2.6 bottom). The average thickness of PAH and PSS is 0.96 nm and 1.2 nm, respectively. The overall thickness of the film is determined to be 12.6 nm, including the SAM surface.
Figure 2.6 SPR angular scan curves of reflectivity versus incident angle recorded after each layer of polyelectrolyte (top) and linear relationship between experimental and theoretical SPR minimum angle shifts for polymer films on SPR gold substrates (bottom).
3) Electrochemical Behavior of the Nanostructured Multiple Layers

To seek thin organic films exhibiting ideal electrochemical behavior, nanostructure of the FUT/MUA monolayer and polyelectrolyte multilayer were studied for their electron transfer property. After the deposition of the polymer layers, it is expected that these layers block or slow down electron transfer because of the polymer attachment.\textsuperscript{49-51} To our surprise, facile electron transfer can be achieved through the carefully prepared PAH/PSS thin films continuously. The physical stability of the electrode surface and polymer build-up appears to be good, with little to no deterioration after multiple scans. Figure 5 shows the CV results on a 1:1 FUT/MUA SAM system with polymer deposition. The ferrocene on the FUT/MUA surface showed well-defined, reversible response after each layer was attached, suggesting the PAH/PSS films provide a favorable environment/membrane that does not interfere with the transfer of electrons and movement of counter ions. There is little or no effect on the magnitude and shape of the electrochemical signals even after the buildup of multiple layers of polymer. More detailed results are summarized in Table 2.1. The average current ($i_p$), peak separation ($\Delta E_p$), and formal potential ($E^\circ$) was found to be $8.6\pm0.3$ µA, $31\pm5$ mV, and $398\pm2$ mV for all layers, respectively. The amount of adsorbed FUT remained constant at $4.7\pm0.9\times10^{-10}$ mole·cm$^{-2}$, all of which strongly indicates there is unconstrained redox communication between the working electrode and the ferrocene interface even after adding 10 polymer layers.
Table 2.1 Voltammetric results for peak current ($i_p$), formal potential ($E^\circ$), and peak separation ($\Delta E_p$) obtained before and after each layer.

<table>
<thead>
<tr>
<th>Layer</th>
<th>$i_p/\mu A$</th>
<th>$E^\circ/V$</th>
<th>$\Delta E_p/mV$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUT/MUA</td>
<td>7.9</td>
<td>0.400</td>
<td>27</td>
</tr>
<tr>
<td>1</td>
<td>9.1</td>
<td>0.395</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>8.3</td>
<td>0.398</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>8.8</td>
<td>0.396</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>8.5</td>
<td>0.400</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>8.9</td>
<td>0.398</td>
<td>31</td>
</tr>
<tr>
<td>6</td>
<td>8.7</td>
<td>0.397</td>
<td>34</td>
</tr>
<tr>
<td>7</td>
<td>8.8</td>
<td>0.400</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>8.9</td>
<td>0.400</td>
<td>37</td>
</tr>
<tr>
<td>9</td>
<td>8.4</td>
<td>0.400</td>
<td>37</td>
</tr>
<tr>
<td>10</td>
<td>8.7</td>
<td>0.396</td>
<td>32</td>
</tr>
</tbody>
</table>
Modified electroactive surfaces have been studied extensively. However, to sufficiently have facile counterion diffusion through a redox inactive film, efficient relay of electrons is often required within the film using redox active polymers.\textsuperscript{52} Clearly effective ionic movement through the organic film above the redox SAM plays an important role in the observed electrochemical properties.\textsuperscript{53-54} To further understand the ionic movement, we evaluated the effect of scan rate on response. A plot of the average peak current versus the scan rate for all layers is shown in the insert of Figure 2.7 with the appropriate error bars. The peak current ($i_p$) is directly proportional to the scan rate ($v$), not $v^{1/2}$, which is characteristic of ET for species immobilized on the surface of the electrode. Plotting log($i_p$) vs. log($v$) yields an average slope of 0.92, which is slightly less than the anticipated value of 1.0 for surface-localized electroactive species. Nevertheless, this value is large and consistent with films of different thicknesses, further proving that the diffusion process of the non-electroactive species through the nanostructures is unhindered. We also observed the insensitivity of the average peak separation versus the scan rate up to 800 mV$\cdot$s$^{-1}$ for all layers, further illustrating an unobstructed mass transport through the film and facile electron tunneling at the surface (Figure 2.8). To our knowledge, this is the first report on unhindered redox chemistry through a 13 nm polyelectrolyte nanosystem without the use of a redox relay.
Figure 2.7 Overlay of the cyclic voltammograms taken after each layer of polyelectrolyte was adsorbed on a 1:1 FUT/MUA surface in 0.1 M NaClO$_4$. The scan rate is 100 mV·s$^{-1}$. Inset: The dependence of the $i_{p,a}$ on the scan rate after each layer. (n=11)
Figure 2.8 The average peak separation versus the scan rate up to 800 mV/s for all layers (n=11).
SPR is sensitive to the structural change on the Au surface, which offers a useful tool to probe continual functionalization of the nanoassembly. The electrochemical reaction of the ferrocene headgroup introduced a local change in the double layer zone and the result of this local change can cause an increase in refractive index. The ESPR technique employed here appears to be ideal to probe the changes within the dielectric layer from the applied electric field, especially for layers covered with redox-active material. We observed that when a modulating potential (cyclic) is applied, the SPR signal responds to it and offers good measurement of the surface property pertinent to the deposited molecules. Upon a CV potential applied to the 1:1 FUT/MUA surface, there is an angular change in the SPR, which returns back to the baseline when the potential is returned (Figure 2.9a). However, when there is no redox material present on the electrode surface, no SPR response was observed (Figure 2.9b). Three contributing factors to the SPR response caused by cyclic voltammetry have been suggested, including a change in dielectric constant (Δε), a change in average thickness of the molecular layer (Δd), and/or a change in surface charge density of the electrode (Δq).\textsuperscript{55-56} In this case, it appears to be the change of charge density that has a large and direct impact on SPR signal due to the presence of a redox active film. It was observed that the SPR signal is proportional to the potential applied, indicating the method could be used to probe the extent of redox reaction on the surface. It also indicates that the polymer film has no blockage to the establishment of the double layer at the headgroup region. The deprotonation and protonation of ferrocene occurs rapidly, allowing for anionic electrolytes to move into and out of the porous polymer films (doping/dedoping) freely.
Figure 2.9  (a) Zoomed-in SPR sensogram of a 10-layer nanostructure on a 1:1 FUT/MUA SAM surface in a 0.1 M NaClO\(_4\) solution. The spikes indicate SPR response to CV cycles. Inset is the overlay of cyclic voltammograms at different scan rates (100-800mV). (b) Zoomed-in SPR sensogram of a 10-layer nanostructure on a MUA SAM surface in a 0.1 M NaClO\(_4\) solution. Inset is the overlay of cyclic voltammograms at different scan rates (100-800mV).
4) **AFM and Ellipsometry Characterization**

Further studies have been conducted using AFM and ellipsometry to confirm the ESPR results. The AFM image presented in Figure 2.10a shows that the Au surface consists of flat grains, ~200 nm in size, and is characterized by the root-mean-square (rms) roughness of $1.2 \pm 0.1$ nm over an area of $5 \mu m^2$, which is in good agreement with previously reported Au substrates.\textsuperscript{26} After incubation with 1:1 FUT/MUA the electrode surface becomes homogenously covered with ferrocenyl-carboxylic alkanthiolates. The rms roughness for 1:1 FUT/MUA was found to be $1.3\pm0.5$ nm, with little structural heterogeneity observed on the surface. The ellipsometric measurement for the FUT/MUA monolayer had an effective film thickness of $1.5\pm0.1$ nm. The theoretical thickness of a COOH(CH$_2$)$_{10}$SAu and Fc(CH$_2$)$_{10}$SAu monolayer is found to be 1.5 nm and 1.7 nm, respectively. The theoretical and actual values are in good agreement with previous values found in the literature.\textsuperscript{26}

A 5-layer and a 10-layer polymer coating on 1:1 FUT/MUA were characterized by AFM, giving an rms roughness of $6.0 \pm 1.3$ nm and $15.2 \pm 5.6$ nm, respectively. The increase in roughness is a strong indicator that the surface is being built up by the deposition of polyelectrolytes that yield inhomogeneous structures. Ellipsometry measurements taking at layers of 1, 2, 4, 6, 8, and 10 yield thickness of $2.5\pm0.3$, $3.3\pm0.2$, $4.9\pm0.2$, $6.3\pm0.3$, $8.2\pm0.3$, and $10.3\pm0.5$ nm, respectively. Close examination of these values shows that thickness by ellipsometry is much smaller than the value obtained through SPR estimation (for instance, 10.3nm vs. 12.6nm for the 10-layer film). It is important to note that AFM and ellipsometry were performed in air, whereas all ESPR
experiments were carried out in aqueous solutions. While the nanostructure in solution may exist in its fully hydrated form, exposure to air can lead to loss of water and thus shrinkage, and therefore a smaller thickness. The advantage of hydrated polyelectrolyte layer on the surface, as demonstrated here, is that it provides a greater number of “holes” for the ions to move in and out of freely, giving rise to a better movement of ions for compensating the charge imbalance due to ET, and thus presenting facile and unhindered redox chemistry on a covered surface.
Figure 2.10  Atomic force microscopy (AFM) images of (a) bare Au, (b) 1:1 FUT/MUA SAMs, (c) 5 layer build up, and (d) 10 layer build up.
E. Conclusions

Through a highly controlled surface procedure, an in-depth study of the polymer layer buildup and its effect on the electrochemical property of the covered redox moiety of a SAM film was performed. The LbL surface construction with PAH and PSS does not hinder the electron exchange to the redox active electrode. The highly porous films allow for the ionic counter charges to freely diffuse, which significantly enhances the ion conductivity of the films. The measured $\Gamma_{\text{Fc}^+}$ reveals a full-coverage of a FUT/MUA monolayer with the quantity of surface-immobilized ferrocene, $\Gamma_{\text{Fc}}$, at $4.1(\pm 0.3) \times 10^{-10}$ mole$\cdot$cm$^{-2}$, and the current magnitude does not change after a ten layer polymer assembly. The electrochemical characterization shows an average peak separation of $30\pm 5.8$ mV and the peak current is directly proportional to scan rate ($i_p \propto v$). The polyelectrolyte was able to completely adhere with great stability to the surface with no incubation needed. Overall, we have demonstrated a new 3-dimensional nanoassembly interface with the desirable surface properties of facile electron transfer, free counter ion movement, and controlled multilayer build-up. This work opens avenues for a potentially multitude of new applications in the science of depositing thin polymer films over the surface of electron active electrodes such as, studying the mass transport of molecules across these films, thin-film electrochemical detection, and creation of electroactive sites for biological detection. With the significantly increased capacity for hosting capture molecules on the polymer layer while remaining facile electron transfer property, we next plan to build onto this multilayer system biological entities for sensing bacteria and viruses with a redox readout.
F. References


3. CHAPTER THREE: Enzyme-Amplified Electrochemical Detection of Viral Particles

A. Abstract

A fast, reliable, and cost-effective method for virus detection is crucial to early diagnosis of viral infection and management of associated diseases worldwide—especially in developing nations. Current viral detection relies on antibody serology (e.g. immunofluorescence assays and enzyme-linked immunoassays), molecular fingerprinting (e.g. viral nucleic acid amplification), and/or direct sensing of intact viruses (e.g. plaque assay). Many of these methods are expensive, time consuming and require trained personnel to perform. Herein we report the fabrication of an electrochemical biosensor that is technically simple, inexpensive, fast, and suitable for detection of viral particles. The electrode surface is modified with a thin polymer layer built through a bottom-up approach with mixed self-assembled monolayers (SAMs) of 11-ferrocenyl-1-undecanethiol (FUT) and mercaptoundecanoic acid (MUA) on a gold substrate. A subsequent 3D layer of porous polyelectrolytes, poly(allylamine hydrochloride) (PAH) and poly-L-glutamic acid (PGA), are deposited for a higher viral loading capacity using a layer by layer (LbL) method. In this work, antibodies are used for capture of the whole viral particles to the surface of the electrode. The detection is achieved by using an alkaline phosphatase (ALP) tagged antibody that converts p-aminophenylphosphate (APP) into the electroactive p-aminophenol (AP). The amplified AP is oxidized to p-
quinoneimine (QI) by electrochemically generated ferrocenium ions on the electrode surface that affords a low detection limit for poliovirus type 1 at 11 pfu mL\(^{-1}\).

B. **Introduction**

Viruses are responsible for changing the course of civilization, from the 1918 influenza pandemic and U. S. poliovirus epidemic of the 1950s, to the more recent HIV pandemic that has already killed more than 25 million people worldwide since its discovery in 1981.\(^1\) The existence of an ultrasensitive on-the-spot biosensor to identify the presence of viruses in the environment would be very useful and possibly would have resulted in fewer individuals becoming infected. In addition, the presence of a rapid and reliable viral biosensor could improve response time in pandemic outbreaks if it were used for routine monitoring purposes. Currently there are two types of detection methods used, either molecular detection (e.g., polymerase chain reaction (PCR) for viral nucleic acid amplification) or direct detection of the whole infective virus particles (e.g., cell culture assays).\(^2\) While these methods are valuable diagnostic tools, the current state of analytical viral detection suffers from a number of deficiencies. Cell culture detection can take several days to weeks to complete, and not all viruses can be detected using cell culture. Molecular methods, while they are more rapid than culture methods, require specialized equipment and are highly vulnerable to contamination and environmental inhibitors.

An advanced analytical approach for viral detection is through the use of enzymes in combination with electrochemical amperometric measurement. Signal transduction with enzymes as the target component is very attractive for building ultrasensitive
electrochemical detection schemes. The enzymes on the surface retain their structure and catalytic activity while facilitating the acceleration of electron transfer between the substrate and the electrode surface. The best characterized and most intensively studied enzymes showing direct electron transfer properties are the peroxidases, in particular horse radish peroxidase (HRP). HRP has been used for a broad range of sensor designs and for selectively identifying biological pathogens of interest. However, HRP has some disadvantages due to low electron transfer kinetics. The reason for this is due to two factors: first, the active center of HRP sits deep within the hydrophobic globule pocket making the distance for electron transfer too large; second, the electron transfer can be isolated or hindered by glycate residues found on native HRP. These issues limit the use of HRP tags for the ultrahigh sensitivity needed for virus detection.

An enzyme that has shown great promise for a new generation of electrochemical enzymes is alkaline phosphatase (ALP). ALP is commonly employed for enzyme-labeled DNA hybridization colorimetric assays, where ALP catalyzes the substrate 3-indosyl phosphate to generate indigo blue in the presence of nitro blue tetrazolium. It has also shown to be a good substrate enzyme for electrochemical amplification schemes due to its high turnover number, low cost, and broad substrate specificity. The electroactive species, $p$-aminophenol (AP), can be generated by the dephosphorylation of the $p$-aminophenyl phosphate (APP). Electron transfer kinetics have been reported to be greater than 1 s$^{-1}$, illustrating un-hindered electron mediation. This method has been used in electrochemical enzyme immunoassays which the enzymatic properties of ALP provide amplification, leading to detection limits in the femtomolar range.
In this work, we have developed a new viral sensor built upon recent advances in microfabrication and nanotechnology, which have played an important role in improving the sensitivity and operational simplicity for various biosensors. Fabrication of the sensing interface has been the core of sensor research, and in many cases it remains the most challenging task among all components in a sensor design. We have exploited the use of novel surface techniques for these tasks, and were able to directly monitor and optimize the sensor design in “real-time” by using surface plasmon resonance (SPR) spectroscopy in combination with electrochemistry. The availability of these methods allows creation of well structurally controlled, durable, yet simple nanoarchitecture with unique electrochemical property for ultrasensitive measurement. Our sensing interface is constructed with molecular assembly approach that offers precision on dimension and thickness. The initial layers of the electrode surface is developed through a bottom-up approach, using mixed self-assembled monolayers (SAMs) attached to a gold substrate, which creates the needed functionalized scaffolding for following layers. Next, the subsequent layers of 3D porous polyelectrolytes is deposited using an electrostatic layer by layer (LbL) method, which generates a high loading capacity for the bioreceptor (antibody) to be deposited for ultrasensitive transduction. Final viral detection is obtained through a sandwich-type electrochemical immunoassay using an ALP-conjugated antibody as the signal transducer.
C. Experimental Section

1) Chemical and Materials

The goat anti-polyivirus and alkaline phosphatase antibody conjugation kit were purchased from AbD serotec (Raleigh, NC). The p-aminophenyl phosphate (APP) was purchased from BIOSYNTH INTERNATIONAL, Inc. (Itasca, IL). Rabbit IgG antibody, for negative control experiments, was purchased from GeneTex, Inc. (Irvine, CA). N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Fisher Scientific (Rockford, IL). Poly-L-glutamic acid (PGA), poly(allylamine hydrochloride) (PAH), and mercaptoundecanoic acid (MUA) were purchased from Sigma-Aldrich (Milwaukee, WI). Dojindo Molecular Technologies (Japan) supplied the 11-ferrocenyl-1-undecanethiol (FUT). Microscope slides were purchased from Fisher (Pittsburgh, PA) and the platinum wire (0.5 mm, 99.997 %) was purchased from Alfa Aesar (Ward Hill, MA). All buffer salts and other inorganic chemicals were obtained from Sigma-Aldrich, unless otherwise stated. All chemicals were of the highest analytical grade, and were used without further purification.

Milli-Q (>18 MΩ) water was used in the preparation of all buffer solutions, and absolute ethanol was used to prepare all thiol solutions. The incubating buffer (IB) consisted of 50mM Tris, 100mM NaCl and 1% BSA (pH 7.2). The rinsing buffer (RB) comprised of 50mM Tris, 100mM NaCl, 0.05% BSA and 0.05% Tween (pH 7.5). The buffer for electrochemical experiments (EB) consisted of 50mM Tris, 100mM NaCl and 10 mM MgCl₂ (pH 8.0).
2) **Poliovirus type 1 (PV1)**

The viral system tested in this work is poliovirus type 1 (PV-1), which was obtained from Prof. Marylynn Yates group at UCR Environmental Microbiology. PV-1 is safe to use and well characterized, making it an ideal model system for viral sensor development. PV-1 is a small, icosahedral, and non-enveloped RNA virus with a 28-30nm diameter and a dry weight of 8250 kDa. To suppress nonspecific contamination on the electrode surface, we further purified the cultured PV-1 solution using a chloroform extraction method along with a cellulose ester dialysis membrane (MWCO: 100 kDa). This resulted in a final virus stock solution of $5.3 \times 10^7 \text{ pfu mL}^{-1}$, which was determined using a plaque assay (Figure 3.1). A plaque assay is a measure of the number of virus particles capable of forming plaques per unit volume in a cell, or plaque forming units (pfu). This quantity is a functional measurement rather than a measurement of the absolute quantity of infective particles. The PV1 was assayed and cultivated using Buffalo Green Monkey kidney (BGMK) cell lines. After inoculation with virus-containing samples, the plates were incubated at 37°C in an atmosphere supplemented with 5% CO$_2$ and observed for plaques in the BGMK cell line. The final PV1 stock solution used in the electrochemical experiments was then serial diluted using the IB solution to attain 7 sample concentrations of $\sim 10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, and $10^7 \text{ pfu mL}^{-1}$. Subsamples (100 µL) were withdrawn and electrochemically examined in triplicate to determine the number of PV1 particles present.
Figure 3.1 Photographic image of the plague assay for poliovirus type 1 (PV-1) using Buffalo Green Monkey kidney (BGMK) cell lines. The stock solution was determined to be $5.3 \times 10^7$ plaque-forming units per milliliter (pfu mL$^{-1}$).
3) Electrochemical and SPR experiments

The electrochemical measurements were carried out with a three-electrode system using a CHI 650 electrochemical work station (CH Instruments, Austin, TX). The modified Au electrode served as the working electrode, and an Ag/AgCl electrode was used as the reference electrode which was placed at the outlet of the nanoSPR flowcell. The gold counter electrode was machined into the top of the SPR flow cell. The CV data fitting was performed using Origin software (Microcal, Inc.) Once the attachment of anti-PV1 is complete and the sensor is ready for PV1 detection, 7 different concentrations of PV1 are injected into the cell and incubated for 60 min, independently. After rinsing with RB, the PV1 sensor is incubated with 100 μg mL⁻¹ ALP-conjugated goat anti-PV1 for 60 min and then washed with RB (see Figure 3.2). Finally the cell is filed with EB solution containing 1 mM APP (injected) for 10 min and then electrochemically scanned with the CV. The APP solution was prepared daily.

The surface plasmon resonance (SPR) spectrometer employed in the procedure is a dual channel nanoSPR in the Kretschmann configuration (Morton Grove, IL) with a semiconductor laser (λ=670 nm) as the excitation source. Surface interaction and modification were monitored and characterized using the tracking mode of SPR angular scanning at the minimum angle. The SAM modified working electrodes were clamped down to a flow cell on a high-refractive index prism. Once the RB solution had established a stable baseline, the SAM chip is injected in sequential order for the PV1 sensing interface build-up. All electrochemical and SPR experiments were carried out using a constant flow rate of 20 μL min⁻¹.
D. Results and Discussion

1) Design and fabrication of the electrochemical PV1 sensor

The fabrication of the electrochemical viral sensor involves the use of the Au chips prepared the same way as previously discussed in Chapter 2. Prior to modification, the Au substrates is carefully cleaned with piranha solution, then extensively rinsed in Milli-Q water, absolute ethanol, and dried with a stream of N2 gas. The electrodes is then immersed in a 1 mM (1:1) FUT/MUA for 12-24 h and rinsed with ethanol, DI water and dried in a N2 stream.

Figure 3.2 shows a cartoon illustration of the system employed in this work. First, 5 bilayers of 1 mg mL\(^{-1}\) PAH and PGA (in 0.1mM NaClO4) was deposited onto the mixed FUT/MUA SAM with PGA as the final layer, creating a negatively charged carboxylic acid surface. With careful control, the electrode surface continues to present facile redox chemistry even after multiple layers of polymer are attached. This 3D thin layer possesses desirable properties similar to those on a SAM while presenting a ~20 nm exceedingly porous structure for high loading capacity. Due to the fact that antibody contains a net positive charge it can be electrostatically attached to the negatively charged PGA substrate. In this work we covalently linked the goat polyclonal antibody (anti-PV1), which is specific to polio virus types 1, 2 and 3, to the 3D thin film surface. After the PGA carboxylic acid groups were activated using 75 mM EDC and 15 mM NHS for 30 min, a 100 µg mL\(^{-1}\) of anti-PV1 (in IB) was injected. This EDC/NHS step acts as a locking mechanism for the PV1 sensing surface by covalently linking the SAM’s layer to the 5 PAH/PGA bilayers, and the PGA carboxyl group on the surface to the amine groups.
on the antibody. After incubation, the anti-PV1 surface is extensively washed with RB, and the chips are ready for PV1 detection.
Figure 3.2 Schematic representation of the preparation of the amperometric ALP enzyme PV1 sensor based on the mixed SAM (FUT/MUA) and polymer bilayer (PAH/PGA) film.
2) Enzyme activity and loading capacity.

The principle of the enzyme-amplified electrochemical sensor for detecting PV1 is shown in Figure 3.3. The enzyme (ALP) on the antibody that binds to PV1 converts the substrate (APP) into an electroactive product (AP) through dephosphorylation. After AP accumulates for a given incubation time, an electrochemical anodic current is measured by oxidizing AP by the partially ferrocenyl-tethered SAM surface at a gold electrode to 1-p-quinoneimine (QI). The oxidation of AP is mediated by the reduction of ferrocenium back to ferrocene and the result is a measurable electrocatalytic anodic current. As the number of ALP is determined by the viral particle captured on the surface, the electrocatalytic current depends on the concentration of PV1.

For this system, a mixed FUT/MUA SAM monolayer was prepared on a gold electrode for two important reasons. First, the MUA has an unreacted carboxylic acid moiety, which provides an efficient site for immobilizing molecules to the sensor surface. Second, the FUT acts as a redox mediator because it can mediate electron transfer between the electrode and the enzyme-generated electroactive products. Without the ferrocenyl-tethered SAM (FUT) the electrode would not be able to oxidize the soluble redox reagent (AP).\textsuperscript{12-13} This compact electroactive mixed SAM monolayer also reduces the background current of the electrode, which results from capacitive current. It should be noted that the mixed SAM surface alone will not provide the needed loading capacity to generate high sensitivity for the enzymatic relay to the electroactive surface. Therefore a larger surface area (3D) and thicker layer for higher loading capacities would be desirable for this biosensor operation. Biosensing devices with a highly active
immobilized enzyme system allow effective maintenance of connection between the target molecules and the transduction component.
**Figure 3.3** Enzymatic signal enhancement using an alkaline phosphatase (ALP) labeled anti-PV1. The ALP label converts $p$-aminophenyl phosphate (APP) into the electroactive compound $p$-aminophenol (AP). This product is electrooxidized to quinoneimine (QI) by the reduction of ferrocenium back to ferrocene, producing an electrocatalytic anodic current.
In order to achieve a 3-D multi-layer molecular nanostructure for higher loading capacity, we utilized the LbL method, which works by alternating physiosorption of oppositely charged molecules.\textsuperscript{14} The negatively charged MUA is the anchor for the architecture, whereas PAH is the first positively charged layer and PGA is the second negatively charged layer. One advantage of the LbL method is that the amount of material deposited on each cycle approaches a constant and reproducible value, allowing a large number of controllable layers and specific properties to be incorporated into the thin films. In this system the specific properties of interest are the amine (NH$_2$) and carboxylic (COOH) moieties, which are not only electrostatic but also suitable for further biomolecular attachment via standard EDC/NHS chemistry. Another major advantage of the LbL method is that the film it generates offers a higher loading capacity than traditional SAM methods, which can only produce a 2D monolayer.\textsuperscript{15-16} In our previous study (Chapter 2), a 5 bilayer system was shown to be the optimal amount of polymer attachment needed to reach the reproducible and constant loading capacity, while at the same time maintaining facile electron communication.\textsuperscript{10}

To qualitatively monitor and compare the loading capacities of a SAM surface vs. a polyelectrolyte 3D multilayer surface, SPR measurements were carried out (Figure 3.4). For the mixed SAM surface (1:1 FUT/MUA) that is immobilized with anti-PV1 (100 $\mu$g mL$^{-1}$), there was an increase of only 20 millidegrees between 60 min and 100 min of incubation. For the 3D multilayer of PAH/PGA, there was a significant enhancement in loading capacity, with an increase in 100 millidegrees between 60 min and 100 min of incubation. The immobilization yield demonstrated a 500% increase when the LbL
method was used. Clearly, the enzyme loading capacity can be considerably increased when a relatively thick densely packed layer of polymers is employed for immobilization.
Figure 3.4 SPR sensorgrams showing the difference in loading capacity of anti-PV-1 (100 μg mL\(^{-1}\)) on a SAM monolayer surface (left) and a polyelectrolyte 3D multilayer surface (right).

\[\Delta 20 \text{ millidegrees} \quad \Delta 100 \text{ millidegrees} \]

3D Multilayer >> 2D Monolayer
3) **Signal amplification and detection of PV-1**

To characterize the electrocatalytic performance of the sandwich-type virus sensing transducer, the ALP-conjugated anti-PV-1 was bound to the captured PV-1 followed by incubation for 10 min in EB containing 1 mM APP (Fig. 3.5). In the presence of ALP, an irreversible anodic peak was observed near 0.2 V vs. Ag/AgCl, indicating redox mediated oxidation of AP (Fig. 3.5C). The peak current increased with incubation time and leveled off after 30 min. In contrast, the oxidation peak was not present in the absence of APP (Fig. 3.5B). The oxidation potential for the 5 bilayer-mixed SAM (FUT/MUA) electrode was near 0.4 V (Fig. 3.5A). As discussed in previous reports, the anodic peak potential shifts from the oxidation potential of AP (0.8 V vs. Ag/AgCl) to the oxidation potential of the FUT ferrocenyl groups (0.4 V vs. Ag/AgCl).\(^{17-18}\) The shift of potential can be explained by the following mechanism,

\[
\text{Fc} \rightarrow \text{Fc}^+ + e^- \\
\]

\[
2 \text{Fc}^+ + \text{AP} \rightarrow \text{QI} + 2 \text{Fc}
\]

where QI (quinoimide) is the oxidation product of AP by the loss of two electrons. When the potential scan is reversed, no cathodic peak corresponding to reduction of QI was observed even in an extended positive potential sweep to 0.8 V. The surface-immobilized ferricenium (Fc\(^+\)) is capable of oxidizing solution-phase AP via a two-step mechanism because the oxidation reaction is thermodynamically favorable. However, the reverse reaction does not occur because it involves the thermodynamically unfavorable electron transfer from ferrocene to QI. The absence of a cathodic peak for QI reduction and the shift of the anodic peak for AP oxidation strongly suggest that AP oxidation is occurring
via the proposed redox reaction involving ferrocene. Other groups have reported similar irreversible redox behavior for this system.\textsuperscript{13,19-20} The electrocatalytic reactions are attractive means to effectively enhance the electric signal for biological detection as reported by several groups.\textsuperscript{21-23}

As a negative control, cyclic voltammetry was carried out in the presence of rabbit IgG instead of PV-1 (Fig. 3.5D). Without the PV-1, the catalytic current was almost non-detectable. The disappearance of the anodic current indicated that the binding of ALP-conjugated anti-PV-1 cannot take place due to the lack of PV-1 on the sensor surface. In addition, the absence of a peak current indicates little to no non-specific binding in our method.

The analytical performance of the virus sensor was characterized by carrying out cyclic voltammetric study of the virus sensing electrodes at various concentrations of PV-1 in EB solution containing 1 mM APP (Fig. 3.6). Three cyclic voltammograms were obtained at each concentration from 0 $\text{pfu mL}^{-1}$ to $5.3 \times 10^7$ $\text{pfu mL}^{-1}$. The peak current increased with the concentration of PV-1 from $5.3 \times 10^1$ $\text{pfu mL}^{-1}$, and did not level off at the highest available PV-1 concentration, $5.3 \times 10^7$ $\text{pfu mL}^{-1}$. Figure 3.7 shows the calibration plot using anodic currents measured at 0.2 V in the cyclic voltammograms. To better reveal the correlation and large dynamic range, we used the logarithm of the number of viruses as the x value and the current ($\mu\text{A}$) as the y value. A good correlation was obtained over 7 orders of magnitude, indicating a very large working range. Linear regression was attempted for data points in the low concentration range, which yields a reasonably well defined linear relationship. Using the 3$\sigma$ cutoff, the detection limit was
determined to be 11 pfu mL⁻¹. It’s interesting to note that the lower limit of quantitation current magnitude at 5.3×10¹ pfu mL⁻¹ PV-1 was considerably higher than that of 0 pfu mL⁻¹ PV-1. We believe that the reason for a high current at 5.3×10¹ pfu mL⁻¹ PV-1 is because we are using pfu (plaque forming units) to report the quantity of virus particles. As pfu is a functional measurement rather than a measurement of the absolute quantity of virus particles, virus particles that fail to infect BGMK cell will not produce a plaque and thus are not counted. Therefore our limit of detection for virus particles is much greater than reported.
Figure 3.5 Cyclic voltammograms of the enzyme-amplified electrochemical detection of poliovirus type 1 (PV-1) with (B-C) normal (5.3×10^6 pfu mL⁻¹ PV-1) and (D) negative (10 μg mL⁻¹ rabbit IgG) control samples. A is the cyclic voltammograms of 5 bilayers (PAH/PGA) on a mixed SAM (FUT/MUA) modified Au electrode. In (B-C), cyclic voltammograms of the (ALP) enzyme-amplified sandwich type detection (B) before and (C-D) after the incubation of 1mM APP for 10 min. In (D), rabbit IgG was used instead of PV-1. All cyclic voltammograms were obtained using EB solution and at a scan rate of 50 mV s⁻¹.
Figure 3.6 Cyclic voltammograms of a sandwich-type electrochemical virus sensor for poliovirus type 1 (PV-1) at various concentrations of PV-1: (A) 0 pfu mL⁻¹, (B) 5.3×10¹ pfu mL⁻¹, (C) 5.3×10² pfu mL⁻¹, (D) 5.3×10³ pfu mL⁻¹, (E) 5.3×10⁴ pfu mL⁻¹, (F) 5.3×10⁵ pfu mL⁻¹, (G) 5.3×10⁶ pfu mL⁻¹, and (H) 5.3×10⁷ pfu mL⁻¹. Cyclic voltammograms were obtained 10 min after incubation in EB containing 1 mM APP and at a scan rate of 50 mV s⁻¹.
Figure 3.7 Calibration plot showing the correlation between the anodic peak current at 0.2 V and the concentration of target poliovirus type 1 (PV-1) from $5.3 \times 10^1$ pfu mL$^{-1}$ to $5.3 \times 10^7$ pfu mL$^{-1}$ after incubation in EB containing 1 mM APP and at a scan rate of 50 mV s$^{-1}$. 
E. Conclusions

In this work, we have demonstrated a fast, reliable, and cost-effective method for virus (PV-1) detection using a sandwich-type electrochemical enzymatic amplification scheme. In our method a 3D polyelectrolyte substrate (PAH/PGA) is fabricated and functionalized on a mixed electroactive SAM (FUT/MUA) surface. The attachment and covalent stabilization of the substrate and bioreceptor (anti-PV-1) via EDC/NHS scheme are monitored by SPR spectroscopy. The use of LbL to build a 3D structure not only provides an increased capacity for the immobilization of the bioreceptor (anti-PV-1) but also an improved stability of the sensor molecule while still maintaining excellent electron communication at the electrode surface. In addition to high loading capacity, nonspecific interaction is minimal on the 3D polymer substrate, which is important for the excellent virus sensor performance. The redox mediated oxidation of AP with FUT is facile, allowing for the determination of peak current based on target virus concentration. The technique described here can be further optimized for better performance, and provide significant enhancement for biosensors using an ultrathin 3D sensing interface.
F. Reference


4. CHAPTER FOUR: Nanofibers Doped With Dendritic Fluorophores for Protein Detection

A. Abstract

We report a solid-state, nanofiber-based optical sensor for detecting proteins with an anionic fluorescent dendrimer (AFD). The AFD was encapsulated in cellulose acetate (CA) electrospun nanofibers, which were deacetylated to cellulose to generate secondary porous structures that are desirable for enhancing molecular interactions, and thus better signaling. The protein sensing properties of the fibers were characterized by monitoring the fluorescent behaviors of cytochrome c (cyt c), hemoglobin (Hgb), and bovine serum albumin (BSA) as a function of concentration. Effective quenching was observed for the metalloproteins, cyt c and Hgb. The effect was due primarily to energy transfer of the imbedded fluorescent dendrimers to the protein as both proteins contain heme portions. Electron transfer, caused through the electrostatic effects in the binding of the anionic dendrimer to the positive patches of globular proteins, could be responsible as well. BSA, on the other hand, triggered a “turn-on” response in fluorescence, suggesting the negatively charged BSA reduces the $\pi-\pi$ stacking of the partially dispersed, negatively charged dendritic fluorophores through repulsion forces, resulting in an increase in fluorescence. Stern-Volmer constants ($K_{sv}$) of the electrospun fibers were found to be $3.4 \times 10^5$ and $1.7 \times 10^6$ M$^{-1}$ for cyt c and Hgb, respectively. The reusability of the nanofibers is excellent: the nanofibers demonstrated less than 15% change of fluorescence intensity signal in a 5-cycle test.
B. Introduction

The development of solid-state optical biosensors continues to be of major interest within the nanotechnology field because of the many practical and potential functions.\(^1\) For instance, these solid-state sensors can exhibit advantages of versatility, sensitivity, selectivity, simplified optical setup, and a large dynamic range. However, desirable mechanical, electronic, and optical properties can be difficult to realize at the sensing interface because they require sophisticated synthesis routes and tend to use a broad range of discontinuous objects such as carbon nanotubes, nanorods and wires.\(^2\) In the past decade, electrospun polymer nanofibers have proven to contain many of the unique properties desirable for biotech development. Recent work using electrospun nanofibers covers a wide range of applications, including optical sensors and biosensors, filtration membranes, drug delivery devices, and scaffolding for stem cell growth.\(^3\)-\(^7\) In this strategy a straightforward top-down in-situ electrospinning approach was utilized along with an anionic fluorescent dendrimer (AFD) to fabricate an optical sensor for the detection of low concentrations of proteins via a fluorescence resonance energy transfer (FRET) principle. Herein we report on a reusable solid-state fluorescent biosensor, using nanofibers, with pattern specific chemical diversity.

Electrospinning is a polymer processing technique used to create fibers with diameters ranging from a few nanometers to micrometers.\(^8\) The electrospinning process works when a flowing polymer solution is subject to a high electric field. When the repulsive electrostatic force overcomes the surface tension of the polymer solution a stable jet is formed and a membrane-like web of small fibers is emitted onto the ground.
Moreover, the use of electrospun nanofibers for chemical sensors using fluorophores has been previously reported. For instance, Samuelson and co-workers have demonstrated that pyrene methanol and hydrolyzed poly[2-(3-thienyl) ethanol butoxy carbonyl-methyl urethane] (H-PURET) can be immobilized to the surface of electrospun membranes for the detection of metal ions (Fe$^{3+}$ and Hg$^{2+}$), 2,4-dinitrotoluene (DNT), and methyl viologen (MV$^{2+}$).\textsuperscript{10-11} Tao et al. reported the use of sol-gel chemistry to make porphyrin-doped nanofibrous membranes for the detection of 2,4,6-trinitrotoluene (TNT) vapor.\textsuperscript{12} Unfortunately a major drawback of these platforms is that they require multiple fabrication steps, resulting in inhomogeneous dispersion of sensing molecules within the membrane and potential fluorescent leakage, and ultimately compromising sensitivity, stability, and reproducibility of the solid-state optical sensors. More recently, Yang et al. demonstrated that secondary porous structures could be added to 9-chloromethylanthracene (9-CMA)-doped cellulose acetate (CA) nanofibers for the detection of MV$^{2+}$.\textsuperscript{13} While Yang et al. provided a simple approach to creating secondary pores within the nanofiber there is still a need for new fluorescent units that demonstrate distinctive FRET properties and better process characteristics, such as improved retention. All of which are important for generating new protein sensors.

Fluorescent dendrimers are highly effective receptors for fluorescent optical sensors for many different target analytes, such as explosives (TNT and DNT) and biomarkers.\textsuperscript{14-18} In comparison with molecular fluorophores, the numbers of fluorophore units in dendrimers can be controlled by simple synthetic means. This convergent approach allows for more predictable structure-related and fluorescent properties within
the sensor. In this work a diphenylacetylene dendritic compound containing negatively charged peripheral groups is used as an effective bioreceptor. In the past, Thayumanavan and coworkers have utilized dendritic scaffolding to generate fluorescence-based patterns for both metalloprotein and non-metalloprotein sensing using solution based detection schemes.\textsuperscript{19-22} The quenching of their sensor was reported to be caused by the charge density around the protein, as well as quantity and position of the heme within the metalloproteins. While the use of fluorescent dendrimers for solution-based protein detection exists,\textsuperscript{19} there is still a lack of research that uses simple approaches to creating reusable solid-state devices that can respond differentially to a variety of proteins.

C. Experimental Section

All chemicals were of the highest analytical grade, purchased from Sigma-Aldrich (Milwaukee, WI) and were used without further purification, unless otherwise stated. Milli-Q (>18 MΩ) water was used in the preparation of all buffer solutions. Steady-state fluorescence measurements were performed on a HORIBA FluoroLog spectrofluorometer using the excitation at 370 nm. Fluorescence image analysis was performed on a Leica TCS SP2/UV confocal microscope using the excitation wavelength at 364 nm. The scanning electron microscope (SEM) used is a Phillips XL30-FEG. Fiber analysis was performed using fourier transform infrared (FTIR) spectroscopy on a Equinox 55/S FTIR spectrometer with a Bruker A590 microscope.

CA is used as the host matrix in our nanofiber fabrication due to its chemical resistance, thermal stability, low non-specific absorption, and capacity to be easily functionalized with recognition elements.\textsuperscript{23} In order to further improve the surface area-
to-volume ratio and overall performance a simple deacetylation treatment was used to create specific secondary structures within our electrospun nanofibers. In fact, Park and coworkers have recently demonstrated that secondary porous structures can easily be inserted into the backbone of electrospun CA fibers by the homogenous deacetylation treatment of CA to cellulose using a more practical processing step while at the same time maintaining the nanofibers physical properties.\textsuperscript{24-26} This deacetylation treatment is used in our work to generate evenly distributed secondary pores throughout the nanofiber backbone of cellulose to improve the sensing performance. A schematic illustration of the electrospinning setup, encapsulation of the fluorescent dendrimer, and deacetylation process is shown in Figure 4.1.

To find the best protein-sensing receptor, 5 water-soluble fluorescent dendritic compounds (AFD-1, AFD-2, AFD-3, AFD-4 and AFD-5) composing of phenylene-ethynylene repeating units (Figure 4.1) were synthesized according to published procedures and screened in solution for the highest visible fluorescence (Figure 4.2).\textsuperscript{27} These dendrimers commonly demonstrated low visible fluorescent emission, but AFD-3 was an exception that gave visibly high fluorescence. The variability of the fluorescent emission among the dendrimers is due to aggregation, which is caused by small differences in the charge distribution among the different AFDs.

In this study an electrospinning solution was prepared by dissolving 17 % CA and 0.1 % AFD-3 (by weight) in 8:1 (v/v) Acetone/H\textsubscript{2}O, and then placed into a plastic syringe. A high-voltage DC power supply (Glassman High Voltage Inc. Series EH) was connected to a 25-gauge blunt nose needle attached to the syringe containing the electrospinning
solution. The electrospun fibers were collected on a grounded aluminum plate. The CA/AFD-3 solutions were electrospun at a voltage of 21 kV, a tip-to-collector distance of 10 cm, and a solution flow rate of 1.2 mL/h. All of the electrospinning procedures were carried out at 25°C with a collection time of approximately 90 s. In order to create secondary porous structures the CA fibers were deacetylated in a 50 mM NaOH ethanol solution at 25°C for 24 h, thoroughly rinsed with water, and dried using N₂. The chemical reaction of CA to cellulose was traced by using FT-IR spectroscopy (Figure 4.3). The characteristic absorption peaks attributed to the vibrations of the acetate group at 1745(νC=O), 1375(νC-CH₃), and 1235 cm⁻¹(νC-O-C) disappeared after deacetylation of CA. An absorption peak at 3500 cm⁻¹ (νO-H) was observed, indicating successful deacetylation. The FT-IR spectrum obtained before and after deacetylation, which agrees with that of pure cellulose fibers.
Figure 4.1 A schematic illustration of the electrospinning setup, encapsulation of the fluorescent dendrimer, and deacetylation process used in this study are shown. The 5 water-soluble fluorescent dendritic compounds (AFD-1, AFD-2, AFD-3, AFD-4 and AFD-5) composing of phenylene-ethynylene repeating units are illustrated. The circled AFD-3 was the fluorescent dye used to dope the CA nanofibers for detection of metalloproteins.
Figure 4.2 Photographic image of the fluorophore solutions (10 µM) in phosphate buffer saline (10 mM, pH 7.4) under black light (360 nm).
Figure 4.3 FT-IR spectra of cellulose acetate (CA) and cellulose fibrous materials (top, before deacytalation; bottom, after deacytalation). The characteristic adsorption peaks attributed to the vibrations of the acetate group at $1745(\nu_{\text{C=O}})$, $1375(\nu_{\text{C-CH}_3})$, and $1235 \text{ cm}^{-1}(\nu_{\text{C-O-C}})$ disappeared after deacetylation of CA. An adsorption peak at $3500 \text{ cm}^{-1}(\nu_{\text{O-H}})$ was observed, further indicating successful deacetylation. The FT-IR spectrum obtained after deacytalition agrees with that of pure cellulose fibers.
D. Results and Discussion

The electrospun fibers exhibited well defined fibrous morphology without bead formation and good structural stability. An electrospun AFD-3-doped nanofiber nonwoven mat is shown in the SEM image in Figure 4.4, further illustrating the large surface area-to-volume ratio formed within the electrospun nonwoven film. The fibers were continuous, uniform, and had a diameter ranging from approximately 400-2000 nm, similar to those reported by Xiang et al.\textsuperscript{28} One-dimension (1D) nanostructures are distributed evenly throughout the electrospun membrane using a simple electrospinning approach. It is assumed that the nanofibers are 3D, due to their inherent porosity created by the deacytelation of CA to cellulose. Consequently, unique secondary porous structures are homogenously distributed throughout the backbone of the nanofibers creating a larger surface area-to-volume ratio and in-turn substantially improving sensitivity.
Figure 4.4  SEM image of electrospun AFD-doped deacetylated cellulose fibers (17% CA/ 0.1% AFD dissolved in 8:1 acetone/water, 10,000× magnification).
The protein sensing properties of the fibers were characterized by monitoring the quenching behaviors of cytochrome c (cyt c), hemoglobin (Hgb), and bovine serum albumin (BSA) as a function of concentration. All proteins were bovine specific, where cyt c is positively charged (pI = 10.2-10.7), Hgb is neutral/slightly negative (pI = 7.0-7.4), BSA is negatively charged (pI = 4.8-4.9), and AFD-3 is negatively charged at physiological pH. The fluorescent spectrum of the fiber varying with the concentration of cyt c is illustrated in Figure 4.5a. The fluorescence intensity decreases proportionally with increase in cyt c concentration. Similar behavior was observed with Hgb (Figure 4.5b). The efficient quenching effects of the metalloproteins, cyt c and Hgb, are due primarily to energy transfer of the imbedded fluorescent dendrimers with the protein as both cyt c and Hgb contain heme portions within the protein. Some of the quenching effect for proteins can be attributed to electron transfer, caused through the electrostatic effects in the binding of the anionic dendrimer to the positive patches of globular proteins. When BSA was used, however, an increase in fluorescence was observed (Figure 4.5c). The slight increase in local fluorescence of the dendritic fluorophore within the high-surface-area of the nanfibers suggested that the negatively charged BSA proteins reduce the π-π stacking of the partially dispersed negatively charged dendritic fluorophores through repulsion forces, resulting in an increase in fluorescence. It is expected that two main factors contribute to protein detection; one, the charge distribution density on the proteins surface, and two, the location of the metalloproteins secondary structure. The intricate nature of the interaction of these two factors should result in protein-dependent patterns allowing for good sensing capabilities.
Figure 4.5 Fluorescence emission spectra of the AFD-functionalized nanofibers in response to varied concentrations of (A) cyt c, (B) Hgb, and (C) BSA ($\lambda_{Ex}$/$\lambda_{Em}$ = 370/475 nm).
The fluorescence dynamic quenching sensitivity can be quantified through the measurements with the Stern-Volmer equation:\(^{32}\)

\[
\frac{I_0}{I} = 1 + K_{sv}[Q]
\]

(1)

where \(I_0\) and \(I\) are the fluorescent intensities in the absence and presence of quencher, respectively; \(K_{sv}\) is the Stern-Volmer quenching constant, and \([Q]\) is the concentration of quencher. The quenching data are usually presented as plots of \(I_0/I\) versus \([Q]\) with a slope equal to \(K_{sv}\). The higher the \(K_{sv}\), the lower the concentration of quencher is required to quench the fluorescence and thus the greater detection sensitivity.

The Stern-Volmer analysis of the electrospun sensors for cyt c and Hgb is shown in Figure 3. At concentrations between 100 nM and 6.4 \(\mu\)M, a linear relationship between quencher concentration and \(I_0/I\) was obtained, showing homogeneous quencher-accessible sites in the electrospun fibers under the experimental conditions. The sensitivity, \(K_{SV}\), of the electrospun fibers were found to be \(3.4\times10^5\) and \(1.7\times10^6\) M\(^{-1}\) for cyt c and Hgb, respectively. BSA tested under the same experimental conditions demonstrated a small negative linear relationship. The inset in Figure 4.6 shows the analyte-dependent pattern from the fluorescence intensity changes at 200 nM. Differential responses for different proteins are demonstrated and therefore illustrated anlyte-specific patterns. The results clearly point to an effective approach to the solid-state fabrication of biosensors by embedding selective receptors into electrospun fibers.

The sensitive protein detection was further visualized using a high resolution UV confocal microscope. Figure 4.7 shows the fluorescence images of the AFD-3-doped cellulose nanofibers before and after incubation with 10 \(\mu\)M cyt c solution for 15 min,
further illustrating the remarkable quenching effect of the nanofiber sensors. The fluorescence images before the quenching process indicate the evident fluorescence emission and the uniform dispersion of fluorophores in cellulose, which is beneficial to sensing performance.
Figure 4.6 Stern-Volmer plots of the nanofibers for cyt c (■) and Hgb (●). Inset: Analyte-dependent pattern for 200 nM of bovine metalloproteins (cyt c, Hgb) and non-metalloprotein (BSA) in PBS buffer solution (pH 7.4).
Figure 4.7  Confocal fluorescence images of the electrospun nanofibers before (left) and after (right) incubation in a 10 μM cyt c solution for 15 minutes (λ<sub>Ex</sub> = 364 nm).
The reusability, reproducibility and stability of the nanofiber material were also investigated. In order to demonstrate the reusability of the sensor the cellulose nanofibers were immersed in a 25μM solution of cyt c for 5 min. Then in a 50 mM NaOH ethanol solution for 15 minutes followed by rinsing in water and drying in air, the fibers were then re-used for sensing the same cyt c solution. Figure 4.8 demonstrates that the used nanofibers contain similar quenching ability as the pristine fibers. In the tested 5 cycles, the nanofibers exhibited less than 15% loss of fluorescence intensity signal, indicating outstanding reusability. We attribute this to the noncovalent nature of the interaction, which is largely based on the electrostatic interaction between the fluorophore and protein. In fact, that weak binding events and striping process do not denature the core, which allows for excellent sample recovery. The reproducibly of the sensor is reflected by low batch-to-batch variation of 5.3% for the CA/AFD-3 nanofibers using 3 separate measurements produced on different days. For the stability experiments we tested the buffer solutions before and after each test and no leakage of the fluorophore were found in the aqueous solutions.
Figure 4.8 Repeated switching of normalized fluorescence emission of the nanofibers for 5 cycles of 25 μM cyt c of quenching/regenerating process. Quenching time: 15 min, regenerated byimmersing into 50 mM NaOH ethanol solution for 5 min, PBS buffered solution (pH 7.4) for 5 min and dried with N₂ gas, λₑₓ/λₑₘ = 370/475 nm.
E. Conclusions

In conclusion, a reusable, solid-state fluorescent biosensor was developed using electrospun nanofibers and anionic dendrimers for quantifying various proteins in solution via a FRET mechanism. The selectivity and specificity of the sensor is displayed in the repeated specific response each protein has with the fluorescent fibers. In particular, the quenching effect is due to the energy/electron transfer processes between iron containing proteins (i.e. cyt c and Hgb) and the fluorescent core resulted in rapid fluorescence quenching. While, the increase in fluorescence by the BSA is due to the increase in π-π stacking of the AFD-3 fluorophore localized on the surface of the nanofibers. A relatively large quenching sensitivity was obtained, which is demonstrated with the Stern-Volmer constants. The electrospun doped fibrous material exhibited large surface area due to small diameter and porosity of the nanofibers. This porosity stems from two factors: the deacetylation of CA to cellulose and the disorderly arrangement of fibers onto the substrate. The ability to homogenously embed the fluorophore into the core of the fiber allows for better reproducibility, reversibility, and durability of the sensor. Future efforts will focus on exploring a nanofiber sensor array containing different fluorescent dendrimers for the detection and identification of protein targets via distinct fluorescence response patterns.
F. References


(9) Huang, Z. M.; Zhang, Y. Z.; Kotaki, M.; Ramakrishna, S. *Compos. Sci. Technol.* 2003, 63, 2223.


5. CHAPTER FIVE: FRET detection of proteins using fluorescently doped electrospun nanofibers and pattern recognition

A. Abstract

This paper reports the fabrication of solid-state nanofiber sensor arrays and their use for detection of multiple proteins using principal component analysis (PCA). Four cationic and anionic fluorescently embedded nanofibers are generated by an electrospinning method, yielding unique patterns of fluorescence change upon interaction with protein samples. Five metal and nonmetal containing proteins, i.e., hemoglobin, myoglobin, cytochrome c, BSA and avidin, have been investigated and the results show that distinct fluorescent patterns can be formed upon the addition of protein samples to the array of solid nanofiber substrates, allowing their unambiguous identification. The nanofiber films are highly repeatable with a batch-to-batch variation of approximately 5% and demonstrated outstanding reusability with less than a 15% loss of fluorescence intensity signal after 5 regenerations of test cycles. For a more practical visualization a cluster map was generated using PCA of the change-in-fluorescence (ΔI) composite patterns, demonstrating the potential of the method for diagnostic applications.
B. Introduction

There has been considerable effort in the development of novel approaches toward highly sensitive detection techniques with the concept of increasing the interface surface area of the integrated sensor systems. Of the techniques that exist, electrospinning has proven effective to generate materials containing many unique properties desirable for creating large surface area-to-volume ratios; continuous 1D nanostructures with 3D porosity, flexibility in surface functionalities, and superior mechanical performance. As a result, this simple technique has been successfully applied to a broad range of studies such as sensors, filtration, drug delivery, tissue engineering, and energy research. However, the development of efficient protein sensors using electrospun nanofibers is still a challenge due to the inherent complexity of biological samples and the lack of highly selective and specific binding agents that interact with protein surfaces.

A key requirement in the development of protein-sensing methods for use in the early diagnosis of cancer or other disease states is the ability to rapidly “detect” and “identify” the presence of certain biomarker proteins and/or irregular protein concentrations. Currently, the most widely used analytical method for protein detection is the enzyme-linked immunosorbent assay (ELISA). Though commercially successful, it is limited by cumbersome preparation procedures, chemical instability and a need to label analytes with an enzyme-coupled antibody. Recently, a number of indirect detection methods have been proposed, including the chemical nose approach that may have potential for analyzing a complete protein expression profile with the speed and
sensitivity required.\textsuperscript{12-13} Bajaj \textit{et al.} has used the chemical nose approach to not only indicate the presence of cancer cells (cervical, liver, testis and breast) in human serum samples but also to distinguish between primary cancer and metastatic disease.\textsuperscript{14-15} However, the current chemical nose technique is still limited by poor selectivity, low sensitivity and complex sample preparation. Herein we report on a novel fluorescent sensing material that contains a high surface-to-volume area and can easily be regenerated for high throughput fingerprint analysis.

We set out to develop novel nanofiber materials for fluorescence detection of biomolecules including proteins and peptides. The use of electrospun nanofibers for chemical sensors using fluorophores has been previously reported for various analytes, such as metal ions (Fe\textsuperscript{3+} and Hg\textsuperscript{2+}), 2,4-dinitrotoluene (DNT), 2,4,6-trinitrotoluene (TNT), and methyl viologen (MV\textsuperscript{2+}).\textsuperscript{16-18} Recently, we reported a solid-state fluorescent sensor using fluorescent dendrimers (AFD) for detection of cytochrome c (cyt c), hemoglobin (Hgb), and bovine serum albumin (BSA).\textsuperscript{19} The dynamic change in fluorescence via FRET mechanism was found to depend upon the complementary electrostatic and hydrophobic fluorophore-protein pair interactions, similar to the literature results.\textsuperscript{20} In this paper, we extend the development of this sensor and describe the detection of multiple protein targets using different cationic and anionic fluorescent dendrimers in combination with pattern recognition. The diphenylacetylene dendritic compounds containing either negatively charged carboxylic acid peripheral groups or positively charged quaternary ammonium peripheral groups are used as the bioreceptor for proteins (Figure 5.1). We chose to use these fluorescent diphenylacetylenic systems
because they have previously demonstrated to be highly effective transducers for solution based optical sensors for many different target analytes, such as explosives (TNT and DNT) and protein biomarkers.\textsuperscript{21-25} Compared to linear polymeric fluorophores, the number of fluorophore units and selective receptor peripheral groups in dendrimers can be controlled by relatively simple stepwise synthesis via Sonogashira cross coupling reaction. Using the fluorescent dendrimers embedded into electrospun nanofibers, we have created an effective assortment of simple and reusable fluorescent sensors allowing distinct fluorescence fingerprinting for proteins.
Figure 5.1 Structures of anionic and cationic fluorescent dendritic molecules AFD-3, AFD-6, CFD-3 and CFD-6.
C. Experimental Section

1) Materials

All chemicals were of the highest analytical grade, and were used without further purification. Cellulose acetate (CA) (M_n ~30,000), acetone and N,N-dimethylacetamide (DMAc) were obtained from Sigma-Aldrich (St. Louis, MO). Fluorescent dendrimers were synthesized according to a published procedure. Microscope slides were purchased from Fisher (Pittsburgh, PA) and the ethyl alcohol (200 proof) was purchased from Gold Shield Chemical Co. (Hayward, CA). Bovine serum albumin (BSA), cytochrome c (cyt c) from bovine heart, myoglobin (Mb) from equine skeletal muscle, hemoglobin (Hgb) from bovine blood and avidin from egg white were purchased from Sigma-Aldrich. All protein solutions were prepared in a 10 mM phosphate buffered saline (PBS; 150 mM NaCl; pH 7.4) using Milli-Q (>18 MΩ) water.

2) Preparation of Polymer Solutions.

Solutions of the dendritic fluorophores (AFD-3, AFD-6, CFD-3 and CFD-6) at a concentration of 0.1% were first prepared in 10:1 acetone/DMAc and sonicated for 30 minutes. Then 17% CA was added to each of the fluorophore solutions under vigorous stirring until uniform polymer solutions were attained.

3) Electrospinning of Dendritic Fluorophore-doped CA fibers.

The polymer solution was placed into a syringe for electrospinning. A high-voltage DC power supply (Glassman High Voltage Inc. Series EH) was connected to a 25-gauge blunt nose needle attached to the syringe containing the electrospinning
solution. The electrospun fibers were collected on a grounded aluminum plate covered with aluminum foil. Solutions were electrospun at a voltage of 20 kV, a tip-to-collector distance of 15 cm, and a solution flow rate of 1.2 mL/h. All of the electrospinning procedures were carried out at room temperature (~25°C) with a collection time of 10 min. Finally, the electrospun fiber mats were dried in an oven at 60-70 °C for 12 h to remove the trace solvent.

4) Preparation of Sensor Interface.

The as-prepared electrospun fiber mats were cut to 10 × 10 mm rectangles and deacetylated on 11 × 50 mm microscope glass slides. Prior to modification, the glass substrates were cleaned with piranha solution (Caution!), then extensively rinsed in D.I. water, absolute ethanol, and dried with a stream of N₂ gas. In order to create secondary porous structures the CA fibers were deacetylated in a 50 mM NaOH ethanol solution at 25°C for 12 h, thoroughly rinsed with water, and dried using N₂. The chemical reaction of CA to cellulose was verified by using FT-IR spectroscopy. The characteristic absorption peaks attributed to the vibrations of the acetate group at 1745 (νC=O), 1375 (νC-CH₃), and 1235 cm⁻¹ (νC-O-C) disappeared after deacetylation of CA. An absorption peak at 3500 cm⁻¹ (νO-H) was observed, indicating successful deacetylation. The morphology of the fibers was evaluated by scanning electron microscopy (SEM) using a Phillips XL30-FEG.
5) **Lifetime Fluorescence Measurements.**

Time resolved fluorescence measurements were performed on the nanofibers in buffer solution, and also on dry nanofibers under vacuum in a Janis ST100 cryostat. The laser pulses were generated using a 40 kHz Spectra-Physics Ti:Sapphire regenerative amplifier. The 800 nm output pulse was doubled to 400 nm and residual light was removed with two BG39 filters. The fluences used for typical scans were $5.0 \times 10^{-7}$ J cm$^{-2}$ in solution and $4.1 \times 10^{-10}$ J cm$^{-2}$ in vacuo. Fluorescence was detected at a $54.7^\circ$ angle relative to the pump with a Hamamatsu C4334 Streakscope. Two OG420 color filters were used to eliminate scattered light from the pump.

6) **Characterization of Protein Sensing.**

In the fluorescent protein sensing study, fluorescence spectra were obtained from samples placed in a cuvette (12 × 12 × 45 mm). All fluorescence measurements were recorded on a HORIBA FluoroLog spectrofluorometer at room temperature (~25°C) and the emission spectrum were recorded with the excitation at 370 nm. During each calibration, 3 mL of PBS buffer was placed in the cuvette containing the optical sensor interface and the initial emission spectrum was recorded with excitation at 370 nm. Aliquots of the protein stock solution (100 µM) were subsequently added to the PBS buffer solution in the cuvette and the fluorescence intensity values at 460 nm were recorded after each addition. The difference between the two readings before and after the addition of protein was treated as the fluorescence response. This process was repeated to generate three replicates of data. Five proteins were tested against the four fluorescent cellulose fiber mats (**AFD-3, AFD-6, CFD-3 and CFD-6**) to give a $3 \times 4 \times 5$
training data matrix. The raw data matrix was processed for principal component analysis with software MYSTAT (version 12).

D. **Results and Discussion**

1) **Electrospun Nanofibers with Fluorescent Dendrimers.**

Cellulose acetate (CA) was used as the host matrix in the nanofiber fabrication due to its chemical resistance, thermal stability, low non-specific adsorption, and capacity to be easily functionalized with recognition elements.\(^{27}\) Other benefits include biodegradability, low cost, natural polymer and thus a renewable resource.\(^{28}\) To obtain the optimal electrospinning conditions, several solution parameters have been investigated, including the matrix concentration and solvent system ratios. Figure 5.2 shows the SEM images for 15% and 17% CA solutions in various mixed solvents of acetone/DMAc. The needle tip could be blocked when only acetone was used as the solvent. The gelation of the CA polymer solution is due to a low boiling point of acetone, which causes rapid evaporation when high voltage is applied to the capillary. However, when only DMAc was used a continuous jet of fibers seemed to form but when further investigated under the microscope no fibers were observed. In addition, distinctively unfavorable beads were formed when the concentrations of CA were less than 17% and the ratio of acetone/DMAc dropped below 10:1 (Figure 5.2a-e). In general, long and uniform nanofibers without any beads were readily obtained at CA concentration of 17% in 10:1 acetone/DMAc (Figure 5.2f). For all further experiments, dendritic CA fibers
were electrospun from 10:1 acetone/DMAc solutions containing 17% of CA and 0.1% of the dendritic fluorophore.
Figure 5.2  SEM images of pure dendritic fluorophore-doped cellulose sensing materials produced from (a, b, c) 15% and (d, e, f) 17% CA solutions in various solvents of acetone/DMAc: (a, d) 5:1, (b, e) 8:1, and (c, f) 10:1 (10,000× magnification).
After approximately 12 hours of drying, the nonwoven fiber mat is deacetylated overnight using a 50 mM NaOH ethanol solution. Secondary porous structures are homogenously distributed throughout the backbone of the nanofibers, creating a larger surface area-to-volume ratio. The nanofibers exhibit strong fluorescent signal. Figure 5.3a shows the image of the as-spun AFD-6-doped fibers as a free standing paper in the dark under a black light. This paper was robust and could be easily handled and manipulated. Figure 5.3b shows the SEM image of an AFD-6 doped nanofiber nonwoven mat, further illustrating the well defined fibrous morphology and structures. The fibers were continuous, uniform, and had a diameter ranging from approximately 400-600 nm, similar to those reported by Yang et al. Clearly, one-dimension (1D) nanostructures are evenly distributed throughout a three-dimensional (3D) nano-architecture using a simple electrospinning approach. It is expected that this large amount of surface area, around and within the porous nanofiber film, has the potential to provide unusually high sensitivity and fast response time in sensing applications.
Figure 5.3 Pure electrospun dendritic fluorophore-doped cellulose fiber sensing interface: (a) digital image of as-spun electrospun fibers as a free standing paper under a black light; (b) SEM images of electrospun dendritic fluorophore-doped cellulose fibers (17% CA/0.1% AFD-6 dissolved in 10:1 acetone/DMAc, 10,000× magnification).
The fluorescence properties of the four doped cellulose fiber mats were characterized. Figure 5.4 shows the emission spectra. When excited at 370 nm, the AFD-3, AFD-6, CFD-3 and CFD-6 doped fibers show an emission maximum at 445, 475, 480 and 450 nm in PBS buffer, respectively. Interestingly, when the fluorescent dendrimers are dissolved in aqueous PBS solution, AFD-3, AFD-6, CFD-3 and CFD-6 displayed maximum emission at 454, 489, 485 and 446 nm, respectively. Going from solutions to electrospun fibers, the fluorescent dendrimer CFD-6 exhibited a small red shift, conversely AFD-3, AFD-6 and CFD-3 exhibited a small blue shift in emission maximum. The blue shifts generally imply disaggregation caused by the addition of the fibrous nanoarchitectures for the dendritic fluorophore to homogenously inhabit. Furthermore, the AFD electrospun nanocomposites emission maximum red shifted from first generation (AFD-3) to second generation (AFD-6) dendrimers, whereas the CFD electrospun nanocomposites blue shifted from first generation (CFD-3) to second generation (AFD-6) dendrimers 30 nm each, further illustrating the wavelength tunability. The size and charge state of the ligands suggest that not only does charge transfer take place, but also self-collision plays a major role in the fluorescent properties of the fibrous sensor surface. Early studies by Niamnont et al. demonstrated that the fluorescent quantum yields ($\Phi_F$) in aqueous solutions of the first generation dendrimers (AFD-6 = 4% and CFD-6 = 3%) were significantly lower than those of their zeroth generation analogs (AFD-3 = 9% and CFD-3 = 14%). The lower $\Phi_F$ is due to their larger hydrophobic cores, which results in self associative interaction of the fluorophores. Strong fluorescence, suggesting high fluorescent quantum efficiency,
visually observed for the nanofiber sheets substantiates the disaggregation state of the dendritic fluorophores within the fiber.
Figure 5.4 Fluorescence emission spectra of the different electrospun dendritic fluorophore-doped cellulose fibers in PBS buffer solution (pH 7.4): (a) AFD-3 (solid line) and AFD-6 (dashed line) doped cellulose sensor interface; (b) CFD-3 (solid line) and CFD-6 (dashed line) doped cellulose sensor interface ($\lambda_{Ex} = 370$ nm).
2) **Protein-Dependent Fluorescence Quenching.**

The protein sensing properties of the fibers were characterized by monitoring the quenching behaviors by five proteins with diverse structural features including molecular weight, isoelectric point (pI) and paramagnetic iron core. The molecular weights of the protein set range from approximately 12 to 66 kDa, where Mb (16 kDa) and cyt c (12 kDa) are light proteins while Hgb (65 kDa), BSA (66 kDa) and avidin (66 kDa) are considered heavy proteins. At physiological pH, Hgb (pI = 6.8) and Mb (pI = 7.2) are slightly negatively charged, cyt c (pI = 10) and avidin (pI = 10) are positively charged, and BSA (pI = 4.8) is negatively charged. Both cyt c and Mb contain one paramagnetic iron core, while Hgb contains four paramagnetic iron cores. The sensitivity, affinity, and differential recognition of the protein towards the dendritic nanofiber interfaces are expected to depend on how well the fluorescent cores of the dendrimers complement the hydrophobic, neutral, and charged amino acid residues found on the surface of the proteins.

Understanding the mechanism of fluorescence quenching is important for optimizing sensor performance. In general, one expects two possible limiting cases for the fluorescence quenching in the nanofibers: dynamic vs. static. In the dynamic case, the dendrimer is randomly distributed within the nanofiber, and proteins can diffuse freely throughout the system. When a protein encounters a dendrimer, it can shorten the fluorescence lifetime due to intermolecular interactions like energy or electron transfer. The observed fluorescence lifetime decreases in an amount directly proportional to the quencher concentration, resulting in Stern-Volmer kinetics. Such dynamic quenching
between the diffusing proteins and embedded dendrimers can be contrasted with a static mechanism, where the protein becomes closely associated with the dendrimer molecules. The degree of static fluorescence quenching depends on the fraction of dendrimers that have associated proteins. In this scenario, there are only two types of emitters, quenched and unquenched. The total fluorescence decreases, but the measured lifetime is unaffected, since only emitters without an associated quenching molecule can contribute to the observed fluorescence.

To distinguish between these two types of quenching mechanisms, we have directly measured the fluorescence decay of the dendrimers under different conditions. Figure 5a shows the results for four different AFD-3 samples. For all the samples, the decays were fit using the biexponential function \( a_1 \exp[t/\tau_1] + a_2 \exp[t/\tau_2] \). We also define the average fluorescence decay time \( \tau_{\text{avg}} \) as

\[
\tau_{\text{avg}} = \frac{a_1 \tau_1 + a_2 \tau_2}{a_1 + a_2}
\]

The results of our fits are summarized in Table 5.1. When AFD-3 is dissolved in buffer solution without the nanofiber host, we observe a subnanosecond fluorescence decay with a strong multi-exponential character. When AFD-3 is embedded in a dry polymer nanofiber, we must use a biexponential decay to fit the data, and \( \tau_{\text{avg}} \) lengthens to about 1.7 ns. When the nanofiber is immersed in an aqueous solution, the fluorescence decay has a more pronounced biexponential character with and more rapid, with \( \tau_{\text{avg}} = 1.3 \) ns. Adding the protein solution has no measurable effect on the fluorescence lifetime, even at the highest concentration, as shown in Figure 5.5a. Figure 5.5b plots the average AFD-3
fluorescence lifetime $\tau_{\text{avg}}$ as a function of myoglobin concentration. It is constant over the whole range. Fluorescence lifetimes for the other dendrimers were also measured with similar results, although their absolute fluorescence lifetimes were shorter and their decays tended to be more multi-exponential than AFD-3. None of the dendrimer-doped nanofiber samples exhibited fluorescence lifetimes that changed with protein concentration, ruling out the dynamic mechanism of fluorescence quenching. The sensing effect therefore must rely on static adsorption of protein molecules to dendrimer sites within the nanofibers.
Table 5.1  Fluorescence lifetime decay data (biexponential) of fibrous and nonfibrous AFD-3 dendrimers under four different conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$a_1$</th>
<th>$\tau_1$ (ns)</th>
<th>$a_2$</th>
<th>$\tau_2$ (ns)</th>
<th>$&lt;\tau&gt;$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry Fiber (in vacuo)</td>
<td>0.34</td>
<td>1.02</td>
<td>0.66</td>
<td>2.05</td>
<td>1.69</td>
</tr>
<tr>
<td>Fiber in PBS buffer w/o Protein</td>
<td>0.21</td>
<td>0.198</td>
<td>0.79</td>
<td>1.53</td>
<td>1.25</td>
</tr>
<tr>
<td>0.4 μM Mb</td>
<td>0.25</td>
<td>0.196</td>
<td>0.75</td>
<td>1.54</td>
<td>1.20</td>
</tr>
<tr>
<td>0.8 μM Mb</td>
<td>0.28</td>
<td>0.197</td>
<td>0.72</td>
<td>1.53</td>
<td>1.15</td>
</tr>
<tr>
<td>1.6 mM Mb</td>
<td>0.31</td>
<td>0.203</td>
<td>0.69</td>
<td>1.65</td>
<td>1.20</td>
</tr>
<tr>
<td>3.2 mM Mb</td>
<td>0.31</td>
<td>0.221</td>
<td>0.69</td>
<td>1.69</td>
<td>1.23</td>
</tr>
<tr>
<td>10 μM AFD-3 Only</td>
<td>0.52</td>
<td>0.170</td>
<td>0.48</td>
<td>0.826</td>
<td>0.485</td>
</tr>
</tbody>
</table>
Figure 5.5  (a) Fluorescence lifetime decay curves ($\lambda_{\text{Ex}} = 400$ nm) of the AFD-3 dendrimer fibers under four different conditions [(solid line) dry fiber, (dashed line) wet fiber, (dotted line) wet fiber with 3.2 $\mu$M of Mb, and (dashed-doted line) 10 $\mu$M AFD-3 only in PBS buffer solution.] (b) Plots of the average AFD-3 fluorescence lifetime $\tau_{\text{avg}}$ as a function of Mb concentration.
The static quenching model yields the same functional dependence on quencher concentration \([Q]\) as the more familiar Stern-Volmer model,

\[
\frac{I_0}{I} = 1 + K_S [Q]
\]

(2)

where \(K_S\) is the association constant for the quencher with the fluorescent species.\(^{35}\)

Figure 6 shows a plot of \(\frac{I_0}{I}\) versus the protein concentration, demonstrating the predicted behavior at lower concentrations. At higher concentrations, the ratio appears to saturate. Given a distribution of such sites, some would be expected to be inaccessible to the proteins, and dendimers occupying such sites would never be quenched. This would explain the saturation of the quenching efficiency seen in Figure 5.6. Once all the accessible sites have been quenched, adding more protein has no effect on the fluorescence signal, in contrast to the dynamic Stern-Volmer model, which does not predict this type of saturation behavior. Thus Figure 5.6 provides additional evidence for the static quenching model that involves protein binding to dendrimer-occupied sites within the nanofiber.
Figure 5.6 Plots of $I_0/I$ of four different electrospun dendritic fluorophore-doped cellulose fibers (AFD-3, AFD-6, CFD-3 and CFD-6) against Mb concentration. Inset: Stern-Volmer plots of fluorophore-doped sensing membranes for Mb. ($\lambda_{Ex}/\lambda_{Em} = 370/460$ nm).
Figure 5.7 shows the Stern-Volmer association constants, $K_{sv}$, calculated from the slopes of the plots for all 5 proteins and the 4 fluorescent nanofibers. Most of the proteins exhibited generation-dependent fluorescence quenching. Within this set of five analytes there are several pairs of proteins having comparable molecular weights and/or pI values, providing a challenging test bed for protein discrimination. The varying sizes and shapes of the proteins will lead to different amounts of contact with the near planar scaffold of the fluorescently doped nanofiber interface. One of the primary quenching effects for metalloproteins (Hgb, Mb and cyt c) and fluorescent dyes is believed to be due to energy transfer of the imbedded fluorescent dendrimers with the protein, as metalloproteins contain heme portions within the protein. For hemoglobin, the highest $K_{sv}$ values were observed and primarily due to the four paramagnetic iron compounds within the protein. However, we observed similar quenching effects when the non-metalloproteins (BSA and avidin) were used. We attribute the non-metalloproteins quenching effect to the energy transfer, which is caused by the electrostatic effects in the binding of the anionic or cationic dendrimers to the positive and negative patches of the various globular proteins. Additionally, fluorescence is very sensitive to quenching by direct resonance transfer of proteins whose absorption spectrum overlaps with the tryptophan, found in avidin, and is believed to be the main reason for the fluorescence quenching caused by avidin. This electrostatic effect is best demonstrated by the increase in fluorescence when BSA is added to the AFD-3 and AFD-6 fiber interface. Instead of a decrease (quenching), a slight increase in local fluorescence of the dendritic fluorophore was observed for BSA, suggesting that the negatively charged BSA proteins reduce the π-π
stacking of the partially dispersed negatively charged dendritic fluorophores through repulsion forces, and thus resulting in an increase in fluorescence.\textsuperscript{36-37} For a control experiment, an un-doped cellulose fiber was studied, and no fluorogenic responses were found. It is expected that there are multiple factors that contribute to protein detection: one, the charge distribution density on the proteins surface; two, the location of the resonance transfer secondary structure; and three, the amount of resonance transfer secondary structures within proteins. The intricate nature of these interactions results distinguishable and highly reproducible protein-dependent patterns, which yield good sensing capabilities.\textsuperscript{38-40}
Figure 5.7 Histogram plot of the Stern-Volmer constants ($K_{sv}$) of the arrangement of electrospun dendritic fluorophore-doped cellulose fibers against five target proteins (Hgb, Mb, cyt c, BSA, avidin) in PBS buffer solution (pH 7.4).
3) Protein-Specific Response Pattern by PCA.

Chemical sensor arrays gather data which have broad, overlapping sensitivity profiles and require substantial data analysis. In order to address these problems the computational method of PCA is used to better interpret the fluorescent data. The normalized change-in-fluorescence intensities ($\Delta I = I - I_0$) obtained from three replicated measurements for each pair of protein and fluorescently doped-nanofiber were used to construct a histogram plot (Figure 5.8). As shown in Figure 5.4 all fluorescence spectra overlapped at approximately 460 nm, suggesting this wavelength presents the most informative pattern for protein discrimination. Notably, each protein possesses a unique response pattern over a large dynamic range with little to no overlap. The classification of different types of proteins can be recognized after processing the data matrix using mathematical transformations, such as PCA. This involves a statistical procedure that transforms a number of correlated variables into a number of uncorrelated variables called principal components. Therefore, the first two principal components frequently account for 80% or more of the complete data variance. In our case shown below, the first three principle components contained nearly 98% of the information.
Figure 5.8  Change-in-fluorescence (ΔI) response patterns at 460 nm (λ_{Ex} = 370 nm) of the fluorophore-doped fiber collection against five target proteins (Hgb, Mb, cyt c, BSA, avidin) at 400 nM in PBS buffer solution (pH 7.4). Each value is an average of three independent measurements.
A total of five proteins were tested using a fluorescence displacement assay with four fluorescent nanofiber substrates and three replicate measurements, providing a data set as a $3 \times 4 \times 5$ matrix. Figure 5.9 shows the PCA 3D scatter plot of the analyzed data set obtained from five protein samples against fluorescent sensor substrate. The three canonical factors contain 41.052%, 30.729%, and 26.094% of the variation, respectively. In this canonical score plot, each dot represents the response pattern for a single protein target to the fluorescent nanofiber sensor surface. Five distinct clusters formed for each of the different proteins. This result indicates an array of fluorescent nanofibers can differentiate between five different protein types using a simple solid-state optical sensor array approach.
Figure 5.9  3D Plot of the first three principal components of the change-in-fluorescence ($\Delta I$) response patterns obtained through the fluorophore-doped fiber array against five target proteins (Hgb, Mb, cyt c, BSA, avidin) at 400 nM in PBS buffer solution (pH 7.4). Note that the first three principal components account for 97.875% of the total data variance.
The reusability, reproducibility and stability of the nanofiber material were also investigated. In order to demonstrate the reusability of the sensor the cellulose nanofibers were immersed in a 25 μM solution of cyt c for 5 min. Then in a 50 mM NaOH ethanol solution for 15 minutes followed by rinsing in water and drying in air, the fibers were then re-used for sensing the same protein solution. The sensor interface was regenerated through 5 test cycles and the nanofibers demonstrated less than a 15% loss of fluorescence intensity signal, indicating outstanding reusability. We attribute this to the noncovalent nature of the interaction, which is largely based on the electrostatic interaction between the fluorophore and protein. In fact, that weak binding events and striping process do not denature the core, which allows for excellent sample recovery. The reproducibility of the sensor is reflected by low batch-to-batch variation of approximately 5% for all fluorescently doped interfaces using 3 separate measurements produced on different days. For the stability experiments we tested the buffer solutions before and after each test and no leakage of the fluorophore were found in the aqueous solutions.

E. CONCLUSIONS

In conclusion, we have demonstrated the FRET detection of proteins using multiple fluorescently embedded electrospun nanofibers in combination with principle component analysis. The size and tunability of four different cationic and anionic fluorescent dendrimers are optimized to provide selective interactions with proteins, which extends the scope of nanofiber sensing applications. These fluorescent dendrimers
provide efficient transduction of the binding event and generate fluorescence signal in response to changed concentration of analyte. Through principle component analysis, we are able to cluster more complex data matrices and resolve the different proteins within this data matrix for detection using an electrospun nanofiber sensor substrate. Considering a large number of diphenylacetylene dendritic compounds that can be rapidly synthesized using Sonogashira coupling approach, these types of fluorescent nanofiber films are very promising for the detection of more complex samples. Future work will incorporate multiple fluorescent nanofibers into an optimized microfluidic array chip for on-chip multiplexed high-throughput detection of physiologically related biological samples.
F. References


Niamnont, N.; Mungkarndee, R.; Techakriengkrai, I.; Rashatasakhon, P.; Sukwattanasinitt, M. *Biosens. Bioelectron.** 2010, **26, 863.


Tao, S. Y.; Li, G. T.; Yin, J. X. *J. Mater. Chem.** 2007, **17, 2730.


(24) Woller, E. K.; Walter, E. D.; Morgan, J. R.; Singel, D. J.; Cloninger, M. J. 


(26) Niamnont, N.; Siripornnoppakhun, W.; Rashatasakhon, P.; 


(31) Terenziani, F.; Painelli, A.; Katan, C.; Charlot, M.; Blanchard-Desce, M. 


(35) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; 3rd ed.; 

(36) Lavigne, J. J.; Broughton, D. L.; Wilson, J. N.; Erdogan, B.; Bunz, U. H. 
(37) Turro, N. J.; Lei, X. G.; Ananthapadmanabhan, K. P.; Aronson, M.


6. **CHAPTER SIX: Colorimetric and Fluorescence Detection with Electrospun Polydiacetylene (PDA)-Embedded Nanofibers**

A. **Abstract**

A quantitative approach for fluorescence differentiation of volatile organic solvents and colorimetric detection of protein streptavidin (STA) is demonstrated by using electrospun nanofibers containing polydiacetylene (PDA) units. 1D polydiacetylene-embedded electrospun fibers are prepared with 10,12-Pentacosadiynoic acid (PCDA), 5,7-eicosadiynoic acid (ECDA), and 8-aminoethoxyethoxyethylpentacosa-10,12-diynamide (PCDA-EDEA) monomers, which display a range of distinct solvent sensitive fluorescent transitions when exposed to organic solvents. Analytical performance of the nanofiber sensors has been characterized. The fibers and their unique fluorescent patterns were stable when exposed to common organic solvent vapors. The organic solvent fluorescent response varies in a dependent manner on the structure of the diacetylene monomer. In addition, we synthesized biotin-terminated PCDA monomers for fabrication of electrospun biotin-PDA nonwoven fiber mats. Upon addition of STA, the biotin-PDA mats display a color change from blue-to-red and fluorescence transition that offer highly sensitive measurement of the protein. Importantly, the color change produced by the STA protein is observable by the naked eye, and fluorescent detection is achievable for quantitating low concentrations of the protein. The biotin-based PDA sensor described in this study has a broad potential for the development of a variety of colorimetric/fluorescent biosensors using biotin-avidin interactions.
B. Introduction

The production of new sensor technology is driven by improving the parameters affecting the performance of sensor sensitivity, selectivity, response time, reproducibility, shelf-life, and simplicity, all of which are dependent directly on the property of the sensing interface structure and chemical components.\(^1\) Among the many different sensing technologies that exist electrospun polymer nanofibers have gained much attention due to their inherent large surface area-to-volume ratio, surface functionality, superior mechanical performance, cost, and ease of construction.\(^2\)\(^-\)\(^4\) In fact, electrospun polymer fibers have been incorporated in a variety of different analyte detection techniques such as colorimetric, fluorescent, and electrochemical measurements.\(^5\)\(^-\)\(^6\) Above all, colorimetric recognition is a desirable technique because it is simple and may make a large impact for those working in emergency response and/or field operation. The ability to quickly detect analytes on site with the naked eye would not only save time but lives and money.

For the design of colorimetric detection techniques, conjugated polymers have gained wide spread attention due to their attractive optical and electrical properties.\(^7\) These properties are primarily due to the extensive delocalized π-system and the intrinsic conformational restrictions within the polymer chain. Some examples of conjugated polymers that have been used in sensing matrices include polyaniline, polyphenylene, polypyrrole, polythiophene, and polydiacetylene (PDA).\(^8\) When the backbone of these conjugated polymer chains are perturbed the delocalized π-network will induce changes in electronic absorption and emission properties. Since the backbone of these conjugated
polymers can easily be modified, a variety of different surface ligands and substrate functionalities can be applied to design a chemo/biosensor for specific analyte detection.

Among the conjugated polymers studied to date PDAs are of particular interest because they can change color from blue-to-red upon response to heat (thermochromism), mechanical stress (mechanochromism), organic solvents (solvatochromism), and ligand-receptor interactions (affinochromism).\textsuperscript{9} Due to the topochemical constrain of the PDA backbone, polymerization can only occur in solids or other highly ordered structures. Once diacetylene monomers are assembled into an ordered array wherein the diacetylene units are properly aligned, they can be stabilized by polymerization with UV irradiation. Interestingly, PDA matrices are highly tolerant to the incorporation of biological receptors for specific binding of analytes to the matrix surface. The analyte binding triggers a distortion of the conjugation plane (ene-yn backbone) of the polymer, which leads to chromatic phase transition in the engineered nanostructure, providing colorimetric detection of the target molecules.\textsuperscript{7} This strategy allows molecular recognition and optical reporting to be built with a single supramolecular assembly, and can be conveniently applied to direct, one-step colorimetric detection of a variety of pathogens and other analytes of interest.

PDA-based sensors have been utilized to detect many different biological analytes including viruses, bacteria, lipophilic enzymes, antibacterial peptides, mammalian peptides, ions, antibodies, proteins, and pharmacologically active compounds.\textsuperscript{10} As the diacetylene monomers must be organized in the correct geometry for the topochemical polymerization the PDA material formation has been limited to Langmuir-Blodgett (LB)
films, self-assembled monolayers (SAMs), multilayer coatings, colloids, and immobilized colloids. Each material platforms offer specific advantages for different biosensing applications. For example, LB films and SAMs provide a well-controlled platform for studying the fundamental aspect of PDAs while colloidal solutions offer a better platform for the liquid handling techniques used in drug discovery assay applications. When ruggedness and ease-of-operation is required for the sensor system, a solid supported PDA material is best suited. Recently Kim and coworkers reported a new approach for constructing silica-enforced nanostructured PDA supramolecules in electrospun micro/nanofibers. This work demonstrated that PDA conjugated polymers embedded into electrospun fibers could easily be used as new colorimetric sensor based on a combinatorial approach. Although this work established a qualitative approach for the blue-to-red colorimetric transition, no effort was made to differentiate the change using fluorescence properties of the red polymer, which would give a better quantitative analysis of the PDA sensor system.

Furthermore, to our best knowledge, no work has been reported on using PDA for the detection of protein interactions with electrospun fibers as the solid support. We describe here a PDA-biotin based colorimetric biosensor for detection of the biotin-STA interactions using nonwoven electrospun fiberous mats. The structures of the diacetylene monomers investigated for the fabrication of PDA-embedded fibers are shown in Figure 6.1. Biotin-STA interactions is one of the most widely used recognition schemes for bioanalytical sensors because the association constant is one of the highest known in biochemistry (10^{15}). In this work, we first study the fluorescence response of the PDA.
nanofibers and demonstrate a more quantitative approach to determine organic solvents using the fluorescence changes in a combinatorial sensor approach. Next, we investigate the colorimetric and fluorescent transitions of the biotin-STA interactions. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images were obtained to characterize the morphology of the electrospun PDA-biotin embedded fibers. The research goal of this work is to show new strategies that allow for molecular recognition and optical reporting to be built within a single supramolecular assembly for direct, one-step colorimetric biological detection. Also, the organic solvent-dependent fluorescent change properties of the PDA-embedded nanofibers demonstrate a straightforward fluorescent pattern procedure to differentiate between several common organic solvents.
Figure 6.1 Structures of diacylene monomers investigated for electrospun fibrous colorimetric sensors.
C. **Experimental Section**

1) **Materials**

All chemicals were of the highest analytical grade, and were used without further purification. 10,12-Pentacosadiynoic acid (PCDA) and 5,7-eicosadiynoic acid (ECDA) were purchased from GFS chemicals (Powell, OH). 8-aminoethoxyethoxyethylypentacosa-10,12-diynamide (PCDA-EDEA) and PCDA-Biotin were prepared according to the literature procedures. Polyethylene oxide (PEO; $M_w = 100,000$ g mol$^{-1}$), Tetraethyl orthosilicate (TEOS; reagent grade, 98%), and streptavidin (STA) form *Streptomyces avidinii* (affinity-purified, lyophilized powder, salt-free) were obtained from Sigma-Aldrich (St. Louis, MO). Microscope slides were purchased from Fisher (Pittsburgh, PA) and the ethyl alcohol (200 proof) was purchased from Gold Shield Chemical Co. (Hayward, CA). All protein solutions were prepared in a 10 mM phosphate buffered saline (PBS; 150 mM NaCl; pH 7.4) using Milli-Q (>18 MΩ) water.

2) **Preparation of PDA-Embedded Electrospun Fibers**

The typical procedure for fabrication of the PDA-embedded electrospun polymer fibers is as follows. A 3:1 EtOH/CHCl$_3$ solution containing 1.6 wt% of the diacetylene (DA) monomer and 4 wt% PEO is prepared. A TEOS solution was prepared in a separate vial with a 1:4:4 molar ratio of TEOS/EtOH/H$_2$O (pH 1.1). The two solutions were mixed with a 10:1 wt ratio between the DA monomer and TEOS solution and stirred for 60 min. The resulting solution was pumped through a capillary connected with a 25-gauge blunt nose needle (Braintree Scientific, Inc.) at a constant rate of 0.12 mL h$^{-1}$ by a syringe
pump (KD Scientific, Model 200 series). The application of a high voltage (18-20 kV) to the metal syringe needle enabled the generation of nanofibers which were collected on the surface of a grounded aluminum plate (distance from the tip of the syringe to the plate is 10 cm). The collected fibers were stored in a dark environment. Photopolymerization of DA monomer fibers was carried out by irradiation with 254 nm UV light (1 m W cm$^{-2}$) for 5 minutes.

3) PDA Fiber Fluorescence Measurements

For the fluorescence measurements the DA-monomer fibers were collected on 11 × 50 mm cut microscope glass slides glass for precisely 45 seconds. In the organic solvent vapor tests, the photopolymerized ECDA, PCDA, and PCDA-EDEA fibers were exposed to the solvent vapor by placing the glass slides on the top cover (inside part) of a glass petri dish. Each glass petri dish contained 2 mL of the organic solvent (THF, Chloroform, Methanol, Hexane) and the fluorescent change of the fibers was monitored after 1 h of incubation at 25 °C. For the STA-Biotin tests, the photopolymerized fibers were immersed into a 250 μg mL$^{-1}$ STA solution and allowed to dry at 25 °C. All fluorescence spectra were measured in a disposable polymethyl methacrylate (acrylic) cuvette (12 × 12 × 45 mm) purchased from Fisher. Fluorescence measurements were recorded on a HORIBA FluoroLog spectrofluorometer at room temperature (~25°C). The fluorescence emission profiles were monitored from 500 to 700 nm ($\lambda_{\text{Ex}} = 490$ nm). Each organic solvent vapor test value is an average of three independent measurements.
4) Colorimetric Experiments

For the colorimetric detection of STA a nonwoven mat of the DA-monomer fibers were collected for 10 minutes onto aluminum foil and stored in a dark environment until use. Photopolymerization of the DA monomer fiber mats was carried out by UV irradiation for 2.5 minutes on both sides. The colorimetric changes of the PCDA-Biotin fiber mat was achieved by using 100 nL deposits of a 500 and 250 µg mL\(^{-1}\) STA solutions. Control experiments were performed using 100 nL deposits of a 500 and 250 µg mL\(^{-1}\) BSA solutions. The colorimetric transition of the fiber mats were monitored using a digital microscope (MicroXplore™ PC200) after 1 minute.

5) SEM and TEM studies.

Scanning electron microscopy (SEM) images of the PDA-embedded fibers were obtained on a Phillips XL30-FEG. The PDA-embedded fibers were electrospun directly onto a carbon-coated copper mesh and then characterized before and after UV-irradiation using a Tecnai T12 tunneling electron microscope (TEM).

D. Results and Discussion

1) Organic Solvent Vapor Tests

The fabrication of the 1D PDA-embedded fibers involves the use of a viscous solution of DA monomer, PEO and TEOS placed into a syringe connected to a metal needle. PEO was used as a carrying polymer because it is water soluble and non-toxic. TEOS, an alkoxide precursor, was used to enhance the stability of the fibers. A high-voltage is applied to the tip of the metal needle while the viscous solution is slowly
pumped out the conductive capillary causing the solvent to evaporate and force a charged polymer jet onto a grounded collector. As the collection of the fibers was unpredictable and many samples had to be collected, only the glass slides with similar collection patterns were used for the solvent vapor tests. After collection the fibers were kept in the dark to avoid polymerization of the DA monomer from ambient light. Photopolymerization of the DA monomer fibers occurs under UV irradiation at 254 nm and blue color change indicates DA polymerization occurred. The stability and shelf life of all stages of the fibers was good: over a 6 month span the unpolymerized fibers, polymerized blue fiber, and used red fibers showed no apparent change in color or morphology.

The electrospun 1D nanofibers of PCDA, ECDA, and PCDA-EDEA used for the organic solvent vapor experiments exhibited good structural stability and uniform (bead-free) fibrous morphology. The fibers were produced by continuous 45 second burst of fiber collection on precut glass slides. Importantly, all fluorescence response experiments were performed with similar amounts of fiber distribution, and had a diameter ranging from approximately 400-600 nm. No significant morphological difference was seen in the SEM images before and after UV-irradiation. However, after 1 hour of exposure to the different organic solvent vapors the highly ordered lipid fibers were disrupted at different rates, which promoted the blue-to-red salvatochromism transition. It is important to note that the distance of the fibers from the organic vapor source was essential and to get repeatable patterns this had to be kept constant.
The PDA-embedded fibers undergo a distinct fluorescent (non-to-red) transition that varies in an organic-solvent-dependent manner. This property was used to build a one-step combinatorial optical sensor. The organic solvent vapor sensing responses were characterized by monitoring the fluorescent transition of PCDA (Figure 6.2a), ECDA (Figure 6.2b), and PCDA-EDEA (Figure 6.2c) embedded fibers for one hour using chloroform, tetrahydroduran (THF), hexane, and methanol. The fluorescent increase of the PCDA and ECDA fibers at 640 nm had similar response patterns to the carboxylic acid ligand (Hexane < methanol < chloroform < THF), with THF causing the greatest transition to red. The ECDA has a much greater effect on the fibber as compared to the PCDA because the carboxylic chain is much shorter and thus closer to the backbone of the conjugated polymer. The fluorescent response for the amine-terminated PDA fibers has a slightly different response pattern (Hexane < THF < methanol < chloroform), further illustrating the solvent-dependent optical pattern that can be created. The fluorescent transition properties of the PDA-embedded polymer 1D nanofibers allows for a straightforward “fluorescent pattern” signaling procedure to differentiate between several common organic solvents as shown in Figure 6.3.
Figure 6.2  Fluorescence emission profiles of the polymerized PCDA (a), ECDA (b), and PCDA-EDEA (c) embedded electrospun fibers after exposure of the organic solvent vapor at 25 °C for 1 h. (λ_{Ex} = 490 nm) Organic solvents; THF (black solid), Chloroform (red dash), Methanol (blue dot), and Hexane (green dash-dot).
**Figure 6.3** Fluorescence emission profiles at 640 nm ($\lambda_{\text{Ex}} = 490$ nm) for the polymerized PCDA, ECDA, and PCDA-EDEA embedded electrospun fibers against four organic solvent vapors (THF, Chloroform, Methanol, Hexane) at 25 °C for 1 h. Each value is an average of three independent measurements.
2) **Colorimetric Detection of STA**

During the past several decades a wide array of biosensors have been developed exploiting the rapid, specific, and irreversible non-covalent binding between biotin and streptavidin. Due to these unique protein-protein binding properties detection of other bioanalytes is possible by incorporating biotin onto the biosensor substrate. Figure 6.4 shows the photographs of the electrospun fiber mats encapsulated with PCDA-biotin, before (Figure 6.4a) and after (Figure 6.4b) irradiation with UV light. The distinctive blue color that is developed after UV light irradiation indicates that the diacetylene monomer is completely photopolymerized within the nonwoven fibrous mats. Secondly, this observation confirms that the delocalized π-network and the conformational restrictions within the polymer chain backbone are retained in the electrospinning process. A blue-colored PCDA-biotin nonwoven mat is shown in the SEM image in Figure 6.4c, further illustrating the large surface area-to-volume ratio formed within the electrospun nonwoven film. The electrospun fibers exhibited well defined fibrous morphology without bead formation and good structural stability. The fibers were continuous, uniform, and had a diameter ranging from approximately 400-600 nm, and no significant morphological difference is seen in the SEM images before and after UV-irradiation.

Figure 6.5 shows the TEM images of the PEO/TEOS electrospun nanofibers mats encapsulated with PCDA-biotin, before (Figure 6.5a) and after (Figure 6.5b) UV-irradiation. The internal structure of the nanofiber can be characterized by the diffraction contrast in the dark and bright regions within the TEM images, which is caused by the electron beam diffraction of different crystal structures within the fiber structure.\(^{18}\)
dark aggregates were found within the fiber before or after UV-irradiation indicating the PDA polymer chain is aligned, which is attributed to the process of electrospinning that allows for a more uniform dispersion of PDA within the PEO/TEOS polymer matrix. Furthermore, the TEM images revealed a homogeneous morphology throughout the PEO/TEOS/PDA nanofibers. The diffraction contrast change before (Figure 6.5a) and after (Figure 6.5b) UV-irradiation is because the diacetylene monomers transform from a more rigid bundle of non-crystalline monomeric rods to a more flexible crystalline polymer chains.
Figure 6.4  Photographs of electrospun fiber mat embedded with PCDA-biotin before (a) and after (b) 254 nm UV-irradiation (1 mW cm$^{-1}$) for 5 min; SEM image of the microfibers containing polymerized PCDA-biotin (c).
Figure 6.5 TEM image of PDA-biotin electrospun nanofiber before (a) and after (b) UV-irradiation.
The highly conjugated photopolymerized blue fibers can be converted into the less conjugated red fibers when STA binds to the biotin conjugated diacetylene surface. As shown in Figure 6.6, both colorimetric and fluorescence response can be monitored using PDA-embedded electrospun nanofibers. Figure 6.6a is the colorimetric response after a 1 min exposure to 500 (top), 250 (middle), and 0 ng (bottom) of 100 nL STA spots. Clearly the blue fibers are rapidly converted into red fibers when STA is applied to the surface of the biotin-PDA fibers. No color change was visible when only PBS buffer solution is used as shown in Figure 6.6a (bottom). Two control experiments were performed. First, a 100nL of a 1 mg mL$^{-1}$ BSA solution was spotted onto a photopolymerized biotin-PCDA-embedded mat. Secondly, STA solutions were tested on fibers without biotin terminuses using polymerized PCDA, ECDA, and PCDA-EDEA electrospun mats and no visible color change was seen. Lastly, we investigated the fluorescence response of 1D biotin-PCDA-embedded fiber upon addition of STA (Fig. 6.6b). Fluorescent (e.g., red) PDA can be excited with wavelengths above 450 nm and emit two broad fluorescent peaks at ca. 560 nm and 640 nm. Upon the addition of STA the fluorescent emission at 560 nm and 640 nm increased indicating the red color change of the fibers. The emission behavior of the biotin-PCDA-embedded fibers parallels the chromic behavior: red PDA is fluorescent while blue PDA is not. While colorimetric detection is the preferred approach the emission properties of the PDA provides an alternative means for detection, verification, and has the potential to deliver greater sensitivity.
Figure 6.6  Optical microscope image showing color change of polymerized PCDA-biotin embedded electrospun fiber mat upon addition of 500 ng (top), 250 ng (middle), and 0 ng (bottom) of streptavidin (a). Fluorescence emission profile of the polymerized PCDA-biotin embedded electrospun fibers after a 5 sec emersion into a 250 μg mL⁻¹ streptavidin solution (b).
E. Conclusions

A new strategy for the differentiation of volatile organic solvents based on PDA 1D-nanofiber fluorescence emission patterns has been demonstrated. The solvent-dependent properties of the nanofibers allows for a straightforward “fluorescent pattern” procedure to differentiate between several common organic solvents. By using silica-enforced PDA nanofiber mats as a recognition system, biotin-STA interactions was investigated for generating colorimetric and fluorescence signals. Using biotinylated PCDA monomers, we have successfully demonstrated that STA molecules could be detected both visually and fluorescently. Future efforts will be placed on (1) optimization of the interface of the 1D-nanofibers by incorporating them into an integrated microarray sensor platform, (2) modification of the substrate for better stability to environmental interactions, and (3) functionalization of the electrospun nanofibers for specific capture and detection of environmental pathogens or other contaminates.
F. References


(4) Huang, Z. M.; Zhang, Y. Z.; Kotaki, M.; Ramakrishna, S. Compos. Sci. Technol. 2003, 63, 2223.


(13) Tieke, B. Advances in Polymer Science 1985, 71, 79.


7. CHAPTER SEVEN: Electrospun Silica Nanofiber Thin Films and Functionalization with Gold Nanoparticles

A. Abstract

In this study we report a simple and efficient method for generating novel silica nanofiber thin films encapsulated with Au nanoparticles (AuNPs). The method is based on electrospinning and thermal decomposition of hybrid nanofibers prepared form a solution of tetraethylorthosilicate (TEOS), polyvinylpyrrolidone (PVP), and AuNPs. The fibers are electrospun onto a silica substrate, followed by high-temperature pyrolysis. The morphologies, surface roughness, and optical properties are characterized using SEM, EDX, AFM, TEM and high confocal microscopy. The study revealed humidity plays a critical role in achieving morphological consistence of the electrospun precursor nanofibers. In addition, to achieve defect free silica nanofibers a protective shield covering the fiber mat during thermal treatment is required to prevent electrostatic discharge. Finally, the AuNP embedded silica nanofibers exhibited a photosensitive response under the illumination wavelength around the surface plasmon resonance (SPR) absorption band. The photosensitive Au/silica nanofiber thin films can have important applications in sensor technology, waveguide devices, optoelectronics, composites, as well as catalyst support and adsorption.
B. Introduction

One-dimensional (e.g. fiber, rod, ribbon, tube and ring) silica mesoporous nanomaterials are extremely attractive due to their nano-confined optical interface properties. First reported by Mobil scientists in 1992, silica nanomaterials have been used in many areas of research including sensors, catalysis, separations and drug delivery. Perhaps the most advantageous property of silica nanomaterials is the chemical and thermal stability, thus allowing for these nanomaterials to be used under cruel ambient conditions. Moreover, silica nanomaterials are inexpensive, well-characterized with desirable bio-compatible surface properties (hydrophilic and non-toxic), and contains a variety of attachment chemistries, all of which are important for biomaterials research and development. Recently the preparation and characterization of continuous silica mesoporous nanofibers has been demonstrated using the electrospinning technique.

The past decade has witnessed tremendous advancements in the development of the electrospinning technique. Some examples of this development include hollow and coaxial fibers, alignment of electrospun fibers, and the fabrication of continuous carbon and ceramic fibers. Additionally, the amount of functional materials that can be embedded into the fibers has expanded greatly, including titanium dioxide, SBA-15, fluorescent dendrimers, and noble metallic nanoparticles. As discussed in previous chapters, electrospinning is a polymer processing technique used to create continuous fibers with diameters ranging from a few nanometers to micrometers. The electrospinning process works when a flowing polymer solution is subject to a high
electric field. When the repulsive electrostatic force overcomes the surface tension of the polymer solution a stable jet is formed and a membrane-like web of small fibers is emitted onto the ground electrode.\textsuperscript{13} For silica nanofibers, post-electrospinning pyrolysis procedures are required to achieve the desirable morphological properties, which entails treating the fibers in a furnace at 600 °C for 4 h. Because of the post-electrospinning pyrolysis procedure, the ability to manipulate and attach the nanofibers onto transducer substrates is limited. To our best knowledge, there have been no reports on electrospun silica nanofiber thin films calcinated and fused onto a transducer substrate for biosensor applications.

In this work, we report the fabrication of hierarchical silica nanofiber system that is transformed into a 2D silica nanofiber network as new and advanced sensing materials. The nanofibers are fused onto a transducer substrate (Fig. 7.1) and embedded with AuNPs for enhanced optical properties. A sol-gel process to create the spin dopant has been adopted, which involves the formation of a colloidal suspension (sol) from a precursor followed by gelation of the sol to form a network in continuous phase (gel).\textsuperscript{14} Specifically the sol-gel was made using the alkoxide precursor, tetraethyl orthosilicate (TEOS), and the carrying polymer, polyvinyl pyrrolidone (PVP). After the solution was prepared, electrospun and calcinated we characterized the nanofiber network using SEM, EDX, and AFM. Once the 2D silica nanofiber network procedure was optimized and fused onto a transducer substrate optical optimization experiments were conducted using AuNPs embedded silica nanofibers. The optical properties have been investigated using TEM and confocal microscopy.
Figure 7.1 Illustration of 3D silica nanofiber system that is transformed into a 2D silica nanofiber network and fused onto a transducer substrate.
C. Experimental Section

1) Materials

All chemicals were of the highest analytical grade, and were used without further purification. Tetraethyl orthosilicate (TEOS, 98%), polyvinyl pyrrolidone (PVP, \( M_w = 1,300,000 \)), hydrochloric acid (HCl), and acetic acid (HAc, glacial) were obtained from Sigma-Aldrich (St. Louis, MO). The ethyl alcohol (EtOH, 200 proof) was purchased from Gold Shield Chemical Co. (Hayward, CA).

2) Preparation of Fused Mesoporous Silica Nanofibers

Sol-gel process is a wet-chemical technique widely used in the synthesis of glass (silica) and ceramics. The most popular silicon alkoxide used for the synthesis of silica is tetraethyl orthosilicate \( \text{Si(OC}_2\text{H}_5)_4 \) (TEOS). The simplified pyrolysis reaction schemes are as below.

**At ambient temperatures (<600 °C)**

\[
\text{Si(OC}_2\text{H}_5)_4 + 2\text{H}_2\text{O} \rightarrow \text{SiO}_2 + 4\text{C}_2\text{H}_5\text{OH}
\]

**At elevated temperatures (>600 °C)**

\[
\text{Si(OC}_2\text{H}_5)_4 \rightarrow \text{SiO}_2 + 2\text{O(C}_2\text{H}_5)_2
\]

At ambient temperature hydrolysis and condensation occur through the absorption of moisture in the air, causing ethanol to be generated, which leads to the loss of fiber morphology. However, if the fibers are dehydrated to elevated temperatures of \( \sim 600 ^\circ \text{C} \), the volatile organic compound diethylether is the byproduct, which will quickly
evaporate into the atmosphere and produce clean defect free silica material. A polycondensation reaction takes place and creates a siloxane polymer chain, with varying concentration of byproducts.

Numerous preparation procedures have been investigated in this study. The optimal spin dopes were prepared using the following procedure: (1) the carrying polymer, 14% PVP, was dissolved in the spinning solvent, EtOH/HAc (15:1) to prepare mixture I; (2) the silica precursor, 14 % TEOS, was mixed drop wise into mixture I; and (3) the electrospinning mixture was sealed in a container, and magnetically stirred for at least 3h to allow the pre-gelation of TEOS. For optical enhancement experiments approximately 2% of AuNPs (13 nm ±2, weight concentration) were added into mixture I. The AuNP mixture was stirred under ambient conditions for 4 h to attain the homogeneity required for electrospinning.

The electrospinning mixture was placed into a syringe for electrospinning. A high-voltage DC power supply (Glassman High Voltage Inc. Series EH) was connected to a 25-gauge blunt nose needle attached to the syringe containing the electrospinning solution. The electrospun fibers were collected on aluminum foil. All solutions were electrospun at a voltage of 20 kV, a tip-to-collector distance of 10 cm, and a solution flow rate of 1.2 mL/h. All of the electrospinning procedures were carried out at ambient temperature (~25°C), a relative humidity of ~30%, and a collection time of 90 s. The electrospun fiber mats were dried in an oven at 50-70 °C for 12 h to remove any trace solvent.
The electrospun precursor nanofibers were carefully covered with an envelope of aluminum foil and placed in a furnace for calcination into final silica nanofiber network. Several calcinations procedures were investigated, and the optimal one based upon fiber properties was identified as follows: (1) increasing the temperature at 5 °C/min from ~25 °C to 600 °C, (2) keep the temperature at 600 °C for 4 h, and (3) allow for natural cooling to ambient conditions, usually 12 h. A constant flow of air was maintained through the furnace during the calcinations.

3) Characterization

A Philips XL30 FEG scanning electron microscope (SEM) with energy dispersive X-ray spectroscopy (EDS) was employed to examine the morphologies of the nanofiber networks. The SEM measurements were performed with a beam power of either 10 or 20 kV and magnification ranging from 10× to 200 000×. Fluorescence image analysis was performed on a Leica TCS SP2/UV confocal microscope using the excitation wavelength at 543 nm. The AuNP-embedded silica fibers were electrospun directly onto a carbon-coated copper grid and then characterized using a Tecnai T12 transmission electron microscope (TEM). Atomic Force Microscopy (AFM) images were obtained using a Veeco Dimension 5000 atomic force microscope (Santa Barbara, CA) with manufacturer-provided software. All images were obtained in tapping mode, and rms surface roughness values were obtained by averaging multiple 10 μm² areas across the entire calcinated substrate at a scan rate of 1.5 Hz.
D. Results and Discussion

1) Effects of Humidity and Electrostatic Discharge

The procedures for preparation of precursor electrospun silica nanofibers have been optimized using 14% PVP (carrying polymer) and 14% TEOS (alkoxide precursor) in EtOH/HAc (15:1). The environmental conditions during electrospinning played an important role in determining morphologies of the precursor nanofibers. The SEM images in Fig. 7.2 A and B show the representative morphologies of electrospun precursor nanofibers made from the above spin dope with a relative humidity greater than 50% (Fig. 7.2 A) and a relative humidity less than 50% (Fig. 7.2 B). At high humidity (greater than 50%), the water condenses on the surface of the fiber and cause circular pores to form on the fiber surfaces, especially when volatile solvents are used. More specifically, during the electrospinning process the water vapor condenses on the surface of the jet, due to cooling of the surface of the jet and the rapid evaporation of the volatile solvent. The size and depth of the circular pores increases with increasing humidity. At a humidity less than 50%, the fiber surfaces were smooth, containing no noticeable morphological defects. However, Ramakrishna and coworkers have reported that at very low humidity (less than 5%), the evaporation of the solvent is extremely rapid.\textsuperscript{15} This causes the needle to clog due to the solvent evaporating before the removal of the solvent form the tip of the needle.
**Figure 7.2** SEM images of precursor silica nanofibers electrospun with a relative humidity (A) greater than 50% and (B) less than 50%. SEM images of calcinated nanofibers (C) without a protective foil cover and (D) with a protective foil cover.
The SEM image in Fig. 7.2 C shows the effects of electrostatic discharge largely caused by the change in high and low temperatures of the furnace on the electrospun silica nanofibers. Triboelectric charging (friction) is the primary cause of the static build-up on the surface of the fibers. It occurs as a result of the flow of hot and cold air across the electrospun fibers. In particular, cold air, due to its low humidity, can cause an accumulation of residual charges on the non-conductive fiber surface and consequently cause strong electric field discharges, and thus the fibers will fold on itself. This phenomenon of electrostatic discharge is very common in the development of nanotechnology and many problems can occur because of these static charges such as particle contamination, material degradation, and/or damage to the material. There are 3 ways to prevent electrostatic discharge: 1) ground the material, which can be very difficult with nano-size materials; 2) use a conductive cover/shield on and around the material; 3) supply ion balanced air to the material under development. In our work we have addressed the problem by simply covering the spun fibers with an aluminum foil envelope. Using this method, defect-free silica nanofibers have been successfully obtained (Fig. 7.2 D).

2) Morphology and Durability of Silica Nanofibers

The chemical composition of the electrospun nanofibers before and after calcination has been examined using energy-dispersive X-ray spectroscopy (EDX) (Figure 7.3). The EDX spectra of the electrospun fibers before calcination indicated the presence of carbon, oxygen, nitrogen and silica, all of which are consistent with chemical composition of the TEOS and PVP in the fibers (Fig. 7.3 A). The EDX spectra of the
electrospun fibers after calcination indicated the presence of only silica and oxygen, confirming a pure silica chemical composition of the fibers (Fig. 7.3 B).

Fig. 7.4 (A) shows an SEM image of a typical 3D precursor silica nanofiber system created using 14% PVP (carrying polymer) and 14 % TEOS (alkoxide precursor) in EtOH/HAc (15:1). PVP has previously been shown to be a good carrying polymer for preparing silica nanofibers due to its low toxicity, high solubility and good thermal behavior.\(^3\) In our early experiments, the pyrolysis temperature was set at 400 °C for 4, 6 and 12 h periods. When this temperature was used the nanofibers often contained a trace amount of black solid, which we presumed to be carbon. The black solid indicated that the PVP was not completely burnt out and the pyrolysis temperature of 400 °C was insufficient. When 600 °C was used, the PVP was completely pyrolized and therefore this was used as the pyrolysis temperature. As shown in Fig. 7.4 (A) the as-electrospun fibers were uniform without beads and/or beaded fibers. The average diameter of the fibers was approximately 1 μm. After pyrolysis, the fiber surface morphology was well retained (Fig. 7.4 B) and the average diameter decreased to approximately 300 nm. It’s important to note that after pyrolysis the 3D nanofibers fused with each other to form a 2D thin film nanofiber network.
Figure 7.3  EDX energy spectra of electrospun silica nanofibers (A) before and (B) after calcination.
**Figure 7.4** SEM images of the electrospun silica nanofibers (A) before and (B) after calcination. The insets are SEM images with higher magnifications.
The calcinated silica nanofibers were further characterized using atomic force microscopy (AFM). The AFM images in Figure 7.5 show the fibers were without beads and/or beaded fibers, consistent with the SEM results. The peak-to-valley surface roughness was calculated to be 3.95 ± 0.08 nm, indicating that a relatively thin film of 2D fused nanofibers was created. Moreover, the silica nanofibers peak-to-valley surface roughness indicates the fibers are randomly orientated within the silica fiber mat: some are stacked while others are parallel and perpendicular to each other. The root-mean-square surface roughness of the nanofibers was 0.84 ± 0.05 nm, demonstrating the silica fiber surface is extremely smooth. The surface qualities of the nanofibers can greatly enhance the performance as sensing materials. In fact a common everyday glass slide has a RMS surface roughness of approximately 3 nm. From the height profiles (Figure 7.5 upper), a fibrous cylindrical like structure is observed, with an average width of approximately 300 nm, which compares well with diameters found within the SEM images in Fig. 7.4. The average height is ~500 nm, further indicating the fibers are randomly layered and fused within the silica fiber film.

3) Fusing Silica Nanofibers onto a Glass Substrate

The ability of the silica nanofibers to fuse onto a transducer substrate was further investigated. Pyrolysis of the fibers were carried out on a typical silica surface (glass slide) (Fig. 7.6 B and C), as well as on an aluminum surface (Fig. 7.6A) for comparison. Fig. 7.6 (A) indicates silica nanofibers do not adhere well to the aluminum surface, and in fact they showed cracking upon calcination, creating an undesirable surface interface. However, when a silica substrate is used the silica nanofibers were evenly calcinated onto
the simulated surface (Fig. 7.6 B) with no visible defects. The SEM image in Fig. 7.6 (C) shows how the silica nanofibers fuse into the silica substrate forming a mechanically stable layer of entangled 2D nanofibers together with the glass substrate.
Figure 7.5 AFM image (lower) and line profile (upper) of electrospun silica nanofibers after calcination.
Figure 7.6  Photographic images of electrospun silica nanofibers after calcination on an aluminum substrate (A) and a silica substrate (B). C is the SEM image of the calcinated silica nanofibers fused onto a silica substrate.
4) Silica Nanofibers Functionalized with AuNPs

We then studied the functionalization of nanofibers with AuNPs and demonstrated that unique optical properties can be obtained when AuNPs are used as components for construction of nanofiber devices. It has been well documented that optical device applications greatly benefit from minimizing the diameter of the waveguide device and adding noble metallic nanoparticles. Some benefits include tight-confinement ability, enhanced evanescent fields and large waveguide dispersions.\textsuperscript{17-18} Unfortunately fabrication of nanosized optical waveguides with metallic particles remains challenging because of the high precision requirements needed to create these nanomaterials. Preliminary work has been carried out with electrospun calcinated silica nanofibers and AuNPs. Figure 7.7A is the TEM micrograph of electrospun Au/silica nanofibers after calcination. The AuNPs were self organized during the electrospinning process and the original size and morphology of AuNPs were maintained. The AuNPs have a tendency towards non-homogeneous distribution when a high electric field is applied to the spinning solution. Therefore the distance between the AuNPs ranged from several nanometers to several hundreds of nanometers, creating patches of AuNPs throughout the nanofiber mat. Fig 7.7B demonstrates the optical properties of these silica nanofibers embedded with AuNPs using fluorescence microscopy ($\lambda_{\text{Ex}} = 543$ nm). When the AuNPs were doped into the silica nanofibers fluorescent properties were clearly detected, while the pure silica nanofibers did not show any emission. We speculate that the refractive index contrast between the nanofiber and air makes the excited light
randomly scatter in the electrospun AuNP embedded silica nanofibers, resulting in the observed phenomenon. These properties are currently under further investigation.
Figure 7.7 TEM micrograph (A) and confocal fluorescence image (B) of electrospun AuNP embedded silica nanofibers after thermal treatment ($\lambda_{ex} = 543$ nm).
E. Conclusions

In summary, we have successfully prepared silica nanofiber thin films using the electrospinning method. The results indicated that at low humidity (<50%) uniform (bead-free) amorphous nanofibers with diameters of approximately 300 nm can be effectively fabricated by selecting PVP as the carrying polymer, TEOS as the alkoxide precursor, and EtOH/HAc as the solvent. The PVP was removed and the TEOS calcinated into silica after thermal treatment at 600 °C for 4h. We also demonstrated that in order to create defect free nanofibers an electrostatic protective cover is required. Additionally, the surface-roughness of the silica nanofiber thin films was controllable and is almost 3 times smoother than that of typical glass slides. The nanofiber films showed enhanced optical properties when AuNPs were embedded. Optical and fluorescence microscopy was used for characterizing the optical scattering effect, evanescent wave properties, and the localized SPR of the noble metallic particles. The hybrid nanofibers may find a range of sensing applications due to increased surface area, high permeability, and improved optical properties. They may also find applications in waveguide devices, optoelectronics, as well as catalyst support.
F. References


(13) Huang, Z. M.; Zhang, Y. Z.; Kotaki, M.; Ramakrishna, S. *Compos. Sci. Technol.* 2003, 63, 2223.


(15) Ramakrishna, S. *An introduction to electrospinning and nanofibers*; World Scientific, 2005.


8. CHAPTER EIGHT: Conclusion and Outlook

The studies outlined in this thesis have emphasized the fabrication and application of new electrochemical and optical biosensor technologies using novel nanomaterials (SAMs, polyelectrolytes, polydiacetylene, dendrimers and electrospun nanofibers) as the building blocks. A majority of the work presented in this dissertation involved the characterization of these new materials and how the materials can be better used as viable sensor platforms. In order to characterize the physical make-up and performance of these unique nanostructures a number of instrumental methods were employed, including cyclic voltammetry, surface plasmon resonance (SPR) spectroscopy, atomic force microscopy, ellipsometry, scanning electron microscopy, tunneling electron microscopy, confocal microscopy, Fourier transform infrared spectroscopy, ultraviolet-visible spectroscopy, and fluorescence spectroscopy. The thesis is organized in the following order: First, the fundamentals, background and groundwork of the dissertation are presented in Chapter One. Each chapter thereafter introduces a unique and innovative nanostructure for sensor technology development and applications: Chapter Two, unobstructed electron transfer on porous polyelectrolyte nanostructures; Chapter Three, enzyme-amplified electrochemical detection of poliovirus; Chapter Four and Five, FRET detection of proteins using fluorescently doped electrospun nanofibers and pattern recognition; Chapter Six, optical response of electrospun polydiacetylene (PDA)-embedded nanofibers, and Chapter Seven, two-dimensional (2D) silica nanofiber thin films.
The electron transfer mediation and long range electron transfer properties of a mixed electro-active SAM and porous polyelectrolyte nanostructure are the focus of Chapter Two.\textsuperscript{1} The nano-architecture build-up is monitored in real time with SPR spectroscopy and the electrochemical behavior is characterized by cyclic voltammetry in the same flow cell. This method of analysis is unique because of its ability to simultaneously probe the interface properties on two different physical quantities (refractive index and electron movement), at the same surface, from inside an electrochemical cell.\textsuperscript{2-3} The study revealed a new three-dimensional (3D) nano-assembly interface with the desirable surface properties of facile electron transfer, free counter ion movement, and controlled multilayer build-up. It opens avenues for a potentially multitude of new applications with the ultra-thin polymer films on an electrode surface that span from the study of mass transport of molecules across thin films, thin-film electrochemical detection, to creation of electroactive sites for biological detection.

Using the mixed electro-active SAM and porous polyelectrolyte nanostructure developed in Chapter Two, a fast, reliable, and cost-effective method for detecting whole viral particles is demonstrated in Chapter Three. Specifically, a sandwich-type electrochemical enzymatic amplification scheme was developed to detect poliovirus type 1. The study reveals that by using the LbL method to build a 3D structure provides an increased capacity for the immobilization of the bioreceptor (anti-PV-1) while still maintaining desirable electrode surface properties: simple electron transfer, free counter ion movement, and controlled multilayer build-up. Viruses will remain a health threat.
New techniques like this open the door for early diagnosis and management of health problems worldwide—especially in developing nations.

Chapters Four, Five, Six and Seven focus on a new area of optical biosensor research using electrospun polymer nanofibers doped with a variety of specific and nonspecific bio-receptors. In the last few years, electrospinning technology has emerged with substantial potential for biomedical and biotechnology applications. These include tissue engineering, controlled drug release, wound dressing, medical implants, dental composites, molecular filtration, and biosensors. A survey of open publications and the distribution around the world using the term “electrospinning” in the SciFinder Scholar demonstrates that the electrospinning technique has attracted increasing attention in the global scientific community. However, as with any emerging technology, there is room for improvements, novel developments, and incorporation of a wide range of techniques and instrumentation. While significant advancements are needed before the clinical usage or commercialization can be realized, the work discussed in this thesis leads the way.

Fluorescence resonance energy transfer (FRET) detection of proteins using fluorescently doped electrospun nanofibers and pattern recognition is the focus of Chapters Four and Five. To date, fluorescence spectra of organic materials represent one of the most sensitive optical properties that can be used as a sensory medium for the detection of biological molecules and agents in the environment, and was therefore the focus of our analytical studies. The size and tunability of four different cationic and anionic fluorescent dendrimers were optimized to provide selective interactions with proteins, which extends the scope of nanofiber sensing applications. These fluorescent
dendrimers provided efficient transduction of the binding event and generated fluorescence signal in response to a changed concentration of analyte. Through principle component analysis, we are able to cluster more complex data matrices and resolve the different proteins within this data matrix for detection using an array of electrospun nanofiber sensor substrates. In order to improve the sensory efficiency of fluorescent biosensors future studies must include the incorporation of the nanofibers onto a more complex multiplexed sensor array chip and the detection of other multifaceted (real-world) biological samples.

A new strategy for the colorimetric and fluorescence differentiation of proteins and volatile organic solvents (VOCs) based on silica-enforced polydiacetylene (PDA) 1D-nanofibers has been demonstrated in Chapter Six. Above all, these studies reveal colorimetric transduction is a simple and desirable sensor technique, because the ability to quickly detect analytes on site with the naked eye would not only save time but also lives and money. The future outlook of PDA based nanofiber sensors includes 1) optimization of the sensing interface of the 1D-nanofibers by incorporating them into a more durable multiplexed microarray sensor platform, (2) modification of the substrate for better stability against environmental perturbations, and (3) functionalization of the nanofibers for specific capture and detection of more pathogens and other biological targets.

In Chapter Seven details are provided on a simple, efficient, and low-cost method for generating functional 2D silica nanofiber thin films. These nanofibers have been embedded with gold nanoparticles for realization of new optical properties. This study
develops a new method and fabrication technique for material sensor science using electrospinning and calcination. One of the exciting properties of the silica nanofibers is the chemical and thermal stability, which in turn allows for their broad use under demanding ambient conditions. Moreover, silica fabrication is inexpensive, well-characterized with desirable bio-compatible surface properties (hydrophilic and non-toxic), and already contains a variety of attachment chemistries, all of which are advantageous for biomaterials research and development.8 This work opens avenues for a multitude of future applications in sensor technology, waveguide devices, optoelectronics, composites (dental), as well as catalyst support and adsorption.

The research presented in this thesis on new sensing concepts has opened the door to many innovative biomedical and biotechnology applications of electrochemical and optical sensing devices. Such devices will be useful for delivering the diagnostic information in a fast, simple, and low-cost fashion, and are thus uniquely qualified for meeting the demands of medicine, homeland security, and defense technology worldwide. The future of sensor is bright, and the only limitation is our imagination. Future biosensor development can benefit from integration of cutting-edge technologies from different disciplines and search of new resources that support testing of novel sensor technology. Therefore the objective of the research performed in this dissertation that is to further advance biosensor technology through demonstrating new and innovative options in electrochemical and optical biosensor design is simply on the right direction.
A. References

(1) Davis, B. W.; Linman, M. J.; Linley, K. S.; Hare, C. D.; Cheng, Q.

*Langmuir* **2009**.


