ENGINEERING NOVEL SUBSTRATES FOR ADVANCED
BIOMOLECULAR DETECTION

A Dissertation in
Chemical Engineering
by
Nitin Kumar
© 2008 Nitin Kumar

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2008
The dissertation of Nitin Kumar was reviewed and approved* by the following:

Jong-In Hahm  
Assistant Professor of Chemical Engineering  
Dissertation Advisor  
Chair of Committee

Darrell Velegol  
Associate Professor of Chemical Engineering

Jian Xu  
Assistant Professor of Engineering Science and Mechanics

Andrew L. Zydney  
Walter L. Robb Chair and Professor of Chemical Engineering  
Head of the Department of Chemical Engineering

*Signatures are on file in the Graduate School
ABSTRACT

Detection of biological species is central to many areas of biology and life sciences such as ultra sensitive disease detection, biochemical sensing and targeted drug delivery. Currently, detection of biomolecules like proteins and DNA is done by fluorescence detection, however, enhancing detection sensitivity and increasing signal to noise ratio still remains a major challenge. Novel techniques are presently required which can provide reliable, low cost, and ultra-sensitive detection of proteins and DNA. This thesis will focus on two approaches in this context: the use of diblock copolymer templates and zinc oxide nanorods, with an aim to provide rapid, sensitive and accurate biomolecular detection. While complementary, these approaches are not necessarily interlinked.

First approach will focus on utilizing the microphase separation behavior of diblock copolymers to pattern proteins with nanometer periodicity. The structural variety and chemical heterogeneity of polystyrene-block-poly(methylmethacrylate) (PS-b-PMMA) and polystyrene-b-poly(4-vinylpyridine) (PS-b-PVP) template surfaces were successfully exploited to spontaneous formation of self-assembled, linear and hexagonally-ordered protein arrays that exhibit repeat spacings in a nanoscopic dimension. More importantly, protein molecules on the polymeric templates maintained their natural conformation and activity for several months. Our results demonstrate that self-assembling, chemically heterogeneous, diblock copolymer templates can be used as excellent, high payload, high density protein templates making them highly suitable as functional substrates in many proteomics applications.
Second approach will focus on the remarkably enhanced optical detection of DNA and proteins which is enabled by the use of nanoscale zinc oxide platforms. Using model protein and nucleic acid systems, we demonstrate that engineered nanoscale zinc oxide nanostructures can significantly enhance the detection capability of biomolecular fluorescence. Without any chemical or biological amplification processes, nanoscale zinc oxide platforms enabled increased fluorescence detection of these biomolecules when compared to other commonly used substrates such as glass, quartz, polymer, and silicon. We also demonstrate the easy integration potential of zinc oxide nanostructures into periodically patterned platforms which, in turn, will promote the assembly and fabrication of these materials into multiplexed, high-throughput, optical sensor arrays.
# TABLE OF CONTENTS

## LIST OF FIGURES

viii

## Acknowledgements

xiii

## Introduction

1

1.1 Background ................................................................. 2
   1.1.1 Diblock Copolymers ................................................ 2
   1.1.2 Zinc oxide micro/nanostructures ................................ 6
   1.1.3 Magnetotactic Bacteria ............................................ 7
   1.1.4 Protein Arrays ........................................................ 8
   1.1.5 Biomolecular Fluorescence Detection ......................... 9

1.2 Thesis Overview ........................................................ 11

1.3 References ............................................................... 17

## Nanoscale Protein Patterning Using Self-assembled Diblock Copolymers ..... 25

2.1 Background ................................................................. 25

2.2 Experimental Details .................................................. 28

2.3 Results and Discussions ............................................... 29
   2.3.1 Microdomain formation in PS-b-PMMA thin film templates ... 29
   2.3.2 Protein deposition onto the PS-b-PMMA thin film templates ... 30

2.4 Conclusions ............................................................... 33

2.5 References ............................................................... 33

## Two-Dimensionally Self-Arranged Protein Nanoarrays on Diblock Copolymer Templates ................................................. 41

3.1 Background ................................................................. 41

3.2 Experimental Details .................................................. 44

3.3 Results and Discussions ............................................... 45
   3.3.1 Fabrication and characterization of two dimensional PS-b-PVP templates .................................................. 45
   3.3.2 Protein adsorption onto the PS-b-PVP thin film templates ... 47
   3.3.3 Biological activity assessment .................................... 49

3.4 Conclusions ............................................................... 51

3.5 References ............................................................... 52

## Activity Study of Self-Assembled Proteins on Nanoscale Diblock Copolymer Templates ................................................. 61

4.1 Background ................................................................. 61

4.2 Experimental Details .................................................. 64

4.3 Results and Discussions ............................................... 65
   4.3.1 Fabrication and characterization of PS-b-PMMA templates .... 65
   4.3.2 Enzyme adsorption and activity assessment .................... 66
8.3 Results and Discussions ........................................................................................................ 154
  8.3.1 Bacillus bacteria .............................................................................................................. 154
  8.3.2 Synthesis and characterization of ZnO templates ......................................................... 156
  8.3.3 Non-covalent vs covalent DNA hybridization .............................................................. 157
8.4 Conclusions .......................................................................................................................... 163
8.5 References ............................................................................................................................ 164

**Synopsis** ............................................................................................................................. 170

  9.1 Summary ............................................................................................................................ 170
  9.2 Future Perspectives ............................................................................................................. 174
  9.3 References ........................................................................................................................ 178
LIST OF FIGURES

Figure 1.1: Mean field phase diagram………………………………………………………….24

Figure 2.1: AFM images showing the microphase-separated ultrathin films of PS-b-PMMA diblock copolymer. Bright and dark domains in the topography (A) and phase (B) images represent PMMA- and PS-rich regions, respectively. The average repeat spacing of the microdomains is 45 nm…………………………….38

Figure 2.2: AFM images of 4 µg/mL (A and B) and 20 µg/mL (C and D) IgG molecules deposited selectively on PS domains in the microphase-separated PS-b-PMMA ultrathin films spun on silicon oxide substrates. (E) Line analysis taken along the white line indicated in (C)……………………………………………………….39

Figure 2.3: AFM images of protein G and FITC anti-bovine IgG molecules assembled selectively on PS domains in the microphase separated PS-b-PMMA ultrathin films on silicon oxide substrates. (A and B) 1 by 1 µm topography and phase images respectively taken after deposition of 4 µg/mL protein G. (C and D) 1 by 1 µm topography and phase images respectively………………………………….40

Figure 3.1: Schematic cartoons illustrating the top and side views of various polymeric templates: (A) original, (B) partially open, and (C) reverted two-dimensional PS-b-PVP micelles on silicon substrates. These templates were subsequently used for nanoscale self-assembly of two-dimensional protein arrays……………………………57

Figure 3.2: AFM topology of various self-assembled nanoscale PS-b-PVP templates prepared by various solvent exposures. (A) (1 and 2) 300 × 300 and 180 × 180 nm AFM images showing the “original” hexagonally packed PS-b-PVP micellar film that was spun cast in toluene on a silicon substrate. (3) Height profile along the indicated white line of the original micelle shown in (2). (B) (1 and 2) 300 × 300 and 180 × 180 nm AFM images…………………………………………………58

Figure 3.3: Schematic illustrations and AFM topography images of IgG and MT molecules on PS-b-PVP templates. (A) IgG molecules deposited on open PS-b-PVP templates from a 20 µg/mL solution. The presented AFM images are (2) 300 × 300 nm, (3) 180 × 180 nm, and (4) 60 × 60 nm in size. On open PS-b-PVP micellar templates, IgG molecules occupy preferentially the PS-rich areas which encircle the hexagonally packed, low-lying PVP cores. (B) IgG molecules assembled on reverted PS-b-PVP templates when (2) 4 µg/mL and (3 and 4) 10 µg/mL of IgG solution was added to the templates. (C) MT molecules assembled on reverted PS-b-PVP templates…………………………………………………59

Figure 3.4: Enzymatic activity test of HRP adsorbed on PS-b-PVP ultrathin micellar films. (A) Control experiment without HRP molecules on PS-b-PVP. (1) In the absence of HRP, color change of the assay solution was not observed. (2) A 180 ×
180 nm AFM image of the hexagonally packed PS-b-PVP micelles which was used in the enzymatic assay shown in (1). (3) Each PS-b-PVP micelle in the hexagonal lattice of the AFM image

**Figure 4.1:** (A) Schematic illustration showing selective deposition and self-assembly of enzymes on microphase separated PS-b-PMMA templates with the spatial control on the nanometer scale. (B). AFM images showing HRP segregated selectively on PS domains. (1) 500 x 500 nm phase AFM image taken after deposition of 4 µg/mL of HRP where the inset is 150 x 150 nm, (2) 750 x 750 nm topography AFM image acquired after deposition of 10 µg/mL of HRP, and (3) 500 x 500 nm topography AFM scanned after deposition of 10 µg/mL of HRP where the inset shows a 200 x 200 nm phase AFM image

**Figure 4.2:** (A). Digital images taken after adding 1 mL of pyrocatechol to vials containing (1) as-annealed PS-b-PMMA where no color change was observed and (2) PS-b-PMMA with self-assembled MT molecules where the assay color changed to yellow-brown. (B) Digital images taken after adding 1 mL of TMB solution to vials containing (1) as-annealed PS-b-PMMA substrate where no color change was observed, (2) PS-b-PMMA substrate with self-assembled HRP molecules in which case the assay color changed to blue

**Figure 4.3:** Bound-state HRP activity assays: visualization and quantification of HRP activity bound on PS-b-PMMA surface. (A and B) Digital images of a TMB assay solution after addition to a PS-b-PMMA template (A) without HRP and (B) with effectively immobilized HRP molecules. No color change was monitored in (A), indicating no catalytic activity. In contrast, the assay color rapidly changed from colorless to blue in (B)

**Figure 4.4:** Free-state HRP activity assays. UV-vis absorbance spectra of HRP molecules free in solution. A volume of 25 µL of (A) 0.01, (B) 0.02, (C) 0.05, and (D) 0.15 µg/mL in deionized water, combined with 1 mL of the indicator solution, was used for the UV-vis measurement. Absorbance spectra were recorded at 0, 2, 5, 10, 15, 20, 25, 30, 60, and 120 min after the addition of the TMB assay solution. The time-dependent absorbance spectra are plotted, from the bottom to the top curves, corresponding to the shortest to the longest time delays. In (D), plots show the absorption spectra taken at 0 min (the bottom-most curve) to the absorption saturation point at 30 min (the top-most curve). The absorption spectrum does not change significantly after 30 min

**Figure 4.5:** Evaluation of HRP activity difference between their free state to PS-b-PMMA bound state. In all graphs, blue data points represent activities of HRP molecules freely floating in solution, and red data points represent those of HRP molecules immobilized on PS-b-PMMA surfaces

**Figure 4.6:** AFM images of blank PS-b-PMMA with and without HRP taken after the UV-vis absorption measurements described in Figures 4.3-4.5. (A and B) 700 nm
× 700 nm phase and topographic images of the PS-b-PMMA template without bound HRP exposed to the same........................................................................................................85

**Figure 4.7:** A. Confocal fluorescence data of proteins and interacting proteins collected at 400 times magnification. The contrast and brightness of the presented confocal fluorescence images were adjusted for clarity. (1) Green fluorescence was observed from PS-b-PMMA regions where 100 µg/mL eGFP was deposited whereas other PS-b-PMMA areas with no eGFP on the surface did not show any fluorescence emission. 2) Deposition of a 20 µg/mL FITC-antiIgG droplet onto BSA-incubated PS-b-PMMA........................................................................................................86

**Figure 5.1:** Schematic illustration and AFM images showing various polymeric surfaces before and after deposition of protein molecules. PS-b-PMMA diblock copolymer, PS homopolymer, and PS/PMMA blend ultrathin films were used as templates for protein adsorption........................................................................................................104

**Figure 5.2:** AFM images displaying protein adsorption behavior on diblock copolymer and PS homopolymer templates. Panels (a) through (d) are 400 × 400 nm AFM scans of (a) BSA, (b) Fn, (c) HRP and (d) protein G molecules assembled selectively onto PS-rich regions on PS-b-PMMA or PS/PMMA blend films. 4 µg/mL protein solution was used for samples (a), and (d) whereas 1.5 µg/mL HRP solution........................................................................................................105

**Figure 5.3:** (A) Typical AFM images of IgG molecules on PS/PMMA blend films. The four AFM images are 600, 450, 250, and 300 nm in diameter, respectively. Chemical make-ups specific to each polymeric domain in the films are specified in each AFM image........................................................................................................106

**Figure 5.4:** UV-vis absorbance comparison between HRP molecules bound on PS-b-PMMA versus on PS templates. In all graphs, red data points represent absorbance of HRP molecules immobilized on PS-b-PMMA whereas blue data points represent that of HRP on PS. To guide the eye, solid lines following data points are inserted. (A) UV-vis absorbance values of PS-b-PMMA-bound and PS-bound HRP recorded at λ = 650 nm are displayed with respect to time from the addition of the indicator solution........................................................................................................108

**Figure 6.1:** Schematic illustration showing the two experimental schemes to produce ZnO NR and MR structures using MS-1 as catalysts. (A) Homogeneous deposition process of MS-1 catalysts and (B) patterned deposition process of MS-1 using PDMS stamps........................................................................................................118

**Figure 6.2:** SEM images of hexagonal ZnO NRs and MRs grown over MS-1. (A and B) ZnO NRs prepared by homogeneously depositing the catalysts on silicon substrates. (C through F) ZnO MRs prepared by microcontact printing the bacterial catalysts on selective areas of substrates........................................................................................................119
Figure 6.3: ZnO NRs and MRs fabricated using MS-1 catalysts. (A and B) SEM images of ZnO NRs homogeneously distributed on substrates (A) and ZnO MRs patterned periodically on substrates (B). The inserted scale bars in (A) and (B) are 100 µm and 50 µm, respectively. The insets shown in the upper right corner in (A) and (B) are 5 by 5 µm. The insets shown in the lower right corner in (A) and (B), clearly displaying the hexagonal NR and MR structures of as-grown ZnO, correspond to 1.5 µm and 2 µm, respectively. (C) XRD pattern of ZnO NRs on a silicon substrate. (D) Typical room-temperature PL spectrum of ZnO NRs and MRs grown over MS-1.

Figure 7.1: Schematic illustrations showing the overall experimental procedures. (a) ZnO nanostructures were grown homogeneously on Si wafers using Ag catalysts, (b) periodically spaced, stripe-patterned ZnO nanopmelts were fabricated by microcontact printing the catalysts onto selected locations of the growth wafers, (c) SiNRs were synthesized on Si wafers.

Figure 7.2: Enhanced fluorescence detection facilitated by the use of ZnO nanopmelts: model protein system. (a) When confocal microscopy was employed to measure fluorescence emission, no distinctive contrast was observed in the confocal images taken from nonspecifically adsorbed FITC-antiIgG molecules on Si wafers, SiNRs, and patterned PMMA substrates. (b) Markedly strong fluorescence emission was.

Figure 7.3: Enhanced fluorescence detection facilitated by the use of ZnO nanopmelts: model DNA system. (a) No fluorescence was detected from bce samples before and after hybridization reaction with basr. The absence of fluorescence contrast in the confocal image taken after the hybridization reaction is due to no or poor duplex formation between mismatching strands of bce and basr. (b) Clear fluorescence contrast.

Figure 7.4: Protein fluorescence monitored using ZnO nanopmelts. (1) SEM image of as-grown, patterned ZnO nanoarrays of filled squares. Both the length and repeat spacing of each ZnO square are 5 µm. (2 and 3) Neither as-grown ZnO nanopmelts nor BBSA modified ZnO nanostructures exhibit any detectable fluorescence. (4 and 5) When DTAF-streptavidin was introduced to BBSA on ZnO nanopmelts.

Figure 7.5: Protein-protein interaction investigated by enhanced fluorescence detection with the use of ZnO nanopmelts. a. (1) SEM image of individual ZnO nanorods as grown on a silicon wafer. The average length and width of these ZnO nanorods are 4.1 ± 0.3 µm and 313.3 ± 68.3 nm, respectively. (2 and 3) Fluorescence emission from FITC-antiIgG molecules that were conjugated to the previously PG modified ZnO nanorods through PG-IgG interaction is clearly seen in the confocal images. Green emission is evident from protein molecules on individual ZnO nanorods whereas.
Figure 7.6: Enhanced fluorescence detection facilitated by the use of ZnO nanoplatformation regardless of emission spectra of fluorophores. a. (top panels) The presented confocal images confirm that ZnO nanoplatformation allow enhanced fluorescence detection of TRITC-IgG. The underlying striped patterns of 10 µm ZnO are visible due to the red emission from TRITC-IgG.

Figure 8.1: (a) Schematic illustrations showing simultaneous synthesis and assembly of ZnO nanoplatformation consisting of (left) individual ZnO nanorods and (right) periodically patterned ZnO nanostructures. (b) (Left) SEM image of a patterned ZnO platform with the stripe width and repeat spacing of 50 µm. The inserted SEM image at the bottom left corner shows the lying-down arrangement of ZnO nanostructures.

Figure 8.2: (a) Fluorescence emission monitored using a non-covalent DNA immobilization scheme. (1) SEM image of as-grown, patterned ZnO nanoplatformation consisting of stripes with a repeat spacing of 20 µm. (2) Oligonucleotide probe strands of *bas* were nonspecifically adsorbed onto the ZnO nanoplatformation shown in (1) and subsequently reacted with 20 µM *basr* to form double stranded DNA. (b) Fluorescence.

Figure 8.3: Fluorescence intensity comparison between covalent and non-covalent DNA detection schemes. Striped ZnO sensor arrays and individual ZnO nanorods were utilized as enhanced platforms for identifying DNA sequence. The ZnO sensor arrays consisted of periodically spaced, striped patterns of 20 µm in both width and repeat spacing. The average length and width of the individual ZnO nanorods are 4.1 ± 0.3 µm and 313.3 ± 68.3 nm, respectively. After covalently or non-covalently attaching the oligonucleotide.
Acknowledgements

I express my deep gratitude to my advisor Dr. Jong-In Hahm for her encouragement, support and guidance throughout the course of this work. My experience working with her has had a profound impact on me and I will carry the many words of wisdom she has kindly shared with me throughout my life. I simply cannot overstate the amount of respect I have for her. Next, I would like to extend a special thanks to Dr. Darrell Velegol, Dr. Andrew Zydney and Dr. Jian Xu for agreeing to serve on my committee. I would like to express my sincerest gratitude to Dr. Michael Pishko, Dr. Wayne Curtis and Dr. Joan Redwing for their advice and suggestions. I thank the past and present members of my research group for their invaluable advice and guidance. I am extremely thankful to Neetu Chaturvedi for her encouragement and unconditional support throughout the phase of my graduate school life. I wish her the very best in life. I would like to also acknowledge both personal and professional help extended by all my friends, especially Sujith Nair, Madhukar Dasika, Amit Varshney, and Praveen Depa for making my stay in State College a memorable one. I wish them success in their endeavors. I simply cannot put to words the amount of love and respect I have for my brother Mr. Naveen Kumar, my sister Mrs. Jyoti Khare and my parents Smt. Madhuri Srivastava and Shri. Jai Kumar for their unwavering support and encouragement, and to them, I dedicate this thesis.
CHAPTER 1

Introduction

Detection of biological and chemical species is central to many areas of biology and life sciences such as ultra sensitive disease detection, genetic screening, biochemical sensing and targeted drug delivery. Currently, detection of biomolecules like proteins and DNA is done by fluorescence detection - a widely used technique in both laboratory scale and high throughput genomic research. However, enhancing detection sensitivity and increasing signal to noise ratio still remains a major challenge in fluorescence based detection methods. Novel techniques are presently required which can provide reliable, low cost, and ultra-sensitive detection of proteins and DNA.

Sensitivity and selectivity are the two key aspects to consider while developing new technology for biosensing applications. Though these attributes are primarily a function of the biomolecule chosen for specific sensing application, they are also affected by the choice of the sensing platform. For example, the selectivity of a biosensing device is closely related to the biocompatibility and non specific binding properties of the nanomaterials of interest.

Nanomaterials such as polymeric nanotemplates and zinc oxide nanorods offer unique advantage as sensing platforms in biosensing applications. The dimension of these nanomaterials are comparable to the dimension of the biological species being sensed and thus represents excellent means for detecting signals that can ultimately interface with optical, electronic or mechanical instruments. Moreover, these nanomaterials exhibit unique mechanical, chemical, electrical and optical properties that can be used for biosensing applications. However despite the excellent prospects offered by these
nanostructures, there are some key issues which need to be addressed in order to realize their full potential for use in biosensing applications: 1) how to control the dimension, position and orientation of these nanomaterials 2) how stable are they in biomolecular environment 3) what is their sensitivity limit and 4) how to fabricate these biosensing devices in a fast and cost effective manner.

The central theme of this thesis is to address the questions and challenges raised in the previous paragraph with regards to the polymeric nanotemplates and zinc oxide nanorods and their subsequent use in biomolecular detection. In the first part of the thesis, we attempted the fabrication of various one and two dimensional nanotemplates using diblock copolymers followed by the qualitative and quantitative analysis of protein adsorption behavior onto these templates. In the second part, synthesis of zinc oxide nanorods using magnetic bacteria is attempted. Following the successful synthesis of zinc oxide nanorods these were then subsequently used to detect: proteins and DNA at very low concentrations, protein-protein interactions and discrimination between two genetically related species of bacterium – Bacillus anthracis and Bacillus cereus.

1.1 Background

1.1.1 Diblock Copolymers

Polystyrene-b-polymethylmethacrylate (PS-b-PMMA) and polystyrene-b-poly(4-vinylpyridine) (PS-b-P4VP) copolymers belong to the class of heteropolymers where in two monomers are joined together by a covalent bond to form a chain. Diblock copolymers contains only two block and can be generally represented by the formula

The covalent bond is the most significant factor determining the phase behavior of the diblock copolymers as it restricts macroscopic segregation of the chemically dissimilar polymer blocks, leading to molecular heterogeneities in compounds with length scales ranging from 50 to 1000Å.

**PS-b-PMMA:** The microphase separation behavior of PS-b-PMMA diblock copolymers is well-characterized and, therefore, can be precisely predicted.\(^1,2\) The two chemically distinct, and thus immiscible, polymer blocks in diblock copolymer undergo phase separation and self-assemble into ordered patterns of microdomains whose characteristic packing and size is determined by the polymer compositions and chain lengths, respectively.

Thus when this diblock copolymer is annealed above the glass transition temperature of the two blocks, it phase separates and self-assembles into periodic nanoscale domains. This unique phase behavior of the diblock copolymer can be characterized by three parameters: overall degree of polymerization (N), Volume fraction of A block in the copolymer (f\(_A\)), and the Flory-Huggins segment-segment interaction parameter (χ). The phase separation is governed by the interplay between the entropic and energetic factors.

<table>
<thead>
<tr>
<th>Product (\chi N)</th>
<th>Controls the degree of segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\chi N \ll 10)</td>
<td>Spatially homogeneous state</td>
</tr>
<tr>
<td>(\chi N = 10)</td>
<td>Order Disorder Transition</td>
</tr>
<tr>
<td>(\chi N \gg 10)</td>
<td>Ordered microstructures with narrower interfaces</td>
</tr>
</tbody>
</table>
While the formation of structures is controlled by $\chi N$, their geometry is controlled by $f_a$ the volume fraction of A component in the diblock as is given by the mean field phase diagram. With $f_a$ varying between 0 and 1 a variety of structures are formed ranging from complete disordered phase as $f_a \rightarrow 0$ or 1 to lamellar phase for nearly symmetric diblock ($f_a \sim 0.5$) – Figure 1.1.

The self-organizing nature of the ultrathin films of the diblock copolymer under carefully balanced thermodynamic conditions is exploited to produce alternating polymeric domains of the two chemical compositions of the diblock at the air/polymer interface. The repeat spacing of such microdomains can be precisely controlled on the nanometer scale by varying, for example, the molecular weight of the diblock copolymer. In addition, the microdomains of PS-b-PMMA can be readily aligned using various methods including electric field, shear force, annuli formation, and substrate modification. Therefore, the self-assembling phase behavior of the diblock copolymer is highly predictable and controllable, making the material suitable as nanoscale templates for electronic, optical, magnetic and biosensing devices.

**PS-b-P4VP:** When this diblock copolymer is dissolved in a solvent that is a preferential solvent for one block, the polymer chains undergoes rearrangement and form micelles. The core consists of the insoluble polymer block while the corona consists of the soluble block. Micellization occurs in dilute block copolymer solutions at a fixed temperature above a certain concentration, called the Critical Micelle Concentration (CMC). Amphiphilic polymeric systems such as polystyrene-b-poly(acrylic acid), poly(ethylene propylene)-b-poly(ethylene oxide), polystyrene-b-poly(2-vinylpyridine), and polystyrene-
b-poly(4-vinylpyridine) were extensively studied to understand their fascinating micellar properties and dependence on diblock copolymer characteristics.\textsuperscript{11-14}

The exact structures and configurations of the resulting micelles or aggregates are determined by the composition of the diblock polymer, the length of each polymer segment, the polarity of the solvent, and the relative solubility of each polymer block in the solvent. Depending on the relative block length, the system can form either of the two types of micelles: star or crew cut micelles. Star micelles are characterized by a small core and a large corona and forms when the solvents prefers the longer block whereas the crew cut micelles consist of large core and a thin corona and forms when the solvent prefers the shorter block.

The morphology is determined by the free energy balance among the chain stretching in the core, the core-corona interfacial energy and the repulsion between corona chains.\textsuperscript{15} By varying the corona size, the morphology change can be seen from spherical to cylindrical to bilayered vesicles. This change is because, if the corona size is reduced i.e. block length of the soluble component is shortened, it permits a larger aggregation number leading to larger core spheres resulting in large entropy penalty from stretching the chains in the core domain. Hence the micelles adopt a cylindrical form to satisfy the interfacial curvature requirement and on further reduction in corona size, it changes to bilayer structure.\textsuperscript{16}

Although the nanoscale micellar templates of diblock copolymer films have been used as seed sites for inorganic nanoparticle or nanowire growth as well as nanocarriers for drug molecules in the past,\textsuperscript{17-23} their applications as nanoscale arrayed biotemplates have never been demonstrated before. Yet, these amphiphilic diblock copolymers can
serve as extremely useful guides in organizing biomolecules into two-dimensional arrays since they exhibit a rich spectrum of morphologies and their repeat spacings are tunable in two dimensions.

1.1.2 Zinc oxide micro/nanostructures

Various structures of zinc oxide (ZnO), including thin films and micro/nano structures, have received considerable attention due to their attractive properties, which include a wide band gap of 3.37 eV and a large exciton binding energy of 60 meV at room temperature. The synthesis and assembly of various ZnO micro/nanostructures have been extensively studied using gas-phase or solution-based approaches. The most commonly used gas-phase approaches to synthesize ZnO structures at the nanometer and micrometer scale are carried out via growth methods such as physical vapor deposition, chemical vapor deposition, pulsed laser deposition and epitaxial electrodeposition. In solution based synthesis approaches, methods such as hydrothermal decomposition processes and homogenous precipitation of ZnO in aqueous solutions were pursued.

Depending on specific growth conditions, ZnO exhibits a variety of nano- and micro-structures resembling combs, rings, helixes/springs, bows, belts, wires, rods, propellers and cages. Such variations in morphology are governed by factors such as substrates, carrier gases, local gas concentrations, temperature and time.

ZnO can serve as an ideal candidate material for a broad range of high technology application such as short-wavelength light emitting, field emitting, luminescence, UV lasing, photovoltaic and piezoelectric materials. The use of ZnO has also been demonstrated as surface acoustic waveguides, transistors and sensors. Nanometer
scale ZnO has very good potential for aiding optical detection of target bioconstituents since ZnO nanomaterials are stable in typical biomolecular detection environments, have attractive optical properties, and can be easily processed through many synthetic routes. Despite its demonstrated functions in broad areas and suitability for advanced optical detection, biosensing applications of wide bandgap ZnO have not yet been realized.

1.1.3 Magnetotactic Bacteria

Discovered by Blakemore, magnetotactic bacteria are heterogeneous group of prokaryotes which are omnipresent in aquatic environments. The bacterium contains unique intracellular structures, known as magnetosomes, consisting of magnetite (iron oxide) crystals enclosed within membrane vesicles. The bacterium uses these magnetosomes to orient themselves and swim along the lines of earth magnetic field. These intracellular magnetosomes crystals are characterized by narrow size distributions, typically 35-120 nm, which is within the permanent, single-magnetic-domain size range for magnetite.\(^\text{41, 42}\) The formation of magnetosomes is achieved by biological mechanism that controls the accumulation of iron and the biomineralization\(^\text{43}\) of magnetite crystal with a characteristic size and morphology within membrane vesicles. Since the magnetite produced in this way—biogenic magnetite, has many distinguishing characteristics like single domain size, chemical purity, crystallographic perfection, unusual morphology and elongation, many commercial uses of bacterial magnetosomes are suggested including the manufacture of magnetic tape and printing inks, spintronics, magnetic memory and biology.\(^\text{44}\) However the use of this bacterium as a source of catalyst particles, whose
location and orientation can be controlled using a magnetic field, to grow nanostructures like nanotubes and nanowires have not been shown till date.

1.1.4 Protein Arrays

Protein array or controlled positioning of proteins and other biomolecules to surfaces is critical to basic biological research involving cell biology\textsuperscript{45, 46} as well as to a variety of applications including high-throughput proteomic arrays and combinatorial library screening.\textsuperscript{47-51} Directed protein adsorption onto surfaces is also essential to biosensor development for disease diagnostics and drug discovery as these applications require regulated immobilization of enzymes and antibodies at particular surface interfaces.\textsuperscript{47, 52-56} For these reasons, a variety of methods for protein localization have been previously explored involving ink-jet and pipet deposition,\textsuperscript{47, 57, 58} soft lithography,\textsuperscript{45, 59, 60} dip-pen lithography and related scanning probe patterning methods,\textsuperscript{56, 61-63} focused-ion-beam patterning,\textsuperscript{64} imprint lithography,\textsuperscript{65} and microfluidic channel networks.\textsuperscript{66-68}

Despite the demonstrated utility of these approaches, the development of miniaturized protein arrays is still facing difficulties associated with the fabrication of regularly-spaced protein platforms which display high areal density and natural protein conformation. The micrometer-scale resolution, typically achieved by the previous approaches utilizing ink-jet and pipet deposition, soft and imprint lithography, and microfluidic channel networks, can often limit address density of proteins. Array production using an alternative method such as scanning probe-related lithographic techniques has the advantage of nanometer-scale spatial resolution but the practical
application of these methods at large scale can be hampered by their time-consuming production. Therefore, novel protein patterning techniques are highly warranted to meet demands for the escalating degree of miniaturization in surface-based biotechnologies and facilitate rapid, high-throughput, protein screening and immunoassays requiring smaller volume of sample usage. In addition, the development of protein arrays in nanoscopic dimensions can facilitate new screening techniques and improve fundamental understanding on biomolecular recognition.  

1.1.5 Biomolecular Fluorescence Detection

Proteins are the key components of the cellular machinery responsible for the processing of detailed biological functions decoded from genetic information. The rapid pace in discovery of new gene products by large-scale genomics demands significant improvements in current technology pertaining to quantitative and functional proteomics. Specifically, the design of alternative strategies for detecting and analyzing protein functions via novel high-throughput approaches is highly warranted in this information-rich age of whole genome biology. Biomolecular fluorescence is the most widely used detection mechanism in both laboratory-scale and high-throughput proteomics research, as evidenced by its use in essential techniques such as enzyme-linked immunosorbent assay, fluorescent gel staining, and protein arrays.

So far, the most recognizable contribution of nanoscience to the field of biomolecular fluorescence detection has been made mainly in the areas of developing new fluorescent probes. A well-known example of this contribution is the design of semiconductor nanocrystals and quantum dots whose emission spectrum at specific
wavelength can be tuned by simply changing the size of the nanomaterials. Continuing research efforts in these fields have led to improved fluorophores that are less subject to photo bleaching while displaying high quantum yield.\textsuperscript{71-78}

An alternative step forward to promoting the study of proteins may be achieved by innovative assembly and fabrication of nanomaterials for use as advanced biosensor substrates in biomolecular fluorescence detection. As-grown nanomaterials have not yet been demonstrated as potentially suitable substrates for improved fluorescence detection when used in conjunction with target biomolecules. In order to design such substrates comprised of nanomaterials, four key characteristics of the candidate nanoscale materials should be carefully considered; i) signal enhancement, ii) ease of fabrication, iii) stability and iv) surface chemistry. Candidate nanomaterials should exhibit an appropriate optical property to foster the fluorescence signal from fluorophore-linked biomolecules in order to promote detection at low concentrations even at an ultratrace level. In addition, simple and straightforward synthesis and assembly routes should yield the successful growth and fabrication of these nanomaterials in order to facilitate high-throughput screening of interacting proteins. These nanomaterials should be biocompatible and chemically inert at detection environments involving most protein-protein interactions. Lastly, chemical reactions applicable to derivatize the surfaces of nanomaterials covalently should be widely available in order to link specific protein molecules on nanomaterials and maximize specificity of protein detection.
1.2 Thesis Overview

The following chapters introduced in this thesis are focused in the development of novel substrates for advanced biomolecular detection. Part 1 (Ch 2-5) of this thesis is focused on the fabrication of fully functional and active one and two dimensional protein nanoarrays using diblock copolymers thin film templates while part 2 (Ch 6-8) of this thesis is focused on the synthesis and application of ZnO nanorods towards highly sensitive biomolecular fluorescence detection. While complementary, these approaches are not necessarily interlinked.

Part 1:

The controlled positioning of proteins and other biomolecules to surfaces is critical to basic biological research involving cell biology as well as to a variety of applications including high-throughput proteomic arrays and combinatorial library screening. Directed protein adsorption onto surfaces is also essential to biosensor development for disease diagnostics and drug discovery as these applications require regulated immobilization of enzymes and antibodies at particular surface interfaces. The development of miniaturized protein arrays is still facing difficulties associated with the fabrication of regularly-spaced protein platforms which display high areal density and natural protein conformation. This issue constitutes the core of chapter 2. Specifically, we used PS-b-PMMA diblock copolymer thin film templates to create nanoscale protein array in one dimension. We developed a straightforward and effective technique to immobilize various protein molecules such as bovine immunoglobulin G (IgG), fluorescein isothiocyanate (FITC) conjugated anti-bovine IgG, and protein G using the
microphase separated domains of PS-b-PMMA diblock copolymer ultrathin films. We demonstrate that these proteins selectively self-segregate themselves on the microdomain regions of a specific polymer component due to their preferential interactions with one of the two polymer segments.

In chapter 2 we have exploited a self-assembly property of half cylinder-forming block copolymer, namely, that these systems create arrays of parallel lines with a controllable repeat spacing in the direction perpendicular to the lines, to create one dimensional protein arrays. However, to create protein arrays widely applicable in basic biological research and biotechnology, control over additional degrees of freedom in the spatial arrangement of proteins on the nanometer scale, that is, two-dimensionally controlled periodicity, is highly warranted – the focus of chapter 3. Here, we demonstrate that PS-b-PVP can be effectively used for the self-assembly of surface-bound, two-dimensional nanoscale protein arrays where the periodicity of repeating protein patterns can be effectively controlled by the nearest-neighbor spacing in the underlying hexagonal array. We also establish a straightforward method to produce protein patterns of different geometries and sizes by successfully manipulating topological structures of underlying PS-b-PVP templates. Further, we report our findings on the activity and stability of various model proteins on these PS-b-PVP templates.

For selective and specific protein assays, surface immobilized proteins should be able to present easily accessible binding sites and maintain their specific activities. Critical factors such as protein address density and protein activity need to be carefully considered in developing improved protein arrays. In chapter 4 we evaluate the activity of the patterned proteins on diblock copolymer templates and determine their
effectiveness as highly selective and sensitive nanosensor platforms. We report the findings on the activity and stability of various proteins that were self-assembled onto the microphase-separated domains of PS-b-PMMA diblock copolymer ultrathin films. The focus of this chapter is to investigate the conformational integrity and biological activity of proteins self-assembled selectively onto the nanoscopic diblock copolymer templates. Specifically, we demonstrate that PS-b-PMMA-bound enzymes such as horseradish peroxidase (HRP) and mushroom tyrosinase (MT) are biologically active and maintain their specific catalytic functionality over a long period of time. We determine that our diblock-copolymer guided, nanoscale enzyme assembly method is effective in preserving a large degree of the enzymatic activity in free motion. Surface-bound enzymes retain approximately 85% of their free activity after selective adsorption onto the PS domains of the underlying PS-b-PMMA. Other proteins such as enhanced green fluorescent protein (eGFP) and immunoglobulin G (IgG) keep their structural integrity and binding activity even after their self-assembly on PS-b-PMMA.

High address density of proteins in an array is also very important as increased protein density will improve the signal to noise ratio of a given detection method. In addition, uniformity in density is equally critical since potential variations in protein density from array to array or from spot to spot can significantly hamper quantitative measurements and comparative analysis of protein assays. Therefore, chapter 5 is focused on the areas of high and uniform protein density. The density of proteins in an array is largely governed by the adsorption behavior of proteins to the surface of an underlying array material. In chapters 2 and 3 we have studied adsorption behavior of various proteins on diblock copolymer ultrathin films such as PS-b-PMMA and PS-b-PVP. We
reported that a variety of protein molecules selectively self-segregates onto one of the
two polymeric segments in both PS-b-PMMA and PS-b-PVP cases. When carrying out
these studies, we observed an intriguing phenomenon. Protein molecules have a tendency
to exhibit a close-packing behavior and arrange themselves in an organized, space-filling
manner at optimized protein concentrations. In this chapter, we systematically study
protein adsorption characteristics on different polymeric surfaces. Three different types
of polymeric templates were used in the study: asymmetric PS-b-PMMA, PS
homopolymer, and PS/PMMA blend. We compare protein density on a PS-b-PMMA
diblock copolymer surface to its density on a PS homopolymer surface. We also
investigate the nature of protein assembly on a PS/PMMA blend film in order to assess
the dependence of protein density on the distance from the interface between PS and
PMMA. When compared to the chemically homogeneous surface provided by PS
homopolymer film, we find that the chemically heterogeneous PS-b-PMMA film is much
more effective in high density and high payload protein assembly. The protein density on
the diblock copolymer is approximately three to four fold higher than that on the
homopolymer. This remarkable effect is further confirmed by enzymatic activity
measurements on the chemically homogeneous and heterogeneous polymeric surfaces.

Part 2:

Fluorescence detection of biomolecules is a heavily relied on technique in gene
profiling, proteomics, drug discovery, disease diagnostics, and environmental analysis.
For many biomolecular detection techniques exploiting fluorescence, enhancing detection
sensitivity and increasing the signal-to-noise ratio still remain as major challenges in
carrying out the much needed, system-wide study of proteins and population-level genetic screening. To improve the fluorescence detection capability and resolution, numerous research efforts have been made in parallel on three main aspects of biomolecular fluorescence detection: (1) molecular design of better fluorophores, (2) development of improved detection apparatus, and (3) engineering of advanced substrates. Focusing on the development of novel bioarray substrates suitable for fluorescence detection, part 2 of this thesis is devoted to the development and utilization of nanoscale ZnO platforms for use as attractive substrates in enhanced fluorescence detection of biomolecules such as proteins and DNA.

Chapter 6 is focused on the synthesis and assembly of various ZnO micro/nanostructures. In this chapter we describe a straightforward method to produce ZnO nanorods (NR) and microrods (MR) using a novel catalyst, *Magnetospirillum magnetotacticum* (MS-1). The MS-1 bacterium synthesizes intracellular, linear, single-domain magnetite nanoparticles through highly regulated biomineralization. We utilize the highly monodisperse iron oxide (Fe₃O₄) nanoparticles inside MS-1 as catalysts to grow ZnO nano- and micro-structures with high optical quality. MS-1 has been previously used as effective biocatalysts to grow multiwalled carbon nanotubes showing a narrow distribution in diameter.⁷⁹ Here, we demonstrate that MS-1 can serve as effective catalysts to produce atomic defect-free ZnO NR and MR structures with unparalleled optical properties which, in turn, can promote new opportunities in photonic-based applications on the nanometer and micrometer scale.

In chapter 7, using model protein and nucleic acid systems, we demonstrate that engineered nanoscale ZnO structures can significantly enhance the detection capability of
biomolecular fluorescence. Without any chemical or biological amplification processes, nanoscale ZnO platforms enabled increased fluorescence detection of these biomolecules when compared to other commonly used substrates such as glass, quartz, polymer, and silicon. The use of ZnO nanorods as fluorescence enhancing substrates in our biomolecular detection permitted sub-picomolar and attomolar detection sensitivity of proteins and DNA, respectively, when using a conventional fluorescence microscope. We also demonstrate the easy integration potential of zinc oxide nanorods into periodically patterned nanoplatfroms which, in turn, will promote the assembly and fabrication of these materials into multiplexed, high-throughput, optical sensor arrays.

In chapter 8, we shift our focus on the use of nanoscale ZnO structures in the identification of the biothreat agent, *Bacillus anthracis*, by successfully discriminating its DNA sequence from other genetically related species. We explore both covalent and non-covalent linking schemes in order to couple probe DNA strands to the ZnO nanostructures. Hybridization reactions are performed with various concentrations of target DNA strands whose sequence is unique to *Bacillus anthracis*. The use of ZnO nanomaterials greatly enhances the fluorescence signal collected after carrying out duplex formation reaction. Specifically, the covalent strategy allows detection of the target species at sample concentrations as low as a few femtomolar levels as compared to the detection sensitivity in tens of nanomolar range when using the non-covalent scheme. We also demonstrate the easy integration potential of nanoscale zinc oxide into high density arrays by using various types of ZnO sensor prototypes in the DNA sequence detection.
1.3 References


Figure 1.1: Mean field phase diagram
2.1 Background

The controlled positioning of proteins and other biomolecules to surfaces is critical to basic biological research involving cell biology\textsuperscript{1,2} as well as to a variety of applications including high-throughput proteomic arrays and combinatorial library screening.\textsuperscript{3-7} Directed protein adsorption onto surfaces is also essential to biosensor development for disease diagnostics and drug discovery as these applications require regulated immobilization of enzymes and antibodies at particular surface interfaces.\textsuperscript{3,8-12} For these reasons, a variety of methods for protein localization have been previously explored involving ink-jet and pipet deposition,\textsuperscript{3,13,14} soft lithography,\textsuperscript{1,15,16} dip-pen lithography and related scanning probe patterning methods,\textsuperscript{12,17-19} focused-ion-beam patterning,\textsuperscript{20} imprint lithography,\textsuperscript{21} and microfluidic channel networks.\textsuperscript{22-24}

Despite the demonstrated utility of these approaches, the development of miniaturized protein arrays is still facing difficulties associated with the fabrication of regularly-spaced protein platforms which display high areal density and natural protein conformation. The micrometer-scale resolution, typically achieved by the previous approaches utilizing ink-jet and pipet deposition, soft and imprint lithography, and microfluidic channel networks, can often limit address density of proteins. Array production using an alternative method such as scanning probe-related lithographic techniques has the advantage of nanometer-scale spatial resolution but the practical
application of these methods at large scale can be hampered by their time-consuming production. Therefore, novel protein patterning techniques are highly warranted to meet demands for the escalating degree of miniaturization in surface-based biotechnologies and facilitate rapid, high-throughput, protein screening and immunoassays requiring smaller volume of sample usage. In addition, the development of protein arrays in nanoscopic dimensions can facilitate new screening techniques and improve fundamental understanding on biomolecular recognition.\textsuperscript{18,25,26}

Here, we report a straightforward and effective technique to immobilize various protein molecules such as bovine immunoglobulin G (IgG), fluorescein isothiocyanate (FITC) conjugated anti-bovine IgG, and protein G using the microphase-separated domains of polystyrene-block-polymethylmethacrylate (PS-b-PMMA) diblock copolymer ultrathin films. We chose these protein molecules as model systems for their importance in biological research as well as biomedical applications such as purifying antibody molecules and detecting the presence of antigen-antibody complexes using enzyme-linked immunosorbent assays or Western blots. We demonstrate that these proteins selectively self-segregate themselves on the microdomain regions of a specific polymer component due to their preferential interactions with one of the two polymer segments. The surface-patterned protein molecules using our approach maintain their conformation and display their natural function. In this Letter, we present a rapid and convenient technique for controlled protein localization on surfaces at the nanometer level. Our new approach involving diblock copolymer thin films represents a step forward for facile, nanometer-spaced protein immobilization with high areal density that could provide a pathway to high-throughput proteomic arrays and biosensors.
The microphase separation behavior of PS-b-PMMA diblock copolymers is well-characterized and, therefore, can be precisely predicted. The two chemically distinct, and thus immiscible, polymer blocks in diblock copolymers undergo phase separation and self-assemble into ordered patterns of microdomains whose characteristic packing and size is determined by the polymer compositions and chain lengths, respectively. The self-organizing nature of the ultrathin films of the diblock copolymer under carefully balanced thermodynamic conditions was exploited to produce alternating polymeric domains of the two chemical compositions of the diblock at the air/polymer interface. The repeat spacing of such microdomains can be precisely controlled on the nanometer scale by varying, for example, the molecular weight of the diblock copolymer. In addition, the microdomains of PS-b-PMMA can be readily aligned using various methods including electric field, shear force, annuli formation, and substrate modification. Therefore, the self-assembling phase behavior of the diblock copolymer is highly predictable and controllable, making the material suitable as nanoscale templates. Unlike optical and scanning lithographic techniques used to attain spatial control of proteins on surfaces involving multiple and time-consuming fabrication steps, the use of diblock copolymers enables easy and rapid construction of nanometer-scaled templates via self-assembly. Such polymeric templates permit control of the spatial resolution of adsorbed proteins on the nanometer scale by site-selective adsorption of proteins onto template polymeric surfaces displaying a spatially-defined, chemical heterogeneity. When combined with previously reported methods of polymer alignment, this polymer-guided method can be extended to produce highly periodic and aligned patterns of proteins on large areas of substrates.
2.2 Experimental Details

Asymmetric PS-b-PMMA diblock copolymer with an average molecular weight of 71,400 Da was obtained from Polymer Source Inc. (Montreal, Canada). The diblock contained 71% of PS by weight with a polydispersity of 1.06. Silicon wafers (resistivity < 1 Ωcm, thickness: 0.017 inch) were obtained from Silicon Inc. (Boise, Idaho). Silicon substrates, 1 by 1 cm, were cleaned with ethanol, acetone, and toluene and spun dry before coating ultrathin polymer films. Polymeric templates with lying-down cylindrical microdomains were produced by spin coating 2% (w/v) PS-b-PMMA in toluene at 3500 rpm for 1 minute, followed by annealing in an argon atmosphere at 240 °C for 6 hours with a transient ramp-up rate of 5 K/min and a cooling rate of 2 K/min. Whole molecule bovine IgG, goat FITC-conjugated anti-bovine IgG, and protein G were purchased from VWR Scientific Inc. (West Chester, PA). The lyophilized powders of these protein molecules were diluted in a buffer (140 mM NaCl and 3 mM KCl in 10 mM PBS pH 7.4) to varying concentrations, ranging from 4 to 20 µg/mL. 100 µL of protein solution was deposited onto the microphase separated diblock polymer surfaces for 20 sec at room temperature. The sample surfaces were rinsed several times with the PBS solution, followed by a thorough rinsing with deionized water. The samples were then dried gently under a stream of nitrogen gas prior to atomic force microscope (AFM) imaging. AFM measurements were carried out using Digital Instruments, Dimensions 3100, in tapping mode at a scan speed of 1.5 Hz. Silicon tips with a resonant frequency of 60 kHz and a spring constant of 5 N/m were used in the measurements.
2.3 Results and Discussions

2.3.1 Microdomain formation in PS-b-PMMA thin film templates

At the polymer/silicon oxide interface, PMMA favors the silicon oxide substrate because of its lower wetting energy.\textsuperscript{29} At the polymer/air interface, the surface tension difference between PMMA and PS is small enough to produce the appearance of both components at the upper interface of certain film areas when the film is prepared to form ‘L-thick’ regions as described in the experimental procedures.\textsuperscript{29-31} ‘L-thick’ regions are film areas where the film height supported on a substrate is commensurate with the characteristic long period of PS-b-PMMA. The PS-b-PMMA diblock copolymer in such areas forms one layer of lying-down cylindrical microdomains parallel to the substrate. Upon thermal annealing in an Ar atmosphere, microphase separation yielded microdomains with the average cylinder repeat spacing on the nanometer scale. AFM topographical measurements of these films identified that PMMA is higher than PS by 1 nm.\textsuperscript{29-32} The repeat spacing of the microdomains of the diblock copolymer used in our experiments is 45 nm where L corresponds to 39 nm. Figure 2.1 shows topography and phase AFM images of PS-b-PMMA microdomains formed after annealing the ultrathin block copolymer films at 240 °C for 6 hours under Ar environment. Bright and dark domains in the topography and the corresponding phase images in Figure 2.1 represent PMMA- and PS- rich regions, respectively. These periodically-spaced polymeric domains served as nanometer-scaled, chemically-heterogeneous templates for subsequent protein adsorption.
2.3.2 Protein deposition onto the PS-b-PMMA thin film templates

Protein deposition onto these spatially-defined, chemically-distinctive, polymeric templates was investigated by AFM using topography and phase imaging modes. Deposition of 4 µg/mL of IgG in PBS buffer resulted in selective segregation of these protein molecules into PS microdomains only. This phenomenon is displayed in the phase images of Figures 2.2(A) and (B) where these phase images clearly resolve individual IgG molecules of 15 nm in diameter localized exclusively to PS regions. When a higher concentration of IgG (20 µg/mL) was applied to the polymeric templates, IgG molecules filled in the PS domains completely and formed nanochains of individual IgG molecules while faithfully following the underlying patterns of PS microdomains. The accumulation of IgG molecules in the PS regions leads to the inversion of AFM topographic contrast: Bright, and therefore higher, areas in Figure 2.2(C) correspond to IgG molecules assembled on PS domains which were initially 1 nm lower in height than the neighboring PMMA domains. Figure 2.2(E) displays a height analysis along the line indicated in Figure 2.2(C). IgG molecules on PS domains exhibit an average height of 3.5 nm greater than the neighboring PMMA domain regions. Within the concentration range of IgG molecules in PBS buffer which was used in the experiments, no accumulation of IgG molecules beyond a monolayer has been monitored on PS domains. The width of the underlying PS domains is approximately commensurate with two IgG molecules assembled side by side. At the monolayer-forming IgG concentration of 20 µg/mL, a maximum of two IgG molecules was observed on the PS domains along the direction of the repeat spacing, see the inserted image in Figure 2.2(C). The selective protein localization on the PS domains was persistent over large areas of sample substrates. This
new approach signifies a facile and convenient means to immobilize effectively IgG protein molecules on substrates in a highly periodic manner, while achieving spatial precision at the nanometer level.

In order to evaluate the broad applicability of diblock copolymer microdomains to protein patterning, other protein molecules were also deposited on the microphase-separated diblock copolymer templates and carefully monitored with AFM. Figure 2.3 summarizes AFM results from patterning protein G (A through D) and FITC anti-bovine IgG (E and F) using these polymeric templates. Similar to the outcome monitored from IgG deposition, both protein G and FITC anti-bovine IgG molecules also interact preferentially with PS domains. This preferential interaction constrains the adsorption of these protein molecules only to PS regions as displayed in the phase images of Figures 2.3(B), (D), and (F). The corresponding topography contrasts shown in Figures 2.3(A), (C), and (E) confirm that the assembled protein molecules closely mimic nanometer-spaced patterns of the underlying PS domains. Figure 2.3 (A) is the topography image of protein G molecules formed on PS domains after 4 µg/mL protein was applied. When a higher concentration of 20 µg/mL was used, protein G molecules configured into film-like structures covering the entire PS domains as shown in the topography image of Figure 2.3(C). The magnified image of a 150 by 150 nm region, inserted in Figure 2.3(C), shows the detailed structures of assembled protein G molecules on PS domains. These film-like arrangements of protein G molecules were ~1.6 nm taller than the neighboring PMMA domains, as displayed in the height profile taken along the white line marked in Figure 2.3(C). The topography and phase AFM images in Figures 2.3 (E) and (F) were obtained after 20 µg/mL of FITC anti-bovine IgG was applied to the diblock copolymer
film. The height difference between the assembled molecules of FITC anti-bovine IgG on PS domains above neighboring PMMA domains ranged from 2.5 to 3 nm, as measured in Figure 2.3(H).

For all protein molecules under investigation, AFM line measurements of the height difference between PS and PMMA domains after protein adsorption yielded much lower values than expected from known diameters of these proteins. We believe that this effect is induced by AFM tip-sample interactions as compliant biological samples were probed intermittently under our imaging conditions. Similar effects to our observed height reduction have been previously reported in tapping mode AFM imaging studies of soft biological molecules such as proteins and DNA.\textsuperscript{39,40}

The exact driving force for the selective segregation of these proteins into PS domains needs further investigation. However, earlier studies have reported that hydrophobic surfaces adsorb larger amounts of various proteins than hydrophilic surfaces.\textsuperscript{41-43} As PS is more hydrophobic than PMMA, we hypothesize that hydrophobic interactions govern the preferential segregation of the proteins used in our experiments into PS domains. As a control, ultrathin polymeric films consisting of either PS or PMMA homopolymers on silicon oxide were used as substrates for adsorption of IgG, protein G, and FITC anti-bovine IgG molecules. When investigated by AFM, a significant amount of adsorbed protein was detectable only on PS films whereas no protein absorption on PMMA films was traceable under the deposition and rinsing conditions used in our experiments.
2.4 Conclusions

Here, we have demonstrated a novel method for immobilizing protein molecules on surfaces using microphase-separated PS-b-PMMA diblock copolymers. Bovine IgG, protein G, and FITC anti-bovine IgG molecules were used as model proteins. The use of polymeric templates under carefully balanced thermodynamic conditions enables convenient and effective protein immobilization on surfaces with spatial control on the nanometer scale. Our method exploits the well-characterized, self-assembling nature of the diblock copolymers in the assembly of nanometer-sized guides with chemical heterogeneity on substrates. The model proteins selectively adsorb and self-organize on the PS microdomain regions by faithfully following the underlying, nanometer-spaced PS microdomains, due to their preferential interactions with PS over PMMA. This diblock copolymer-based approach represents a step forward for facile, nanometer-spaced protein localization with high areal density, and has the potential to impact high-throughput proteomic arrays and biosensors.

2.5 References


Figure 2.1: AFM images showing the microphase-separated ultrathin films of PS-b-PMMA diblock copolymer. Bright and dark domains in the topography (A) and phase (B) images represent PMMA- and PS-rich regions, respectively. The average repeat spacing of the microdomains is 45 nm.
Figure 2.2: AFM images of 4 µg/mL (A and B) and 20 µg/mL (C and D) IgG molecules deposited selectively on PS domains in the microphase-separated PS-b-PMMA ultrathin films spun on silicon oxide substrates. The scan size and mode of the four panels correspond to (A) 2 by 2 µm phase, (B) 1 by 1 µm phase, (C) 1 by 1 µm topography, and (D) 500 by 500 nm phase AFM images. The selective segregation of IgG molecules on PS domains is clearly visible in the inset of a 150 by 150 nm phase image in (B). The inset in (C), a 100 by 100 nm topography image, shows the packing of individual IgG molecules on the PS domains in detail. (E) Line analysis taken along the white line indicated in (C).
Figure 2.3: AFM images of protein G and FITC anti-bovine IgG molecules assembled selectively on PS domains in the microphase separated PS-b-PMMA ultrathin films on silicon oxide substrates. (A and B) 1 by 1 µm topography and phase images respectively taken after deposition of 4 µg/mL protein G. (C and D) 1 by 1 µm topography and phase images respectively obtained after deposition of 20 µg/mL protein G. (E and F) 1 by 1 µm topography and 500 by 500 nm phase images respectively taken after deposition of 20 µg/mL FITC anti-bovine IgG. The zoomed-in images of protein molecules on PS domains are inserted in (C) and (E) in order to clearly show the structures formed by protein G and FITC anti-bovine IgG, respectively. The image sizes of the insets in (C) and (E) are 150 by 150 nm and 200 by 200 nm, respectively. (G and H) Height profiles measured along the white lines marked in (C) and (E), respectively.
CHAPTER 3
Two-Dimensionally Self-Arranged Protein Nanoarrays on Diblock Copolymer Templates

3.1 Background

Protein arrays are utilized as active supports for a variety of diagnostic assays in tissue engineering, pharmacology, and proteomics. High-density protein arrays, consisting of active and surface-immobilized protein molecules in a periodic manner, can greatly benefit these applications. Ideal protein sensors should be capable of delivering efficient, sensitive, parallel, and automated analyses that can be applied to large numbers of samples with greatly reduced volume of sample and reagent usage. Such biosensor platforms can, in turn, facilitate rapid, high-throughput screening and immunoassays by enabling an escalated degree of miniaturization in surface-based biotechnologies. Successful development of these protein arrays would require a straightforward method to immobilize proteins on a solid surface with a precise and nanoscale spatial control. However, inherent difficulties associated with positioning these small protein molecules have restricted to date both the study and application of nanoscaled protein arrays. To this end, reliable placement of protein molecules in well-defined nanoscale patterns while retaining their native functionality is essential for overcoming the current limitations of protein arrays. Thus, a novel method that can conveniently and rapidly organize proteins into high-density arrays with nanoscale resolution is highly warranted.
A variety of methods for protein localization have been previously explored. Surface deposition techniques for proteins include ink-jet and pipette deposition, soft lithography, dip-pen lithography and related scanning probe patterning methods, focused-ion-beam patterning, imprint lithography, and microfluidic channel networks. As a step forward toward overcoming limitations associated with these techniques such as low protein address density and slow assembly speed, we have previously developed nanoscale protein arrays by exploiting the self-organizing nature of polystyrene-block-poly(methyl methacrylate) (PS-b-PMMA) diblock copolymer templates and the self-segregating tendency of proteins to preferential PS domains. In this previous work, nanometer-scale, surface-bound protein patterns were instantly achieved via self-assembly where nanoscale spatial control of proteins was achieved without undergoing a series of time-consuming fabrication or modification processes. This previous study exploited a self-assembly property of half cylinder-forming block copolymer, namely, that these systems create arrays of parallel lines with a controllable repeat spacing in the direction perpendicular to the lines. However, to create protein arrays widely applicable in basic biological research and biotechnology, control over additional degrees of freedom in the spatial arrangement of proteins on the nanometer scale, that is, two-dimensionally controlled periodicity is highly warranted.

In this paper, we turn to the rich morphology of amphiphilic diblock copolymers to achieve rapid, large-area self-assembly of two-dimensionally controlled protein arrays with a periodic repeat spacing in nanoscopic dimensions. Micellar assembly of amphiphilic diblock copolymers above a critical polymer concentration is a well-known behavior. Amphiphilic polymeric systems such as polystyrene-b-poly(acrylic acid),
poly(ethylene propylene)-b-poly(ethylene oxide), polystyrene-b-poly(2-vinylpyridine), and polystyrene-b-poly(4-vinylpyridine) were extensively studied to understand their fascinating micellar properties and dependence on diblock copolymer characteristics.\textsuperscript{31-34}

The exact structures and configurations of the resulting micelles or aggregates are determined by the composition of the diblock polymer, the length of each polymer segment, the polarity of the solvent, and the relative solubility of each polymer block in the solvent. Although the nanoscale micellar templates of diblock copolymer films have been used as seed sites for inorganic nanoparticle or nanowire growth as well as nanocarriers for drug molecules in the past,\textsuperscript{35-44} their applications as nanoscale arrayed biotemplates have never been demonstrated before. Yet, these amphiphilic diblock copolymers can serve as extremely useful guides in organizing biomolecules into two-dimensional arrays since they exhibit a rich spectrum of morphologies and their repeat spacing are tunable in two dimensions.

Herein, we demonstrate that polystyrene-b-poly(4-vinylpyridine) (PS-b-PVP) can be effectively used for the self-assembly of surface-bound, two-dimensional nanoscale protein arrays where the periodicity of repeating protein patterns can be effectively controlled by the nearest-neighbor spacing in the underlying hexagonal array. We also establish a straightforward method to produce protein patterns of different geometries and sizes by successfully manipulating topological structures of underlying PS-b-PVP templates. Further, we report our findings on the activity and stability of various model proteins on these PS-b-PVP templates. The model proteins chosen in our study include bovine immunoglobulin G (IgG), mushroom tyrosinase (MT), and horseradish peroxidase (HRP). These proteins are commonly used not only in biological research but also in
biomedical applications where their flexibility of usage can range widely from purifying antibody molecules, to detecting antigen-antibody complexes, and to investigating enzymatic activity. Our polymer-guided self-assembly approach enables facile, two-dimensional control of nanoscale protein patterns. Equally important, our new approach leads to protein arrays that maintain their biological activity and specific functionality over a long period of time. Such advantages of our polymer-based method are crucial in creating much needed, high-density, functional protein arrays which, in turn, can be extremely beneficial for improved basic biological assays and high-throughput biomedical tests.

### 3.2 Experimental Details

Asymmetric PS-b-PVP diblock copolymer with an average molecular weight of 68,500 Da was obtained from Polymer Source Inc. (Montreal, Canada). The diblock contained 70% of PS by weight with a polydispersity of 1.14. Upon dissolution of 0.5% (w/v) PS-b-PVP in toluene, micelles consisting of a PVP core and a PS corona readily formed, as toluene is a preferential solvent for PS. This micellar solution was then spun on silicon substrates at 3500 rpm for 1 min. Silicon substrates, obtained from Silicon Inc. (Boise, Idaho), were cleaned with ethanol, acetone, and toluene and spun dry before coating ultrathin PS-b-PVP films. Atomic force microscopy (AFM) measurements were carried out using Digital Instruments Multimode, Nanoscope IIIa, in tapping mode at a scan speed of 1 Hz. Silicon tips with a resonant frequency of 60 kHz and a spring constant of 5 N/m were used in our measurements.
Whole molecule bovine IgG was purchased from VWR Scientific Inc. (West Chester, PA), and mushroom tyrosinase was obtained from Sigma-Aldrich (St. Louis, MO). Lyophilized powder of these protein molecules were reconstituted in a PBS buffer (10 mM mixture of Na$_2$HPO$_4$ and NaH$_2$PO$_4$, 140 mM NaCl, 3 mM KCl, pH 7.4) to varying concentrations. A total of 100 µL of desired protein solution was then deposited onto the ultrathin PS-b-PVP micelle films for 20 s at room temperature. The sample surface was then thoroughly rinsed with PBS buffer followed by gentle blow-drying under a stream of nitrogen gas.

HRP and 3, 3’, 5, 5’-tetramethylbenzidine (TMB) solution containing 1.25 mM TMB and 2.21 mM H$_2$O$_2$ were purchased from VWR Scientific Inc. (West Chester, PA). The lyophilized powder of HRP was reconstituted in deionized water to a final concentration of 10 µg/mL. An amount of 100 µL of the enzyme solution was deposited onto the original PS-b-PVP templates for 20 s at room temperature. The sample surfaces were thoroughly rinsed with deionized water. Visualization of HRP activity was carried out in the presence of TMB. A digital camera, Sony Cybershot DSC-P92, was used to capture the assay color changes. UV-vis spectra were recorded on a Hewlett-Packard 8452A Diode Array spectrophotometer.

3.3 Results and Discussions

3.3.1 Fabrication and characterization of two dimensional PS-b-PVP templates

To demonstrate that diverse surface PS-b-PVP geometries can be easily self-assembled on the nanoscopic scale and subsequently employed to construct nanoscale protein arrays with spatial control in two dimensions, we first describe the simple and
robust methods which we used to create an assortment of PS-b-PVP micellar templates. The protein assembly demonstrated in this paper utilizes the three PS-b-PVP micellar thin film templates, illustrated schematically in Figure 3.1. We categorize the two-dimensionally periodic nanoscale PS-b-PVP templates as (i) original, (ii) open, and (iii) reverted PS-b-PVP micelles.

Upon dissolution of 0.5% (w/v) PS-b-PVP in toluene, micelles consisting of a PVP core and a PS corona readily formed, as toluene is a preferential solvent for PS. Figure 3.2A displays topographic AFM images of these PS-b-PVP micelles on a silicon substrate. The topographical AFM images clearly show a two-dimensional array of PS-b-PVP micelles that are hexagonally packed. We refer to these spherical PS-b-PVP micelles as “original” PS-b-PVP templates. Individual micelles in the original template are approximately 50 nm in diameter. The height difference between the lower region surrounding the micelles and the top of the micelles is measured as ~7-8 nm. The core and corona of these micelles can be partially opened by exposing the original PS-b-PVP templates on silicon substrates to a solvent favoring PVP such as ethanol. Micelles with partially open PS coronae exposing their PVP cores were produced by introducing an ethanol vapor to the original template for 15 min. The resulting “open” PS-b-PVP templates are shown in the topographic AFM images in Figure 3.2B. The elevations in Figure 3.2A turned into depressions in Figure 3.2B as the exposure of the original PS-b-PVP template to ethanol vapor leads to rearrangements of the PS chains at the air/polymer interface, partially uncovering the core PVP chains of the original micelles, as illustrated in Figure 3.1B. The array of PVP depressions that are surrounded by taller PS chains persisted over a large area of the substrate. Each hole, seen as dark depression
spots in Figure 3.2B, in the hexagonally packed open PS-b-PVP template measures approximately 37 nm in diameter and ~8-9 nm in depth. Upon further treatment of the open PS-b-PVP micelles with a toluene vapor for 15 s, these open templates were switched to yet another type of micellar morphology shown in the topographic AFM images of Figure 3.2C. The hexagonally packed micelles in the resulting “reverted” PS-b-PVP template resemble the original template. To minimize the unfavorable interaction between PVP and toluene, the partially exposed PVP cores are recovered by PS in the reverted template. However, the micelles in the reverted PS-b-PVP are larger in width and shorter in height than the original micelle, where individual micelles in the reverted template measure approximately 60 nm in diameter and ~3-4 nm in height. The center of the reverted micelle is slightly lower in height than its surrounding, as shown in the height profile along a reverted micelle in Figure 3.2C. This observation may possibly be explained by incomplete refolding of PS chains to enclose the PVP core.

3.3.2 Protein adsorption onto the PS-b-PVP thin film templates

AFM data shown in Figure 3.3 correspond to typical topography images of (A and B) IgG and (C) MT deposited on various PS-b-PVP templates. Figure 3.3A clearly demonstrates that IgG molecules adsorb preferentially on the PS-rich regions of the open PS-b-PVP template when a 20 µg/mL solution of IgG molecules is deposited on the PS-b-PVP template as shown in Figure 3.2B. As PS chains occupy the neighboring regions between holes in the open PS-b-PVP template, individual IgG molecules assemble in the perimeter areas of holes as seen in the zoomed-in image of Figure 3.3A-4. Since PS regions of the reverted PS-b-PVP space-fill around the hexagonally arrayed PVP cores,
IgG molecules form structures surrounding the hexagonal spaces. Due to the adsorption of IgG molecules onto PS domains of the open template, the height difference between the center of the hole and the IgG-bound region is ~13-14 nm. Figure 3.3B displays AFM images of IgG molecules self-assembled on reverted PS-b-PVP templates. A single layer of IgG molecules cover PS-rich regions in the reverted templates at a concentration of 10 µg/mL where typically three IgG molecules assemble into close-packed structures on top of the refolded PS chains, as shown in the zoomed-in image of Figure 3.3B-4. The onset of the close-packing individual protein molecules on PS domains is seen on the sample prepared with 4 µg/mL protein concentration; see Figure 3.3B-2. AFM data in Figure 3.3C show self-assembly of MT molecules on the reverted PS-b-PVP templates. Similar to IgG molecules, they selectively segregate onto PS-rich areas of the underlying template. Individual protein molecules are clearly resolved on the micellar surface of the reverted PS-b-PVP templates in parts C-2 and C-3 of Figure 3.3 which were prepared at MT concentrations of 4 and 20 µg/mL, respectively. The compact packing nature of MT, arrangement of protein molecules on PS domains, and the number of protein molecules occupying the surface of a single micelle are very similar to that of IgG as they have comparable molecular weights.

For all protein molecules under investigation, AFM line measurements of the height difference between bare polymer templates and adsorbed proteins on these polymer templates yielded much lower values than expected from known diameters of these proteins. This effect is induced by AFM tip-sample interactions as compliant biological samples were probed intermittently under our tapping mode imaging conditions. Similar effects to our observed height reduction have been previously
reported in tapping mode AFM imaging studies of soft biological molecules such as proteins and DNA.\textsuperscript{46,47}

### 3.3.3 Biological activity assessment

To assess biological activity of the two-dimensional protein nanoarrays prepared using PS-b-PVP templates, we carried out an enzymatic activity measurement of HRP molecules. HRP molecules were first immobilized on the original PS-b-PVP micellar template. The enzymatic activity of these surface-bound HRP molecules was subsequently monitored over a long period of time. HRP is a widely used, highly specific enzyme that catalyzes reduction of hydrogen peroxide at a high turnover rate in a short period of time.\textsuperscript{48} Visualization of HRP activity was carried out in the presence of TMB. This chemical process transfers electrons from TMB to peroxidase, changing the solution color from colorless to blue.

Figure 3.4 summarizes the results of our HRP activity test on original PS-b-PVP templates. When PS-b-PVP micellar templates were exposed to the TMB assay solution without any surface-bound HRP as a control, no color change was observed, as shown in Figure 3.4A-1. On the contrary, when the TMB assay solution was added to PS-b-PVP micellar templates containing HRP molecules, the color of the solution rapidly turned from colorless to blue; see Figure 3.4B-1. The density of the enzyme molecules bound on the original PS-b-PVP templates was $\sim 3 \times 10^{10}$ molecules/cm$^2$ when they were adsorbed from a 10 µg/mL HRP solution. The AFM images in parts A-2 and B-2 of Figure 3.4 clearly display the surface morphology of the micellar templates used in our activity test without and with HRP molecules, respectively. As shown in Figure 3.4B-2,
HRP molecules organize themselves on top of the underlying, hexagonally packed PS-b-PVP micelles which, in turn, yields periodic, two-dimensional nanoscale protein arrays. HRP molecules occupying the top sites of the original PS-b-PVP micellar template are higher than the original micelles by ~2.5-3 nm. Figure 3.4C displays UV-vis spectra of the two assay solutions prepared for the HRP enzymatic activity test as shown in parts A-1 and B-1 of Figure 3.4; no absorption peaks were observed in the absence of HRP whereas, in the presence of HRP, characteristic absorption peaks corresponding to oxidized TMB were monitored. The enzyme molecules of HRP on PS-b-PVP templates remained active for reduction of hydrogen peroxide even after 100 days when kept at 4 °C.

We also monitored differences in HRP activity between free vs PS-b-PVP micelle-bound HRP molecules by performing time-dependent UV-vis measurements. Two samples containing comparable amounts of the enzyme molecules, approximately $7.5 \times 10^9$ molecules, were used for this comparison experiment. In one sample, HRP molecules were prepared in a solution whereas, in the other sample, the enzyme molecules were bound on a PS-b-PVP template. UV-vis absorbance was then monitored over time, and the absorption maximum at 650 nm was recorded as a function of time; see Figure 3.4D. Data from the free HRP sample are shown in blue and those from the bound HRP on PS-b-PVP micelles are shown in red. When compared with the activity of unbound HRP molecules in solution, HRP molecules bound on PS-b-PVP retained 78% of the activity measured in solution. These results indicate that much of the enzyme molecules indeed maintain their natural conformation and activity when they are effectively immobilized on the underlying PS-b-PVP templates via self-assembly.
Our data also suggest that amphiphilic diblock copolymer templates permit control of the spatial resolution of adsorbed proteins on the nanometer scale by site-selective adsorption of proteins onto template surfaces displaying a spatially defined, chemical heterogeneity. When their rich, extensively characterized, and precisely controlled surface morphology is exploited, these polymeric surfaces can serve very efficiently as two-dimensional nanoscale guides for spontaneously constructed protein arrays that feature high areal density, stable protein conformation, and no loss of protein activity. We envision that immediate applications of our protein nanoarrays will involve the use of currently available microsample handling and detection apparatus, where a group of multiple micelles will serve as a single, independently addressable unit in a given protein array. However, ideal application of our protein nanoarrays would be able to address each micelle as an independent unit on the nanoscale where a single protein array would contain a large number of different proteins to be examined. Therefore, to take full advantage of our diblock copolymer-based nanoscale protein arrays, conventionally available methods of sample delivery as well as detection systems need to be improved to provide a nanoscopic spatial resolution.

3.4 Conclusions

The use of amphiphilic PS-b-PVP polymeric templates under carefully balanced thermodynamic conditions enables convenient and effective protein immobilization on surfaces with two-dimensional spatial control on the nanometer scale. We have demonstrated that the structural variety and chemical heterogeneity of PS-b-PVP template surfaces can be successfully exploited to enable spontaneous formation of self-
assembled, hexagonally ordered protein arrays. These protein arrays were rapidly created over a large area on the substrate without the use of external fields. Protein molecules self-assembled exclusively to the PS domains with a nanoscale periodicity. More importantly, protein molecules on the PS-b-PVP templates maintained their natural conformation and activity for several months. Our methods involving amphiphilic diblock copolymers to create highly periodic, high-density, surface-bound protein patterns also have the unique capability of controlling the exact repeat spacings of protein nanoarrays two-dimensionally by tuning the size of the underlying PS-b-PVP micellar templates. This additional advantage makes our platforms highly suitable as functional sensor substrates or protein arrays which demand precise spatial arrangements of protein molecules in multiple directions. Therefore, our novel two-dimensional protein assembly method will be greatly beneficial for high-throughput proteomic assays and multiplexed biosensing applications.

3.5 References


Figure 3.1: Schematic cartoons illustrating the top and side views of various polymeric templates: (A) original, (B) partially open, and (C) reverted two-dimensional PS-b-PVP micelles on silicon substrates. These templates were subsequently used for nanoscale self-assembly of two-dimensional protein arrays.
Figure 3.2: AFM topology of various self-assembled nanoscale PS-b-PVP templates prepared by various solvent exposures. (A) (1 and 2) 300 × 300 and 180 × 180 nm AFM images showing the “original” hexagonally packed PS-b-PVP micellar film that was spun cast in toluene on a silicon substrate. (3) Height profile along the indicated white line of the original micelle shown in (2). (B) (1 and 2) 300 × 300 and 180 × 180 nm AFM images displaying surface morphology changes of the original PS-b-PVP templates into open micelles due to ethanol exposure. (3) Height profile measured along the open micelle as indicated with the inserted white line in (2). PS chains of the original micelle at the polymer/air interface spread open away from the micelle center and exposing the low-lying PVP core shown as darker circles in the AFM images. (C) (1 and 2) 300 × 300 and 180 × 180 nm AFM images revealing reverted micellar templates upon further toluene vapor treatment of samples shown in B. (3) Topographical profile analyzed along the inserted line on the reverted micelle in (2). Hexagonally packed nanoscale micelles resembling the original PS-b-PVP templates were formed spontaneously over a large area.
Figure 3.3: Schematic illustrations and AFM topography images of IgG and MT molecules on PS-b-PVP templates. (A) IgG molecules deposited on open PS-b-PVP templates from a 20 µg/mL solution. The presented AFM images are (2) 300 × 300 nm, (3) 180 × 180 nm, and (4) 60 × 60 nm in size. On open PS-b-PVP micellar templates, IgG molecules occupy preferentially the PS-rich areas which encircle the hexagonally packed, low-lying PVP cores. (B) IgG molecules assembled on reverted PS-b-PVP templates when (2) 4 µg/mL and (3 and 4) 10 µg/mL of IgG solution was added to the templates. (C) MT molecules assembled on reverted PS-b-PVP templates when (2) 4 µg/mL and (3 and 4) 20 µg/mL of MT solution was added to the templates. Individual protein molecules are visible on the micellar surface of the reverted PS-b-PVP templates in panels B and C at the 4 µg/mL concentration, whereas the compact packing nature of those individual protein molecules on the micelles due to increased protein concentration is clearly resolved in the AFM images of the 10 µg/mL IgG and 20 µg/mL MT samples. The scan size of the topographical AFM images shown in B and C corresponds to (2) 180 × 180 nm, (3) 180 × 180 nm, and (4) 60 × 60 nm.
Figure 3.4: Enzymatic activity test of HRP adsorbed on PS-b-PVP ultrathin micellar films. (A) Control experiment without HRP molecules on PS-b-PVP. (1) In the absence of HRP, color change of the assay solution was not observed. (2) A 180 × 180 nm AFM image of the hexagonally packed PS-b-PVP micelles which was used in the enzymatic assay shown in (1). (3) Each PS-b-PVP micelle in the hexagonal lattice of the AFM image shown in (2) is circled for clarity. (B) Enzymatic activity experiment with 10 µg/mL HRP on PS-b-PVP. (1) Digital image of a TMB assay solution after addition to HRP-bound PS-b-PVP micelles on a silicon substrate. The assay color rapidly changed from colorless to blue. (2) 180 × 180 nm AFM image revealing the sample surface employed in the enzymatic test shown in (1). (3) To guide the eye, black and white circles are inserted in the AFM image shown in (2) to indicate the surface-bound HRP molecules and the underlying PS-b-PVP micelles, respectively. HRP molecules organize themselves on top of the underlying, hexagonally packed PS-b-PVP micelles which, in turn, form periodic, two-dimensional nanoscale protein arrays. (C) UV-vis absorbance spectra of the TMB assay solutions shown in A and B. (A) No absorbance peaks were observed when TMB solution containing hydrogen peroxide was introduced to a PS-b-PVP micellar thin film containing no HRP on its surface. (B) Characteristic UV-vis absorbance peaks were monitored from the blue assay solution. (D) UV-vis absorbance of HRP molecules monitored over time in solution (data shown in blue) and on PS-b-PVP micelles (data shown in red). When compared with the activity of HRP molecules in solution, HRP molecules bound on PS-b-PVP showed 78% of the activity measured in solution.
CHAPTER 4
Activity Study of Self-Assembled Proteins on Nanoscale Diblock Copolymer Templates

4.1 Background

Protein arrays are presently utilized in a variety of screening and diagnostic assays encompassing protein-antibody, protein-protein, protein-ligand, protein-small molecule, enzyme-substrate and multianalyte proteins.\(^1\)\(^-\)\(^3\) To meet these demands, various methods are being heavily pursued to create surface bound proteins with high areal density.\(^4\)\(^-\)\(^9\) High protein address density is crucial in achieving efficient, parallel and automated analysis that can screen large numbers of samples. The aforementioned applications of protein arrays require controlled immobilization of proteins at particular surfaces without affecting their natural functionality. For selective and specific protein assays, surface immobilized proteins should be able to present easily accessible binding sites and maintain their specific activities.\(^1\)\(^-\)\(^4\), \(^7\)\(^-\)\(^11\) Therefore, critical factors such as protein address density and protein activity need to be carefully considered in developing improved protein arrays. Further, understanding precise effects of protein adsorption onto common proteomics substrates such as polymers is extremely crucial to accurate and meaningful interpretation of the outcome of protein assays. Systematic studies are highly warranted in order to elucidate potential conformational and functional changes of proteins upon their adsorption onto polymeric surfaces.

Enzymes, proteins that catalyze chemical reactions, can be employed as a model system to explore the potential perturbations in activity caused by binding to solid
surfaces. Enzymes exhibiting high selectivity and a turnover rate can be conveniently used to study their activity changes upon surface adsorption. The active sites of an enzyme, typically consisting of a set of amino acid residues in a specific geometry, mediate its catalytic activity. In order to achieve their particular catalytic activity, these small interactive sites need to be retained in a precise position even after adhering to a solid surface. Additionally, the orientation of active sites with respect to solid supports may influence protein activity since the presence of solid supports can physically block accessible pathways to active sites. Therefore, enzymes can serve as responsive and sensitive indicators in assessing whether a particular surface will preserve native structures and unique functionalities of proteins.

We have previously developed nanoscale protein arrays by exploiting the self-organizing nature of diblock copolymer templates and the self-assembling nature of proteins onto preferential polymeric domains – chapter 2 & 3. In this previous work, nanometer-scale spatial resolution of surface bound proteins was spontaneously achieved over large areas via self-assembly, without undergoing time-consuming fabrication or modification processes. En route to successful applications of these diblock-copolymer guided, nanoscale protein assembly as protein arrays, it is extremely important to evaluate the activity of the patterned proteins on diblock copolymer templates and determine their effectiveness as highly selective and sensitive nanosensor platforms.

Herein, we report the findings on the activity and stability of various proteins that were self-assembled onto the microphase-separated domains of polystyrene-block-polymethylmethacrylate (PS-b-PMMA) diblock copolymer ultrathin films. Model proteins chosen for this activity study are horseradish peroxidase (HRP), mushroom
tyrosinase (MT), enhanced green fluorescent protein (eGFP), bovine immunoglobulin G (IgG), fluorescein isothiocyanate conjugated anti-bovine IgG (FITC-antiIgG), bovine serum albumin (BSA) and protein G. We also did a quantitative activity study of HRP enzymes immobilized on self-assembled PS-b-PMMA. We directly compare the enzymatic activity of PS-b-PMMA surface-bound HRP to its activity in free solution by carrying out UV-vis absorbance studies of a HRP-catalyzed substrate. In our activity assays, the same number of surface-bound HRP molecules and of HRP molecules in free solution were compared. In order to study systematically the biological functionality of diblock copolymer-bound proteins, the focus of this paper is to investigate the conformational integrity and biological activity of proteins self-assembled selectively onto the nanoscopic diblock copolymer templates.

In this paper, we demonstrate that PS-b-PMMA bound enzymes such as HRP and MT are biologically active and maintain their specific catalytic functionality over a long period of time. We further determine that our diblock-copolymer guided, nanoscale enzyme assembly method is effective in preserving a large degree of the enzymatic activity in free motion. Surface-bound enzymes retain approximately 85% of their free activity after selective adsorption onto the PS domains of the underlying PS-b-PMMA. Other proteins such as eGFP and IgG keep their structural integrity and binding activity even after their self-assembly on PS-b-PMMA. Substantiated by the fact that our surface-patterned protein molecules maintain their conformation and display their natural function, our block copolymer-guided, nanoscale-patterned proteins represents a direct step forward for a pathway to much needed, selective and sensitive, high-throughput protein arrays with high areal density.
4.2 Experimental Details

Asymmetric PS-b-PMMA diblock copolymer with an average molecular weight of 71,400 Da was obtained from Polymer Source Inc. (Montreal, Canada). The diblock contained 71% of PS by weight with a polydispersity of 1.06. Silicon substrates, obtained from Silicon Inc. (Boise, Idaho), were cleaned with ethanol, acetone, and toluene and spun dry before coating ultrathin polymer films. Polymeric templates with lying-down cylindrical microdomains were produced by spin coating 2% (w/v) PS-b-PMMA in toluene at 3500 rpm for 1 minute, followed by annealing in an argon atmosphere at 240 °C for 6 hours with a transient ramp-up rate of 5 °C/min and a cooling rate of 2 °C/min.

HRP was purchased from VWR Scientific Inc. (West Chester, PA). 3, 3’, 5, 5’-tetramethylbenzidine (TMB) solution containing 1.25 mM TMB and 2.21 mM H₂O₂ was also received from VWR Scientific Inc. MT and pyrocatechol were obtained from Sigma-Aldrich (St. Louis, MO). The lyophilized powder of HRP and MT were reconstituted in deionized water and PBS buffer (10 mM mixture of Na₂HPO₄ and NaH₂PO₄, 140 mM NaCl, 3 mM KCl, pH 7.4), respectively, to varying concentrations ranging from 4 to 10 µg/mL. 100 µL of these enzyme solutions was deposited onto the microphase separated diblock copolymer surfaces for 20 sec at room temperature. The sample surfaces were rinsed thoroughly and then dried gently under a stream of nitrogen gas prior to AFM imaging. AFM measurements were carried out using Digital Instruments Multimode, Nanoscope IIIa, in tapping mode at a scan speed of 1 Hz. Silicon tips with a resonant frequency of 60 kHz and a spring constant of 5 N/m were used in the measurements.

Enhanced green fluorescent protein (eGFP), obtained from BioVision Inc. (Mountain View, CA), and was diluted to a 100 µg/mL concentration in PBS. Whole
molecule IgG, protein G and fluorescein-conjugated antobodyne IgG (FITC-antiIgG) were purchased from VWR Scientific Inc. The lyophilized powder of these protein molecules were reconstituted in PBS buffer and diluted to various concentrations ranging from 4 to 200 µg/mL. Confocal images were collected using a conventional confocal laser scanning microscope (Olympus Fluoview 300) with an Ar (488 nm, 40 mW) laser at a resolution of 512 pixels. The fluorescence from biosamples was separated from the excitation light by a dichroic beam splitter and a 510-535 nm bandpass filter. The microscope was also equipped with a 100 W mercury arc lamp (Osram) which allowed overall inspection of a large sample area in a single view frame.

UV-vis spectra were recorded on a Hewlett Packard 8452A Diode Array Spectrophotometer in order to monitor characteristic absorption peaks of oxidized TMB or pyrocatechol and to confirm the presence of active and functional enzymes on PS-b-PMMA diblock copolymer templates.

4.3 Results and Discussions

4.3.1 Fabrication and characterization of PS-b-PMMA templates

The two chemically distinct, and thus immiscible, polymer blocks in diblock copolymers undergo phase separation and self-assemble into ordered patterns of microdomains whose characteristic packing and size is determined by the polymer compositions and chain lengths, respectively. The self-organizing nature of the ultrathin films of the diblock copolymer under carefully balanced thermodynamic conditions\textsuperscript{13-16} was exploited to produce alternating polymeric domains of the two chemical compositions of the diblock at the air/polymer interface. The highly predictable and
controllable self-assembling phase behavior of the diblock copolymer makes the material suitable as nanoscale templates.

At the polymer/silicon oxide interface, PMMA favors the silicon oxide substrate because of its lower wetting energy. At the polymer/air interface, the surface tension difference between PMMA and PS is small enough to produce the appearance of both components at the upper interface of certain film areas when the film is prepared to form ‘L-thick’ regions as described in the experimental procedures. ‘L-thick’ regions are film areas where the film height supported on a substrate is commensurate with the characteristic long period of PS-b-PMMA. The PS-b-PMMA diblock copolymer in such areas forms one layer of lying-down cylindrical microdomains parallel to the substrate. Upon thermal annealing in an Ar atmosphere, microphase separation of the diblock copolymer used in our experiments yielded microdomains with the average cylinder repeat spacing of 45 nm. Atomic force microscopy (AFM) topographical measurements of these films identified that PMMA is higher than PS by 1 nm and, therefore, PMMA rich regions appear lighter than PS domains in AFM topography. By directly comparing the topographical and phase AFM images acquired from the same area of the diblock copolymer thin film, we determined that PMMA domains also appear lighter in phase AFM imaging.

4.3.2 Enzyme adsorption and activity assessment

Figure 4.1A is a schematic illustration showing our nanoscale protein patterning process where enzymes are preferentially segregated onto certain domains of microphase-separated PS-b-PMMA ultrathin films via self-assembly. Enzyme deposition onto these
spatially-defined, chemically-distinctive, polymeric templates was investigated by AFM in both topography and phase imaging modes. Figure 4.1B-1, a 500 x 500 nm phase AFM image, clearly displays that adsorption of HRP is localized selectively to PS domains when the enzyme was deposited from a 4 µg/mL solution. As-prepared PS-b-PMMA templates exhibit alternating PS and PMMA stripes at the air/polymer interface with the repeat spacing of 45 nm. HRP molecules seen as circular features in Figure 4.1B-1 prefer the darker, i.e. PS-rich, areas of the underlying PS-b-PMMA templates. Figures 4.1B-2 and 4.1B-3 are 750 x 750 nm and 500 x 500 nm topographical AFM images, respectively, of HRP molecules deposited from a 10 µg/mL solution. When increasing the concentration of HRP to 10 µg/mL, individual HRP molecules lined up inside each available PS domain while forming a linear chain of deposited enzyme molecules. The phase images displayed in the insets of Figures 4.1B-1 and 4.1B-3 clearly resolve individual HRP molecules with a molecular weight of ~ 44 kDa that were localized exclusively to PS regions. These AFM data show that the periodically-spaced diblock copolymer domains of PS-b-PMMA can effectively serve as nanometer-scaled, chemically-heterogeneous templates for selective HRP deposition to PS regions.

When using another enzyme, MT, the same discriminatory effect of enzyme segregation onto PS domains was monitored. Figures 4.1C-1 through 4.1C-3 clearly exhibit the biased self-assembling nature of MT molecules onto PS domains. At a concentration of 4 µg/mL, the onset of MT self-assembly on the underlying PS domains was captured in the phase AFM image of Figure 4.1C-1. At the monolayer-forming MT concentration of 10 µg/mL MT, individual MT molecules with a molecular weight of ~ 130 kDa close packed and filled in the PS domains completely, while faithfully following
the underlying patterns of PS microdomains, see Figures 4.1C-2 and 4.1C-3. The close packing nature of MT on the underlying PS domains is very similar to that of IgG reported earlier in chapter 2, possibly due to the comparable sizes of the two proteins. Roughly two MT molecules assemble along the short axis of the PS domain as the width of the underlying PS domains is approximately commensurate with the length of two MT molecules positioned side by side. Self-assembly of enzyme molecules onto PS-b-PMMA results in the inversion of AFM topographic contrast; bright, and therefore higher, areas in Figures 4.1B and 4.1C correspond to HRT or MT molecules assembled on PS domains. Prior to enzyme deposition, PS domains were 1 nm lower in height than the neighboring PMMA domains. After HRP and MT deposition, PS domains are higher than PMMA domains by 2.3 nm and 4.1 nm, respectively. The width of individual HRP and MT molecules on the PS domains are measured approximately as 7 and 18 nm, respectively. These values agree with the estimated diameters of HRP and MT from their known molecular weights. However, the measured heights of these proteins are much smaller than the expected values. This discrepancy is explained by intermittent contact made by the AFM tip on the flexible protein during imaging. Similar effects to our observed height reduction have been previously reported in tapping mode AFM imaging studies of soft biological molecules such as proteins and DNA.\textsuperscript{18, 19} When using the protein concentration of 4 µg/mL, the protein coverage is determined as $\sim 2 \times 10^{10}$ molecules/cm$^2$ for HRP and $\sim 4 \times 10^{10}$ molecules/cm$^2$ for MT on PS-b-PMMA.

Active sites of enzymes, typically consisting of a few specific amino acid residues held in specific geometries, mediate biological activities of proteins. In order to achieve their specific activity, these small interactive sites for substrates or regulators need to be
retained in the precise positions required for activity even after adhering to a solid support. The relative orientation of active sites relative to solid supports is extremely important as little irregularities affecting protein folding processes can result in significant alterations in bioactivity. Attachment of enzymes to solid supports may result in changes in the natural conformation of proteins. Any abnormal conformational change of enzymes may hinder biological substrates from fitting into active sites of enzymes. This undesirable structural change of enzymes may lead to reduction in enzymatic activity. Steric hindrance due to the presence of solid supports may also influence protein activity by physically blocking accessible pathways to active sites. Due to these reasons, it is extremely critical to consider the effectiveness of proteins on such platforms in carrying out biological functions when assessing the full potential of our nanometer scale diblock copolymer templates in biosensing applications.

Subsequently, we have carried out enzymatic activity measurements using the effectively immobilized HRP and MT molecules on PS-b-PMMA with a spatial resolution on the nanometer scale. The two enzymes are chosen for our protein activity study as they are highly specific and powerful catalysts that accelerate particular biochemical reactions. HRP is a widely used, highly specific, enzyme that catalyzes reduction of hydrogen peroxide at a high turnover rate in a short period of time. Visualization of HRP activity was carried out in the presence of a TMB/H₂O₂ indicator solution where the enzymatic activity was monitored by a color change of the indicator solution from colorless to blue. MT, in the presence of oxygen, oxidizes pyrocatechol to hydroquinone. MT activity was monitored by a change in assay color from colorless to yellow-brown.
A HRP or MT immobilized PS-b-PMMA sample was placed at the bottom of a glass vial and 1 mL of TMB or pyrocatechol was added to the vial, respectively. At the same time, we performed a control experiment where as-annealed PS-b-PMMA, without any enzymes deposited on the film surface, was incubated with TMB or pyrocatechol. We also carried out additional control experiments where enzymes on microphase separated PS-b-PMMA surfaces were denatured purposely either by exposing them to an acid or heat. In the case of the acid exposure, HRP on the sample surface was denatured by directly depositing 100 µL of 0.1 N HCl onto the HRP for 1 h. In the case of the heat treatment, HRP on the sample surface was denatured by keeping the sample at 75 ºC for 12 h. Subsequently, similar to prior procedures, 1000 µL of TMB was then added to the denatured sample surfaces for 30 min in order to observe any change in assay color.

The series of digital images shown in Figures 4.2A and 4.2B display the activity of the surface-bound MT and HRP in the oxidation reaction of their specific substrates of pyrocatechol and TMB, respectively. Active tyrosinase, assembled selectively on the PS domains of PS-b-PMMA thin films, changed the assay color to yellow-brown (Figure 4.2A-2) whereas as-annealed PS-b-PMMA substrates did not yield any color change upon adding pyrocatechol (Figure 4.2A-1). Addition of TMB solution to peroxidase molecules, segregated exclusively to the PS domains of the diblock copolymer thin film, resulted in change of the assay color to blue, Figure 4.2B-2. The color of the added TMB solution did not change in the presence of as-annealed PS-b-PMMA without adsorbed HRP, Figure 4.2B-1. The results from our control experiments involving denatured HRP using acid and heat are displayed in Figures 4.2B-3 and 4.2B-4. No change in color is monitored in both cases. Absence of enzymes, loss of enzyme activity, and alterations of
natural enzyme structures are responsible for no change in color of the assays shown in Figures 4.2B-1, 4.2B-3, and 4.2B-4. Figures 4.2C and 4.2D show UV-vis data of sample solutions presented in Figure 4.2A and 4.2B, respectively. Samples containing no or denatured enzymes, as shown in Figures 4.2A-1, 4.2B-1, 4.2B-3, and 4.2B-4, exhibited no absorbance peak. On the other hand, UV-vis spectra of samples containing PS-bound enzymes in Figures 4.2A-2 and 4.2B-2 displayed characteristic absorbance peaks that correspond to the oxidized substrates of MT and HRP, respectively. These results clearly indicate that the enzyme molecules indeed maintain their biological function when they are effectively immobilized on the underlying microphase separated PS-b-PMMA via self-assembly. The enzymatic activity of HRP and MT maintained over a long period of time where, when kept at 4 °C, no loss in their activity was observed even after 100 days.

4.3.3 Quantitative activity analysis

Quantitative activity measurements were carried out using UV-vis spectroscopy in order to gain insight on the time-and concentration-dependent activity of HRP molecules. Measurements were taken on a variety of HRP concentrations immobilized on PS-b-PMMA templates (bound-state, data shown in Figure 4.3) and HRP in solution (free-state, data shown in Figure 4.4). Figure 4.3, parts C-F, shows bound-state absorbance spectra measured from PS-b-PMMA samples that were pretreated with HRP solution concentrations of (C) 0.1, (D) 1, (E) 1.5, and (F) 2 µg/mL. At each concentration, absorbance spectra were collected at 0, 2, 5, 10, 15, 20, 25, 30, 60, and 120 min after the addition of the indicator solution to PS-b-PMMA-bound HRP molecules until saturation in absorbance was reached.
By determining the spot size and the surface density of the attached HRP molecules on PS-b-PMMA, the total number of enzyme molecules used in each of the bound state assays in Figure 4.3 was calculated. Conditions for HRP solution assays were then chosen to yield comparable numbers of HRP molecules in the free-state assay to those of HRP from the bound-state assay. Figure 4.4 displays the results of UV-vis absorbance taken from various concentrations of free HRP assays. A volume of 25 µL of (A) 0.01, (B) 0.02, (C) 0.05, and (D) 0.15 µg/mL HRP in solution was added to 1 mL of the indicator solution described earlier. Similarly to the UV-vis data shown in Figure 4.3, parts C-F, the absorbance peak of the oxidized TMB was monitored over time at each of the different HRP concentrations in the free-state assays shown in Figure 4.4.

The activity change of HRP molecules that are immobilized on PS-b-PMMA versus free in solution was further compared and evaluated. For qualitative comparison to our bound-state HRP experiments, we chose free-state solutions that contain similar numbers of HRP molecules at the four concentrations tested. Using the concentrations in the bound-state assays (shown in red), we determined the total number of HRP molecules to be $6.1 \times 10^9$, $10.1 \times 10^9$, $24.4 \times 10^9$, and $45.7 \times 10^9$ for our specified experimental conditions. We then chose concentrations and volume used in the free-state assays (shown in blue) to correspond to a total number of HRP molecules of $3.3 \times 10^9$, $6.6 \times 10^9$, $16.5 \times 10^9$, and $49.5 \times 10^9$. Figure 4.5 displays UV-vis absorbance data comparing the activity between PS-b-PMMA-bound and free HRP molecules. In all graphs, data from the free-state assays are shown in blue and those from the bound-state assays are shown in red. The time-dependent absorbance data are displayed for all concentrations evaluated in both the free-and bound-state HRP assays. Figure 4.5, parts A and B, clearly
shows that the measured absorbance intensity increases as the total number of HRP molecules increases regardless of the type of assays. Figure 4.5B shows absorbance maximum values versus total numbers of HRP molecules in each experiment. From the linear fits of data in Figure 4.5B, bound-state HRP retained $85 \pm 8\%$ of its free-state activity.

Physical integrity of bare PS-b-PMMA templates and bound HRP molecules on PS-b-PMMA was examined after the UV-vis assays. Figure 4.6 shows morphology of the polymer templates as well as the enzymes on the templates after undergoing the exact experimental conditions used for the UV-vis assay. Figure 4.6, parts A and B, displays 700 nm × 700 nm AFM scans of bare PS-b-PMMA templates taken after UV-vis absorption measurements. Figure 4.6, parts C and D, shows 700 nm × 700 nm and 300 nm × 300 nm topographic scans of PS-b-PMMA-bound HRP molecules imaged after the enzymatic activity assay. We ensure that the surface-bound HRP does not leach out to the assay solution during the UV-vis measurements by stringent washing of the sample after protein deposition. This step removes loosely bound proteins from the polymer surface after protein adsorption. Stringent washing procedures are determined by repeated rinsing of the sample and subsequent absorbance measurements of the rinsed elution until no more enzymes are present in the rinsed elution. The AFM images indicate that the HRP molecules are still bound selectively on PS domains of the underlying PS-b-PMMA templates without migrating to the neighboring PMMA domains in the presence of the indicator solution in the assay. The AFM images also demonstrate that HRP molecules remain on the PS-b-PMMA surface even after 2 h in the UV-vis assay conditions and the total number of HRP molecules bound on the polymeric templates does not change.
significantly before and after the assay. The change in the density of bound HRP molecules after the 2 h assay is less than 5% of the total density of HRP before the assay, which is well within the tolerance range reported earlier.

4.3.4 Study of protein-protein interactions

In order to evaluate the broad applicability of diblock copolymer templates as self-organizing nanoscale protein arrays, additional protein molecules such as eGFP, IgG, FITC-antiIgG, BSA, and protein G on PS-b-PMMA were assessed for their natural activity towards other binding proteins.

Enhanced GFP, derived from the Pacific jellyfish *Aequoria victoria*, is a recombinant, spontaneously fluorescing protein. The fluorophore located centrally in the green fluorescent protein is extremely well protected from collisional quenching by oxygen or photochemical damage. The remarkable cylindrical fold of protein exhibits a protective, tightly woven, barrel-like structure around the fluorophore. When this natural conformation is maintained, eGFP absorbs and emits at maximum absorbance and emission wavelengths comparable to those of a commonly used dye, FITC. Figure 4.7A-1 displays a representative confocal image taken from eGFP on PS-b-PMMA. A 20 μL droplet of 100 μg/mL eGFP was deposited to the center of a PS-b-PMMA thin film substrate. The circular edge formed by the droplet served as a convenient interface for distinguishing substrate areas with and without immobilized proteins during our confocal measurements. The green contrast in the confocal image of Figure 4.7A-1 is due to fluorescence emission from the eGFP molecules on the PS-b-PMMA templates whereas
the neighboring black contrast area corresponds to PS-b-PMMA area with no adsorbed eGFP molecules.

Figures 4.7A-2 through 4.7A-4 summarizes our confocal data involving multiple protein-protein interactions. A control experiment was carried out using BSA and FITC-antiIgG, see Figure 4.7A-2. 20 µL of 200 µg/mL BSA was first deposited on a PS-b-PMMA thin film for 5 min. Subsequently, 100 µL of 20 µg/mL FITC-antiIgG was incubated with BSA for 30 min and excess proteins were removed by thorough PBS rinsing. No fluorescence was monitored anywhere on this control sample surface as displayed in Figure 4.7A-2. In a sandwich assay shown in Figure 4.7A-3, a 100 µL droplet of 20 µg/mL protein G was first deposited on a PS-b-PMMA thin film for 5 min. After rinsing with an ample amount of PBS, a 20 µL droplet of 20 µg/mL IgG was added to the protein G-modified sample surface for 30 min in a humidity controlled chamber. Unbound IgG was removed by rinsing with PBS thoroughly. Subsequently, 100 µL of 20 µg/mL FITC-antiIgG was incubated with the protein pairs immobilized on the PS-b-PMMA for 30 min in a humidity controlled environment. Loose proteins were removed by careful PBS rinsing and the sample was gently blow dried with nitrogen before imaging with a confocal microscope. The confocal image shown in Figure 4.7A-3 displays green fluorescence from the area where the sequential reaction of proteins led to sandwich reactions of protein G/IgG/FITC-antiIgG.

In order to study the activity of an interacting protein pair of IgG/FITC-antiIgG, a 20 µL droplet of 200 µg/mL IgG was deposited to a PS-b-PMMA substrate for 5 min. Unreacted proteins were rinsed off with ample amounts of PBS. Immediately following, 20 µL of 20 µg/mL FITC-antiIgG were deposited onto the IgG-modified PS-b-PMMA
substrates for 30 min. Prior to confocal imaging, the sample surfaces were rinsed multiple times with PBS and gently blow dried with nitrogen. Figure 4.7A-4 shows confocal images taken after IgG/FITC-antiIgG interactions where green fluorescence is clearly resolved from the substrate regions containing the protein molecules.

Activity loss of surface bound enzymes is often associated with slow leaching of enzyme molecules to a surrounding liquid medium. We carried out a control experiment to confirm the presence and integrity of enzyme molecules on PS-b-PMMA domains before and after our enzymatic activity measurements. Evaluating possible structural variations of enzymes on PS-b-PMMA templates enables us to assess whether our diblock copolymer-guided immobilization technique can retain the stability and activity of surface bound proteins. Figure 4.7B-1 displays a typical topographic AFM image of HRP molecules self-assembled on PS-b-PMMA template immediately after they were deposited. Figure 4.7B-2 shows a topographic AFM image of the same HRP molecules on the PS-b-PMMA template after 4 days in TMB solution. These images are comparable to each other and indicate that no significant changes in the density and stability of surface-bound HRP molecules occurred during our measurements. Figure 4.7B-3 is the topographic AFM image of denatured HRP on PS-b-PMMA after 0.1N HCl was placed directly onto the protein molecules assembled on PS domains. Unlike the structures shown in Figures 4.7B-1 and 4.7B-2, HRP no longer maintains its structural integrity after HCl treatment. Small denatured protein fragments accumulate onto the polymeric template into flat band-like structures where the height difference between the bright and dark areas in Figure 4.7B-3 is ~1.3 nm.
4.4 Conclusions

It has been demonstrated that protein molecules, selectively self-assembled on the nanoscale PS domains of the microphase-separated PS-b-PMMA thin films, maintain their natural activity and conformational integrity. Specific catalytic functions of HRP and MT were evaluated over a long period of time where these self-organized enzymes on PS-b-PMMA kept their activity and stability well over three months. We also demonstrated that PS-b-PMMA bound HRP maintains a large degree of their activity of their free state by directly comparing the activity of PS-b-PMMA bound HRP to that in free solution. Further, by utilizing protein G, IgG, and FITC-antiIgG as model systems for our protein/protein interaction studies, we have also demonstrated that binding domains of these proteins are fully available for interacting with other proteins when they are affixed to PS-b-PMMA templates. Our diblock copolymer-based approach is a straightforward localization method for creating highly-periodic, high density, surface-bound protein patterns with the spatial control on the nanometer scale. The main advantage of our approach lies in the spontaneous, self-assembled, nanoscale organization of proteins over a large substrate area. Equally beneficially, our diblock copolymer-based strategy maintains the natural activity, conformation and stability of surface-immobilized proteins. The confluence of the two very important attributes, precise nanoscale spatial control while retaining natural activity of proteins, can make our platforms highly suitable as functional sensor substrates or protein arrays for high-throughput proteomic assays and multiplexed biosensing applications.
4.5 References


Figure 4.1: (A) Schematic illustration showing selective deposition and self-assembly of enzymes on microphase separated PS-b-PMMA templates with the spatial control on the nanometer scale. (B) AFM images showing HRP segregated selectively on PS domains. (1) 500 x 500 nm phase AFM image taken after deposition of 4 µg/mL of HRP where the inset is 150 x 150 nm, (2) 750 x 750 nm topography AFM image acquired after deposition of 10 µg/mL of HRP, and (3) 500 x 500 nm topography AFM scanned after deposition of 10 µg/mL of HRP where the inset shows a 200 x 200 nm phase AFM image. The phase images shown as insets in (1) and (3) clearly show individual HRP molecules selectively occupying PS regions. (C) AFM images displaying MT molecules located exclusively on PS domains. (1) 750 x 750 nm phase AFM image taken after deposition of 4 µg/mL of MT where the inset is 100 x 100 nm, (2) 750 x 750 nm topography AFM image acquired after deposition of 10 µg/mL of MT, and (3) 500 x 500 nm topography scanned after deposition of 10 µg/mL of MT where the inset shows a 30 x 30 nm topography AFM image. The phase and topography images shown as insets in (1) and (3), respectively, display clearly individual MT molecules and their packing behavior on top of the PS domains.
Figure 4.2: (A) Digital images taken after adding 1 mL of pyrocatechol to vials containing (1) as-annealed PS-b-PMMA where no color change was observed and (2) PS-b-PMMA with self-assembled MT molecules where the assay color changed to yellow-brown. (B) Digital images taken after adding 1 mL of TMB solution to vials containing (1) as-annealed PS-b-PMMA substrate where no color change was observed, (2) PS-b-PMMA substrate with self-assembled HRP molecules in which case the assay color changed to blue, (3) PS-b-PMMA substrate with acid-denatured HRP where no color change was monitored, and (4) PS-b-PMMA substrate with heat-denatured HRP in which case no color difference was observed. (C) UV-vis spectra of tyrosinase test series as pictured in A. (D) UV-vis spectra of peroxidase test series as pictured in B. Samples containing no or denatured enzymes, as shown in Figures A-1, B-1, B-3, and B-4, exhibited no absorbance peak. On the other hand, UV-vis spectra of samples containing PS-bound enzymes pictured in Figures A-2 and B-2 clearly displayed characteristic absorbance peaks that correspond to the oxidized substrates of MT and HRP, respectively.
Figure 4.3: Bound-state HRP activity assays: visualization and quantification of HRP activity bound on PS-b-PMMA surface. (A and B) Digital images of a TMB assay solution after addition to a PS-b-PMMA template (A) without HRP and (B) with effectively immobilized HRP molecules. No color change was monitored in (A), indicating no catalytic activity. In contrast, the assay color rapidly changed from colorless to blue in (B). (C-F) HRP concentration-dependent UV-vis absorbance spectra monitored over time. HRP molecules were adsorbed onto PS-b-PMMA surfaces from the solution concentration of (C) 0.1, (D) 1, (E) 1.5, and (F) 2 µg/mL. Enzymatic activity of the PS-b-PMMA surface-bound HRP molecules was recorded at various time intervals by observing the characteristic absorbance peak of the oxidized TMB at 650 nm. Absorbance spectra were recorded at 2, 5, 10, 15, 20, 25, 30, 60, and 120 min after the addition of the TMB assay solution. The time-dependent absorbance spectra were then plotted where the absorbance plots, from the bottom to the top, correspond to data from the shortest to the longest time. In (F), plots show the absorption spectra taken at 0 min (the bottom-most curve) to the absorption saturation point at 30 min (the top-most curve). The absorption spectrum does not change significantly after 30 min. To guide the eye, solid lines following data points are inserted in each plot.
Figure 4.4: Free-state HRP activity assays. UV-vis absorbance spectra of HRP molecules free in solution. A volume of 25 µL of (A) 0.01, (B) 0.02, (C) 0.05, and (D) 0.15 µg/mL in deionized water, combined with 1 mL of the indicator solution, was used for the UV-vis measurement. Under these conditions, the numbers of HRP molecules are comparable to their counterpart bound-state assays in Figure 4.3. Solid lines following data points are inserted in each plot to guide the eye. Enzymatic activity of the free HRP molecules was recorded at various time intervals by observing the characteristic absorbance peak of the oxidized TMB at 650 nm. Absorbance spectra were recorded at 0, 2, 5, 10, 15, 20, 25, 30, 60, and 120 min after the addition of the TMB assay solution. The time-dependent absorbance spectra are plotted, from the bottom to the top curves, corresponding to the shortest to the longest time delays. In (D), plots show the absorption spectra taken at 0 min (the bottom-most curve) to the absorption saturation point at 30 min (the top-most curve). The absorption spectrum does not change significantly after 30 min.
Figure 4.5: Evaluation of HRP activity difference between their free state to PS-b-PMMA bound state. In all graphs, blue data points represent activities of HRP molecules freely floating in solution, and red data points represent those of HRP molecules immobilized on PS-b-PMMA surfaces. (A) UV-vis absorbance at $\lambda = 650$ nm is plotted for free-and bound-state HRP. From top to bottom, plots correspond to 0.15(f), 2(b), 1.5(b), 0.05(f), 1(b), 0.02(f), 0.1(b), and 0.01(f) µg/mL of either free-or bound-state HRP concentration, where (f) stands for free-state and (b) denotes bound-state. These concentration conditions correspond to the total number of HRP molecules of $49.5 \times 10^9$, $45.7 \times 10^9$, $24.4 \times 10^9$, $16.5 \times 10^9$, $10.1 \times 10^9$, $6.6 \times 10^9$, $6.1 \times 10^9$, and $3.3 \times 10^9$ from top to bottom plots. (B) Absorbance maximum values are plotted against the total number of either free-or bound-state HRP molecules. When the enzymatic activity of the same number of HRP molecules in the free versus the bound state was compared, bound-state HRP retained approximately 85% of its free-state activity.
Figure 4.6: AFM images of blank PS-b-PMMA with and without HRP taken after the UV-vis absorption measurements described in Figures 4.3-4.5. (A and B) 700 nm × 700 nm phase and topographic images of the PS-b-PMMA template without bound HRP exposed to the same UV-vis measurement conditions as the control. (C and D) Topographic AFM of HRP molecules bound selectively on PS domains of the underlying PS-b-PMMA templates scanned after the enzymatic activity assay. Image sizes are (C) 700 × 700 nm, and (D) 300 × 300 nm. HRP molecules remained on the PS-b-PMMA surface even after 2 h in the UV-vis assay conditions, and the total number of HRP molecules does not change significantly before and after the assay.
Figure 4.7: A. Confocal fluorescence data of proteins and interacting proteins collected at 400 times magnification. The contrast and brightness of the presented confocal fluorescence images were adjusted for clarity. (1) Green fluorescence was observed from PS-b-PMMA regions where 100 µg/mL eGFP was deposited whereas other PS-b-PMMA areas with no eGFP on the surface did not show any fluorescence emission. 2) Deposition of a 20 µg/mL FITC-antiIgG droplet onto BSA-incubated PS-b-PMMA led to no observable fluorescence signal from the entire diblock surface and (3) Green fluorescence signal was captured after a sandwich reaction between protein G/IgG/FITC-antiIgG where the concentration of the all three proteins was 20 µg/mL. (4) Fluorescence emission was monitored after the reactions between 20 µg/mL IgG and 20 µg/mL FITC-antiIgG. B. 500 x 500 nm AFM images comparing the density and structural integrity of HRP molecules 1) immediately after they were deposited on microphase-separated PS-b-PMMA microdomains, 2) after 4 days of enzyme activity tests in TMB solution and 3) after HRP on PS-b-PMMA was intentionally denatured in 0.1 N HCl for 1h.
CHAPTER 5

Elucidation of Protein Adsorption Behavior on Polymeric Surfaces: Towards High Density, High Payload, Protein Templates

5.1 Background

The burgeoning area of proteomics creates increasing demands for miniaturization, high throughput, and high sensitivity of protein assays. Protein arrays can assist small-volume assays in a parallel fashion and, therefore, meet such needs to some degree. However, even state-of-the-art protein arrays still face crucial challenges in keeping up with ever growing demands of proteomics. Main difficulties associated with protein arrays can be identified in four areas; i) attachment of protein onto a solid surface with retained protein functionality, ii) development of a universal method to attach protein on an array surface, iii) achievement of a high protein density for increased detection signal, and iv) attainment of uniform protein density between arrayed spots. In protein array applications, retaining the native structure and full biological activity of proteins after surface attachment is essential. When compared to DNA chips, reliable protein arrays are much more difficult to produce and to use because proteins are much more diverse in chemical and physical properties than DNA. The structure and functionality of proteins can be affected by the presence of a solid surface as well as by chemical linking processes. Standardized procedures that can effectively and simultaneously handle multiple proteins are not currently available. High address density of proteins in an array is also very important as increased protein density will improve the signal to noise ratio of a given detection method. In addition, uniformity in density is
equally critical since potential variations in protein density from array to array or from spot to spot can significantly hamper quantitative measurements and comparative analysis of protein assays. Among these current challenges associated with protein arrays, the focus of this paper is on the areas of high and uniform protein density.

The density of proteins in arrays is largely governed by the adsorption behavior of proteins to the surface of an underlying array material.\textsuperscript{5, 6} We have previously studied adsorption behavior of various proteins on diblock copolymer ultrathin films such as polystyrene-block-polymethylmethacrylate (PS-b-PMMA) and polystyrene-b-poly(4-vinylpyridine) (PS-b-PVP).\textsuperscript{7, 8} We reported that a variety of protein molecules selectively self-segregates onto one of the two polymeric segments in both PS-b-PMMA and PS-b-PVP cases. When carrying out these studies, we observed an intriguing phenomenon. Protein molecules have a tendency to exhibit a close-packing behavior and arrange themselves in an organized, space-filling manner at optimized protein concentrations.

In this study, we systematically study protein adsorption characteristics on different polymeric surfaces. Three different types of polymeric templates used in our study are ultrathin films of asymmetric PS-b-PMMA, PS homopolymer, and PS/PMMA blend. We compare protein density on a PS-b-PMMA diblock copolymer surface to its density on a PS homopolymer surface. We also investigate the nature of protein assembly on a PS/PMMA blend film in order to assess the dependence of protein density on the distance from the interface between PS and PMMA. When compared to the chemically homogeneous surface provided by PS homopolymer film, we find that the chemically heterogeneous PS-b-PMMA film is much more effective in high density and high payload protein assembly. The protein density on the diblock copolymer is approximately
three to four fold higher than that on the homopolymer. This remarkable effect is further confirmed by enzymatic activity measurements on the chemically homogeneous and heterogeneous polymeric surfaces. Combined with the self-assembling nature of proteins arranged in a nanoscopic spatial dimension, our diblock copolymer-based protein assembly method may provide a much needed means to achieve protein arrays with high protein density and protein payload.

5.2 Experimental Details

Asymmetric PS-b-PMMA diblock copolymer with an average molecular weight of 71,400 Da was obtained from Polymer Source Inc. (Montreal, Canada). The diblock contained 71% of PS by weight with a polydispersity of 1.06. PS with a molecular weight of 152,000 Da and PMMA with a molecular weight of 120,000 Da were obtained from Alfa Aesar (Ward Hill, MA). PS with a molecular weight of 70,400 Da, which is similar to the molecular weight of the diblock copolymer, was also received from Polymer Source Inc. Si wafers (resistivity < 1 $\Omega$cm, thickness: 0.017 inch) were obtained from Silicon Inc. (Boise, Idaho). Si substrates were cleaned with ethanol, acetone, and toluene and spun dry before coating ultrathin polymer films. Diblock copolymer templates with lying-down cylindrical microdomains were produced by spin coating 2% (w/v) PS-b-PMMA in toluene at 3500 rpm for 1 min, followed by annealing in an argon atmosphere at 240 $^\circ$C for 6 h with a transient ramp-up rate of 5 $^\circ$C/min and a cooling rate of 2 $^\circ$C/min. Ultrathin PS homopolymer films were prepared by spin casting 2% (w/v) PS in toluene at 3500 rpm for 1 min onto Si substrates. For PS/PMMA blend films on Si substrates, 2%
(w/v) of PS/PMMA blend solution containing 1:1 weight ratio of the two homopolymers in toluene was spin-coated at 3500 rpm for 1 min.

Whole molecule immunoglobulin G (IgG), fibronectin (Fn), bovine serum albumin (BSA), protein G and horseradish peroxidase (HRP) were purchased from VWR Scientific Inc (West Chester, PA). The lyophilized powder of these protein molecules were reconstituted in PBS buffer or in deionized water as recommended by the manufacturer. The reconstituted protein solutions were further diluted to various concentrations ranging from 0.01 to 20 µg/mL. 40 µL of these protein solutions was deposited onto various polymeric surfaces for 10 min at room temperature. The sample surfaces were rinsed thoroughly with an appropriate buffer and then dried gently under a stream of nitrogen gas prior to AFM imaging. AFM measurements were carried out using a Veeco Multimode, Nanoscope IIIa, in tapping mode at a scan speed of 1 Hz. Si tips with a resonant frequency of 60 kHz and a spring constant of 5 N/m were used in the measurements.

5.3 Results and Discussions
5.3.1 Fabrication and characterization of protein loaded polymeric templates

Figure 5.1 displays AFM data showing topologies of various polymeric surfaces before and after deposition of protein molecules. PS-b-PMMA diblock copolymer, PS homopolymer, PMMA homopolymer, and PS/PMMA blend ultrathin films were used as templates for protein adsorption. Figures 5.1(a), (c), (e) and (g) show typical topographic AFM scans of 750 x 750 nm PS-b-PMMA, 2 x 2 µm PS, 2 x 2 µm PMMA, and 2.5 x 2.5 µm PS/PMMA blend surfaces, respectively. Upon thermal annealing, PS-b-PMMA
diblock copolymer thin film microphase-separates into alternating regions of PS and PMMA at the air:polymer boundary\textsuperscript{7, 9-11} which, in turn, provides chemically heterogeneous templates with a repeat spacing of 45 nm, as seen in Figure 5.1(a). Homopolymer thin films of PS and PMMA, shown in Figure 5.1(c) and (e), present smooth, uniform surfaces that are morphologically and chemically homogeneous. Upon spin casting, PS/PMMA blend film forms circular structures, as shown in Figure 5.1(g), where the chemical immiscibility of the PS and PMMA leads to the assembly of micrometer-sized circular domains in which each domain contains only one type of polymer.\textsuperscript{12-14} The average film thickness of PS/PMMA blend is determined as 92 nm by ellipsometry. In the diblock and blend AFM images, PMMA-rich areas result in the brighter (higher) regions and PS-rich regions yield the darker (lower) regions. Figures 5.1(b), (d), (f), and (h) display typical morphologies of the three templates after protein adsorption and they correspond to AFM images of IgG on PS-b-PMMA (380 x 380 nm), IgG on PS (500 x 500 nm), IgG on PMMA (500 x 500 nm), and IgG on PS/PMMA blend (750 x 750 nm), respectively. Both the AFM images of the diblock and blend samples in Figures 5.1(b) and (h) show clearly the selective adsorption of IgG molecules onto the PS regions.

We note that this selective segregation of proteins onto the PS regions is not limited to IgG only. In fact, this phenomenon was observed for all proteins used in our study including IgG, HRP, Fn, protein G, and BSA. Figures 5.2(a) through 5.2(d) display typical AFM images of proteins assembled on chemically heterogeneous films of PS-b-PMMA and PS/PMMA blend templates.
We also observed that the surface density of protein molecules increases considerably when PS-b-PMMA templates are used for protein adsorption as opposed to homopolymer templates of PS or PMMA. Surface density of protein molecules is defined as the total number of adsorbed protein molecules per unit surface area. We refer to this surface density simply as density throughout this paper. When comparing the number of adsorbed protein molecules on the diblock copolymer versus homopolymer PS samples prepared by using the same concentration of a protein solution, the number of protein molecules on the PS-b-PMMA template is higher by multifold per unit PS area than the number on the homopolymer template. In order to evaluate possible dependence of protein assembly on the molecular weight of polymer films, we studied protein adsorption behavior using two different PS homopolymer films, one with the molecular weight of 152,000 Da and the other of 70,400 Da. The results are displayed in Figures 5.2(e) and 5.2(f). The latter film contains polymer chains with a comparable molecular weight as that of the diblock copolymer. We find that similar numbers of IgG molecules adsorb on the two PS homopolymer templates, regardless of the molecular weight of the polymer. On both PS templates, less protein molecules adsorb when compared to their assembly on diblock copolymer templates shown in Figure 5.2(g).

5.3.2 Study of protein adsorption behavior

In addition to these new findings, we observed in an earlier study that protein molecules tend to close-pack into the PS domains of PS-b-PMMA templates in an organized, space-filling manner at a monolayer loading concentration.\(^7\) Therefore, we reason that the chemical heterogeneity of the PS-b-PMMA diblock copolymer surface
may result in tight, space-filled protein adsorption at a monolayer loading concentration. As a result, diblock copolymer films may serve as better protein loading templates than chemically homogeneous, homopolymer surfaces. In order to test this hypothesis, PS/PMMA blend films were used as a model system. PS/PMMA blend surfaces provide self-assembled circular domains and interfaces on the micrometer scale. These circular domains and interfaces permit two-dimensional analysis of the density of adsorbed proteins. Therefore, PS/PMMA blend templates can be conveniently used to investigate distance-dependent protein adsorption behavior from the interfacial regions of PS and PMMA.

As seen in the arrangement of protein molecules near the innermost circular line in Figures 5.3(A) and 5.1(h), a large number of proteins gather tightly on the PS regions next to concentric circular lines. These circular lines correspond to the interfaces between PS and PMMA, where the brighter and darker areas in the AFM images are PMMA and PS regions, respectively. Proteins prefer PS regions near the PS/PMMA interfaces to PS regions away from the interfaces. The number of protein molecules for a given PS area decreases as the distance along the R axis in Figure 5.3(A) increases from the PS:PMMA interface. In order to substantiate this effect, the total number of proteins is analyzed per each ring defined by the white circle inserted in the left AFM image in Figure 5.3(A). Figures 5.3(B) and 5.3(C) summarize our analysis on the protein adsorption behavior on PS/PMMA blend films. The circular line marked as 0 in the image was used as the reference point when determining the distance of proteins from the PS:PMMA interface. Figure 5.3(B) displays a plot of relative protein density versus distance from the point 0. Protein density in each white ring is normalized with respect to the protein density in ring
I. Ten topographic AFM images of either IgG or Fn bound PS/PMMA blend films were used to obtain the plot in Figure 5.3(B). For both IgG and Fn, the protein density decreases exponentially as the distance from the PS:PMMA interface increases. This trend confirms the tendency of proteins preferring PS regions adjacent to the PS:PMMA interfaces.

The repeating interfacial unit present on a PS-b-PMMA surface consists of two neighboring PS:PMMA interfaces separated by a PS region where proteins adsorb. The separation between the two PS:PMMA interfaces, i.e. the width of the PS region with adsorbed protein molecules, is on the order of tens of nanometers. To gain some insight on protein adsorption characteristics when the separation between the PS:PMMA interfaces increases, we calculated the number of adsorbed protein molecules when varying the separation distance between two PS:PMMA interfaces. This calculation was carried out by assuming the presence of two PS/PMMA interfaces, each of which follows the relationship of protein density to distance shown in Figure 5.3(B). Figure 5.3(C) displays a plot showing the correlation between protein density and interfacial separation distance. A larger number of adsorbed proteins is expected when the separation between two PS:PMMA interfaces is smaller. Separation of the two PS:PMMA interfaces on the nanometer scale, seen towards the left side of the plot in Figure 5.3(C), mimics the PS:PMMA interfaces on the PS-b-PMMA diblock copolymer surface. Homogeneous PS surface may be considered to have an infinitely large separation distance between the two PS:PMMA interfaces. As shown in Figure 5.3(C), protein density is inversely proportional to the separation distance between two neighboring PS:PMMA interfaces. Therefore, a smaller number of protein molecules on PS than on PS-b-PMMA is expected.
from the result of our analysis. This analysis is consistent with our experimental observation stated earlier. Higher protein density is observed on PS-b-PMMA than on PS, arising from the fact that chemically heterogeneous PS-b-PMMA presents periodically-spaced PS:PMMA interfaces on the nanometer scale whereas no such interfaces are present on PS homopolymer.

In order to evaluate systematically the density of protein molecules adsorbed on chemically homogeneous versus heterogeneous polymeric templates, enzymatic activity of HRP molecules is used as a convenient and quantitative density indicator. HRP is a widely used, highly specific, enzyme that catalyzes the reduction of hydrogen peroxide at a high turnover rate in a short period of time. HRP is chosen as a density indicator for two specific reasons; its importance in biomedical applications and its facilenes in quantitative activity measurement. It is commonly used not only in biological research but also in biomedical applications where its flexibility of usage can range widely from purifying antibody molecules, to detecting antigen-antibody complexes, and to investigating protein activity. In addition, the catalytic activity of HRP can be easily measured and quantified by using a chromogenic agent. Quantification of HRP activity can be straightforwardly carried out in the presence of a 3, 3’, 5, 5’-tetramethylbenzidine (TMB)/H2O2 indicator solution where the enzymatic activity is monitored by a color change of the indicator solution from colorless to blue. Active HRP catalyzes the oxidation process of TMB by H2O2. The presence of oxidized TMB in the assay is responsible for the distinctive color change which can be measured using a UV-vis spectrophotometer.
HRP molecules were first deposited onto the polymer substrates consisting of either PS homopolymer or PS-b-PMMA diblock copolymer. The samples were then thoroughly rinsed with deionized water in order to remove unbound enzymes and dried using a gentle stream of nitrogen. Then, a HRP-immobilized polymeric substrate was placed at the bottom of a UV-vis cuvette and 1 mL of as-received TMB solution was added to the vial. UV-vis absorbance spectra were subsequently recorded on a Hewlett Packard 8452A Diode Array Spectrophotometer at different time intervals. The characteristic absorption peak of oxidized TMB appears at 650 nm and, therefore, changes in the absorbance intensity were monitored for various HRP solution concentrations at the wavelength range between 500 and 750 nm. UV-vis absorbance data measured from varied concentrations of HRP on the two templates; chemically heterogeneous PS-b-PMMA and chemically homogeneous PS.

Further analysis of UV-vis absorption data was carried out in order to substantiate the unique and intriguing behavior of proteins where protein molecules adsorb in higher density on chemically heterogeneous surfaces than on chemically homogeneous surfaces. The absorbance intensity of the indicator solution is directly related to the amount of oxidized TMB in the solution which, in turn, is associated with the number of HRP molecules on polymeric surfaces. Figure 5.4 illustrates our comparative analysis of UV-vis absorbance between HRP molecules bound on PS-b-PMMA versus on PS templates. In both graphs in Figure 5.4, data points shown in red represent absorbance of HRP molecules immobilized on a PS-b-PMMA surface whereas those in blue represent absorbance of HRP on a PS surface. Figure 5.4(A) displays plots containing time-dependent absorbance values at $\lambda = 650$ nm. From top to bottom, plots correspond to
10(b), 2(r), 1.5(r), 2(b), 1(r), 1.6(b), 1.2(b), and 0.2(r) µg/mL of HRP. The notation of (r) and (b) specifies enzyme deposition onto PS-b-PMMA and onto PS, respectively. Figure 5.4(A) displays that, in order to attain the same absorbance intensity, deposition using a higher HRP concentration is needed on PS than on PS-b-PMMA templates. For all types of polymeric surfaces used in our template comparison study, proteins adsorb only onto the PS regions under our experimental conditions, see Figure 5.1. Due to this exclusive adsorption of proteins onto PS regions, structural conformation and integrity of HRP on both chemically heterogeneous and homogeneous templates can be considered comparable to each other after surface adsorption. Hence, the enzymatic activity of HRP in our template comparison study is only affected by the number of enzyme molecules on the surfaces. Data shown in Figure 5.4(A) clearly indicate that higher density protein substrates can be obtained on a chemically heterogeneous polymeric template than on a chemically homogeneous template when other experimental conditions are kept the same.

Figure 5.4(B) displays absorbance maxima plotted against the number of HRP molecules on the two types of polymeric surfaces. The total number of HRP molecules and the density of HRP molecules on the diblock and homopolymer templates are obtained by statistical analysis of AFM data. The total number of enzyme molecules on the PS-b-PMMA template (shown in red in Figure 5.4(B)) corresponds to $6.1 \times 10^9$, $10.1 \times 10^9$, $24.4 \times 10^9$, and $45.7 \times 10^9$ for the four specified HRP concentrations of 0.2, 1, 1.5, and 2 µg/mL, respectively. When taking into account the template area exposed to HRP adsorption, the average density of HRP on the PS-b-PMMA template is $16.9 \times 10^9$, $28.1 \times 10^9$, $67.8 \times 10^9$, and $127.0 \times 10^9$ molecules/cm$^2$ for the four HRP concentrations, respectively. For the PS homopolymer template, the total number of enzyme molecules
(shown in blue in Figure 5.4(B)) corresponds to $7.0 \times 10^9$, $8.2 \times 10^9$, $17.5 \times 10^9$, and $52.8 \times 10^9$ for the four specified HRP concentrations of 1.2, 1.6, 2, and 10 µg/mL, respectively. These values correspond to the average density of HRP on the PS of $19.4 \times 10^9$, $22.8 \times 10^9$, $48.6 \times 10^9$, and $146.7 \times 10^9$ molecules/cm², respectively. For the same HRP concentration, the number of adsorbed HRP molecules on the chemically heterogeneous diblock surface is greater than that of HRP on the chemically homogeneous PS surface. The density of HRP molecules on the heterogeneous PS-b-PMMA surface is approximately three to four fold higher than on the homogeneous PS surface. These measurements confirm that chemically heterogeneous templates are more effective in immobilizing protein molecules with a higher loading density than chemically homogeneous templates.

The underlying physical and chemical mechanism for the interesting adsorption behavior of proteins is currently under investigation. Diblock copolymer templates have been used in the past to assemble inorganic nanoparticles onto one of the polymer domains. Inorganic nanoparticles were delivered to diblock copolymer templates through an evaporation method or from a colloidal solution. Further annealing under an inert environment was necessary for achieving organized assembly of some inorganic particles. In these cases, nanoparticles situate at the center of their preferred polymeric domain, rather than favoring interfacial areas. Protein molecules in our experiments were delivered to diblock copolymer templates from a buffer solution by simply placing a droplet of solution onto a sample surface. The assembly of proteins on the polymeric templates and the segregation of proteins onto PS domains occur instantaneously at room temperature without any thermal treatment.
Protein adsorption can be governed by many factors such as hydrophobic/hydrophilic interaction, electrostatic interaction, hydrogen bonding, and van der Waals interaction. Hydrophobic index of a protein is an indication of the hydrophobic versus hydrophilic balance between all amino acid residues within a protein. The hydrophobic index of the proteins used in our experiment varies from 67 to 85. As PS-rich domain is more hydrophobic than a PMMA-rich domain due to the phenyl group present in PS, we speculate the dominating mechanism of the observed protein assembly to PS domains is through the hydrophobic interaction. Electrostatic interaction can also cause the preferred protein segregation to PS. The isoelectric point of the proteins used in our study ranges from 4.7 to 8. At our experimental condition of pH 7.4, these protein molecules exhibit neutral to negative net charges. These net charges of proteins may direct them towards PS and avoid the carboxylic acid ester group present in PMMA. The size of the proteins does not seem to play a significant role in their adsorption behavior. All proteins used in our experiment prefer PS domains, regardless of the molecular weight of the proteins used in our experiment which ranges from 45 to 580 kDa.

The presence of underlying polymeric films should also be considered in reasoning possible mechanisms for the observed phenomenon where proteins favor PS regions closer to the PS:PMMA interfacial areas. Topological variations such as film roughness within homopolymer films and height difference between different chemical regions within the diblock films can be a factor. The average film roughness of ± 0.5 nm is observed from homopolymer films of PS and PMMA samples and the height difference between PS and PMMA regions in diblock copolymer films is measured to be
1 nm, see Figure 5.1. When the topological variations of polymeric templates are compared to the known diameters of the protein molecules, variations of such film height are less than 10% of the physical sizes of the proteins. Therefore, we believe that the PS-favoring protein assembly is not related to height variations of the polymeric templates. The difference in the water contact angle between different polymer templates may serve as another factor. As the water contact angle on PMMA will be lower than that on PS, more proteins can be in immediate contact with the polymer surface in a more spread-out droplet on PMMA. However, more proteins adsorb on PS than on PMMA. In the case of protein assembly on the diblock films, the nanoscale repeat spacing provided by the diblock copolymer templates ensures that a single droplet covers a large number of alternating PS and PMMA domains and that the same number of protein molecules is in contact with the two domains equally. For these reasons, we do not believe that the difference in water contact angles can explain our observed phenomena. Rather, we hypothesize that the surface free energy lowered by proteins assembling on PS may explain the observed effects. The surface free energy of PS is slightly lower than that of PMMA. Further work is in progress in order to understand precisely the effect of proteins adsorbed onto different polymeric phases on the total surface free energy.

The ramification of our study is directly related to multiple crucial areas in designing array materials for biology and medicine. Polymers are often chosen as a candidate platform material for a variety of surface-based protein assays of optical, electrical, and mass-sensitive detection. Obtaining bioplatforms with a high protein loading density is one of the most essential design factors for the success of these applications. Our efforts point to a simple method that can be easily implemented to yield
a high protein payload by providing a chemically heterogeneous surface instead of a homogeneous surface. As protein adsorption behavior prefers PS regions that are near PS:PMMA interface areas, high payload protein templates can be better obtained from a chemically heterogeneous surface that presents densely populated interfaces of alternating polymer segments at the air:polymer boundary. Ultrathin films of diblock copolymers under carefully balanced thermodynamic conditions reveal alternating, chemically heterogeneous, nanometer-spaced interfaces on their film surfaces. In addition, this assembly of nanoscale templates with two distinctive chemical components is achieved via self-assembly. Therefore, our results indicate that diblock copolymers can serve as excellent, self-assembling, high payload protein templates.

5.4 Conclusions

In summary, we have evaluated and elucidated protein adsorption behavior on chemically homogeneous and heterogeneous polymeric surfaces by employing PS-b-PMMA diblock copolymer, PS homopolymer, PMMA homopolymer, and PS/PMMA blend as protein templates. Selective protein adsorption onto PS areas is observed for the chemically heterogeneous PS-b-PMMA and PS/PMMA blend templates. We have also investigated distance-dependent protein adsorption behavior from the interfacial regions of PS:PMMA using PS/PMMA blend templates. On blend films, protein adsorption is highly favored to the PS regions located near the PS:PMMA interface than the PS areas situated away from the interface. Specifically, we observed that protein density is inversely proportional to the separation distance between two neighboring PS:PMMA interfaces. We also observed that higher protein density is achieved on the PS-b-PMMA
than on the PS. This effect arises from the fact that chemically heterogeneous PS-b-PMMA presents periodically-spaced PS:PMMA interfaces on the nanometer scale whereas no such interfaces are present on PS homopolymer. The density of protein molecules on the heterogeneous PS-b-PMMA surface is approximately three to four fold higher than those on the homogeneous PS surface for the identical experimental conditions. In addition, these diblock copolymer templates are formed by spontaneous, self-assembly of protein patterns with a spot-to-spot spacing on the nanometer scale. Our results indicate that self-assembling, chemically heterogeneous, nanoscale domains of PS-b-PMMA diblock copolymers can be used as excellent, high payload, high density protein templates. The unique advantages of the diblock copolymer templates make the spontaneously-constructed protein nanoarrays highly suitable as functional substrates in many proteomics applications.

5.5 References


Figure 5.1: Schematic illustration and AFM images showing various polymeric surfaces before and after deposition of protein molecules. PS-b-PMMA diblock copolymer, PS homopolymer, and PS/PMMA blend ultrathin films were used as templates for protein adsorption. AFM images correspond to (a) PS-b-PMMA (750 x 750 nm), (b) IgG on PS-b-PMMA (380 x 380 nm), (c) PS (2 x 2 µm), (d) IgG on PS (500 x 500 nm), (e) PMMA (2 x 2 µm), (f) IgG on PMMA (500 x 500 nm), (g) PS/PMMA blend (2.5 x 2.5 µm), and (h) IgG on PS/PMMA blend (750 x 750 nm). Images (a), (b), (c), (e) and (g) are topography scans whereas (d), (f) and (h) are phase scans. Samples (b), (d) and (f) were prepared by depositing 4 µg/mL IgG on each polymeric template while 10 µg/mL IgG was used for the protein assembly in (h). Line profiles along the inserted white lines in (a), (c), (e), and (g) are also shown to display the topographic roughness of each polymeric template. PMMA-rich areas result in the brighter (higher) regions and PS-rich regions yield the darker (lower) regions in the diblock and blend AFM images. The AFM images acquired from the diblock and blend samples show clearly that IgG molecules segregate selectively onto the PS regions of the polymeric surfaces which present both PS and PMMA at the air:polymer boundary. This PS-favoring phenomenon was observed for all proteins used in our study.
Figure 5.2: AFM images displaying protein adsorption behavior on diblock copolymer and PS homopolymer templates. Panels (a) through (d) are 400 x 400 nm AFM scans of (a) BSA, (b) Fn, (c) HRP and (d) protein G molecules assembled selectively onto PS-rich regions on PS-b-PMMA or PS/PMMA blend films. 4 µg/mL protein solution was used for samples (a), and (d) whereas 1.5 µg/mL HRP solution was used for sample (b) and (c). All protein molecules used in our study favor PS domains. Panels (e) and (f) are 500 x 500 nm topographic images of IgG molecules adsorbed on two types of PS homopolymer films prepared from PS with a molecular weight of (e) 152,000 Da and (f) 70,400 Da. For a comparison, a 400 x 400 nm AFM image of IgG accumulated on PS-b-PMMA is displayed in (g). Regardless of the molecular weight of PS homopolymers, less protein molecules adsorb onto PS than on diblock copolymer templates when they were deposited from a 10 µg/mL solution.
Figure 5.3: (A) Typical AFM images of IgG molecules on PS/PMMA blend films. The four AFM images are 600, 450, 250, and 300 nm in diameter, respectively. Chemical make-ups specific to each polymeric domain in the films are specified in each AFM image. In all cases, IgG molecules selectively adsorb onto the PS areas and concentrate near the PS:PMMA interfaces. Inside the box, an AFM image (850 nm in diameter) of IgG molecules on a PS/PMMA blend template is shown as an example in order to indicate reference points and different areas used in the density analysis. Two conical sections of the 850 nm-wide AFM image are shown separately on the right in order to demonstrate clearly the tendency of proteins to prefer PS regions close to PS:PMMA interfaces. These sectioned images are shown along the R axis (away from the center of the circle) as marked in the left AFM image. Dotted white lines in the conical AFM images indicate the PS:PMMA interfaces. (B) A graph illustrating relative protein density versus spatial location of proteins away from the interface of PS and PMMA. Protein...
density from each area defined by two neighboring concentric circles (shown in white in the AFM image in (A)) is analyzed and plotted against the distance from the reference point 0 in (A). The number of protein molecules within each band of 40 nm in band width (indicated with Roman numerals in (A)) is obtained per given PS area for determining protein density. The protein density is subsequently normalized with respect to that of ring I. (C) Correlation of protein density to the interfacial separation distance between two PS:PMMA interfaces is shown. The relationship between the protein density and distance from a PS:PMMA interface, shown in (B), is used to calculate the number of protein molecules while varying the separation distance between two neighboring PS:PMMA interfaces. Protein density is inversely proportional to the separation distance between two neighboring PS:PMMA interfaces.
Figure 5.4: UV-vis absorbance comparison between HRP molecules bound on PS-b-PMMA versus on PS templates. In all graphs, red data points represent absorbance of HRP molecules immobilized on PS-b-PMMA whereas blue data points represent that of HRP on PS. To guide the eye, solid lines following data points are inserted. (A) UV-vis absorbance values of PS-b-PMMA-bound and PS-bound HRP recorded at $\lambda = 650$ nm are displayed with respect to time from the addition of the indicator solution. From top to bottom, plots correspond to 10(b), 2(r), 1.5(r), 2(b), 1(r), 1.6(b), 1.2(b), and 0.2(r) $\mu$g/mL of HRP. The notation of (r) and (b) specifies enzyme deposition onto PS-b-PMMA and onto PS, respectively. (B) Absorbance maxima are plotted against the number of HRP molecules on the two types of surfaces. The adsorbed amount of HRP on the chemically heterogeneous diblock surface is much greater than that of HRP on the chemically homogeneous PS surface at the same HRP concentration. These measurements confirm that chemically heterogeneous templates are more effective in achieving a higher loading density of protein molecules on surfaces.
CHAPTER 6
Fabrication of Optically Enhanced ZnO Nanorods and Microrods using Novel Biocatalysts

6.1 Background

Various structures of zinc oxide (ZnO), including thin films and micro/nano structures, have received considerable attention due to their attractive properties, which include a wide band gap of 3.37 eV and a large exciton binding energy of 60 meV at room temperature. ZnO can serve as an ideal candidate material for a broad range of high-technology applications such as short-wavelength light-emitting, field-emitting, luminescence, UV lasing, photovoltaic, and piezoelectric materials. The use of ZnO has been also demonstrated as surface acoustic waveguides, sensors, nanoresonators, transistors, and cantilevers.

For their rich potential in such applications, the synthesis and assembly of various ZnO micro/nanostructures have been extensively studied using gas-phase or solution-based approaches. The most commonly used gas-phase approaches to synthesize ZnO structures at the nanometer and micrometer scale are carried out via growth methods such as physical vapor deposition, pulsed laser deposition, chemical vapor deposition, metal-organic chemical vapor deposition, vapor-liquid-solid epitaxial mechanisms, and epitaxial electrodeposition. In solution-based synthesis approaches, methods such as hydrothermal decomposition processes and homogeneous precipitation of ZnO in aqueous solutions were pursued.
We focus our efforts on gas phase synthetic methods since as-grown ZnO structures using gas phase approaches are relatively atomic defect-free when compared to ZnO materials produced using solution-based synthetic methods. Depending on specific growth conditions, ZnO exhibits a variety of nano- and micro-structures resembling combs, rings, helixes/springs, bows, belts, wires, rods, propellers, and cages. Such variations in the morphologies of ZnO are governed by factors such as substrates, carrier gases, local gas concentrations, temperatures, and time. Catalysts also play an important role in their synthesis and a wide variety of catalysts such as Au, Ag, Co, Sn, Ni, NiO, Ge, Al, Zn, and Pt have been previously used for gas-phase growth to produce the rich family of ZnO structures.

Herein, we describe a straightforward method to produce ZnO nanorods (NR) and microrods (MR) using a novel catalyst, *Magnetospirillum magnetotacticum* (MS-1). The MS-1 bacterium synthesizes intracellular, linear, single-domain magnetite nanoparticles through highly regulated biomineralization. We utilize the highly monodisperse iron oxide (Fe$_3$O$_4$) nanoparticles inside MS-1 as catalysts to grow ZnO nano- and micro-structures with high optical quality. MS-1 has been previously used as effective biocatalysts to grow multiwalled carbon nanotubes showing a narrow distribution in diameter. In this paper, we demonstrate that MS-1 can serve as effective catalysts to produce atomic defect-free ZnO NR and MR structures with unparalleled optical properties which, in turn, can promote new opportunities in photonic-based applications on the nanometer and micrometer scale.
6.2 Experimental Details

*Magnetospirillum magnetotacticum* (ATCC #31632) was grown microaerobically in a chemically defined growth medium. The presence of succinic acid and sodium nitrate served as a principal source of carbon and nitrogen, while iron was supplied to the bacteria by adding 0.01 M ferric quinate to the culture medium. Silicon wafers (resistivity < 1 Ω cm, thickness: 0.017 inch) were obtained from Silicon Inc. Poly-L-lysine (PLL) in H$_2$O (0.1% w/v) was obtained from Ted Pella, Inc. and was diluted (1:100) in de-ionized water before use. Zinc Oxide (99.999%) and graphite (99 %) powders were obtained from Alfa Aesar. To pattern substrates with catalysts at predetermined locations, PDMS stamps containing periodic patterns of 10 µm in width were constructed. They were fabricated by casting and curing an elastomeric polymer, Sylgard 184 (Dow Corning), against a photoresist micropatterned silicon master which was fabricated using standard photolithography procedures.

For homogeneous catalyst deposition, 50 µL of bacterial solution (optical density: 0.129) was deposited on silicon substrates for 30 min in the presence of a magnetic field by placing a bar magnet (170 mT) under the growth substrates. For patterned catalyst deposition, PDMS stamps were inked with 50 µL PLL and gently blow-dried with N$_2$ to remove excess solution. Subsequently, 50 µL of bacterial solution was placed on the PLL inked PDMS stamps. The catalysts were immediately transferred onto growth substrates by microcontact printing MS-1 for 30 sec. The periodically-patterned bacterial catalysts on silicon surfaces were then treated with distilled water. This process led to cell lysis by osmotic shock and exposed Fe$_3$O$_4$ nanoparticles within the bacterial cells. Remaining
cellular membranes and debris on silicon substrates were removed by rinsing with deionized water and then by annealing at 800 °C in air for 1 h.

Various ZnO structures were then grown on silicon substrates by heating a 2:1 mixture of graphite and zinc oxide powder at 900 °C for 30 min under a 100 standard cubic centimeters per minute (sccm) argon flow. A source boat containing the mixture powder was kept at the center of a horizontal tube furnace and substrates were kept at 5 inches away in the downstream from the source boat. Light grey material was found on the substrates after growth.

Atomic force microscope (AFM) was used to investigate the morphology of individual catalyst nanoparticles. The measurements were carried out using Digital Instruments, Dimensions 3100, in tapping mode at a scan speed of 1 Hz. Silicon tips with a resonant frequency of 60 kHz and a spring constant of 5 N/m were used in the measurement. Scanning electron microscope (SEM) was used to determine the morphology of various as-grown ZnO structures as well as their size distribution. SEM was performed using FEI/Philips XL 20 operating at 20 kV. The optical properties of as-synthesized ZnO were examined by carrying out room-temperature PL measurements. A pulsed nitrogen laser (3.5 ns, 337 nm) was used as an excitation source. The synthesized materials were also characterized by X-ray diffraction (XRD). XRD measurements were made on a Bruker AXS P4 using Cu Kα radiation under an accelerating voltage of 50 kV.

6.3 Results and Discussions

In order to assess the catalytic effect of MS-1 on producing semiconducting ZnO nano- and micro-structures, we used the two following experimental schemes. We first
demonstrate that MS-1 can serve as active catalysts to grow ZnO by depositing MS-1 homogeneously on silicon substrates and, secondly, that these ZnO structures can be effectively and conveniently arranged in periodic patterns on substrates over large areas by microcontact printing the bacterial catalysts. Microcontact printing of MS-1 over a large area of substrates was carried out using polydimethyl siloxane (PDMS) stamps prepared with standard photolithography techniques. These fabricated PDMS stamps contained regularly-spaced rectangular patterns of 10 μm in width and 3 cm in length. A schematic representation of the two experimental procedures employed in our experiments is shown in Figure 6.1. ZnO structures varying in size were then grown over MS-1 catalysts on silicon substrates by heating a 2:1 mixture of ZnO and graphite powder at 900 °C for 1 hour using Ar as a carrier gas.

The specified growth conditions led to the growth of ZnO NR structures on samples containing homogeneously deposited MS-1 catalysts whereas ZnO MR structures were observed from samples prepared by microcontact printing MS-1 catalysts. Figures 6.2 and 6.3 present typical SEM images of the nano- and micro-structures of ZnO that were synthesized using the two catalyst deposition schemes. Figures 6.2(A)-(B) and 6.2(C)-(F) display SEM images taken from ZnO structures that were produced using the former and the latter method, respectively. The one-dimensional structures of both ZnO NRs and MRs reveal hexagonal morphologies, which are clearly visible in the SEM images of Figures 6.2(E and F) as well as the insets in Figures 6.3(A and B).

As-synthesized ZnO NRs exhibited an average width of 590 ± 190 nm and ZnO MRs dispalyed an average width of 1068 ± 52 nm. ZnO NRs had a higher aspect ratio in width:length (~ 1:7) than ZnO MRs (~ 1:15) whereas ZnO microstructures produced over
microcontact-printed MS-1 showed a much narrower distribution in their width (less than 5% in coefficient of variation). After the transfer of MS-1 to selectively and periodically arranged areas on substrates, the growth of ZnO MRs took place only on patterned catalytic regions on substrates. This effect clearly demonstrates the catalytic effect of Fe₃O₄ nanoparticles inside MS-1 for ZnO growth. The microcontact printing method was highly efficient for the growth of ZnO structures at predetermined locations over large areas of substrates, see Figure 6.3(B).

XRD was used to determine the crystal structure of the ZnO structures fabricated using MS-1 as catalysts on silicon substrates. Figure 6.3(C), displaying the XRD pattern of ZnO NRs, indicates that these ZnO materials exhibit Wurtzite structures. The pronounced peak at 2θ= 34.5° which corresponds to (0002) facet of Wurtzite ZnO specifies <0001> as the preferential growth direction. Room-temperature photoluminescence (PL) effect of these ZnO NR and MR structures was examined in order to assess optical properties of as-grown ZnO materials. Figure 6.3(D) shows a typical PL spectrum of ZnO NRs and MRs grown over MS-1. PL spectrum of as-synthesized ZnO NRs and MRs showed extremely strong and sharp UV emission at 390 nm and very weak green emission at 510 nm. The exceptionally narrow UV emission to this degree (full-width at half maximum of ~ 7 nm) has not been demonstrated previously by using any other synthetic methods.

The UV emission corresponds to near band-edge emission of the semiconductor ZnO NRs and MRs whereas the very weak green emission is due to deep-level or trap-state emission. The green emission results from radiative recombination of a photo-generated hole with an electron in the oxygen vacancy. The ultra-sharp UV emission
obtained from our ZnO samples, in conjunction with their lack of green emission, indicates that MS-1 is capable of producing atomic defect-free ZnO NRs and MRs of enhanced optical quality. These defect-free ZnO nano-and micro-materials grown on MS-1 may open the possibility of creating one-dimensional structures of high optical quality for applications such as optoelectronic devices and chemical/biological sensor arrays.

6.4 Conclusions

In summary, we developed a straightforward method to produce hexagonal ZnO NRs and MRs using a novel catalyst, MS-1. ZnO NRs were synthesized homogeneously on growth substrates when MS-1 catalysts were deposited uniformly on substrates whereas ZnO MRs were formed when MS-1 catalysts were introduced to selective areas of growth substrates using microcontact printing. XRD patterns reveal that these ZnO NRs and MRs exhibit Wurtzite structures with preferential growth along [0001] direction. Room-temperature PL spectra of the as-synthesized ZnO NRs and MRs show extremely strong and sharp UV emission at 390 nm and very weak green emission at 510 nm. Our results demonstrate that MS-1 is an excellent catalyst for the growth of nanometer- and micrometer-sized ZnO structures with high-quality optical properties. These defect-free ZnO nano-and micro-materials, when combined with microcontact printing techniques to achieve patterned growth over large areas of substrates, can facilitate their photonic-based applications as optoelectronic devices and chemical/biological sensors.
6.5 References


Figure 6.1: Schematic illustration showing the two experimental schemes to produce ZnO NR and MR structures using MS-1 as catalysts. (A) Homogeneous deposition process of MS-1 catalysts and (B) patterned deposition process of MS-1 using PDMS stamps. The atomic force microscope image in the middle, 500 by 500 nm, shows intracellular Fe₃O₄ nanoparticles inside MS-1 after cell lysis.
Figure 6.2: SEM images of hexagonal ZnO NRs and MRs grown over MS-1. (A and B) ZnO NRs prepared by homogeneously depositing the catalysts on silicon substrates. (C through F) ZnO MRs prepared by microcontact printing the bacterial catalysts on selective areas of substrates. The scale bars inserted in images A through F correspond to 10, 5, 20, 10, 5, and 2 µm, respectively.
Figure 6.3: ZnO NRs and MRs fabricated using MS-1 catalysts. (A and B) SEM images of ZnO NRs homogeneously distributed on substrates (A) and ZnO MRs patterned periodically on substrates (B). The inserted scale bars in (A) and (B) are 100 µm and 50 µm, respectively. The insets shown in the upper right corner in (A) and (B) are 5 by 5 µm. The insets shown in the lower right corner in (A) and (B), clearly displaying the hexagonal NR and MR structures of as-grown ZnO, correspond to 1.5 µm and 2 µm, respectively. (C) XRD pattern of ZnO NRs on a silicon substrate. (D) Typical room-temperature PL spectrum of ZnO NRs and MRs grown over MS-1.
7.1 Background

Fluorescence detection of biomolecules is a heavily relied on technique in gene profiling, proteomics, drug discovery, disease diagnostics, and environmental analysis. Novel methods which enable rapid, high-throughput, ultrasensitive, and specific optical detection are in great demand for the burgeoning areas of genomics and proteomics.\textsuperscript{1-4} For many biomolecular detection techniques exploiting fluorescence, enhancing detection sensitivity and increasing the signal-to-noise ratio still remain as major challenges in carrying out the much needed, system-wide study of proteins and population-level genetic screening.

To improve the fluorescence detection capability and resolution, numerous research efforts have been made in parallel on three main aspects of biomolecular fluorescence detection: (1) molecular design of better fluorophores, (2) development of improved detection apparatus, and (3) engineering of advanced substrates. New organic, inorganic, and hybrid labels were developed to prevent photobleaching of fluorescing dyes while allowing measurements of multiple fluorophores with a single excitation source at very low concentration levels.\textsuperscript{5-10} Advanced confocal optics and more reliable miniaturized detection devices were developed in order to increase detection sensitivity, in some cases down to the single molecule level.\textsuperscript{11-16} The use of metallized substrates has been explored to increase quantum yield and photostability of fluorophore labels.\textsuperscript{17-19}
Focusing on the development of novel bioarray substrates suitable for fluorescence detection, we herein report for the first time the development and utilization of nanoscaled zinc oxide (ZnO) platforms for use as attractive substrates in enhanced fluorescence detection of biomolecules such as proteins and DNA.

ZnO thin films and micro/nano structures have received considerable attention in the past particularly due to their desirable optical properties, which include a wide band gap of 3.37 eV and a large exciton binding energy of 60 meV at room temperature. ZnO has been previously demonstrated as a candidate material for use in a broad range of optical and optoelectric applications. Examples of ZnO applications in these areas include short-wavelength light-emitters, field-emitters, luminescence, and UV lasers. In addition to their rich potential in these applications, sensitization of ZnO by organic dye molecules has been extensively studied for use as highly efficient solar cells. However, biosensing applications of wide band gap ZnO have not been previously demonstrated although nanometer scale ZnO is stable in typical biomolecular detection environments and an easily processed material which is ideal for aiding optical detection of target bioconstituents.

In this paper, we demonstrate that engineered nanoscale ZnO structures can significantly enhance fluorescence detection capability toward sensing proteins and nucleic acids without any need for amplification. We also report the use of ZnO in the enhanced fluorescence detection of protein interactions. We demonstrate that ZnO nanomaterials can serve as excellent signal-intensifying substrates for a wide range of protein systems. In addition, we demonstrate that these nanostructured ZnO building blocks can be easily assembled into biosensor platforms of predetermined dimensions.
during their synthesis while eliminating additional post synthetic processes. We confirm that this signal enhancing capability of nanoscale ZnO is not influenced by the specific emission wavelength of fluorophores that are linked to protein molecules. Our results suggest that ZnO nanoplatforms can be conveniently fabricated into fluorescence enhancing substrates and straightforwardly assembled into a tailor-made array format which, in turn, will promote highly sensitive, multiplexed, high-throughput, fluorescence detection of target biomolecules.

7.2 Experimental Details

7.2.1 Preparation of substrates

(a) Homogeneous and Patterned ZnO Growth. Silicon wafers (resistivity < 1 Ω cm, thickness: 0.017 in.) were obtained from Silicon Inc. Poly-L-lysine (PLL) in H₂O (0.1% w/v) and Ag colloids (40 nm in diameter) were obtained from Ted Pella, Inc. Zinc oxide (99.999%) and graphite (99%) powders were obtained from Alfa Aesar. A total of 100 µL of 40 nm Ag colloid was deposited on a PLL treated Si wafer for 30 min and the substrate was then gently blow dried with nitrogen. The growth wafer was placed approximately 5-6 in. downstream from a 2:1 mixture of graphite powder and zinc oxide which was kept at the center of a horizontal resistance furnace. The sample was subsequently heated to 900 °C for 1 h under a constant flow of 100 standard cubic centimeters per minute (sccm) of Ar. A light gray material was visible on the Si wafer surfaces following the growth. To pattern the substrates with catalysts at predetermined locations, poly-(dimethylsiloxane) (PDMS) stamps containing periodic patterns of 10 or 20 µm in width were constructed by casting and curing an elastomeric polymer, Sylgard 184 (Dow Corning), against a photoresist micropatterned silicon master which was
fabricated using standard photolithography procedures. A total of 50 µL of PLL placed on the PDMS stamp was gently blown dry with nitrogen and was then transferred onto clean growth wafers for 30 s. Following elastomeric stamping, the samples were treated with 100 µL of Ag colloid (40 nm in diameter) for 30 min. ZnO nanomaterials were synthesized using the same conditions as described in the homogeneous growth.

(b) SiNR Growth. A total of 10 µL of 50 nm Au colloid, obtained from Ted Pella Inc., was deposited for 10 min onto a clean Si wafer whose surface was pretreated with a thin layer of PLL. The growth wafer was then placed in a chemical vapor deposition (CVD) reactor for subsequent SiNR synthesis. The reactor was evacuated to a pressure of less than 1 mTorr and heated to 550 °C under a flow of 20 sccm of Ar and 60 sccm of H₂. SiNRs were then grown for 20 min by introducing 3 sccm of SiH₄ (10% in Ar). The total pressure in the reactor was controlled at 70 Torr during growth.

(c) Patterned Polymeric Films. PMMA (Mw = 120,000 g/mol) was obtained from Alfa Aesar. Si substrates were cleaned with ethanol, acetone, and toluene and spun dry before coating the polymer films. Ultrathin homopolymer films were prepared on Si substrates by spin casting 2% (w/v) PMMA in toluene at 3500 rpm for 1 min. Patterned polymeric films were then constructed by imprinting periodic stripe patterns of 10 or 20 µm on a PDMS stamp to the ultrathin polymer films. Subsequent annealing of the polymer films at 150 °C for 6h in a resistance furnace under Ar atmosphere yielded patterned PMMA films.

(d) ZnO Thin Film. ZnO thin films have been prepared by radio frequency magnetron deposition of pure ZnO (99.999%) at 300 °C in a multisource sputtering system CMS-18 supplied by Kurt J. Lesker Co. The base pressure in the process chamber
was $1.7 \times 10^{-7}$ Torr. High purity Ar/15% O$_2$ at 5 mTorr in a continuous flow mode was used as a working gas. The final film thickness was 100 nm as measured by a Tencor Alfa Step surface profiler.

### 7.2.2 Preparation of proteins and DNA

(a) **Model Protein System.** A fluorescein-conjugated antibovine IgG (FITC-antIgG) was obtained from Rockland Inc. and diluted to various concentrations ranging from 200 µg/mL to 2 mg/mL in PBS buffer (10 mM mixture of Na$_2$HPO$_4$ and NaH$_2$PO$_4$, 140 mM NaCl, 3 mM KCl, pH 7.4). Tetramethyl rhodamine isothiocyanate conjugated bovine IgG (TRITC-IgG) was obtained from Rockland Inc. and reconstituted in deionized water as recommended by the manufacturer. Cyanine 3 human serum albumin (Cy3-HSA) was received from Jackson ImmunoResearch Inc. and the freeze-dried protein was reconstituted in deionized water to various concentrations. As needed, 100 µL of protein solution at desired concentration was deposited onto various ZnO substrates for 30 sec to 1 min. Following the protein deposition, unbound proteins were removed by thoroughly rinsing with ample amounts of PBS and/or deionized water. The sample was then gently blow dried with nitrogen before imaging with a confocal microscope.

Immunopure biotinylated bovine serum albumin (BBSA) was obtained from Pierce Inc. Dichlorotriazinylaminofluorescein (DTAF-streptavidin) was received from Beckman-Coulter Inc. The lyophilized BBSA and DTAF-streptavidin powder were reconstituted in deionized water as recommended by the manufacturer. 100 µL of 200 µg/mL of BBSA was deposited onto a ZnO substrate for 1 min. The substrate was rinsed
with ample amounts of deionized water multiple times and subsequently dried under a gentle stream of nitrogen. Immediately following BBSA deposition, 100 µL of 200 µg/mL of DTAF-streptavidin was placed onto the BBSA covered ZnO surface for 40 min. Any loose proteins were rinsed away with multiple rinsing of deionized water and the sample was dried under a stream of nitrogen for fluorescence imaging.

Simultaneous monitoring of multiple protein/protein pairs was carried out on the same ZnO substrate by using a PDMS elastomer that contained two reaction chambers. Bovine IgG whole molecule (IgG) and fibronectin bovine plasmon (Fn) were obtained from Rockland Inc. and EMD Biosciences Inc., respectively. The PDMS piece conformed to the underlying ZnO substrate and provided isolated compartments for conducting two independent protein/protein reactions. 5 µL of Fn and IgG at a concentration of 200 µg/mL were first deposited into the two separate chambers. After 5 min, the protein solutions were carefully removed from the chambers and the protein-covered ZnO surfaces in the chambers were rinsed with deionized water multiple times. Subsequently, 5 µL of 200 µg/mL FITC-IgG was added to both chambers and the protein pairs were allowed to react for 20 min at room temperature. Before disengaging the PDMS piece from the ZnO substrate, excess FITC-IgG was carefully removed and unbound protein molecules were rinsed with an ample amount of deionized water.

(b) Model DNA System. DNA oligonucleotides specific to *B. anthracis* (bas:5'-AGTGCAGGAGGCT-3') and *Bacillus cereus* (bce:5'-GTTACCGAAAGACCA-3') were custom synthesized from Eurogentec Inc. A compliment oligonucleotide to bas was custom-made with a 6-carboxyfluorescein (FAM) labeled on its 5' end (basr:5'-FAM-TCACGCGCTCTCGCA-3'). All oligonucleotides were reconstituted in TE
buffer solution (10 mM Tris, 1 mM EDTA, pH 8.0). ZnO nanoplatforms were first treated with either *bas* or *bce*. For subsequent hybridization reaction, 100 µL of *basr* in reaction buffer (10 mM Tris, 1 mM EDTA, 10 mM MgCl₂, 50 mM NaCl, pH 8.0) of desired concentration was deposited onto the single strand-modified ZnO nanoplatforms and incubated for 1 h at room temperature in a humidity controlled environment. Immediately before confocal and fluorescence imaging, the substrates were rinsed thoroughly with TE and blown dry under a gentle stream of nitrogen.

### 7.2.3 Sample characterization

FEI/Philips XL 20 operated at 20 kV was used in the SEM characterization of as-grown nanomaterials of ZnO and SiNRs. The confocal microscope data was collected using a conventional confocal laser scanning microscope (Olympus Fluoview 300) with an Ar (488 nm, 40 mW) laser at a resolution of 512 pixels. The fluorescence from biosamples was separated from the excitation light by a dichroic beam splitter and a 510-535 nm band-pass filter. The microscope was also equipped with a 100 W mercury arc lamp (Osram) which allowed overall inspection of a large sample area in a single view frame. An epi-fluorescent upright microscope (Olympus BX-60), interfaced with a computer controlled focus drive and a Hamamatsu Orca-100 camera, was used to capture digital images of samples. Image Pro Plus software was used for subsequent image analysis.
7.3 Results and Discussions

7.3.1 Detection of proteins

We first determined the suitability of the wide band gap ZnO nanomaterials as optical signal enhancing platforms in biomolecular detection. The ZnO-mediated fluorescence signal was measured and compared with other nanoscale materials such as silicon nanorods (SiNRs) as well as other commonly used biosensor substrates such as glass slides, quartz slides, silicon surfaces, and polymers. The overall experimental designs to examine various substrates systematically are illustrated schematically in Figure 7.1. ZnO (Figure 7.1a, b) and SiNRs (Figure 7.1c) were grown on Si wafers using chemical vapor deposition on Ag and Au catalysts, respectively. For regularly patterned ZnO synthesis (Figure 7.1b), microcontact printing techniques were employed to deliver the catalysts selectively at predetermined locations on Si wafers which subsequently led to directed ZnO growth only on patterned areas. The average diameter of as-grown ZnO and SiNRs measured by scanning electron microscopy (SEM) are 677 ± 65 and 547 ± 17 nm, respectively. Ultrathin films of poly (methyl methacrylate) (PMMA) were produced by spincoating and subsequent thermal annealing under Ar environment. The average thickness of the PMMA films, determined by ellipsometry, is 98 ± 2 nm. Nanoimprint lithographic techniques were used to generate periodic, micrometer scale patterns on these polymeric films (Figure 7.1d). These substrates were then incubated with fluorophore-labeled proteins or DNA to allow direct adsorption of biomolecules onto surfaces. A commercially available confocal microscope was used for fluorescence detection, and the excitation and detection wavelengths were chosen according to the specific emission properties of the fluorophores that were employed in our proof-of-concept
experiments. As our experimental scheme involves nonspecific adsorption of these biomolecules onto substrates, biomolecules were randomly distributed over the entire surface area upon deposition.

A fluorescein-conjugated antibovine IgG (FITC-antiIgG) was used as a model system as these protein molecules are commonly used for many basic biological and biomedical applications such as detecting the presence of antigen-antibody complexes via enzyme-linked immunosorbent assays or Western blots. No measurable fluorescence signal, under our experimental conditions, was detected from the model protein molecules deposited on various control substrates which include Si, SiNR, glass, quartz, and PMMA surfaces (Figure 7.2a, d). Even at higher concentrations up to 2 mg/mL, no fluorescence was observed from proteins prepared on these surfaces under our experimental conditions. Direct comparison of the fluorescence signal observed by the use of two different one-dimensional systems, comparably sized ZnO nanorods versus SiNRs, resulted in extremely strong fluorescence emission from ZnO nanoplatforms but negligible emission from SiNR platforms (Figure 7.2d). Therefore, the observed fluorescence enhancement is attributable to the inherent material properties of ZnO and not due to the larger surface area of the nanomaterials as compared to glass or quartz surfaces. To gain better insight into possible size effects of planar ZnO versus ZnO nanomaterials on fluorescence enhancement, biomolecular fluorescence emission was measured and compared on two different types of the same ZnO material, two-dimensional ZnO thin films, and one-dimensional ZnO nanorods. FITC-antiIgG molecules on 100 nm thick ZnO thin films show increased fluorescence relative to the other planar materials such as glass, silicon, and quartz (Figure 7.2d). However, the effect
is ~1/5 of the enhancement observed from ZnO nanoplat forms. This result suggests that the reduced dimensionality of ZnO nanorods, when compared to ZnO thin films, plays a very important role in the fluorescence enhancement effect.

To ascertain the ability of ZnO nanomaterials in promoting biomolecular fluorescence detection, various concentrations of FITC-antiIgG were applied to substrates containing individual ZnO nanomaterials. Fluorescence from the biomolecules adsorbed on individual ZnO nanomaterials were clearly visible using confocal microscopy (Figure 7.2b). In contrast to the intense green emission observed from FITC-antiIgG molecules on individual ZnO nanomaterials, no fluorescence was detected from the same biomolecules that were adsorbed elsewhere on the substrates. To substantiate our findings, substrates fabricated with periodically patterned and densely grown ZnO nanomaterials were then employed as substrates. Confocal microscope measurements confirmed that fluorescence signal was observable only from FITC-antiIgG supported on ZnO nanomaterials. The confocal image of the protein fluorescence displayed in Figure 7.2c faithfully mimics the corresponding scanning electron microscope images of the underlying ZnO nanoplat forms containing regularly striped and micrometer-spaced ZnO patterns.

The fluorescence intensity and detection limit were investigated and compared between various substrates including ZnO nanoplat forms (Figure 7.2d, e). Fluorescence intensities emitted from FITC-antiIgG molecules on ZnO nanoplat forms were 3 orders of magnitude higher when compared to that on Si, SiNR, glass, quartz, and PMMA substrates at the same protein concentration of 200 µg/mL. When using FITC-antiIgG on ZnO nanoplat forms as a model system, the lowest concentration limit for fluorescence
detection using a 40 mW scanning Ar laser was 2 ng/mL under our confocal measurement conditions (Figure 7.2e) and 0.02 ng/mL using a 100 W mercury arc lamp (data not shown). The lowest detection limit is determined by the protein concentration for which the observed fluorescence signal exceeds the baseline noise by a factor of 3. For protein concentrations higher than 2 µg/mL, the fluorescence intensity data collected from ZnO NR platforms display a shallower slope than those from lower concentrations, possibly due to an increased self-quenching effect of the fluorophores (Figure 7.2e).

7.3.2 DNA detection

Rapid differentiation and accurate identification of bioagents such as *Bacillus anthracis* (*B. anthracis*) is crucial to planning timely and appropriate measures for public safety. To evaluate the broad applicability of ZnO nanoplanforms in the enhanced fluorescence detection of DNA sequence variations, two oligonucleotides were designed where each sequence is specific to either *B. anthracis* (**bas**:5′-AGTGCGCGAGGAGCCT-3′) or *Bacillus cereus* (**bce**:5′-GTTACGGAAACCA-3′). In addition, 6-carboxyfluorescein modified oligonucleotide (**basr**: 5′-TCACGCGCTCCTCGGA-3′) that is fully complementary to the DNA sequence of **bas** was synthesized. DNA hybridization reactions were subsequently carried out, and the fluorescence signal from duplex formed DNA was examined using confocal and fluorescence microscopy.

Two control experiments were carried out. As-grown ZnO substrates were examined for fluorescence emission in the detection wavelength range in order to confirm that they are devoid of autofluorescence, and ZnO nanoplanforms modified with **bas**
strands alone were also tested to verify no fluorescence emission. For the subsequent hybridization experiments involving fully matching versus noncomplementary DNA sequences of the same concentration, no fluorescence was detected from samples containing the two strands of *bce* and *basr* due to the DNA sequence mismatch (Figure 7.3a). On the contrary, intense fluorescence signal was apparent when complementary *basr* strands were introduced to *bas* on patterned ZnO platforms as the two fully complementary oligonucleotide strands of *bas* and *basr* formed duplex DNA (Figure 7.3b). Without any need of amplification, ZnO nanoplatforms permit successful fluorescence detection of fluorophore labeled DNA molecules at DNA concentrations as low as tens of attomolar range when detecting with the mercury arc lamp and a few nanomolar range when using the Ar laser. Plots in Figure 7.3 display fluorescence intensity as a function of DNA concentration measured with the Ar laser. Our data indicates that ZnO nanostructures facilitate and permit differentiation of the anthrax causing isolate of *B. anthracis* from its closely related species which does not pose a biological threat. This proof-of-concept experiment verifies that ZnO nanoplatforms can effectively differentiate nucleic acid sequence variations even at ultratrace-level DNA concentrations by serving as excellent fluorescence enhancing substrates.

### 7.3.3 Study of protein-protein interactions

Four types of ZnO nanoplatforms were used as needed in our experiments: i) individual ZnO nanorods, ii) striped ZnO arrays, iii) open square ZnO arrays, and iv) filled square ZnO arrays. To evaluate the use of ZnO nanoplatforms in the enhanced fluorescence detection of interacting proteins, 200 μg/mL of biotinylated bovine serum
albumin (BBSA) was deposited onto a ZnO substrate containing periodic patterns of filled square arrays and the substrates were subsequently rinsed multiple times with a phosphate saline buffer in order to remove unbound proteins. Figure 7.4-1 displays an SEM image of as-grown ZnO nanomaterials composing filled square arrays of 5 µm in size with the same repeat spacing. The confocal images taken from the as-grown ZnO substrate as well as the BBSA-covered ZnO nanoplatform did not yield any fluorescence in the detection wavelength range as shown in Figures 7.4-2 and 7.4-3. 200 µg/mL of streptavidin molecules conjugated with dichlorotriazinylaminofluorescein (DTAF-streptavidin) was then introduced to the ZnO nanoplatform in order to initiate biomolecular reactions between the two proteins. When ZnO samples were examined under a confocal fluorescence microscope after thorough rinsing to remove unreacted protein molecules, intense green emission was present from BBSA/DTAF streptavidin molecules supported by the underlying ZnO nanomaterials. The markedly strong fluorescence emission is due to the protein complex formation between BBSA and DTAF-streptavidin proteins via highly favored biotin/streptavidin reaction, Figures 7.4-4 and 7.4-5.

In order to determine broad applicability of ZnO nanoplatforms to screen for wide-ranging protein-protein interaction, protein G (PG) and FITC-antiIgG reacted on individual ZnO nanorods were used as a model system. The concentration of both proteins used in this experiment was 200 µg/mL. Figure 7.5a-1 displays a SEM image of individual ZnO nanorods as grown in a lying-down orientation on silicon wafers. The average length and width of the ZnO nanorods are 4.1 ± 0.3 µm and 313.3 ± 68.3 nm, respectively. Neither as-synthesized ZnO nanorods nor PG-adsorbed ZnO nanorods
resulted in any fluorescence emission in the detection wavelength range (data not shown). When PG-covered ZnO nanorods were further reacted with FITC-antiIgG, intense green emission was clearly visible in the confocal images, as displayed in Figures 7.5a-2 and 7.5a-3. The acquired fluorescence signal is attributable to the PG/FITC-antiIgG protein complex formed on individual ZnO nanorods.

Our approach involving ZnO nanostructures in the enhanced fluorescence detection was then extended to identify the presence or absence of multiple protein/protein interactions on the same substrate. This experiment was designed to examine carefully two possible contributing factors in positive protein fluorescence detection other than the signal enhancing effect of ZnO nanoplatforms. Specifically, the experiment allows us to eliminate potential errors from any substrate to substrate variations that may be introduced during the growth of ZnO. The experiment also permits us to ascertain that the observed fluorescence signal from the model systems of two interacting protein pairs in our study is solely due to the formation of the protein/protein complex and not attributable to possible nonspecific adhesion of fluorophore-labeled protein to the underlying ZnO nanostructures. A patterned substrate comprised of striped ZnO nanostructures of 20 µm in width and repeat spacing was used as a test bed, Figure 7.5b-1. A PDMS piece which contained two reaction chambers was placed on top of the ZnO test bed before the introduction of protein solutions.

200 µg/mL of two different proteins, fibronectin (Fn) and IgG were subsequently introduced to the chambers 1 and 2, respectively. After rinsing these proteins in their chambers multiple times, 200 µg/mL of FITC-antiIgG solution was added to both chambers for a possible protein/protein interaction to take place. After the reaction,
samples were rinsed carefully and thoroughly with the buffer solution and unreacted protein molecules were removed from the ZnO nanoplatform. No discernable fluorescence signal was observed from the chamber 1 due to lack of protein interaction between Fn and FITC-antiIgG whereas strong green signal was identified from ZnO nanostructures in the chamber 2 owing to a protein complex formation between the interacting pairs of IgG and FITC-antiIgG. Similarly, 200 µg/mL of BBSA and IgG were introduced to the chambers 3 and 4, respectively. After rinsing these proteins in their chambers multiple times, 200 µg/mL of DTAF-streptavidin solution was added to both chambers in order to investigate interacting protein systems. Strong fluorescence emission was unambiguously detected in chamber 3 as displayed in the corresponding confocal image in Figure 7.5b. However, no discernable fluorescence signal was monitored from chamber 4 due to the lack of interaction between the two protein pairs of IgG and streptavidin. The confocal images displayed in Figure 7.5b clearly demonstrate that ZnO nanoplatforms can be successfully used in the enhanced fluorescence detection for screening presence or absence of specific protein/protein interactions. With the combined use of microfluidic chambers to provide many protein reaction chambers, our approach using ZnO nanostructures can serve as ideal substrates for multiplexed, high throughput, enhanced fluorescence detection.

Lastly, versatility of ZnO nanoplatforms in the enhanced fluorescence detection was assessed by utilizing various fluorophores in our model systems. Commonly used dyes for protein detection such as tetramethyl rhodamine isothiocyanate (TRITC) and cyanine 3 (Cy3) were exploited in our experiment in order to evaluate the fluorescence signal enhancing effect of ZnO nanoplatforms. The emission properties of these
fluorophores are distinct from the previous examples of FITC and DTAF. ZnO substrates of various patterns including 10 µm striped (top panels in Figure 7.6a) and open square (bottom panels in Figure 7.6a) were employed in order to monitor enhanced fluorescence signal from 200 µg/mL of TRITC-IgG. In addition, individual ZnO nanorods (top panels in Figure 7.6b) and filled square ZnO arrays (bottom panels in Figure 7.6b) were used to monitor the fluorescence emission from 200 µg/mL of Cy3-human serum albumin (Cy3-HSA). Regardless of the emission properties of the dyes, the underlying ZnO nanoplatorms significantly enhanced the detection signal of fluorophores, as evidenced by the confocal images presented in Figure 7.6. Similar to the results obtained from the use of FITC and DTAF, only the protein molecules that were supported by the underlying ZnO nanomaterials displayed strong fluorescence emission which led to fluorescence images that faithfully mimic the underlying nanoscale ZnO patterns as shown in the confocal images of Figure 7.6.

The exact mechanism governing the observed ZnO NR-enabled fluorescence need to be explored further. Our control experiment involving SiNRs did not yield enhancement in fluorescence emission although they present similar amount of surface areas as ZnO NRs. In addition, our planar control substrates did not lead to any detectable increase in fluorescence signal, even for samples whose surfaces were determined by atomic force microscopy as densely saturated with protein molecules. Therefore, the observed fluorescence enhancement is not due to possible variations in surface protein density but is rather related to the inherent property of ZnO. Enhanced fluorescence emission in the presence of ZnO NRs may be explained by changes in photonic mode density and/or reduction in self-quenching of fluorophores. Changes in photonic mode
density and subsequent alterations in radiative decay rates have been previously observed in metal enhanced fluorescence. The presence of ZnO NRs may lead to modifications in the decay rates of radiative and nonradiative pathways, leading to dominantly fast radiative decay. The fluorophores used in our experiment display a self-quenching property due to the presence of traps in their energy levels. The presence of ZnO NRs may disable these traps and reduce self-quenching, resulting in enhanced fluorescence.

The key advantages of using ZnO nanoplatforms as substrates in biomolecular fluorescence detection are 3-fold. First, nanoscale wide band gap ZnO substrates enable reliable and enhanced fluorescence detection of proteins and DNA at very low concentrations without the need for sample or signal amplification. Moreover, the enhancement effect of ZnO nanomaterials is persistently present for various fluorescence detection systems ranging from green to red emission which, in turn, permits reliable and multipurpose optical detection of interacting protein molecules. Our results also demonstrate that ZnO nanoplatforms are chemically inert at detection environments involving many protein/protein systems. For increased detection specificity of protein reactions, ZnO nanoplatforms can be covalently modified in order to host specific protein molecules of interest only. Second, these nanomaterials can be conveniently assembled into an array format during their growth in order to promote seamless biotechnology applications by combining with a conventional automatic sample handling apparatus and computerized fluorescence detection equipment. Last, the advanced biomolecular detection capability of ZnO nanoplatforms can be exploited for the detection and screening of biomolecules at the single-molecule level such as analyzing unamplified genomic DNA from a single cell and probing protein localization in a single cell.
7.4 Conclusions

Engineered nanoscale ZnO structures can significantly enhance the detection capability of biomolecular fluorescence using proteins and nucleic acids as model systems. When compared to other commonly used substrates, nanoscale ZnO platforms enabled increased fluorescence detection of these biomolecules without any chemical or biological amplification processes. ZnO nanomaterials served as ideal signal enhancing substrates in order to facilitate biomolecular fluorescence detection which is evidenced by subpicomolar and attomolar detection sensitivity of proteins and DNA, respectively. We also demonstrated the easy integration potential of the ZnO nanoplatforms which will greatly promote the assembly and fabrication of these materials into multiplexed, high-throughput optical sensor arrays. These ZnO nanoplatforms will be extremely beneficial in accomplishing highly sensitive and specific detection of biological samples involving nucleic acids, proteins and cells, particularly involving detection environments involving extremely small sample volume of ultratrace-level concentrations.

7.5 References

Figure 7.1: Schematic illustrations showing the overall experimental procedures. (a) ZnO nanostructures were grown homogeneously on Si wafers using Ag catalysts, (b) periodically spaced, stripe-patterned ZnO nanoplatforms were fabricated by microcontact printing the catalysts onto selected locations of the growth wafers, (c) SiNRs were synthesized on Si wafers using Au catalysts, and (d) regularly spaced, stripe-shaped polymeric substrates were prepared by carrying out nanoimprint lithography on spin-castted PMMA ultrathin films. For subsequent fluorescence detection, these substrates were treated with fluorophore-modified biomolecules consisting of proteins or DNA.
Figure 7.2: Enhanced fluorescence detection facilitated by the use of ZnO nanoplateforms: model protein system. (a) When confocal microscopy was employed to measure fluorescence emission, no distinctive contrast was observed in the confocal images taken from nonspecifically adsorbed FITC-antiIgG molecules on Si wafers, SiNRs, and patterned PMMA substrates. (b) Markedly strong fluorescence emission was detected from FITC-antIgG molecules that were adsorbed on the surfaces of single ZnO nanostructures. Owing to the strong fluorescence signal, topological profiles of each ZnO nanomaterial are clearly visible in the confocal image. Confocal images of (a) and (b) are taken from a $25 \times 25 \mu m^2$ area. (c) The same fluorescence enhancing effect was observed from FITC-antiIgG that was deposited on regularly patterned ZnO nanoplateforms on the micrometer scale. The repeat spacing of the underlying ZnO nanoplateforms is $20 \mu m$ and the concentration of FITC-antiIgG used in the confocal measurement was $200 \mu g/mL$. (d) Relative fluorescence intensity from nonspecifically adsorbed FITC-antiIgG molecules prepared on various control substrates such as PMMA film, quartz, glass, Si, ZnO thin film, and SiNRs. The plot displays fluorescence intensity measured from $200 \mu g/mL$ FITC-antiIgG on these control substrates which was normalized with respect to that on ZnO nanoplateforms. (e) The plot of fluorescence intensity versus protein concentration displays the detection sensitivity limit of protein molecules on ZnO nanoplateforms using
a conventional confocal microscope. Fluorescence data from protein molecules of the same composition and concentration on patterned PMMA platforms are plotted for comparison. ZnO data points at low concentration are rescaled and displayed in the inset for clarity. The lowest concentration of FITC-antiIgG on ZnO nanomaterials which were readily detectable using a 40 mW Ar laser was 2 ng/mL.
Figure 7.3: Enhanced fluorescence detection facilitated by the use of ZnO nanoplateforms: model DNA system. (a) No fluorescence was detected from bce samples before and after hybridization reaction with basr. The absence of fluorescence contrast in the confocal image taken after the hybridization reaction is due to no or poor duplex formation between mismatching strands of bce and basr. (b) Clear fluorescence contrast was obtained from bas samples after hybridization with the fully complementary basr. Well-structured fluorescing stripes were clearly visible in the confocal image which was taken after duplex formation of fully sequence-matching bas and basr. This effect is the result of enhanced fluorescence emission of 6-carboxyfluorescein (FAM) molecules linked to basr due to the presence of underlying, periodically patterned, ZnO nanoplateforms. The repeat spacing of the underlying ZnO nanoplateforms is 10 µm and the concentration of basr shown in the confocal image was 1 nM. (c) Fluorescence intensity from FAM-modified basr was monitored as a function of basr concentration using the specified Ar laser. ZnO nanoplateforms allow successful fluorescence detection of FAM-basr at DNA concentrations as low as a few nanomolar range without any amplification using a conventional confocal microscope. (d) Data points at low concentration, shown in the blue box in the graph (c), are rescaled and displayed for clarity.
Figure 7.4: Protein fluorescence monitored using ZnO nanoplatorms. (1) SEM image of as-grown, patterned ZnO nanoarrays of filled squares. Both the length and repeat spacing of each ZnO square are 5 µm. (2 and 3) Neither as-grown ZnO nanoplatorms nor BBSA modified ZnO nanostructures exhibit any detectable fluorescence. (4 and 5) When DTAF-streptavidin was introduced to BBSA on ZnO nanoplatorms, bright green fluorescence emission from periodically-spaced, square-patterned ZnO nanostructures were clearly visible in the confocal images.
Figure 7.5: Protein-protein interaction investigated by enhanced fluorescence detection with the use of ZnO nanoplatforms. a. (1) SEM image of individual ZnO nanorods as grown on a silicon wafer. The average length and width of these ZnO nanorods are 4.1 ± 0.3 µm and 313.3 ± 68.3 nm, respectively. (2 and 3) Fluorescence emission from FITC-antIgG molecules that were conjugated to the previously PG modified ZnO nanorods.
through PG-IgG interaction is clearly seen in the confocal images. Green emission is evident from protein molecules on individual ZnO nanorods whereas the same pairs of interacted biomolecules elsewhere on the substrate do not show any fluorescence. 

b. (1) SEM image of as-grown, patterned ZnO nanoplateforms consisting of 20 µm stripes with a repeat spacing of 20 µm. A PDMS piece containing two hollow chambers was placed on top of the ZnO nanoplateforms in order to carry out simultaneous reactions of multiple proteins. Four pairs of proteins were investigated for possible biomolecular interaction; Fn and FITC-antiIgG, IgG and FITC-antiIgG, BBSA and DTAF-streptavidin, and IgG and DTAF-streptavidin. Fn and IgG were placed into chambers 1 and 2, respectively. Then, FITC-antiIgG was introduced simultaneously to both chambers. (Chamber 1) No discernable fluorescence signal was monitored from chamber 1 due to the lack of interaction between the two proteins of Fn and FITC-antiIgG. (Chamber 2) Green fluorescence emission is unambiguously monitored in the confocal image due to the presence of interacting protein pairs of FITC-antiIgG and IgG. Similarly, BBSA and IgG were placed into chambers 3 and 4, respectively. Then, DTAF-streptavidin was introduced simultaneously to both chambers. (Chamber 3) Green fluorescence emission is unambiguously monitored in the confocal image due to the formation of strongly interacting protein complex of BBSA and DTAF-streptavidin. (Chamber 4) No detectable fluorescence signal was monitored from chamber 4 due to the lack of interaction between the two protein pairs.
Figure 7.6: Enhanced fluorescence detection facilitated by the use of ZnO nanoplatforms regardless of emission spectra of fluorophores. a. (top panels) The presented confocal images confirm that ZnO nanoplatforms allow enhanced fluorescence detection of TRITC-IgG. The underlying striped patterns of 10 µm ZnO are visible due to the red emission from TRITC-IgG. (bottom panels) Red emission from TRITC-IgG molecules adsorbed onto open-square ZnO arrays of 10 µm in size and spacing is clearly visible in the displayed confocal images. b. (top panels) Individual ZnO nanorods facilitate and enhance fluorescence detection of Cy3-HSA when they were nonspecifically adsorbed onto the surfaces of ZnO. (bottom panels) Red fluorescence emission from Cy3-HSA adsorbed on the ZnO nanoarrays containing 5 µm filled squares is apparent in the confocal images whereas the same biomolecules elsewhere on the substrate surface yields no red emission.
CHAPTER 8

Ultrase nsitive DNA Sequence Detection using Nanoscale ZnO Sensor Arrays

8.1 Background

DNA sequence analysis is widely applied to the areas of mapping genes, determining genetic variations, detecting genetic diseases, and identifying pathogenic micro-organisms. The rapidly increasing numbers of sequencing data have revealed a large number of single nucleotide polymorphisms and other mutations in the human genome and in the genomes of other organisms.\textsuperscript{1-5} Subtle differences in DNA sequence due to these polymorphic sites can lead to considerable changes in disease susceptibility and drug response in humans.\textsuperscript{1, 2, 6-8} Similarly, small disparity in genetic code can cause significant variations in phenotypes and biological activities of micro-organisms. Therefore, the development of improved DNA sequencing technologies is critical in correlating specific DNA sequences with the particular biological function of an organism. Novel techniques which can perform rapid and accurate genetic sequence analyses on a large scale are specially warranted as the need for fast, inexpensive, ultrasensitive, and high-throughput DNA detection escalates in the areas of medicine, public health, forensic studies, and national security.

Biomolecular fluorescence is the most widely used detection mechanism in both laboratory-scale and high-throughput genomics research. Fluorescence detection is the dominant mechanism and extensively utilized in state-of-the-art DNA sensors such as DNA arrays and gene chips.\textsuperscript{5-12} The emerging need for high-throughput genetic detection will continue to push the limit of fluorescence detection sensitivity. These sequencing
assays require the use of lower DNA concentrations as well as smaller amounts of
fluorophores in order to cope better with the increasing demands for effectively screening
human genes or biological agents at large scale. At the same time, these DNA sensor
platforms need to eliminate high costs associated with large numbers of samples and
biomedical reaction steps. Therefore, novel techniques are currently warranted in order to
facilitate cataloguing genetic variants and enhance the fluorescence detection sensitivity
of DNA beyond the limits that current technologies offer.

Innovative assembly and fabrication of nanomaterials for use as advanced
biosensor substrates can be greatly beneficial in increasing the detection sensitivity of
biomolecular fluorescence. Zinc oxide (ZnO) nanostructures have received considerable
attention, particularly due to their desirable optical properties, which include a wide
bandgap of 3.37 eV and a large exciton binding energy of 60 meV at room temperature.
ZnO has been previously demonstrated as a candidate material for use in a broad range of
technological applications. Examples of ZnO materials in these areas include short-
edge fields, field-emitters, luminescence devices, UV lasers, and solar cells. Nanometer scale ZnO has very good potential for aiding optical detection
of target bioconstituents, as ZnO nanomaterials are stable in typical biomolecular
detection environments, have attractive optical properties, and can be easily processed
through many synthetic routes. Despite its demonstrated functions in broad areas and
suitability for advanced optical detection, biosensing applications of wide bandgap ZnO
have not yet been extensively realized.

Herein, we report the use of nanoscale ZnO materials in the enhanced
fluorescence detection of genetic materials. We demonstrate that ZnO nanomaterials
exhibit an optical property useful in fostering the fluorescence signal from fluorophore-linked DNA molecules and promoting detection at ultratrace concentrations. Specifically, we show that ZnO nanomaterials can serve as excellent signal-enhancing substrates for hybridization reactions of model DNA systems which involve genetically related Bacillus bacteria. Enhanced detection limits of ZnO nanoplatforms in the identification of a harmful Bacillus species were explored using both covalent and non-covalent schemes of DNA immobilization. In addition, in order to facilitate high-throughput screening of genetic variants, we establish simple and straightforward assembly routes which yield successful growth and fabrication of these useful nanomaterials in a dense array format directly upon their synthesis. Lastly, we show that arrayed ZnO nanomaterials allow unambiguous detection of the presence/absence of fluorescence signal from duplex-formed DNA, which, in turn, enables rapid and accurate identification of genetic mutation sites and discrimination of genetically similar bacterial species.

8.2 Experimental Details
8.2.1 Individual and patterned ZnO growth

Silicon wafers (resistivity < 1 Ω cm, thickness 0.017 inch) were obtained from Silicon Inc. Poly-L-lysine (PLL) in H2O (0.1% w/v) and Ag colloids (40 nm in diameter) were obtained from Ted Pella, Inc. Zinc oxide (99.999%) and graphite (99%) powders were obtained from Alfa Aesar. 100 µL of Ag colloid was deposited on a PLL treated Si wafer for 30 min. The substrate was then rinsed with deionized water and gently blow dried with nitrogen. The growth wafer was placed approximately 5–6 inches downstream from a 2:1 mixture of graphite powder and zinc oxide, which was kept at the centre of a
horizontal resistance furnace. The sample was subsequently heated to 900 °C for 30 min under a constant flow of 100 standard cubic centimetres per minute (sccm) of Ar.

In order to pattern the substrates with catalysts at predetermined locations, polydimethylsiloxane (PDMS) stamps containing periodic stripe or square patterns of 10, 20, or 50 µm in width were constructed by casting and curing an elastomeric polymer, Sylgard 184 (Dow Corning), against a photoresist micropatterned Si master, which was fabricated using standard photolithography procedures. 42 50 µL of PLL placed on the PDMS stamp was gently blow dried with nitrogen and was then transferred onto clean growth wafers for 30 s. Following elastomeric stamping, the samples were treated with 100 µL of Ag colloid (40 nm in diameter) for 30 min. ZnO nanomaterials were synthesized at 900 °C for 1–2 h under a constant flow of 100 standard cubic centimetres per minute (sccm) of Ar.

8.2.2 Preparation of DNA

Three custom synthesized probe oligonucleotides were used in our experiments. The three oligonucleotides are 5'-AGTGCgCGAGGAGCCT-3' (bas), 5'-GTTACGGAAAGAACCA-3' (bce), and 5'-AGTGCgCGAGGAGCCT-C6-NH2-3' (basA). In addition, 6-carboxyfluorescein modified oligonucleotide that is fully complementary to the DNA sequence of bas as well as that of basA was synthesized, 5'-TCACGCGCTCCTCGGA-3' (basR). All oligonucleotides were reconstituted in TE buffer solution (10 mM Tris, 1 mM EDTA, pH 8.0).

When using the covalent detection scheme, ZnO nanoplateforms were first silanized through the following process. The ZnO platforms were first submerged in a
0.1% (v/v) solution of 3-glycidoxypropyltrimethoxysilane (GOPS) in 95% ethanol for 1 h. Following the GOPS incubation, the nanoplatforms were rinsed with ethanol in order to remove excess silane and then gently blow dried. A mixture of 0.01% (v/v) of poly-L-lysine (PLL, 0.1% solution) was deposited onto the ZnO substrates and allowed to sit for 1 h. The samples were then rinsed thoroughly with deionized water and dried. Probe oligonucleotides, *basa*, of desired concentrations were prepared in a reaction mixture (10 mM Tris, 1 mM EDTA, 10 mM MgCl\(_2\), 50 mM NaCl, and 0.1 M KOH) and deposited on the ZnO nanoplatforms. After *basa* deposition, the ZnO substrates were placed in a humidity chamber at 37 °C and incubated for 6 h. Upon covalent attachment of *basa*, the samples were then rinsed with de-ionized water and dried. Complementary strands of DNA were then added to the probe strands. Varying concentrations of *basr* in hybridization buffer (10 mM Tris, 1 mM EDTA, 10 mM MgCl\(_2\), 50 mM NaCl, pH 8.0) were deposited onto the modified ZnO platforms for 1 h at room temperature. The concentration of *basr* used in our experiments ranged from 2 fM to 20 µM. After duplex formation reaction, the samples were then rinsed multiple times with TE and dried with a gentle stream of nitrogen. For a noncovalent detection scheme, 50 µL of 20 µM *bas* were deposited onto ZnO striped arrays or nanorods for 5 min and then rinsed with TE. The compliment strand, *basr*, of varying concentrations in reaction buffer (10 mM Tris, 1 mM EDTA, 10 mM MgCl\(_2\), 50 mM NaCl, pH 8.0) was deposited onto the platforms for 1 h at room temperature. The samples were rinsed with ample TE.

Simultaneous monitoring of DNA interactions were carried out on the same ZnO striped substrate by using a PDMS elastomer that contained two reaction chambers. The PDMS piece conformed to the underlying ZnO substrate and provided isolated
compartments for conducting two independent DNA hybridization reactions. 5 µL of *bas* and *bce* at a concentration of 20 µM were first introduced into the two separate chambers. After 5 min of deposition, the solutions were removed and then rinsed with TE solution. Subsequently, 5 µL of 20 µM *basr* was added to both chambers and the oligonucleotides were allowed to hybridize for 1 h at room temperature. Before disengaging the PDMS piece from the ZnO substrate, excess DNA was carefully removed and unbound oligonucleotides were rinsed with an ample amount of TE.

### 8.2.3 Sample characterization

FEI/Philips XL 20 operated at 20 kV was used in the SEM characterization of as-grown nanomaterials of ZnO. The confocal microscope data were collected using a conventional confocal laser scanning microscope (Olympus Fluoview 300). Samples were excited with the 488 nm line of a 40 mW Ar laser. The fluorescence emission from biosamples was separated from the excitation light by a dichroic beam splitter and a long-pass filter with cut-off wavelengths of 510 nm. Scanned fluorescence signals from samples were collected at a resolution of 512 pixels. The confocal microscope was also equipped with a 100 W mercury arc lamp (Osram), which allowed overall inspection of a large sample area in a single view frame.

### 8.3 Results and Discussions

#### 8.3.1 Bacillus bacteria

Gram-positive *Bacillus* bacteria are commonly found in soil, water, and airborne dust. Although most species of *Bacillus* are harmless saprophytes, two species are
considered medically significant: *Bacillus anthracis* (*B. anthracis*) and *Bacillus cereus* (*B. cereus*).\(^4,31,32\) *B. anthracis* is an endospore-forming bacterium that causes inhalational anthrax. It is considered to be one of the most potent biological weapons because the spores are highly pathogenic, easily transmissible, and very resistant to environmental stress. In the suspected case of a biological attack, the accurate detection of a biological agent such as *B. anthracis* will provide the most direct and effective pathway in devising appropriate treatment and containment plans in a timely manner since the first appearance of noticeable anthrax symptoms can take up to two months in humans. *B. cereus*, a genetically closely related bacterium to *B. anthracis*, is motile and it can cause toxin-mediated food poisoning. Health risks associated with *B. cereus* are non-lethal, whereas *B. anthracis* can potentially prompt a widespread fatal threat to public health. When assessing impending health risks and threats, effective DNA sequence analysis targeting specifically the genetically differentiating regions of *B. anthracis* from its closely related species is imperative in accurate identification of *B. anthracis* among many *Bacillus* species with similar genetic sequences.

Three, custom-synthesized, probe oligonucleotides were used in our experiments. The three oligonucleotides are 5'-AGTGCGCGAGGAGCCT-3' (*bas*), 5'-GTTACGGAAA GAACCA-3' (*bce*), and 5'-AGTGCGCGAGGAGCCT-C6-NH2-3' (*bas*). The sequences of *bas* and *bas* probes are specific to *B. anthracis*, whereas the sequence of *bce* probes is specific to *B. cereus*. In addition, 6-carboxyfluorescein modified oligonucleotide that is fully complementary to the DNA sequence of *bas* as well as that of *bas* was synthesized, 5'-TCACGCGCTCCTCGGA-3' (*basr*). Upon covalently or non-covalently linking the three probe oligonucleotides on ZnO nanoplatorms, hybridization
reactions were carried out using various concentrations of *basr* in order to detect duplex formation of fully matching DNA pairs and, therefore, to discriminate the biothreat agent of *B. anthracis* from its genetically closely related but non-fatal *B. cereus*. A commercially available confocal microscope was used for fluorescence detection. The excitation and detection wavelengths were chosen according to the specific emission properties of the fluorophore that was employed in our proof-of-concept experiments.

### 8.3.2 Synthesis and characterization of ZnO templates

Three types of ZnO nanoplateform were used as needed in our experiments: individual ZnO nanorods, striped ZnO arrays, and open square ZnO arrays. Individual ZnO nanorods were produced by using Ag colloids as catalysts. In order to assemble striped ZnO nanoplateforms, microcontact printing was used to deliver catalysts to predetermined locations of substrates. The open square ZnO platforms were obtained by first inking catalysts onto an elastomer stamp which contained square arrays of desired dimensions and then transferring the catalysts onto growth wafers via overpressure contact printing. Subsequently, ZnO nanomaterials were grown from the patterned catalytic sites.

Figure 8.1(a) displays our experimental design in order to synthesize and assemble simultaneously nanoscale ZnO materials into various platforms. Low-density synthesis on Ag catalysts led to the growth of individual ZnO nanorods whose average size is $4.1 \pm 0.3 \, \mu m$ in length and $313.3 \pm 68.3 \, nm$ in width. Regularly spaced, stripe or square, platforms consisting of nanoscale ZnO materials were constructed directly upon their synthesis by microcontact printing catalyst particles on the selective locations of
growth substrates with the help of pre-fabricated polydimethylsiloxane (PDMS) stamps. These ZnO nanorods in the striped array platforms were grown lying-down parallel to the growth substrate, as shown in the left panel of Figure 8.1(b). The diameter of ZnO nanorods is larger than the diameter of the Ag catalyst particles. This effect is likely due to high mobility of the Ag catalyst at our growth temperature of 900 °C, leading to the formation of catalyst aggregates, which, in turn, serve as catalytic clusters for ZnO nanorod growth.

8.3.3 Non-covalent vs covalent DNA hybridization

A patterned substrate comprised of striped ZnO nanostructures, 20 µm in width and 20 µm in repeat spacing, was used as a test bed in order to discriminate \textit{B. anthracis} from \textit{B. cereus}. The as-synthesized ZnO platforms do not show any fluorescence in the visible range as shown in Figure 8.1(b). A PDMS vessel containing two reaction chambers was placed on top of the ZnO test bed (Figure 8.1(c)). Two solutions of 20 µM \textit{bce} and 20 µM \textit{bas} in TE buffer were subsequently introduced to the chambers 1 and 2, respectively. After incubating the two oligonucleotide strands on the exposed substrate surface in each chamber, unbound \textit{bas} and \textit{bce} were removed from their chambers by thoroughly rinsing with TE buffer. 20 µM \textit{basr} solution was then added to both chambers for a possible duplex DNA interaction to take place. After the hybridization reaction, the sample was rinsed carefully and thoroughly with the buffer solution and unreacted DNA molecules were removed from the substrate. The confocal fluorescence data of the two reaction chambers are displayed in Figure 8.1(c). No discernable fluorescence signal was observed from chamber 1 due to lack of DNA hybridization between the sequence...
mismatching strands of *bce* and *basr*. In contrast, strong green emission was identified from ZnO nanostructures in chamber 2 owing to *bas/basr* duplex formation between the fully complementary pairs of *bas* and *basr*. As the above experimental scheme involves nonspecific adsorption of oligonucleotide probe molecules, single stranded DNA probe molecules of *bce* or *bas* were randomly distributed over the entire exposed surface area in each chamber upon deposition. Despite the homogeneous distribution of the DNA on both the exposed silicon oxide and patterned ZnO regions in chamber 2, hybridization reactions between *bas* and *basr* always led to fluorescence emission only from the surface areas where ZnO is present. Therefore, the presence of the underlying ZnO nanomaterials is evident in achieving enhanced fluorescence detection of hybridized DNA. This effect is clearly seen in the fluorescence patterns observed from chamber 2 in Figure 8.1(c). The striped patterns of fluorescence emission monitored from chamber 2 faithfully mimic the underlying geometry of the ZnO nanoplatform. On the other hand, sample chambers containing *bce* showed no observable fluorescence emission after hybridization reactions, regardless of the chemical composition of the exposed surface area. All oligonucleotides used in our experiments contain similar ratios of pyrimidines to purines in their DNA sequences and, thus, they exhibit similar adsorption behaviour to the underlying surfaces. Therefore, the results shown in Figure 8.1 are not due to possible differences in the adsorption behaviour of the oligonucleotides. Rather, the results in Figure 8.1 clearly suggest that the two critical factors leading to successful fluorescence emission are DNA duplex formation between the fully complementary strands and the presence of ZnO nanoplatfor

In addition to the non-covalent scheme used in the DNA detection discussed
above, we have also explored a covalent strategy in immobilizing oligonucleotide probes and compared the fluorescence detection capability of ZnO nanoplatforms systematically at various target DNA concentrations. Figures 8.2 and 8.3 summarize our results gathered from fluorescence emission of DNA duplexes of \textit{bas/basr} and \textit{basa/basr} when the oligonucleotide probes were non-covalently and covalently linked to the underlying ZnO nanoplatforms. The specific DNA sequences of \textit{bas} and \textit{basa} are identical to each other. In comparison to the \textit{bas} probe containing non-modified 5' end, the \textit{basa} probe has an amine group at the 3' end linked through a short spacer. This primary amine group of \textit{basa} was used to couple \textit{basa} strands directly and covalently to an epoxy group of a silane-treated ZnO nanoplatforms.\textsuperscript{9,34-37}

Figure 8.2(a) displays the result from the non-covalent linking method where \textit{bas} was nonspecifically deposited onto the underlying ZnO nanoplatform before introducing \textit{basr}. Figure 8.2(a)-1 represents a typical SEM image of a striped ZnO sensor array before coupling any biomolecules. Probe oligonucleotide strands were adsorbed onto the nanoplatform by incubating a 20 µM solution of \textit{bas} for 5 min. Then, loosely bound strands were removed by multiple washing with TE buffer. A 20 µM solution, containing the target strands of \textit{basr}, was added to \textit{bas}-ZnO stripe arrays in order to carry out DNA hybridization for 1 h at room temperature. After the hybridization reaction, the sample was rinsed with an ample amount of TE buffer and gently blow dried before imaging with a confocal microscope. Figure 8.2(a)-2 shows the fluorescence emission monitored from the sample after duplex DNA formation of \textit{bas/basr}. Figure 8.2(b) displays fluorescence emission monitored by first covalently linking \textit{basa} through an epoxy terminus on the glycidoxypropyltrimethoxysilane (GOPS) modified ZnO surface and
then performing hybridization reactions with a 2 µM solution of basr. The target DNA concentration used in this covalent coupling scheme is an order of magnitude lower than that of the previously described non-covalent detection. In addition, the PMT setting for the confocal measurements was 20% lower for Figure 8.2(b)-2 than the value used to collect the fluorescence image of Figure 8.2(a)-2. Despite the reduced DNA concentration and detection setting, fluorescence signal from the covalently conjugated basa/basr pairs was much stronger than physically adsorbed basa/basr pairs. Figure 8.2(c) displays fluorescence emission monitored on an open square ZnO array after carrying out a hybridization reaction between covalently bound basa strands and the fully sequence matching basr strands. The underlying ZnO nanostructures exhibit squares of 10 µm in length with a repeat spacing of 10 µm. In the series of confocal images of Figure 8.2(c) taken after the hybridization reaction of 20 µM basa/20 µM basr, open square patterns of fluorescence emission are clearly visible, which closely follows the underlying ZnO square sensor array.

As our covalent attachment scheme is effective for derivatizing both silicon oxide and ZnO surfaces, basa strands are present not only on the ZnO surface but also on silicon oxide after the covalent linking procedure. However, we monitored fluorescence only from the surface areas where nanoscale ZnO materials are present, i.e., DNA fluorescence images closely mimic the underlying ZnO patterns. This observation again demonstrates the importance of ZnO nanoplatforms in the enhanced fluorescence detection resulting from DNA duplex formation.

We performed a control experiment using silicon nanorods as substrates. These silicon nanorods exhibit similar dimensions as ZnO nanorods used in our experiments
and, thus, present similar amounts of surface area as ZnO nanoplatf orms. Yet, after hybridization reactions between the strands of \textit{bce/basr} as well as \textit{bas/basr}, the silicon nanorod samples did not yield any fluorescence emission even though higher DNA concentrations than 20 µM were used to carry out the duplex formation reactions on silicon nanorod surfaces. Therefore, we do not believe that the observed fluorescence enhancement is due to possible variations in exposed surface area.

The fluorescence effect is likely to be related to the inherent optical property of ZnO. Enhanced fluorescence emission in the presence of ZnO nanorods may be explained by changes in photonic mode density and/or reduction in self-quenching of fluorophores. Changes in photonic mode density and subsequent alterations in radiative decay rates have been previously observed in metal enhanced fluorescence.\textsuperscript{38-40} The presence of ZnO nanorods may lead to modifications in the decay rates of radiative and non-radiative pathways, leading to dominantly fast radiative decay. The fluorophores used in our experiment display a self-quenching property due to the presence of traps in their energy levels.\textsuperscript{40, 41} The presence of ZnO nanorods may disable these traps and reduce self-quenching, resulting in enhanced fluorescence. The exact mechanisms governing the observed ZnO nanoplatfor m enabled fluorescence need to be explored further and are currently under our investigation.

The ZnO nanoarrayed substrates displayed in Figure 8.2(c) can be seamlessly combined with conventional robotic sample deposition apparatus in order to handle many DNA samples for simultaneous screening. Combined with the easy synthetic and integration routes of the materials demonstrated in this paper, our results suggest that ZnO nanoplatf orms can be efficiently used for rapid identification of a large number of
biologically threatening subjects and bioagents from their genetically similar species.

In order to substantiate the increased fluorescence intensity monitored when using the covalent linking strategy, we measured fluorescence intensity of the duplex forming strands of $\text{basa/basr}$ and $\text{bas/basr}$ at various $\text{basr}$ concentrations. The observed fluorescence intensity values between the covalent and non-covalent DNA attachment schemes were then systematically compared against one another (Figure 8.3). Striped ZnO sensor arrays and individual ZnO nanorods were utilized as duplex detection platforms. The sensor arrays consisted of periodically spaced ZnO stripes of 20 µm in width and repeat spacing. 20 µM solutions of $\text{basa}$ and $\text{bas}$ were used for the covalent and non-covalent attachment of oligonucleotides to underlying ZnO platforms, respectively. After covalently or non-covalently attaching the oligonucleotide probes to desired ZnO nanoplatforms, fluorescein-conjugated $\text{basr}$ whose sequence is fully complementary to the probe strands were annealed onto $\text{basa}$ or $\text{bas}$ strands. Solutions ranging from 2 fM to 20 µM $\text{basr}$ were used in these hybridization experiments. Fluorescence intensity was measured and relative fluorescence intensity was then plotted versus the concentration of the target strand, $\text{basr}$. Figure 8.3(a) summarizes the results of fluorescence intensity difference of the DNA duplexes formed on striped ZnO sensor arrays (left) and individual ZnO nanorods (right). All fluorescence signals of both $\text{basa/basr}$ and $\text{bas/basr}$ were normalized to the fluorescence intensity measured from $\text{basa/basr}$ at 20 µM $\text{basr}$ concentration. Data points corresponding to the covalent $\text{basa/basr}$ and non-covalent $\text{bas/basr}$ are shown in red and blue, respectively. Figure 8.3(b) displays typical confocal images taken from (1) 20 µM $\text{basa}$/2 µM $\text{basr}$ and (2) 20 µM $\text{bas}$/20 µM $\text{basr}$ on striped ZnO sensor arrays and Figure 8.3(c) shows representative
images obtained from (1) 20 µM basa/20 µM basr and (2) 20 µM bas/20 µM basr on individual ZnO nanorods. Data provided in Figure 8.3(a) clearly indicate that the covalent linking scheme of DNA to the underlying ZnO nanoplatforms led to much higher fluorescence intensity of the duplex DNA regardless of the target concentration or the type of ZnO nanoplatforms. The combined use of ZnO nanoplatforms and the covalent linking scheme allowed ultrasensitive genetic sequence detection at DNA concentration levels down to 2 fM when using a conventional confocal microscope equipped with a 40 mW Ar laser. On the other hand, the detection limit of ZnO nanoplatforms coupled with DNA through the non-covalent linking scheme was 20 nM. The lowest detection limit is defined by the DNA concentration for which the observed fluorescence signal exceeds the baseline noise by a factor of three.

8.4 Conclusions

We have demonstrated for the first time that engineered nanoscale zinc oxide structures can be effectively used for screening genetic variants of closely related Bacillus species and identifying the biothreat agent B. anthracis. We used both covalent and non-covalent linking schemes in order to attach probe DNA strands to various ZnO nanoplatforms. After carrying out duplex formation reaction with target DNA strands, fluorescence intensity was measured and compared. The presence of the underlying ZnO nanomaterials was critical in achieving increased fluorescence detection of hybridized DNA. When coupled with the covalent attachment strategy of DNA to these nanomaterials, the inherently increased fluorescence detection capability of ZnO nanoplatforms was enhanced even more significantly. This collective approach allowed
detection of the target species \textit{B. anthracis} at sample concentrations as low as a few femtomolar level and, therefore, permitted highly sensitive identification of the biothreat agent. We also demonstrated the easy integration potential of the nanoscale ZnO materials into high-density arrays directly upon their synthesis. When combined with conventional automatic sample handling apparatus and computerized fluorescence detection equipment, our approach can greatly promote the use of ZnO nanomaterials as signal enhancing substrates for multiplexed, high-throughput optical DNA sensor arrays. These ZnO nanoplatforms will be extremely beneficial in accomplishing rapid, multiplexed, high-throughput, highly sensitive detection of genetic variations.

8.5 References


33. Guo Q J, Teng X W and Yang H 2004 *Nano Lett.* **4** 1657


Figure 8.1: (a) Schematic illustrations showing simultaneous synthesis and assembly of ZnO nanoplatfoms consisting of (left) individual ZnO nanorods and (right) periodically patterned ZnO nanostructures. (b) (Left) SEM image of a patterned ZnO platform with the stripe width and repeat spacing of 50 µm. The inserted SEM image at the bottom left corner shows the lying-down arrangement of ZnO nanostructures inside the patterned stripes. (Right) Confocal fluorescence image taken from the as-synthesized, striped, ZnO nanoplatform where no fluorescence emission was detected. (c) Detection scheme to identify *B. anthracis* from *B. cereus* using ZnO nanoplatfoms: PDMS chambers were used in order to carry out simultaneous hybridization reactions on the same ZnO nanoplatfom. The ZnO nanoplatfom contained regularly patterned ZnO stripes with a repeat spacing of 20 µm. Oligonucleotide probes of *bce* and *bas* were first introduced to the reaction chambers 1 and 2, respectively. Subsequently, fluorescein modified *bas* strands were added to both chambers and allowed to form DNA duplex under the same hybridization conditions. Confocal images taken from these samples showed clear fluorescence emission from chamber 2, in contrast to no discernable fluorescence signal from chamber 1. The insets in the upper left corners of the confocal images are the corresponding bright field images taken from each chamber after the duplex formation reaction. Distinctive fluorescence emission monitored from chamber 2 is due to DNA duplex formation between fully complementary strands of *bas* and *bas*, whereas the lack of duplex formation between mismatching sequences of *bce* and *bas* led to no observable fluorescence in chamber 1. The striped patterns of fluorescence emission observed from chamber 2 faithfully mimic the underlying geometry of the ZnO nanoplatfom.
Figure 8.2: (a) Fluorescence emission monitored using a non-covalent DNA immobilization scheme. (1) SEM image of as-grown, patterned ZnO nanoplatorms consisting of stripes with a repeat spacing of 20 µm. (2) Oligonucleotide probe strands of bas were nonspecifically adsorbed onto the ZnO nanoplatform shown in (1) and subsequently reacted with 20 µM basr to form double stranded DNA. (b) Fluorescence emission monitored using a covalent strategy in order to link oligonucleotide probe molecules to striped ZnO arrays. (1) SEM image of as-grown, patterned ZnO nanoplatorms consisting of stripes with a repeat spacing of 20 µm. (2) Amine-terminated oligonucleotide strands of basa were used to covalently link the probe molecules to the ZnO nanoplatform shown in (1) and they were subsequently reacted with 2 µM basr to form DNA duplex. Confocal images taken from these samples indicate that covalent derivatization of probe DNA strands to the nanoplatform, when compared to physical adsorption of the probe strands, leads to higher fluorescence emission even when using a lower target DNA concentration. (c) Fluorescence emission monitored from covalently bound basa strands and the fully sequence matching basr strands on an open square ZnO array. The concentrations of the probe and target strands were the same, 20 µM. The underlying ZnO nanostructures as well as confocal fluorescence patterns exhibit open squares of 10 µm in length with a repeat spacing of 10 µm.
Figure 8.3: Fluorescence intensity comparison between covalent and non-covalent DNA detection schemes. Striped ZnO sensor arrays and individual ZnO nanorods were utilized as enhanced platforms for identifying DNA sequence. The ZnO sensor arrays consisted of periodically spaced, striped patterns of 20 µm in both width and repeat spacing. The average length and width of the individual ZnO nanorods are 4.1 ± 0.3 µm and 313.3 ± 68.3 nm, respectively. After covalently or non-covalently attaching the oligonucleotide probes, fluorescein-conjugated target DNA whose sequence is fully complementary to the probe strands was annealed onto the probe strands. Probe strands used for the covalent and non-covalent attachment schemes were 20 µM solutions of basa and basr, respectively. Concentrations of the target basr, used in each hybridization reaction, ranged from 2 fM to 20 µM. (a) Fluorescence intensity was measured from the two hybridized DNA duplexes, one involving covalently bound basa/basr and the other involving non-covalently attached bas/basr. The relative fluorescence intensity is then plotted versus the concentration of basr. Data shown in red represent the results from the covalent strategy whereas data indicated in blue correspond to those from the non-covalent linking scheme. The two insets display rescaled data points at low concentration and clearly show the lowest detection limits of the two DNA linking schemes observed from the ZnO stripe array and individual ZnO nanorods. (left) Relative fluorescence intensity observed from basa/basr versus bas/basr duplexes on striped ZnO sensor arrays is plotted against basr concentration. (right) Relative fluorescence intensity observed from basa/basr versus bas/basr duplexes on individual ZnO nanorods is plotted against basr concentration. The dashed lines are inserted as a guide to the eye to follow data points. (b) Confocal images taken from (1) 20 µM basa/2 µM basr and (2) 20 µM bas/20 µM basr on striped ZnO sensor arrays. (c) Confocal images obtained from (1) 20 µM basa/20 µM basr and (2) 20 µM bas/20 µM basr on individual ZnO nanorods. The covalent linking scheme to the underlying ZnO nanoplanforms led to much higher fluorescence from duplex DNA in all cases. When using the covalent linking scheme on ZnO nanoplanforms, fluorescence signal was detectable even at as low as 2 fM of the target DNA concentration. The detection limit of ZnO nanoplanforms coupled with DNA through the non-covalent linking scheme was 20 nM. The lowest detection limit is defined by the DNA concentration for which the observed fluorescence signal exceeds the baseline noise by a factor of three.
Synopsis

9.1 Summary

Detection of biological and chemical species is central to many areas of biology and life sciences such as ultra sensitive disease detection, genetic screening, biochemical sensing and targeted drug delivery. Currently, detection of biomolecules like proteins and DNA is done by fluorescence detection - a widely used technique in both laboratory scale and high throughput genomic research. However, enhancing detection sensitivity and increasing signal to noise ratio still remains a major challenge in fluorescence based detection methods. In response to these challenges, this work is focused on two approaches in this context: the use of diblock copolymer templates and the use of zinc oxide nanorods, with an aim to provide rapid, sensitive and accurate detection of bioolecules.

Directed protein adsorption onto surfaces is essential to biosensor development for disease diagnostics and drug discovery as these applications require regulated immobilization of enzymes and antibodies at particular surface interfaces. The development of miniaturized protein arrays is still facing difficulties associated with the fabrication of regularly-spaced protein platforms which display high areal density and natural protein conformation. This issue constitutes the core of chapter 2. Specifically, we used PS-b-PMMA diblock copolymer thin film templates to create nanoscale protein array in one dimension. We demonstrate that the proteins selectively self-segregate themselves on the microdomain regions of a specific polymer component due to their preferential interactions with one of the two polymer segments.
In chapter 3, we have extended our approach to create protein arrays with control over additional degrees of freedom in the spatial arrangement of proteins on the nanometer scale, that is, two-dimensionally controlled periodicity. Here, we demonstrate that PS-b-PVP can be effectively used for the self-assembly of surface-bound, two-dimensional nanoscale protein arrays where the periodicity of repeating protein patterns can be effectively controlled by the nearest-neighbor spacing in the underlying hexagonal array. We also establish a straightforward method to produce protein patterns of different geometries and sizes by successfully manipulating topological structures of underlying PS-b-PVP templates. Further, we reported our findings on the activity and stability of various model proteins on these PS-b-PVP templates.

In chapter 4 we evaluate the activity of the patterned proteins on diblock copolymer templates and determine their effectiveness as highly selective and sensitive nanosensor platforms. We report the findings on the activity and stability of various proteins that were self-assembled onto the microphase-separated domains of PS-b-PMMA diblock copolymer ultrathin films. The focus of this chapter is to investigate the conformational integrity and biological activity of proteins self-assembled selectively onto the nanoscopic diblock copolymer templates. Specifically, we demonstrate that PS-b-PMMA-bound enzymes such as horseradish peroxidase (HRP) and mushroom tyrosinase (MT) are biologically active and maintain their specific catalytic functionality over a long period of time. Surface-bound enzymes retain approximately 85% of their free activity after selective adsorption onto the PS domains of the underlying PS-b-PMMA. Other proteins such as enhanced green fluorescent protein (eGFP) and
immunoglobulin G (IgG) keep their structural integrity and binding activity even after their self-assembly on PS-b-PMMA.

Chapter 5 is focused on the areas of high and uniform protein density. The density of proteins in an array is largely governed by the adsorption behavior of proteins to the surface of an underlying array material. In this chapter, we systematically study protein adsorption characteristics on different polymeric surfaces. Three different types of polymeric templates were used in the study: asymmetric PS-b-PMMA, PS homopolymer, and PS/PMMA blend. We compare protein density on a PS-b-PMMA diblock copolymer surface to its density on a PS homopolymer surface. We also investigate the nature of protein assembly on a PS/PMMA blend film in order to assess the dependence of protein density on the distance from the interface between PS and PMMA. When compared to the chemically homogeneous surface provided by PS homopolymer film, we find that the chemically heterogeneous PS-b-PMMA film is much more effective in high density and high payload protein assembly. The protein density on the diblock copolymer is approximately three to four fold higher than that on the homopolymer. This remarkable effect is further confirmed by enzymatic activity measurements on the chemically homogeneous and heterogeneous polymeric surfaces.

Chapter 6 is focused on the synthesis and assembly of various ZnO micro/nanostructures. In this chapter we describe a straightforward method to produce ZnO nanorods (NR) and microrods (MR) using a novel catalyst, *Magnetospirillium magnetotacticum* (MS-1). We utilize the highly monodisperse iron oxide (Fe₃O₄) nanoparticles inside MS-1 as catalysts to grow ZnO nano- and micro-structures with high optical quality. We demonstrate that MS-1 can serve as effective catalysts to produce
atomic defect-free ZnO NR and MR structures with unparalleled optical properties which, in turn, can promote new opportunities in photonic-based applications on the nanometer and micrometer scale.

In chapter 7, using model protein and nucleic acid systems, we demonstrate that engineered nanoscale ZnO structures can significantly enhance the detection capability of biomolecular fluorescence. Without any chemical or biological amplification processes, nanoscale ZnO platforms enabled increased fluorescence detection of these biomolecules when compared to other commonly used substrates such as glass, quartz, polymer, and silicon. The use of ZnO nanorods as fluorescence enhancing substrates in our biomolecular detection permitted sub-picomolar and attomolar detection sensitivity of proteins and DNA, respectively, when using a conventional fluorescence microscope. We also demonstrate the easy integration potential of zinc oxide nanorods into periodically patterned nanoplatforms which, in turn, will promote the assembly and fabrication of these materials into multiplexed, high-throughput, optical sensor arrays.

In chapter 8, we shift our focus on the use of nanoscale ZnO structures in the identification of the biothreat agent, *Bacillus anthracis*, by successfully discriminating its DNA sequence from other genetically related species. We explore both covalent and non-covalent linking schemes in order to couple probe DNA strands to the ZnO nanostructures. Hybridization reactions are performed with various concentrations of target DNA strands whose sequence is unique to *Bacillus anthracis*. The use of ZnO nanomaterials greatly enhances the fluorescence signal collected after carrying out duplex formation reaction. Specifically, the covalent strategy allows detection of the target species at sample concentrations as low as a few femtomolar levels as compared to the
detection sensitivity in tens of nanomolar range when using the non-covalent scheme. We also demonstrate the easy integration potential of nanoscale zinc oxide into high density arrays by using various types of ZnO sensor prototypes in the DNA sequence detection.

\subsection*{9.2 Future Perspectives}

Although we have addressed several problems with respect to the fabrication and application of diblock copolymer thin film templates and zinc oxide nanorods, it can be seen that there are still limitations that need to be overcome for the advancement of these approaches from a scientific point of view to a technologically viable approach. There are some questions that need to be addressed for a better understanding of the science behind the fabrication and application of these nanomaterials as applied to biomolecular detection. In this section the questions that need to be answered will be outlined and the future work of this thesis will be briefly proposed.

There are several questions which need to be answered with regard to the fabrication and successful application of the diblock copolymer thin film templates as proteomic array substrates. One of the most important questions that need to be answered is the mechanism behind the selective segregation of proteins onto the PS domains of the diblock copolymers as observed in chapters 2-5. In chapter 5, we have looked into many factors like the role of chemical heterogeneity, electrostatic interactions, molecular weight effects and nature of proteins, in order to explain these preferential segregations of proteins onto the PS domains. The exact mechanism behind this observed phenomenon is
still an avenue to be explored in order to truly utilize the potential of our simple and effective approach to create these nanoscale protein arrays.

One of the areas which can be looked into is the variation in charge of proteins by varying pH of the protein solution and the corresponding effect on the protein adsorption behavior. There are very large changes in the total surface charge and the surface charge distribution with pH, which might induce conformational rearrangements exposing either hydrophilic or hydrophobic residues to a given surface. The structure of the protein can also give an idea of the nature of the protein and the various conformations of the protein. Based on the work by Kyte and Doolittle one can generate a hydropathy plot, which gives the distribution of hydrophilic and hydrophobic residues in a protein. Li et. al. have done a similar study on the effect of pH on site preference by carrying out X-ray photoemission electron microscopy studies of protein adsorption on different polymeric surfaces.

In this work we have created one and two dimensional protein arrays with only one type of protein on the surface. However, many applications of protein arrays require more than one protein on the arrayed substrate to carry out detection of large number of proteins from a single experiment. Once we know the exact reasons for this preferential segregation of proteins, further experiments can be devised to create a substrate with large number of proteins on the substrate. This can be done either by tuning the properties of the proteins for e.g charges on them by varying pH or by exploiting the rich family of block copolymers where we can select each block of the diblock copolymer so that each block will prefer only a certain type of protein.
Currently, we envision that immediate applications of our protein nanoarrays will involve the use of currently available microsample handling and detection apparatus, where a group of multiple micelles will serve as a single, independently addressable unit in a given protein array. However, ideal application of our protein nanoarrays would be able to address each micelle as an independent unit on the nanoscale where a single protein array would contain a large number of different proteins to be examined. Therefore, to take full advantage of our diblock copolymer-based nanoscale protein arrays, conventionally available methods of sample delivery as well as detection systems need to be improved to provide a nanoscopic spatial resolution. Techniques like fluorescence microscopy, which is a widely used technique, would not be able to exploit the truly nanoscopic resolution of our protein array. Near-field scanning optical microscopy (NSOM) would be a technique of choice to study systems using our protein arrays. However, this type of imaging is beyond the scope of this work and hence would be an important future step to be taken in the imaging of these nanoscale protein arrays.

With regard to the synthesis and application of zinc oxide nanorods in enhanced biomolecular detection, again there are issues which need to be resolved. In chapter 6 we have developed a novel method to synthesize zinc oxide nanorods and microrods with enhanced optical quality using magnetotactic bacteria but in order to be commercially viable we need to be able to devise ways in order to culture this bacteria in large quantities with low cost and in short amount of time. Presently it takes over 2 months to culture only a 3 mL solution of this bacterium in lab settings.

Next, the exact mechanisms governing the observed zinc oxide nanorod enabled fluorescence need to be explored further. Our control experiment involving silicon
nanorods did not yield enhancement in fluorescence emission although they present similar amount of surface areas as zinc oxide nanorods. In addition, our planar control substrates did not lead to any detectable increase in fluorescence signal, even for samples whose surfaces were determined by atomic force microscopy as densely saturated with protein molecules. Therefore, the observed fluorescence enhancement is not due to possible variations in surface protein density but is rather related to the inherent property of zinc oxide. Enhanced fluorescence emission in the presence of zinc oxide nanorods may be explained by changes in photonic mode density and/or reduction in self-quenching of fluorophores. Changes in photonic mode density and subsequent alterations in radiative decay rates have been previously observed in metal enhanced fluorescence. The presence of zinc oxide nanorods may lead to modifications in the decay rates of radiative and nonradiative pathways, leading to dominantly fast radiative decay. The fluorophores used in our experiment display a self-quenching property due to the presence of traps in their energy levels. The presence of zinc oxide nanorods may disable these traps and reduce self-quenching, resulting in enhanced fluorescence.

Finally, it would be interesting to study the effect of reduced dimensionality on the detection sensitivity for biomolecular fluorescence detection. In chapter 7, we have observed that when compared to planar zinc oxide thin film substrates the fluorescence intensity is five orders of magnitude higher in zinc oxide nanorods. The zinc oxide nanorods synthesized in this work are ~ 300-400 nm in diameter and it would be interesting to know the limits of detection with zinc oxide nanorods diameter approaching to less than 100 nm.
9.3 References


VITA

Nitin Kumar was born on October 30th, 1979 in Allahabad, India. He received his bachelor’s degree in Chemical Engineering from the Indian Institute of Technology, Roorkee, India in 2002. In fall 2003, Nitin joined the graduate school at the Pennsylvania State University, where he pursued a Ph. D. in chemical engineering under the guidance of Dr. Jong-In Hahm. His research focused on the use of nanomaterials as novel substrates for enhanced biomolecular detection and resulted in the following publications.


