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NANOMATERIAL PLATFORMS FOR ENHANCED BIOMOLECULAR DETECTION

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This thesis focuses mainly on two areas; the enzymatic activity and adsorption behavior of proteins onto the diblock copolymer thin film templates and the method of creating high quality ZnO nanorod arrays and using the fluorescence enhancing ZnO for bio-sensing applications. Following an introduction, chapter 2 describes the qualitative and quantitative enzymatic activity of protein arrays on PS-b-PMMA thin film and shows that the deposited proteins retain their characteristic catalytic properties even after surface immobilization. Comparative protein adsorption behavior of proteins on different polymer thin films is discussed in Chapter 3, showing that the PS-b-PMMA is better than homopolymers PS or PMMA and polymer blend PS/PMMA thin films. Chapter 4 describes the method of creating high quality ZnO NR array and further discusses a highly sensitive and effective telomeric repeat elongation (TRE) assay by exploiting the fluorescence signal enhancing ability of ZnO NR platforms. This result shows that the novel ZnO-base TRE assays can be successfully used for detecting active telomerase. As telomerase is a useful biomarker in cancer diagnosis and screening, our ZnO NR-based TRE assays may prove to be not only useful in basic biological research but also in clinical testing. In Chapter 5, ZnO platforms are further exploited in ultrasensitive detection of disease biomarkers cytokines. The detection sensitivity achieved by using ZnO NR method is in the subfemtogram per milliliter level, which is 3-4 orders of magnitude more sensitive than the conventional assay detection limits. This detection sensitivity is achieved without the need for indirect enzyme reactions of specialized instrumentation. The final chapter presents a highly unexpected finding of self-aligned, self-assembled copper silicide nanobeams on silicon substrate which is used as catalyst and support for carbon nanotube cantilever growth.
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Chapter 1

Introduction

This thesis is mainly focused on developing novel substrates for enhanced biomolecular detection. Chapter 2 and 3 focus on the use of diblock copolymer thin film for making high aerial density protein array as well as enzymatic activity of deposited enzyme and adsorption behavior of proteins on polymeric thin film templates. Chapter 4 and 5 are focused on the synthesis and application of ZnO nanorods for ultrasensitive fluorescence detection of biomolecules. Final chapter includes a new method of growing carbon nanotube cantilever (CNT) on self-assembled, self-aligned copper silicide nanobeams.

Enhancing detection limit of bio-molecules is crucial in many areas of research such as disease detection, genetic screening, drug delivery and biochemical sensing applications. Finding suitable bio friendly substrates, increasing signal to noise ratio and the detection limits are some of the areas of concerns as well as a challenge. A variety of methods for protein localization have been previously explored. Surface deposition techniques include ink-jet and pipet deposition\textsuperscript{1, 2, 3}, soft lithography\textsuperscript{4, 5, 6}, dip-pen lithography and related scanning probe patterning methods\textsuperscript{7, 8, 9}, focused-ion beam patterning\textsuperscript{10}, imprint lithography\textsuperscript{11} and microfluidic channel networks\textsuperscript{12}. Despite the improvements in surface protein patterning by these methods, poor protein address density and slow assembly speed remain as major concerns. As a step forward, a unique method to pattern proteins with a nanometer periodicity is demonstrated by using microphase separated diblock copolymer polymethyl-b-polymethylmethacrylate (PS-b-PMMA) thin films. The self-organizing nature of the diblock copolymer was exploited to produce periodically alternating, nanometer spaced polymer domains exposing the two chemical composition of the diblock to the
Model proteins that we have used so far, selectively self organize on the microdomains with one of the two polymer segments.

Understanding structural and functional changes of polymeric surface bound proteins is extremely important as polymers play an increasingly significant role as arrays and substrate in proteomics applications. Chapter 2 demonstrates that the PS-b-PMMA thin film is effective in making functional protein arrays, subsequently, for the first time; the qualitative and qualitative enzymatic activity study of horseradish peroxidase (HRP) enzymes immobilized selectively on the polystyrene domains of microphase separated PS-b-PMMA ultrathin film is discussed. The specific enzymatic activity of HRP adsorbed on the diblock copolymer surface was evaluated and compared to that of HRP in free solution. Atomic Force Microscopy (AFM) and UV-Vis studies show that approximately 85% of surface bound HRP retain their enzymatic activity. In Chapter 3, protein adsorption behavior on polymer thin film is discussed. The effect of molecular weight, topology, and size of the domains and the importance of chemical heterogeneity in protein adsorption are compared. Our result shows that the PS-b-PMMA is a better substrate for making protein array than the homopolymer PS or PMMA or the polymer blend PS/PMMA thin films.

Fluorescence detection is currently one of the most widely used methods in the areas of basic biological research, biotechnology, cellular imaging, medical testing and drug discovery. Using model proteins and nucleic acid system we show that nanoscale zinc oxide can significantly enhanced the detection capability of biomolecular fluorescence without any chemical or biomolecular amplification processes. The untrasensitive detection was due to the presence of ZnO nanomaterial which contributed greatly to the increased signal to noise ratio of biomolecular fluorescence. The ease of fabrication of these ZnO nanostructures into periodically patterned platforms which, in turn, will promote the assembly and fabrication of these materials into multiplexed, high throughput, optical sensor arrays. These ZnO platforms have extremely
beneficial potential application in accomplishing highly sensitive and specific detection of biological samples involving nucleic acids, proteins and cells.

In Chapter 4, the application of ZnO NR platform for enhanced fluorescence detection of Telomeric Repeat Elongation (TRE) assay is discussed, especially, this proof of concept experiment shows that the ZnO platforms are robust and can sustain the multiple steps involving in many bio-assays. Telomere protects the termini of chromosomes from degradation. The natural division processes in normal cells result in reduction of the original telomeric repeats where each cell division progressively shortens the length of the chromosomes by losing about 50-200 nucleotides of telomeric sequence (TTAGGG). When the telomeric sequence reaches a certain short length, cells stop dividing or die. But the cancer cells have a mechanism to keep their telomeric length. Telomerase is a ribonucleoprotein that catalyzes the addition of telomeric repeats to the 3’ end of chromosomal DNA and thus is responsible for the elongation of telomere ends in cancer cells. As telomerase prevents the loss of telomeric sequences after each cell division, it leads to the uncontrollable and indefinite growth often associated with cancer cells. Due to its involvement in carcinogenesis, activity of telomerase can serve as a promising biomarker in cancer diagnosis and therapy.

Early detection of disease markers can provide higher diagnostic power and improve disease prognosis. Acute renal failure (ARF) occurs in 5-7% of hospitalized patients and results in a mortality rate of nearly 50%. It is unlikely that this high mortality and associated cost will be reduced until we have better tools for the early diagnosis of renal injury. Early diagnosis of acute kidney injury (AKI) in hospitalized patients has proved problematic due to the inadequacy of currently available laboratory tests. Cytokines are small proteins produced by a variety of cells and play a central role in coordinating the host response to infectious diseases. Deregulation of cytokine production is implicated in the pathogenesis of such diseases as cancer, diabetes, arthritis and neurodegenerative diseases. Elevated level of these cytokines in body fluids
may serve as markers of either disease severity or diagnosis. Elevated levels of certain chemokines and cytokines such as cytokines interleukin-18 (IL-18) and tumernecrosis factor-\(\alpha\). (TNF-\(\alpha\)) have been reported in blood, kidney tissue, or urine in experimental models of AKI\textsuperscript{25-27} and in patients with AKI\textsuperscript{27-29}. In Chapter 5, focusing on the clinically relevant applications we demonstrate the use of ZnO NR arrays in a straightforward, reliable and ultrasensitive detection of IL-18 and TNF-\(\alpha\).

Chapter 6 covers the highly unexpected new finding of self-assembled, self-aligned copper silicide nanobeams which can be used as catalyst and support for a straightforward in situ route to fabricate CNT cantilevers. This approach eliminates traditional lithographic steps while providing regularly assembled nanostructures over very large areas without the use of external fields. In addition, this new method leads to integration of CNTs with copper silicide directly upon CNT synthesis.

1.1 References


Chapter 2

Evaluation of Enzymatic Activity on Nanoscale Polystyrene-block-Polymethylmethacrylate Diblock Copolymer Domains

2.1 Background

Polymers are used extensively in a myriad of biologically relevant areas such as genomics, proteomics, drug delivery, and medical implants. They represent a choice material for diagnostics and array technologies, and are often used as arrayed supports for biomolecules under investigation. Although many applications of polymers in basic biological research and in biotechnology involve adsorption of biomolecules onto polymeric surfaces, the effect of such adsorption on the physical integrity and biological activity of biomolecules is still poorly understood. One particular field where this understanding plays an imperative role is proteomics. 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 yet 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.

Here, we report both qualitative and quantitative study of enzymes that were self-assembled onto the microphase-separated domains of polystyrene-block-poly(methylmethacrylate) (PS-b-PMMA) diblock copolymer ultrathin films. Model proteins; horseradish peroxidase (HRP), mushroom tyrosinase (MT), enhanced green fluorescence protein (eGFP), bovine serum albumen (BSA), bovine immunoglobulin G (IgG), fluorescein-conjugated antibovine IgG (FITC-antiIgG), and protein G were chosen for the activity and stability study because these proteins have been used on various biological research and biomedical applications such as purifying antibodies, detecting antibody-antigen complexes, investigating enzymatic activity and serving as catalyzed colorimetric/fluorometric substrates. To study the biological functionality of thin film bound proteins, the focus of this work is to investigate the conformational integrity and biological activity of proteins selectively self-assembled onto the diblock copolymer templates. Further, HRP immobilized on the PS-b-PMMA thin film is used for the quantitative analysis.

In this work, we show that the PS-b-PMMA bound enzymes such as HRP and MT are biologically active and maintain their catalytic functionality over a long period of time and other model proteins such as eGEP and IgG keep their binding activity and structural integrity even after their selective self-assembly on PS domain of PS-b-PMMA thin film. We also quantitatively compare the enzymatic activity of PS-b-PMMA surface bound HRP to its activity in free solution by using UV-Vis absorbance studies. Specifically, we first demonstrate that the nanometer scale spatial resolution of surface bound enzymes can be spontaneously achieved over large areas via
self-assembly on PS-b-PMMA templates. This nanoscale enzyme assembly is achieved without undergoing time-consuming fabrication or modification processes often used to make protein arrays. We further determine that our diblock-copolymer guided, nanoscale enzyme assembly method is effective in preserving a large degree of the enzymatic activity. Surface bound enzymes retain approximately 85% of their activity after selective adsorption onto the PS domains of the underlying PS-b-PMMA thin film compare to the activity of enzymes in solution.

### 2.2 Experimental Details

The 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. Si wafers (resistivity < 1 Ω 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. 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 min, followed by annealing in an argon atmosphere at 240 °C for 6 h 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.

UV-Vis absorbance spectra were recorded on a Hewlett-Packard 8452A Diode Array Spectrophotometer. A digital camera, Sony Cybershot DSC-P92, was used to capture changes in assay color.

For quantitative analysis, the lyophilized powder of HRP was reconstituted in deionized water, to varying concentrations ranging from 0.01 to 2 µg/ml. 40 µl of these enzyme solutions was deposited onto the microphase separated diblock copolymer surfaces for 10 min at room temperature. The 40 µl droplet of HRP solution on the PS-b-PMMA forms circular deposition spot of 0.6 cm in diameter which, in turn, yields a deposition spot size of 2.8 cm². The sample surfaces were rinsed thoroughly and then dried gently under a stream of nitrogen gas prior to AFM imaging. UV absorbance spectra were taken in the wavelength range between 500 and 750 nm in order to monitor the characteristic absorption peak of oxidized TMB at 650 nm.

2.3 Result and Discussions

2.3.1 PS-b-PMMA thin film template

The two chemically distinct, and thus immiscible, PS and PMMA 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 thermodymanic conditions was exploited to produce
alternating polymeric domains of the two chemical compositions of the diblock at the air/polymer interface. The advantages of using diblock copolymers for protein assembly are multifold. The microphase separation behavior of PS-b-PMMA diblock copolymers is well-characterized and, therefore, can be precisely predicted.\textsuperscript{21, 22} 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. 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,\textsuperscript{23-27} 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. At the polymer/oxide interface, PMMA favors the silicon oxide layer because of its lower wetting energy\textsuperscript{28} while at the polymer/air interface, the surface tension difference between PS and PMMA 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-thik” regions\textsuperscript{29-30}. 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 surface. After thermal annealing at 240\textdegree C for 6 hours in an Argon atmosphere, PS-b-PMMA used in our experiments yielded microdomains with an average cylinder repeat spacing of 45 nm as shown in Figure 2.1. In AFM topographic images, PMMA rich regions are lighter than PS domains and shows height difference of 1 nm. PMMA domains look lighter in phase AFM imaging also.
2.3.2 Protein array

Figure 2.2A is a carton of a PS-b-PMMA thin film which is used for nanometer scale protein array where proteins are preferentially self-assembled onto the PS domain. Enzyme deposition onto PS-b-PMMA thin film was studied by both AFM phase and topography imaging modes. Figure 2.2B-1 is a 500 x 500 nm phase image, clearly showing the preferential deposition of HRP on darker PS domain when the enzyme is deposited from a 4 µg/ml solution. Figure 2.2B-2 and 2.2B-3 are 750 x 750 nm and 500 x 500 nm topographical images respectively, in which HRP solution was deposited using 10 µg/ml solution. When increased in deposition concentration, HRP molecules formed a linear chain. The phase images shown in the insets of Figure 2.2B clearly display individual HRP molecules with a molecular mass of about 44 kDa that were localized in PS region. While using MT, the same preferential deposition of enzymes onto the PS domain was observed (Figure 2.2C). The self-assembly of enzyme molecules onto the PS-b-PMMA results in the inversion of AFM topographic contrast so after the deposition, the bright and hence higher areas in Figure 2.2B, C correspond to HRP or MT molecules assembled on PS domains and show the height difference of 2.3 nm and 4.1 nm respectively. The widths of individual HRP and MT molecules on the PS domains are about 7 and 18 nm respectively and these values agree with the estimated diameters of HRP and MT with molecular weight of about 44 kDa and about 130 kDa respectively. However, the measured heights of these proteins are much smaller than the expected values which can be explained by the soft nature of proteins and the intermittent contact made by the AFM tip during tapping mode imaging. This result is consistent with the previous studies of soft molecules using tapping mode AFM. The deposited protein concentrations are found to be about 2 x 10^{10} molecules/cm² for HRP and about 4 x 10^{10} molecules/cm² for MT while using 4µg/ml solution on PS-b-PMMA thin film.
2.3.3 Enzymatic activity

It is important that the enzyme should retain its catalytic properties even after deposition onto the polymer thin film. The catalytic active sites usually consist of a few specific amino acids residues forming a specific geometries and it should be accessible for the biological activity. The relative orientation and shape of the active sites of a bound protein is extremely important because slight irregularities could lead it a significant or a total loss of biological activity. Steric hindrance due to the presence of solid supports may also influence the protein activity by physically blocking the pathways to active sites. Therefore, it is extremely important to consider the effectiveness of proteins on such platforms when assessing the full potential of nanometer scale diblock copolymer templates in biosensing applications.

In order to evaluate the enzymatic activity proteins, HRP and MT enzymes were chosen because they are very specific and powerful catalysts that accelerate particular biochemical reactions. HRP is a widely used enzyme that catalyses the reduction of hydrogen peroxide at a high turnover rate in a short period of time. While using colorless TMB/H$_2$O$_2$ indicator solution, the released oxygen from peroxide oxidizes the TMB, converting the solution color into blue. Similarly, MT oxidizes pyrocatechol in presence of oxygen changing the assay solution color from colorless to yellow-brown.

For the qualitative assay as shown in Figure 2.3, a HRP or MT deposited PS-b-PMMA sample was placed in a vial and 1 ml of TMB or pyrocatechol was added respectively. To support the activity results, some control experiments such as blank PS-b-PMMA sample with no enzymes and deposited enzyme denatured with acid or heat were also performed. HRP on the sample was denatured by directly depositing 100uL of 0.1N HCl for 1hour. For heat treatment, HRP deposited sample was kept at 75c for 12 hours. After denaturation, sample was placed in a vial then added 100 uL of TMB and observed for 30 minutes. Digital images in Figure 2.3A and
2.3B visualize the activity of PS-b-PMMA thin film bound MT and HRP. PS-b-PMMA substrate did not change any color upon adding pyrocatechol (Figure 2.3A-1) but the active MT bound on thin films, changed the assay color from colorless to yellow-brown (Figure 2.3A-2). Figure 2.3B-1 to 2.3B-4 show the TMB solution added on PS-b-PMMA substrate, HRP deposited, HRP denatured with acid and HRP denatured with heat respectively. The assay color changed from colorless to blue in case of HRP bound PS-b-PMMA substrate but the color did not change in other control experiments. UV-Vis spectra in Figure 2.3C correspond to the assay solutions in Figure 2.3 A-1 and A-2 and Figure 2.3D correspond to the assay solutions in Figure 2.3 B-1, B-2, B-3, B-4. As seen in the figure, samples with no enzyme or denatured enzyme exhibited no absorbance peak but the samples with the enzyme molecules showed absorbance peaks corresponding to the oxidized form of pyrocatechol and TMB. These results confirm that the PS-b-PMMA bound enzymes maintain their biological functions and when stored at 4C, HRP and MT maintained their enzymatic activity for a long period of time and there were no significant loss in activity even after 100 days. Other protein activities are shown in Appendix A.

2.3.4 Quantitative enzymatic activity

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 2.4) and HRP in solution (free-state, data shown in Figure 2.5). Figures 2.4C through 2.4F show 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 minutes 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 2.4 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 2.5 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. Similar to the UV-vis data shown in Figures 2.4C through 2.4F, 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 2.5.

The activity change of HRP molecules that are immobilized on PS-b-PMMA versus free in solution was further compared and evaluated. For the 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 x 10⁹, 10.1 x 10⁹, 24.4 x 10⁹, and 45.7 x 10⁹ 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 x 10⁹, 6.6 x 10⁹, 16.5 x 10⁹, and 49.5 x 10⁹. Figure 2.6 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. Figures 2.6A and 2.6B clearly show that the measured absorbance intensity increases as the total number of HRP molecules increases regardless of the type of
assays. Figure 2.6B shows absorbance maximum values versus total numbers of HRP molecules in each experiment. From the linear fits of data in Figure 2.6B, bound-state HRP retained 85±8 % of its free-state activity.

The active site of HRP comprises an iron-protoporphyrin IX prosthetic group and a hydrophobic binding pocket. Various amino acid residues in the HRP active site provide the hydrophobic binding pocket for a substrate molecule. The catalytic reaction of HRP results in one-electron as well as two-electron oxidation products of a substrate molecule. After successful TMB oxidation by HRP, the one-electron transfer process produces a free-radical cation and the two-electron process forms a complex of diimine and diamine. These oxidation processes of functional HRP are responsible for the assay color change shown in Figure 2.6.

When immobilized onto a surface at a high concentration, enzymes may not be able to accommodate substrate molecules to their active site due to steric hindrance imposed by neighboring enzymes. Limited access of substrate molecules can prevent HRP from effectively producing oxidation products and cause reduced enzyme activity when compared to its free-state activity. However, the degree of the activity difference between PS-b-PMMA-bound and free HRP molecules in Figure 2.6B remains unchanged regardless of the surface density of HRP molecules. Therefore, steric hindrance resulting from other bound enzyme molecules does not appear to play a critical role in the HRP activity for the concentration regimes used in our experiments. The largely preserved activity of surface-bound enzymes also suggests that the active site of HRP is well protected even after surface adsorption. This observation is probably explained by the centrally located active site in HRP. Despite the orientation of proteins with respect to a solid support after adsorption, surrounding amino acids may effectively provide protection from structural damage of the active site. However, the orientation of enzyme molecules on the polymer surface can still cause reduced enzymatic activity for a different reason. As HRP molecules in our experiments are physisorbed onto diblock copolymer domains
with no preferred conformational direction, the orientation of adsorbed HRP on the diblock
copolymer surface is random. Depending on how the binding pocket may orient with respect to
the polymer surface, access pathways for substrate molecules to the available binding pockets can
be partially blocked due to the underlying solid support. This effect may explain the observed
reduction in the activity of PS-b-PMMA-bound HRP when compared to 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 2.7 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. Figures 2.7A and 2.7B display 700 x 700 nm AFM scans
of bare PS-b-PMMA templates taken after UV-vis absorption measurements. Figures 2.7C and
2.7D show 700 x 700 and 300 x 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 2h 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 2h assay is less than 5% of the total
density of HRP before the assay, which is well within the tolerance range reported earlier.
2.4 Conclusion

In summary, this research shows that the PS-b-PMMMA diblock copolymer thin film offer many advantages when used as novel templates for enzyme assembly. First, the self-organizing nature of the block copolymer can be easily exploited to produce periodically alternating, nanometer-spaced polymeric templates exposing the two chemical compositions of the diblock to the template surface. Second, preferential interactions of enzyme molecules with the PS block allow selective segregation of the biomolecules to the underlying nanoscale PS domain via self-assembly. Further, we carried out enzymatic activity study of MT and HRP enzyme molecules selectively self-assembled on the nanoscale PS domains of micophase-separated PS-b-PMMA thin films. Then, we compared the quantitative activity of HRP adsorbed on PS-b-PMMA to that of HRP in free solution. The results have demonstrated that the HRP molecules on the thin film surface maintain a large degree of their activity and conformational integrity of their free state. The unique advantages of the diblock copolymer templates make the spontaneously constructed enzyme nanoarray highly suitable as functional substrates. Therefore, our novel functional enzyme assembly on diblock copolymer thin films can be greatly beneficial for high-throughput proteomic assays and multiplexed high-density protein array applications.
2.5 References


Figure 2.1: 350 nm x 350 nm AFM images displaying the diblock copolymer templates of PS-b-PMMA. Topographic (A) and Phase (B) images of the nanoscale-spaced, chemically heterogeneous PS-b-PMMA microdomains. In both the topographic and phase scans, the brighter domains corresponds to PMMA and the darker domains are PS regions. The average repeat spacing of the domains is 45nm
Figure 2.2 A. Schematic illustrations 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 2.3 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 2.3 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 2.3A-2 and 2.3B-2 clearly displayed characteristic absorbance peaks that correspond to the oxidized substrates of MT and HRP, respectively.
Figure 2.4 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 through 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 bottommost curve) to the absorption saturation point at 30 min (the topmost 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 2.5 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 2.4. 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 bottommost curve) to the absorption saturation point at 30 min (the topmost curve). The absorption spectrum does not change significantly after 30 min.
Figure 2.6 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) $\mu$g/ml of either free- or bound-state HRP concentration. (f) stands for free-state and (b) denotes for 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 free- versus bound-state was compared, bound-state HRP retained approximately 85% of its free-state activity.
Figure 2.7 AFM images of blank PS-b-PMMA with and without HRP taken after the UV-vis absorption measurements described in Figures 2.4 through 2.6. (A and B) 700 x 700 nm phase and topographic images of the PS-b-PMMA template without bound HRP exposed to the same UV-vis measurement conditions as 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 x 700 nm and (D) 300 x 300 nm. HRP molecules remained on the PS-b-PMMA surface even after 2h in the UV-vis assay conditions and the total number of HRP molecules does not change significantly before and after the assay.
Chapter 3

Comparative Study of Protein Adsorption Behavior on Polymeric Thin Films

3.1. Background

Protein arrays are presently used in a variety of screening and diagnostic assays such as protein-protein, protein-antibody, protein-ligand, protein-small molecule, and enzyme-substrate. For these applications, a variety of protein localization methods have been previously explored. Ink-jet and pipette deposition, soft lithography, dip-pen lithography and related screening probe patterning methods, focused-ion beam patterning, imprint lithography and microfluidic channel networks have already been studied and applied in making protein patterns. Protein arrays can assist small-volume assays in a parallel fashion and, therefore, meet a demand for miniaturization, high throughput, and high sensitivity to some degree. However, even the state of the art protein arrays still face crucial challenges in keeping up with the ever growing demands of proteomics. The main difficulties found in this field can be identified as (i) the attachment of a protein onto a solid surface with retained protein functionality, (ii) the development of a universal method to attach a protein on an array surface, (iii) the achievement of a high protein density for increased detection signal and (iv) the attainment of a 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. A high address density of proteins in an array is also
very important because the increased protein density will improve the signal-to-noise ratio of a
given detection method. In addition, a uniform 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 the underlying array material. We have previously studied the
adsorption behavior of various proteins on diblock copolymer ultrathin films such as polystyrene-
block-polymethylmethacrylate (PS-b-PMMA) and polystyrene-\textit{b}-poly(4-vinylpyridine) (PS-b-
PVP). We reported that a variety of protein molecules selectively self-segregates onto one of
the two polymeric segments in both the PS-b-PMMA and the PS-b-PVP cases. When carrying
out these studies, we observed an intriguing phenomenon. Protein molecules exhibited a
tendency to close-pack and arrange themselves in an organized, space-filling manner at optimized
protein concentrations.

Here, we systematically examine the protein adsorption characteristics on different
polymeric surfaces. The four different types of polymeric templates used in our study are the
ultrathin films of asymmetric PS-b-PMMA, PS homopolymer, PMMA 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 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 payload.

3.2 Experimental Details

3.2.1 Polymer thin films

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 Ωcm, thickness: 0.017 inch) were obtained from Silicon Inc. (Boise, Idaho). The 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 °C for 6 h with a transient ramp-up rate of 5 °C/min and a cooling rate of 2 °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.
3.2.2 Protein solutions

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.

3.2.3 AFM and UV-Vis

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. TMB solution containing 1.25 mM TMB and 2.21 mM H₂O₂ was received from VWR Scientific Inc (West Chester, PA). 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 at 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.

### 3.3 Results and Discussion

#### 3.3.1 Proteins on polymer templates

Figure 3.1 displays AFM images of various polymeric thin films before and after protein deposition. PS-b-PMMA diblock copolymer, PS homopolymer, PMMA homopolymer, and PS/PMMA blend ultrathin films were used as templates for protein adsorption. Figures 3.1a, 3.1c, 3.1e and 3.1g show typical topographic AFM scans of 750 x 750 nm PS-b-PMMA, 2 x 2 \( \mu \)m PS, 2 x 2 \( \mu \)m PMMA, and 2.5 x 2.5 \( \mu \)m 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{22,23-25} which, in turn, provides chemically heterogeneous templates with a repeat spacing of 45 nm, as seen in Figure 3.1a. Homopolymer thin films of PS and PMMA, shown in Figure 3.1c and 3.1e, present smooth, uniform surfaces which are morphologically and chemically homogeneous. Upon spin casting, PS/PMMA blend film forms circular structures, as shown in Figure 3.1g, 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{26-28} 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 3.1b, 3.1d, 3.1f, and 3.1h display typical morphologies of 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 3.1b and 3.1h show clearly the selective adsorption of IgG molecules onto the PS regions. It is important to note that the selective segregation of proteins onto the PS region is not limited to IgG only but this phenomenon was observed for proteins which we have studied so far, including IgG, HRP, MT, Fn, Protein G, and BSA. Figure 3.2a-d display AFM images of various proteins assembled on chemically heterogeneous films of PS-b-PMMA and PS/PMMA blend templates. Previously, we have also studied and reported three types of polymer thin films prepared by using PS-b-PVP and shown that the proteins, indeed, prefer PS region29 (Figure 3.3 and 3.4).

It is also interesting to find that the surface density of proteins increases when PS-b-PMMA templates are used as compared to the homopolymer templates PS or PMMA, even though almost half of the PS-b-PMMA surface is covered by PMMA. The 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 we compare the number of adsorbed protein molecules per unit of PS area on the diblock copolymer versus on the homopolymer PS samples prepared by using the same concentration of a protein solution, we find the density of protein molecules on the PS-b-PMMA template to be multifold greater than the density of protein molecules on the homopolymer template. In order to evaluate the 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 weighing 70,400 Da. The results are displayed in Figures 3.2e and 3.2f. The latter film contains polymer segments with a comparable molecular weight to 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 3.2g.

### 3.3.2 Protein adsorption behavior on polymer thin films

The previous studies have shown that protein molecules tend to close pack into the PS domain of PS-b-PMMA templates in an organized, space-filling manner at a monolayer leading concentration. The height profile shows that the PMMA domain is about 1 nm higher than the PS domain which could lead us to argue that the topography of the surface could play a significant role in protein deposition pattern, however, while using open type PS-b-PVP templates, proteins still preferred PS over PVP even though there is a significant height difference between PS and PVP region and PS being higher as shown in Figures 3.3 and 3.4. So the topography might play some role but it is not the only contributing factor in case of protein adsorption on PS-b-PMMA thin film. As noted earlier, molecular weight also does not play a significant role in case of homopolymer thin films as shown in Figures 3.2e and 3.2f. Therefore, we reason that this phenomenon may be due to the chemical heterogeneity of the PS-b-PMMA diblock copolymer surface. 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 the dependence of protein adsorption behavior on the distance from the interfacial regions of PS and PMMA.

As seen in the Figures 3.5A and 3.1 h, a large number of proteins self assembles on the PS domain next to the concentric circular interfaces between the darker PS and the brighter
PMMA in the AFM images. Proteins prefer PS regions near the PS/PMMA interfaces more than 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 3.5A 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 3.5A. Figures 3.5B and 3.5C summarize our analysis of 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 3.5B displays a plot of relative protein density versus distance from 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 3.5B. 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 to prefer PS regions adjacent to the PS:PMMA interfaces. We do not know at this time the exact reasons for the selective protein segregation that extends beyond the radius of gyration of the PS blocks. We speculate that interfacial energy gradients may drive this exponential decay effect of protein density from PS:PMMA interfaces, while the strong interaction between proteins and PS drives proteins to adsorb only on the PS domains. Further work needs to be done in order to understand precisely the effect of protein assembly on the total surface free energy when protein molecules are adsorbed onto different polymeric templates as well as onto various polymeric 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 Figures 3.5 B. Figure 3.5C 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 3C, 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 3.5C, protein density is inversely proportional to the separation distance between two neighboring PS:PMMA interfaces. Therefore, a smaller number of protein molecules is expected to adsorb on PS than on PS-b-PMMA from our analysis. This analysis is consistent with our experimental observation stated earlier that higher protein density is observed on PS-b-PMMA than on PS. These effects may arise 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 facileness 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. The 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.

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 3.6 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 3.6, the 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 3.6A displays plots containing time-dependent absorbance values at \( \lambda = 650 \text{ nm} \). 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) \( \mu \text{g/ml} \) of HRP. The notation of (r) and (b) specifies enzyme deposition onto PS-b-PMMA and onto PS, respectively. Figure 3.6A 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, as shown in Figure 3.1. 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 3.6A 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 3.6B 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 3.6B) corresponds to 6.1 x 10^9, 10.1 x 10^9, 24.4 x 10^9, and 45.7 x 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 x 10^9, 28.1 x 10^9, 67.8 x 10^9, and 127.0 x 10^9 molecules/cm² for the four HRP concentrations, respectively. For the PS homopolymer template, the total number of enzyme molecules (shown in blue in Figure 3.6B) corresponds to 7.0 x 10^9, 8.2 x 10^9, 17.5 x 10^9, and 52.8 x 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 x 10^9, 22.8 x 10^9, 48.6 x 10^9, and 146.7 x 10^9 molecules/cm², respectively. For the same HRP deposition 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 still under investigation. Diblock copolymer templates have been used in the past to
assemble inorganic nanoparticles onto one of the polymer domains.\textsuperscript{31-35} Once inorganic nanoparticles were delivered to diblock copolymer templates through an evaporation method or from a colloidal solution, further annealing under an inert environment was often necessary for achieving organized assembly of nanoparticles. The 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 additional thermal treatment.

Protein adsorption is governed by many factors such as hydrophobic/hydrophilic interaction, electrostatic interaction, hydrogen bonding, and van der Waals interaction. The 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 the PS-rich domain is more hydrophobic than the PMMA-rich domain due to the phenyl group present in PS,\textsuperscript{21} we speculate that the dominating mechanism of the observed protein assembly to PS domains is through hydrophobic interactions. Electrostatic interactions can also cause the preferred segregation of proteins to PS. The isoelectric points of the proteins used in our study ranges from 4.7 to 8. At our experimental pH of 7.4, these protein molecules exhibit neutral to negative net charges. These net charges of proteins may direct proteins towards PS and cause them to avoid the ester group present in PMMA. The sizes of the proteins do not seem to play a significant role in their adsorption behaviors. All proteins used in our experiment prefer PS domains, regardless of their molecular weights which range from 45 to 580 kDa.

The presence of underlying polymeric films should also be taken into consideration as a possible mechanism for the observed protein assembly. Topological variations such as the homopolymer film roughness and the height difference between different chemical regions within
the diblock films can affect protein adsorption behavior. The observed film roughness is less than 0.5 nm for homopolymer films of PS and PMMA samples. The height difference between PS and PMMA regions in diblock copolymer films is approximately 1 nm, Figure 3.1. When these topological variations of the 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 many proteins we used. 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 polymeric templates may also serve as a factor in protein adsorption. As the water contact angle on PMMA is lower than that on PS, more proteins can be in direct 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. The surface free energy of PS is slightly lower than that of PMMA. Therefore, another intriguing possible mechanism may be the difference in surface free energy of proteins assembling on various regions of polymeric templates.

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.\textsuperscript{38, 39} 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.

\subsection{3.4 Conclusion}

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 to 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.

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3.5 References


Figure 3.1 Schematic illustrations 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 3.2 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 of 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 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 onto diblock copolymer templates when they are deposited from a 10 µg/ml solution.
Figure 3.3 AFM topology of various self-assembled nanoscale PS-b-PVP templates prepared by various solvent exposures. A. (1 and 2) 300 x 300 and 180 x 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 x 300 and 180 x 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 expose the low-lying PVP core shown as darker circles in the AFM images. C. (1 and 2) 300 x 300 and 180 x 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 3.4. 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 x 300 nm, (3) 180 x 180 nm, and (4) 60 x 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 B and C at the concentration of 4 µg/ml whereas the compact packing nature of those individual protein molecules on the micelles due to increase 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 x 180 nm, (3) 180 x 180 nm, and (4) 60 x 60 nm.
Figure 3.5 (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.
Figure 3.5 contd...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 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 3.6 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 absorbance 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 4

Novel Telomeric Repeat Elongation Assay Performed on Zinc Oxide Nanorod Array Supports

4.1 Background

Telomere, a chromosomal structure consisting of tandem GT-rich repeats of (TTAGGG)$_n$, protects the termini of linear chromosomes from degradation. However, the natural division processes in normal somatic cells result in reduction of the original telomeric repeats where each cell division progressively shortens the length of chromosomes by losing about 50~200 nucleotides of telomeric sequence. When the telomeric sequence reaches a certain short length, cells stop dividing and enter a state known as replicative senescence or die. In contrast, immortal cancer cells have a mechanism to keep their telomeric length. Telomerase is a ribonucleoprotein complex that catalyzes the addition of telomeric repeats to the 3’ end of chromosomal DNA and, thus, is responsible for the elongation of telomere ends in cancer cells. As telomerase prevents the loss of telomeric sequences after each cell division, it leads to the uncontrollable and indefinite growth often associated with cancer cells. Therefore, activity of telomerase is not only linked to the maintenance of life span in normal cells but also to the expansive proliferation of abnormal cancer cells.

Due to its involvement in carcinogenesis, activity of telomerase can serve as a promising biomarker in cancer diagnosis and therapy. Accurate and rapid assays for detecting telomerase activity are highly warranted in order to promote its potential uses as prognostic markers in cancer diagnosis and anti-telomerase drugs in chemotherapy. Both solution-based as well as surface-based telomerase assays have been developed in the past. Telomerase activity is usually detected in cellular protein extracts by the telomeric repeat amplification protocol (TRAP) assay which is a solution-based technique. Though still in its infancy, several surface-based
techniques have recently been used for measuring telomerase activity. Detection platforms of
these surface-based techniques include total internal reflection, surface plasmon resonance, and
nanowire field effect transistors.

In this study, we utilize nanoscale zinc oxide (ZnO) materials as a novel candidate
material to construct a highly sensitive surfaced-based technique for detecting telomerase activity.
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. Another important and relatively new area of ZnO
application is in biological detection. Nanometer scale ZnO can be an ideal material for use as
biosensors since ZnO is easily processable through many synthetic routes, stable in typical
biomolecular detection environment, and has the potential for aiding optical detection of
target bioconstituents. Despite these advantages, biosensing applications of wide bandgap ZnO
has not yet been fully demonstrated or extensively assessed using many different biological
systems.

Our group’s previous research efforts using ZnO nanorods (NRs) have revealed that these
materials are capable of enhancing fluorescence signals from biomolecules, when compared to
emission signals from the same biomolecules on typical biosupports such as glass, quartz, silicon,
and polystyrene. The remarkable enhancement effect of ZnO NRs further permitted
ultrasensitive detection of DNA and proteins at ultratrace concentration levels by using
conventional fluorophores and detection apparatus. In order to achieve suitable detection
sensitivity, many biological assays require additional biological processes to effectively amplify target biomolecules and detection signals. Examples of such biological amplification processes include PCR and enzyme-linked immunosorbent assay (ELISA). More recently, some biological assays capitalize on the development of chemical processes to synthesize new fluorescent probes such as quantum dots\textsuperscript{26-28} and metallic nanoparticles\textsuperscript{29-32} which can serve as improved biological labels. The key advantages of our ZnO NR-based biosupports lie in the ability to perform ultrasensitive biological detection without any chemical or biological amplification of target biomolecules or detection signals. In addition, ZnO NR-based detection is direct where the observed signal is entirely from the subject biomolecules under study in contrast to indirect signals acquired from secondary enzyme reactions, as required by ELISA-based methods.

Despite the previous efforts to use as-grown ZnO NRs as optical signal enhancing platforms; those test biological systems were restricted to one type of subject bioconstituents, involving either purified oligonucleotides or proteins only. Yet, common biological and biomedical protocols often include many types of biomolecules in a single analysis, relying on the sensitivity of the given detection platform and the selectivity of the given assay protocol for positively identifying the target biomolecules. In order to utilize ZnO NR-based biodetection platforms effectively and widely in such biosensing applications, it is critical that these ZnO NR platforms need first to be evaluated with a variety of complicated biological systems consisting of multiple biological constituents. For the first time, we demonstrate that nanoscale ZnO biosensor-based platforms can be successfully used for a fast and straightforward assay for determining telomerase activity. Specifically, we focus on developing rapid and highly efficient telomeric repeat elongation (TRE) assays based on nanoscale ZnO platforms for the first time. We exploit our ZnO nanoplatorms in a TRE assay as TRE protocols eliminate PCR-related artifacts as well as post-PCR procedures such as separating PCR products by gel electrophoresis and evaluating them by phosphorimager or densitometry. As a model biological system, we chose HeLa cells to
assess our ZnO-NR based TRE assays as HeLa cells express telomerase.\cite{33,34} HeLa, derived from an adenocarcinoma of the cervix 1952, is the first human epithelial cancer cell line established in continuous culture.\cite{35} We demonstrate that our ZnO NR platforms can successfully enhance fluorescence signal in TRE assays which, in turn, signifies a highly sensitive detection of active telomerase in subject cells. Since the experimental design of our TRE analysis depends on the physical integrity and biological activity of many bioconstituents such as cell lysates, proteins, and deoxyribonucleotide triphosphate (dNTPs) on ZnO NRs, our novel ZnO NR-based TRE assay will be invaluable in the accurate assessment of the applicability of ZnO NR platforms in more biologically meaningful as well as clinically relevant settings.

4.2 Experimental Details

4.2.1 ZnO Substrate

Three types of ZnO nanoplatforms were used in our experiments: individual ZnO nanorods, striped ZnO arrays, and open square ZnO arrays. 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) 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. For creating individual ZnO NR platforms, 100 µL of 40nm Ag colloid was deposited on a PLL treated Si wafer for 30 min and the substrate was then gently blow dried with nitrogen. For synthesizing ZnO NR stripe or open square arrays, catalysts were first transferred to specific locations on growth substrates. In order to pattern the substrates with catalysts at predetermined locations, polydimethylsiloxane (PDMS) stamps containing periodic stripe or square patterns of 10 or 50 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. 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 sec. Following elastomeric stamping, the samples were treated with 100 µL of Ag colloid for 30 min. The open square ZnO patterns were obtained by first pre-inking catalysts onto a PDMS stamp containing the square pattern arrays of desired dimensions and then transferring the catalysts onto growth wafers via overpressure contact printing.36 The growth wafer was then placed approximately 5 to 6 inches downstream from a 2:1 mixture of graphite powder and ZnO which was kept at the center of a horizontal resistance furnace. The sample was subsequently heated to 900 °C for 30 min to 1 h under a constant flow of 100 standard cubic centimeters per minute (sccm) of Ar. A light grey material was visible on the Si wafer surfaces following the growth. FEI/Philips XL 20 operated at 20 kV was used in the SEM characterization of as-grown nanomaterials of ZnO.

4.2.2 Telomeric repeat elongation (TRE) assay

The overall experimental scheme of our TRE assay is illustrated in Figure 4.1. Overall procedures for the ZnO NR-based TRE assays begin by covalently linking oligonucleotides, TS, to our various ZnO nanoplatforms. The oligonucleotide sequence of TS, 5’-NH2-C6-AATCCGTCGAGCAGAGTT-3’, is specifically designed to be recognized by telomerase that are present in HeLa cell lysates. Subsequent addition of a dNTP mixture consisting of dCTP, dGTP, dTTP, and biotinylated dATP, permit telomerases to incorporate the dNTPs and extend the 3’ end of TS sequence with the telomeric repeat unit of TTAGGG. The newly formed dNTPs, specifically biotinylated dATPs, in the elongated portion of TS are further reacted with streptavidin molecules conjugated with dichlorotriazinylaminofluorescein (streptavidin-DTAF). Fluorescence signal from the elongated TS sequence is subsequently detected in order to assess
telomerase activity where successful sequence extension of TS by the presence of active telomerases will lead to fluorescence in our assay.

In order to couple oligonucleotides covalently to various ZnO NR supports, ZnO nanoplatforms were 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) in de-ionized water (DI) was deposited onto the ZnO substrates and allowed to sit for 1 h. The samples were then rinsed thoroughly with DI and dried. A custom synthesized oligonucleotide, TS: 5'-NH2-C6-AATCCGTCGAGCAGAGTT-3’, of desired concentrations was prepared in a reaction mixture (10 mM Tris, 1 mM EDTA, 10 mM MgCl2, 50 mM NaCl, and 0.1 M KOH) and deposited on the ZnO nanoplatforms. After TS deposition, the ZnO substrates were placed in a humidity chamber at 37 °C and incubated for 6 h. Upon covalent attachment of TS, the samples were then rinsed with DI and dried. HeLa cell lysates, received from Rockland Immunochemicals, Inc., were diluted in telomerase assay buffer consisting of 10 µM HEPES buffer, 1.5 mM KCl, and 100 µM MgCl2 to desired concentrations. 100 µL of this mixture was placed on the TS-modified ZnO NR support and kept for incubation in a humidity controlled chamber for 2 h at room temperature (RT). The ZnO NR substrate was then rinsed thoroughly with assay buffer. Biotinylated dNTP was received from New England Biolabs, Inc. where a small fragment of dATP in the dNTP mixture is pre-labeled with biotin. 100 µL of 10 µM dNTP in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was subsequently placed on the HeLa cell-treated ZnO NR substrate and left to incubate for 1 h in a humidity chamber at RT. The sample was then rinsed with TE multiple times. The lyophilized streptavidin-DTAF powder, received from Beckman-Coulter Inc, was reconstituted in DI as recommended by the manufacturer. 100 µL of 10 µM streptavidin-DTAF in DI was deposited onto the dNTP-treated substrate and
incubated for 30 min at RT. The sample was rinsed with DI thoroughly and dried under a gentle stream of N₂ before fluorescence imaging. The fluorescence images were collected using a conventional confocal laser scanning microscope (Olympus Fluoview 300) as well as a fluorescence microscope (Zeiss AxioImager).

4.3 Result and Discussion

Figure 4.2 displays our fabrication processes to create various ZnO NR supports for TRE assays. Individual ZnO NRs used in our assay exhibit the average width and length of 300 nm and 3.5 µm, respectively, as shown in the left SEM panel in Figure 2. The middle and right SEM panels in Figure 2 display regularly patterned ZnO nanoplatforms consisting of striped arrays and open square arrays, respectively, with the unit stripe or square size of 10 µm. Individual ZnO NRs grown at a tilt with respect to the plane of the growth wafer comprise each unit pattern in stripe and open square ZnO NR arrays. These regularly patterned ZnO nanomaterials were directly fabricated upon their synthesis by a catalyst-transfer method using an elastomeric polydimethylsiloxane (PDMS) stamp. This synthetic method, involving combined growth and assembly steps, enables fabrication of arrayed ZnO NR supports directly upon their synthesis. This advantage can be particularly useful for high-throughput and multiplexed TRE assays, as our approach allows ZnO NR biosupports to be seamlessly combined with conventional robotic sample deposition apparatus when handling many cell samples simultaneously.

Figure 3 displays fluorescence data taken after performing TRE assays on both positive and negative samples prepared on the three types of ZnO NR supports. Positive assays were proceeded with all experimental procedures outlined above. On the other hand, negative assays were carried out by omitting either HeLa cell lysates or dNTPs. The underlying ZnO NR arrays used in the assays, Figures 3A and 3B, exhibit the unit stripe width of 50 µm whereas the unit
stripe width of the ZnO NR arrays used in the assays, Figures 3C and 3D, is 10 µm. The open square ZnO NR array employed in Figure 3E shows the unit square width of 10 µm. Figure 3F consists of individual ZnO NRs as assay platforms. In all cases, the as-grown ZnO NR platforms do not emit any fluorescence in the detection wavelength range.

Figures 3A through 3C display typical confocal fluorescence data obtained from the positive samples. In these cases, green fluorescence emission is clearly seen which signifies the presence of active telomerase and the successful incorporation of dNTP in the extension of TS. By contrast, Figure 3D shows a representative fluorescence image collected from the negative samples where no fluorescence is emitted from these control samples. The lack of fluorescence in these samples is due to the absence of active telomerase to extend TS or dNTP to react with streptavidin-DTAF. Figures 3E and 3F display fluorescence images collected from positive samples on an open square ZnO NR and individual ZnO NR array, respectively.

Our data from ZnO NR-based TRE assays verify that ZnO nanoplatforms can effectively determine active telomerase present in sample cells. Since our ZnO nanoplatforms can serve as excellent fluorescence enhancing biosupports, the detection of telomerase would be possible even when using very small number of cells and ultratrace-level concentrations of biological reagents. Our data also confirm that complex biological assays involving many different biocomponents can be successfully carried out on ZnO NR platforms. As telomerase can serve as a useful biomarker in cancer screening, our ZnO NR-based TRE assays can be not only useful in basic biological research but also in clinical diagnosis. The combined advantages of our approach, the one-step synthesis and fabrication process to create arrayed ZnO NRs and their enhanced fluorescence detection capability, eliminates the use of elaborate fluorophores or detection apparatus and allows simple and robust telomerase detection at a very low concentration level even when using a conventional dye and a fluorescence microscope.
4.4 Conclusion

We have developed a highly-sensitive and effective TRE assay by exploiting the fluorescence signal enhancing capability of ZnO NR platforms. We have demonstrated that this novel ZnO NR-based TRE assay can be successfully used for detecting active telomerase. Our new TRE assay relies on the physical integrity and biological activity of many bioconstituents on the ZnO NR supports. Therefore, our effort will be very beneficial in the accurate assessment of the actual applicability of ZnO NR platforms in biologically complex and clinically relevant settings. By carrying out TRE assays on various types of ZnO NR arrays, we have also shown the potential of ZnO NR array-based TRE assay. Our effort can be especially useful in many clinical tests where a large number of samples need to be screened for telomerase activity in a high-throughput and multiplexed fashion.

Acknowledgement: Undergraduate student Adam Dorfman, contributed significantly in fluorescence intensity measurement.

4.5 References


Figure 4.1. Schematic cartoons illustrating the overall procedures for TRE assays performed on ZnO NR platforms. TS, an oligonucleotide whose sequence is recognized by telomerase, is covalently bound onto the surface of ZnO NRs. In the presence of dNTP mixtures, telomerase in HeLa cell lysates adds the telomeric repeat DNA sequence units of TTAGGG to the end of the TS sequence which, in turn, elongates the DNA by incorporating dNTPs. Biotinylated dATPs in the dNTP mixture are then reacted with streptavidin-DTAF. The fluorescence signal from the elongated DNA sequence was subsequently detected.
Figure 4.2. Fabrication processes of various ZnO NR supports for TRE assays and the typical SEM images corresponding to the three types of ZnO NR supports: individual ZnO NR, striped ZnO NR, and open square ZnO NR arrays. SEM panels of these ZnO NR arrays are 10 x 10 µm, 70 x 70 µm and 70 x 70 µm in size from left to right.
Figure 4.3. Fluorescence images acquired from TRE assay performed on ZnO NR arrays. The fluorescence images are (A) 1200 x 1200 µm, (B) 400 x 400 µm, (C) 120 x 120 µm, (D) 120 x 120 µm, (E) 60 x 60 µm, and (F) 25 x 25 µm in size. The underlying ZnO NR platforms used in assays A through D have striped patterns. (A-C) Confocal fluorescence data obtained from positive samples. The green fluorescence emission is clearly seen in these samples which signifies the detection of active telomerase and the successful incorporation of dNTPs in the extension of TS. (D) Typical fluorescence image collected from negative samples. No fluorescence emission is detected from these samples due to the lack of telomerase in the assay. The platforms used in assays E and F are open square and individual ZnO NR arrays, respectively. (E and F) Fluorescence images obtained from positive samples on (E) open square ZnO NR and (F) individual ZnO NR arrays.
Chapter 5

Ultrasensitive Detection of Cytokines Enabled by Nanoscale ZnO Arrays

5.1 Background

Measurement of the concentrations of specific proteins in biological fluids is crucial in many clinical and laboratory settings. Protein biomarkers, for example, are increasingly used to assess risk, to diagnose or monitor the activity of diseases and to guide therapy or assess therapeutic response. Antibody-based assays are convenient and provide good levels of sensitivity and specificity for many proteins. However, conventional antibody-based assays may lack sufficient sensitivity to accurately quantify low abundance proteins. Chemokines and cytokines, for example, are small proteins which are often produced in the setting of inflammation. In normal individuals, the levels of certain cytokines in plasma or urine may be below the detection limits of conventional immunoassays. Therefore, improved sensitivity of protein assays is an area of active investigation. In this report, we demonstrate for the first time the ultrasensitive fluorescence detection of cytokines at extremely low concentrations using a novel zinc oxide nanorod (ZnO NR) platform.

ZnO thin films and micro/nano structures exhibit certain desirable optical properties, including a wide band gap of 3.37 eV and a large exciton binding energy of 60 meV at room temperature, which have led to their use in a broad range of optical and optoelectric applications. However, applications of ZnO in biological or clinical testing schemes have remained largely unexplored, even though many biological assay systems rely heavily on optical detection techniques. Fluorescence is a widely used bioanalytical technique in genomics, proteomics, drug discovery, disease diagnostics, cell studies, and environmental analysis. Major challenges associated with such fluorescence techniques include enhancing detection sensitivity
and increasing signal to noise ratio. Novel methods which overcome current drawbacks and enable rapid, facile, high-throughput, ultrasensitive, and specific optical detection are in great demand.

Our group has previously carried out proof-of-concept studies in order to demonstrate that nanometer scale ZnO can be used successfully in the fluorescence detection of duplex DNA formation and protein interaction.9-11 Although these previous studies employed simplified bioassays which did not involve biological fluids, they demonstrated the potential for ZnO NRs to facilitate the highly sensitive detection of DNA and proteins. In this paper, we assess the lowest detection limits of ZnO NR arrays in more clinically meaningful assays. ZnO NR arrays can be easily assembled as assay platforms upon the material’s synthesis, ready to be employed in bioanalysis without any post-synthetic assembly or purification processes.10, 12 The physical and chemical structures of ZnO NRs remain stable and inert in commonly used bioassay conditions. Detection of a wide range of fluorophores typically utilized in many bioassays can be benefited from ZnO NRs regardless of their spectroscopic characteristics.11 The absorption and emission spectra of our high crystalline and atomic defect-free ZnO NRs do not overlap with those of fluorophores and this property of ZnO NR arrays makes them suitable as non-autofluorecent platforms. Combined with these advantages, the reduced size inherent to ZnO NRs can allow miniaturized, high-throughput, and multiplexed detection.

Acute renal failure (ARF) occurs in 5-7% of hospitalized patients13, 14 and results in a mortality rate of about 50%.15 It is unlikely that this high mortality and associated cost will be reduced until we have better tools for the early diagnosis of renal injury. Early diagnosis of acute kidney injury (AKI) in hospitalized patients has proved problematic due to the inadequacy of currently available laboratory tests. Cytokines are small proteins produced by a variety of cells and play a central role in coordinating the host response to infectious diseases. Deregulation of cytokine production is implicated in the pathogenesis of such diseases as cancer, atherosclerosis,
diabetes, arthritis and neurodegenerative diseases. In some instances, elevated levels of cytokines in body fluids may serve as markers of either disease severity or diagnosis. There is experimental evidence that a number of cytokines and chemokines, including tumor necrosis factor-α (TNFα), keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), interferon-γ inducible protein-10 (IP-10) and interleukin-18 (IL-18), participates in the pathogenesis of AKI. Elevated levels of certain cytokines and chemokines have been reported in blood, kidney tissue, or urine in experimental models of AKI and in patients with AKI. Focusing on the clinically relevant applications of ZnO nanomaterials, we herein report the ultrasensitive detection of cytokines in urine, using IL-18 and TNFα as model systems.

5.2 Experimental Details

5.2.1 ZnO NR Array Platforms and Polymeric substrates

Stripe- and square-arrays of ZnO NR platforms were fabricated using a gas-phase growth method as described in an earlier report. Si wafers (resistivity < 1 Ω·cm, thickness: 0.017 inch) were obtained from Silicon Quest International (Santa Clara, CA). In order to achieve ZnO NR platforms directly upon the material’s synthesis, 40nm Ag colloidal catalysts (Ted Pella Inc. Redding, CA) were first transferred to predetermined locations on Si wafers. The catalyst delivery was carried out by microcontact printing catalyst nanoparticles from a polydimethylsiloxane (PDMS) stamp that contained periodic patterns of 5 or 10 μm in width. ZnO NRs were then synthesized in a home-built chemical vapor deposition reactor at 950 °C for 1h under a constant flow of 100 standard cubic centimeters per minute of Ar. For various polymeric platforms, asymmetric PS-b-PVP diblock copolymer with an average molecular weight of 68,500 Da was obtained from Polymer Source Inc. (Montreal, Canada). 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). The diblock contained 70% of PS by weight with a polydispersity of 1.14. Si substrates were first cleaned with ethanol, acetone, and toluene and spun dry. Ultrathin films of PS-b-PVP, PS, and PMMA were spun cast from 0.5% (w/v) PS-b-PVP, 2% (w/v) PS, or 2% (w/v) PMMA in toluene, respectively, at 3500 rpm for 1 min onto Si substrates.

5.2.2 Various Proteins and Antibodies

Bovine serum albumin (BSA) and horse radish peroxidase (HRP) were purchased from VWR Scientific Inc (West Chester, PA). The lyophilized powder of these protein molecules was reconstituted in PBS (10 mM mixture of Na₂HPO₄ and NaH₂PO₄, 140 mM NaCl, 3 mM KCl, pH 7.4) buffer or in deionized water, as recommended by the manufacturer. The reconstituted protein solutions were further diluted to various concentrations, as needed. 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution containing 1.25 mM TMB and 2.21 mM H₂O₂ was obtained from VWR Scientific Inc (West Chester, PA). Recombinant IL-18 (Biovision, Mountain View, CA) and TNFα (MBL, Woburn, MA) were reconstituted in deionized water and stored in small aliquots at -80°C until use. The recombinant cytokines were added in varying concentrations to PBS or urine obtained from a healthy individual for use in calibrating the assay. A monoclonal anti human IL-18 antibody (clone 125-2H, MBL) was labeled with Alexa 488 using the Microscale Protein Labeling kit (Invitrogen Molecular Probes, Eugene, OR). Briefly, 50 µg of antibody (1 mg/ml) was mixed with 5 µl 1M NaHCO₃. Alexa 488 tetrafluorophenyl ester was added at a molar ratio of 70 and the mixture reacted for 30 minutes in the dark at room temperature. Labeled antibody was separated from free dye by centrifugation through a gel filtration column according to the manufacturer’s instructions. The protein concentration and degree of labeling (DOL) were determined spectrophotometrically at 280 and 494 nm.
wavelengths. Generally, the antibody yield was 60-80% with a DOL of 5-11 molecules of dye/molecule of antibody. This antibody was used as the detection antibody for both the direct and sandwich IL-18 assays. A second, unlabeled IL-18 antibody (clone 159-12B, MBL) was used as the capture antibody in the sandwich IL-18 assay. A polyclonal rabbit anti-TNFα antibody (MBL) was used as the capture antibody for direct and sandwich TNFα assays. A phycoerythrin-labeled (PE-labeled) rat anti-TNFα antibody (BD Biosciences, San Jose, CA) was used as the detection antibody for the TNFα immunoassay.

5.2.3 Direct Cytokine Assays

20µl aliquots of PBS buffer or urine containing various amounts of IL-18 or TNFα were deposited onto a NR platform and incubated for 15 min in a humidity-controlled environment at room temperature. After the incubation step, the platform was rinsed with PBS buffer multiple times. In order to passivate surface areas where no IL-18 or TNFα was bound, 20µl of 5 % BSA (w/v in PBS) was deposited onto the substrates. After 15 min of BSA blocking, the platform was rinsed with an ample amount of PBS buffer. Subsequently, 10µl of 2 µg/ml Alexa 488-labeled IL-18 antibody or 3 µg/ml PE-labeled TNFα antibody was placed onto the substrate and incubated for 30 min to allow the antibodies to bind to their respective target proteins. Following the incubation period, the sample was once again rinsed with PBS buffer multiple times. Immediately before fluorescence measurements, the sample was gently dried under a stream of N2 gas.
5.2.4 Sandwich Cytokine Assays

20µl of 2µg/ml unlabeled IL-18 or TNFα antibody was deposited onto a ZnO NR platform. Following a 15 min incubation period in a humidity controlled chamber at room temperature, the platform was rinsed thoroughly with PBS buffer. 20µl of 5 % BSA (w/v in PBS) was then deposited onto the platform to block any remaining binding sites. After a 15 min of BSA blocking period, the sample was rinsed with an ample amount of PBS buffer. Then, 20µl aliquots of either PBS or human urine containing known amounts of IL-18 or TNFα were deposited onto the platforms. After a 15 min of further incubation, the platform was rinsed with PBS buffer. Subsequently, 10µl of 2µg/ml Alexa 488-labeled IL-18 antibody or 3µg/ml PE-labeled TNFα antibody was deposited to the above platform and incubated for 30 min. After the incubation period, the platform was rinsed with PBS buffer multiple times and gently dried under a stream of N₂ gas just before fluorescence imaging.

5.2.5 Enzyme-Linked Immunosorbent Assays (ELISA)

For comparison with the ZnO NR assays, levels of IL-18 and TNFα were measured using commercially available ELISA kits (IL-18 ELISA kit, MBL, Nagoya, Japan and Quantikine TNFα kit, R and D Systems, Minneapolis, MN) according to the manufacturers’ protocols. Absorbance results, after subtraction of background, were normalized relative to the absorbance obtained using a standard containing 1000 picogram/ml cytokine.
5.2.6 Sample Characterization and Data Analysis

The size and shape of as-grown ZnO nanomaterials in the array platforms were characterized by using a scanning electron microscope, a FEI/Philips XL 20 operated at 20 kV. UV-vis absorbance spectra were subsequently recorded on a Hewlett Packard 8452A Diode Array Spectrophotometer. Fluorescence from the direct and sandwich assay samples was captured using a Zeiss Axio Imager A1m (Carl Zeiss Inc., Thornwood, NY). Fluorescence images were recorded with a Zeiss Axio CAM MRM digital camera and Axio-Vision software. Green fluorescence emission from IL-18 assays was characterized by excitation at 460-500 nm and collection at 510-560 nm. Red fluorescence emission from TNFα assays was obtained by excitation at 528-560 nm and collection at 570-645 nm. A computer software, Image Pro Plus (Media Cybernetics Inc., Bethesda, MD), was used for subsequent image analysis. All concentration-dependent plots presented in this paper display data after subtracting the background signal that is measured by using assay solutions with no cytokine. The lowest detection limit is defined as the cytokine concentration for which the observed fluorescence signal exceeds the standard deviation of zero concentration data by a factor of 3.

5.3 Results and Discussion

5.3.1 Results

Figure 5.1 illustrates our overall assay schemes for detecting cytokines in a pure buffer or in a biological fluid using ZnO NR arrays. Prefabricated square or stripe arrays of ZnO NRs were employed as fluorescence-enhancing detection platforms in the cytokine assays. The periodic arrays of ZnO NRs were fabricated using a gas-phase synthetic route as reported earlier.10,12 By tailoring the dimensions of predetermined patterns used to deliver catalysts for growing the
nanomaterials, the periodicity of the resulting stripe or square arrays was controlled to be either 5 or 10 µm. Each pattern in the array consisted of densely-populated ZnO NRs that grew vertically out of the plane of an underlying Si substrate. Figures 5.2a and 5.2b display typical SEM images of the ZnO NRs in a stripe array sample. ZnO NRs, prepared using our method, are highly uniform in diameter, length, and crystalline structure. Higher magnification images shown in Figures 2c through 2e demonstrate the uniformity in diameter and length of the ZnO NRs where the average diameter and length of the ZnO NRs are 180 ± 12 nm and 1.2 ± 0.3 µm, respectively. The resulting NRs exhibit Wurtzite structures and individual NRs have hexagonal end facets grown preferentially along the Wurtzite c-axis. The end and side facets of an individual ZnO NR are clearly shown in the SEM panels in Figure 5.2f and 5.2g.

Detection of cytokines such as IL-18 and TNFα often involve enzyme-linked immunosorbent assays or other related techniques where microplates are typically used as assay platforms. We first assessed whether ZnO NR arrays are superior to commonly-used polymeric materials found in conventional microplates for ultrasensitive detection of cytokines. For this experiment, identical TNFα sandwich assays were performed on substrates fabricated from PS, PMMA, PS-b-PVP, and ZnO NR arrays. Unlabeled TNFα antibodies were noncovalently adsorbed onto each of these substrates and blocking with BSA followed as described in methods. The substrates were further incubated with known concentrations of TNFα in PBS buffer and then with PE-labeled TNFα antibodies. The fluorescence signal was measured at various concentrations of TNFα after carrying out the identical sandwich assays on the four substrates. The signal was then normalized with respect to the fluorescence intensity detected using 1 µg/ml of TNFα on ZnO NRs. Figure 5.3a displays the plot of relative fluorescence intensity versus TNFα concentration where the concentration of TNFα in PBS buffer is shown on a logarithmic scale. On ZnO NR arrays, the detection sensitivity reached down to the sub-femtogram per
milliliter range. In contrast, only a very weak fluorescence signal was detected from the same assay performed on the polymeric substrates.

One possible explanation for the enhanced fluorescent signal from the ZnO NR arrays might be that ZnO NRs allow greater adsorption of the capture antibody due to greater surface area or other physico-chemical surface properties compared with the polymeric substrates. To evaluate the nonspecific adsorption behavior of proteins on ZnO NR versus polymeric platforms, we exposed ZnO NRs and PS platforms of 0.25 cm² in size to identical HRP adsorption steps. The amount of HRP bound on both platforms was analyzed spectrophotometrically using TMB as a colorimetric reaction substrate. Our results (Figure 5.3b) indicate that more HRP was adsorbed on the PS than on ZnO NR platforms. Therefore, our data suggest that the increased fluorescence signal observed using ZnO NR arrays is not related to the larger surface area of the nanomaterials or owing to increased nonspecific protein adsorption. Using atomic force microscopy, we have also determined that biomolecules adsorb onto the silicon surface between the features of the ZnO arrays, yet do not produce a detectable fluorescent signal (data not shown). These results indicate that the ZnO NRs specifically enhance the fluorescent signal of vicinal fluorophores.

We next employed the ZnO NR arrays for the detection of IL-18 and TNFα in both buffered and biologically relevant solutions of PBS and urine, respectively. Figure 5.4 displays data obtained from direct and sandwich assays of IL-18 and TNFα in PBS buffer performed on striped ZnO NR arrays. Figures 5.4a through 5.4d show typical fluorescence images obtained from IL-18 (panels a and b) and TNFα (panels c and d) sandwich assays at a cytokine concentration of 1 pg/ml. A fluorescent signal is readily detectable above the neighboring background between the stripes of ZnO. No fluorescence was observed from control samples consisting of ZnO NR arrays without any cytokines or antibodies adsorbed onto their surfaces. In general, higher fluorescence signals were obtained from Alexa 488 used in IL-18 assays than from PE used in TNFα assays. This result indicates that different fluorophores result in changes
in the fluorescence intensity profiles on ZnO NR platforms. Many factors may explain this observation such as variations in the source light intensity at the absorption maxima, coupling efficiency of labeling, and quantum yield of the two fluorophores. Therefore, the detection sensitivity reported in this paper may be improved even further with the use of alternative fluorophores.

Figures 5.4e and 4f display relative fluorescence intensity measured at various cytokine levels in PBS. Our results consistently demonstrate that the detection sensitivity of the ZnO NR platform approaches the femtogram/milliliter level for many modes of assay. The linear response range lies in the concentration range between 0.1 pg/ml and 10 ng/ml for IL-18, and between 0.01 pg/ml and 0.1 ng/ml for TNFα, respectively. For all plots shown in this paper, error bars are omitted when they are smaller than the symbols used to indicate data points. The IL-18 direct assay performed without the BSA blocking step led to higher signals than the direct assay with blocking. Sandwich assays yielded higher signals than direct assays for IL-18. Two possible factors can contribute to this outcome. The observation might reflect better binding of the unlabeled antibody than IL-18 to the nanorods or better accessibility of IL-18 to the labeled detection antibody. The unlabeled IL-18 antibody may have a slightly better binding affinity to the surface of ZnO NRs than the protein itself which, in turn, leads to more protein molecules present on the sandwich platform for further reactions. In addition, unlike the direct assay case, IL-18 protein molecules on the sandwich platform are not restricted sterically by the presence of the underlying ZnO NR surface. Therefore, the secondary labeled antibody can have better access to the binding sites present on the protein, thereby increasing the signal. For TNFα, we did not observe much difference in the standard curves between the direct and sandwich assay schemes. In all cases, the slope of the standard curves was similar to one another and the linear detection ranges were comparable between the different assay schemes. The slopes of the linear regions in the semilog plots, corresponding to the various modes of IL-18 and TNFα assays, show
similar values to one another which indicates approximately 20% of the fluorescence intensity change of the saturation value per 10-fold serial dilution.

Figures 5.5 and 5.6 display fluorescence emission data acquired from sandwich assays of IL-18 and TNFα in urine performed on square ZnO NR arrays. The fluorescence images shown in Figures 5 and 6 were obtained using 20 fg/ml IL-18 and 10 fg/ml TNFα, respectively, in urine. As in Figure 5.4, the presence of cytokine, even at these low concentrations, is evident from the fluorescence emission from square-patterned areas of ZnO NRs, while intervening areas of bare silicon oxide yield no signal. Results from two independent assays are presented in Figures 5.5d, 5.6c and 5.6d. Results obtained using commercially available ELISA assays are also shown in Figures 5.5 and 5.6, for comparison. The minimum detection levels for the ELISA assays were in the range of 10 pg/ml which was consistent with the manufacturer’s stated specifications. Our data also demonstrate that the IL-18 and TNFα fluorescence assays are consistent between different batches of ZnO NR arrays. The detection limit for each cytokine was in the 1-10 fg/ml range and exceeded the sensitivity of the conventional assays by 3 to 4 orders of magnitude. Similar to the assays performed in PBS, the slopes of the linear regions in the semilog plots, corresponding to the various modes of IL-18 and TNFα assays in urine, also show similar values to one another which indicates approximately 20% of the fluorescence intensity change of the saturation value per 10-fold serial dilution.

Despite the complexity of the composition and chemical characteristics of urine, our results also suggest that the assay scheme is highly specific and extremely sensitive to each cytokine. The implication of these results is significant in that our assay technique can be applied to measure cytokine levels at ultra low concentrations directly and conveniently in a clinical setting. Furthermore, as demonstrated in the previous results, ZnO NR platforms are capable of enhancing dyes of various spectroscopic characteristics which, in turn, will enable multiplexed detection. The data for a multiplexing assay are provided in the Supporting
Information section. We do not know at this time how the detection sensitivity of the ZnO NR platform may change when samples from individuals with ARF, instead of healthy individuals, are tested. We are currently investigating this important capability for cytokine detection and the results will be reported elsewhere.

5.3.2 Discussion

Measurement of specific protein concentrations is a key tool in clinical medicine and biological research. Techniques, capable of measuring accurately the levels of proteins that are present in very low abundance, are becoming increasingly important for the diagnosis of a variety of diseases such as inflammatory diseases, degenerative diseases and malignancies. The present report describes a simple method for detection and quantitation of biologically and medically relevant proteins with markedly improved sensitivity compared with conventional immunoassays. Specifically, ZnO NR array platforms synthesized in situ provided fluorescence signal enhancement which allowed the detection of proteins at concentration levels 3 to 4 orders of magnitude lower than conventional platforms, even when considering those methods with enzyme-linked signal amplification.

We demonstrate the measurement of two cytokines implicated in kidney disease, IL-18 and TNFα. Levels of both proteins are undetectable in urine from normal individuals but increase in the urine in humans or animals with ARF. It is possible that changes in the levels of these proteins below the detection levels of currently available assays may provide an earlier indication of kidney injury or a sensitive measure of subclinical kidney injury.

The current detection sensitivity for commercially available IL-18 and TNFα assays ranges between the picogram to nanogram per milliliter. ELISA, the standard assay for IL-18 and TNFα measurements, typically shows a sensitivity in the 1-10 picogram per milliliter range.
while assays using cells to measure biologically active IL-18 or TNFα are generally less sensitive. Alternative signal amplification schemes such as rolling chain amplification, or detection methods such as evanescent field fluorescence or electrochemiluminescence increase the sensitivity but require additional steps or specialized instrumentation. While conventional ELISA or related tests may be sensitive enough for proteins which are present in easily detectable levels, they may be inadequate for proteins which are normally present in low levels, such as many cytokines. Moreover, the levels of proteins in certain body fluids, notably urine, may be much lower than in blood due to the glomerular filtration barrier and the capacity of the proximal tubule to reabsorb or catabolize filtered proteins. Yet, as argued above, quantifying such proteins as IL-18 or TNFα at very low concentrations may be extremely important in early detection of disease or in predicting disease progression.

In this paper, we demonstrate for the first time the application of ZnO NR platforms for the ultrasensitive detection of cytokines at extremely low concentrations. The detection sensitivity enabled by the use of ZnO NR platforms is in the sub-femtogram/milliliter range for cytokines in PBS buffer and in the femtogram/milliliter range for cytokines in urine. Such unparalleled detection sensitivity is achieved by directly measuring the target protein levels and bypassing the need for extended downstream biochemical or molecular steps that other assay routes require. Unlike these conventional test methods discussed above, fluorescence signal in our ZnO NR-assisted assay scheme is obtained directly from cytokines interacting with their antibodies. Therefore, the fluorescence measured in our assay is directly related to the presence of target proteins under analysis, not to the secondary enzyme-substrate reactions in ELISA-based assays. In addition to its superior sensitivity, other key advantages include ease of array fabrication, mechanical and chemical robustness, no autofluorescence, multiplexing ability, and direct correlation of observed signal to protein level. Moreover, our data confirm that ZnO nanomaterials function as ideal fluorescence detection platforms, even when involving complex
biological fluids. These ZnO NR arrays can be seamlessly combined with conventional robotic sample deposition apparatus for simultaneous handling and screening of many different proteins in biological fluids. This versatility opens up a new possibility of applying ZnO NR arrays for rapid detection of proteins in a highly parallel manner. These capabilities will be critical and valuable when screening multiple protein markers in large numbers of samples.

The exact mechanism responsible for the enhanced fluorescence signal on ZnO NRs is not clear at this time and requires further study. One pathway leading to enhanced fluorescence results from reducing resonance energy transfer between fluorophores themselves. Electron transfer processes seen in spectral sensitized solar cells of ZnO-fluorophores may occur in our cytokine detection system.41,42 This electron transfer process from the excited levels of fluorophores to the conduction band of ZnO NRs may prevent self-quenching that may have been otherwise widely present in fluorophore-only systems. It is possible that surface enhancement effects, similar to the observations reported earlier in metal-fluorophore systems,43-46 may take place in our ZnO platforms. However, decay lengths of fluorescence enhancement observed in our semiconducting ZnO NRs are much longer ranged than the length scale seen on Au or Ag. For radiative decay mechanism in metal enhanced fluorescence to be effective, fluorophores should be placed approximately between 5-20 nm away from the metal surface. Yet, we observe the fluorescence enhancement effect on ZnO NRs, even when fluorophores are located well beyond 20 nm away from the NR surface. At the same time, we do not observe any quenching of fluorophores when they are placed directly onto ZnO NR surfaces. When considering these decay lengths of surface enhancement effects, factors other than surface plasmon or polariton may play a more important role in our system. Another pathway leading to enhanced fluorescence may originate from the evanescence wave-enhancing and wave-guiding nature of metal oxides. In an earlier work, metal oxide nanostructures such as ZnO have shown as exceptional UV and visible light-guiding mediums.47-49 Metal oxide NRs are capable of guiding
visible light in and out of fluorophores as well as along NRs.\textsuperscript{48} This phenomenon results in direct fluorescence from the fluorophores as well as guided fluorescence on NRs. In another study, a metal oxide-incorporated waveguide has been predicted for enhancing evanescent wave fields up to 1500 times higher than the waveguide without the presence of a metal oxide.\textsuperscript{50} The wave-guiding property of ZnO NRs and their ability to enhance the intensity of evanescent field may explain the remarkable fluorescence sensitivity observed in our system. Other experimental observations that corroborate this explanation include the dimensions of ZnO NRs used in our assays and the relatively large decay length of fluorescence enhancement in our system. The predicted decay distance of the evanescent wave field on the metal oxide NRs for visible wavelengths is much longer than 20 nm.\textsuperscript{48} The average diameter of ZnO NRs that are used in our assay scheme is commensurate to the predicted dimensions of ZnO NRs to guide visible light effectively. The absorption and emission characteristics of common fluorophores operate in this wavelength range. Therefore, it is highly likely that ZnO NRs serve as efficient evanescent waveguides enhancing the absorption and emission processes of fluorophores which, in turn, enables the extremely high detection sensitivity in our bioassay scheme. For testing this hypothesis, experimental variables such as refractive index and physical dimensions of nanorods can be changed for systematically investigating the fluorescence enhancement effect. For elucidating the precise dependence of the length between fluorophores and NRs, fluorescence intensity of a known number of fluorophores on a single nanorod, instead of involving statistical intensity analysis from a large number of fluorophores and NRs, should be assessed.
5.4 Conclusions

In summary, we demonstrate a straightforward and reliable method for the ultrasensitive detection of interleukins and tumor necrosis factor using ZnO NR arrays. We exploit the fluorescence enhancing properties of ZnO NR platforms in assays involving both a pure buffer and a biological fluid. ZnO NR-enabled detection sensitivity in our scheme approaches the sub-femtogram/milliliter level. This unparalleled detection sensitivity, well below the current limitations, is achieved without the need for indirect enzyme reactions or instrumentation improvements. We also highlight many advantages of employing ZnO NR arrays in the ultrasensitive profiling low abundance proteins. Key advantages include robustness of NR arrays, simple and direct assay schemes, high throughput and multiplexing capabilities, and the ability to correlate directly measured signal to the protein level. Combined with the extremely high sensitivity demonstrated in this work, our ZnO NR array-based approach may be highly beneficial in early detection of many diseases for which protein biomarkers exist.

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5.5 References


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Figure 5.1. Schematic illustration showing the overall assay scheme for the detection of cytokines. The illustration displays a sandwich assay scheme on a square ZnO NR platform that is assembled directly upon the NR synthesis in a chemical vapor deposition (CVD) reactor. Prefabricated square or stripe arrays of ZnO NRs are employed as fluorescence-enhancing detection platforms in the cytokine assays. For direct assays, cytokines in the sample are adsorbed onto ZnO NRs and then analyzed after incubating with their primary antibodies labeled with a fluorophore. For sandwich assays, primary antibodies pre-adsorbed onto ZnO NRs are incubated with samples containing cytokines. Then, secondary antibodies labeled with a fluorophore are further allowed to interact with the cytokines. Blocking steps are used both in direct and sandwich assays, unless indicated otherwise.
Figure 5.2. Typical SEM images of a stripe-array ZnO NR platform. ZnO NRs within each array show a uniform size and shape where the majority of the NRs is grown out of the plane of the underlying Si substrate. The average diameter and length of the ZnO NRs are 180 nm and 1.2 µm, respectively. Individual ZnO NRs exhibit Wurtzite structures of high crystallinity and their preferential growths along the c-axis expose hexagonal end and side facets. Images (F) and (G) display high magnification images of (F) an end facet and (G) side facets. The SEM images correspond to scan areas of (A) 70 x 90 µm, (B) 3.5 x 4.5 µm, (C) 2 x 2 µm, (D) 2 x 2 µm, (E) 800 x 800 nm, (F) 200 x 200 nm, and (G) 763 x 1200 nm.
Figure 5.3. (A) Comparison of fluorescence intensity obtained from various detection platforms after sandwich assays involving TNFα. After carrying out identical assays on ZnO NR, PS, PMMA, and PS-b-PVP platforms, the measured fluorescence signals at various TNFα concentrations were normalized with respect to the fluorescence intensity detected using 1 µg/ml of TNFα. The normalized fluorescence intensity was then plotted against the logarithmic value of the cytokine concentration. (B) UV-vis spectra comparing the amount of proteins on ZnO NR versus on PS platforms. Both platforms were treated identically with 50 µg/ml HRP and the characteristic absorbance peak of oxidized TMB product at 650 nm was subsequently recorded after 15 min of HRP-TMB assay.
Figure 5.4. Cytokine Assays in PBS buffer: Fluorescence images of 10 µm-period, stripe-array ZnO NR platforms after carrying out sandwich assays of cytokines diluted in PBS buffer. Panels of (A) 320 x 320 µm and (B) 100 x 100 µm display fluorescence images obtained from a sandwich assay involving 1 pg/ml of IL-18. Panels of (C) 250 x 250 µm and (D) 100 x 100 µm display fluorescence images obtained from a sandwich assay involving 1 pg/ml of TNFα. Brightness and contrast are adjusted in order to show the fluorescence images more clearly. (E and F) After performing various assays on stripe-array ZnO NR platforms, the measured fluorescence signals at various IL-18 concentrations were normalized with respect to the fluorescence intensity detected using 20 µg/ml of IL-18. The measured fluorescence signals at various TNFα concentrations were normalized with respect to the fluorescence intensity detected using 1 ng/ml of TNFα. The normalized fluorescence intensity is plotted against the logarithmic value of the cytokine concentration. Although not shown in the data set of TNFα, data points from direct assays without BSA blocking overlap with the data points from other TNFα assays shown in (F).
Figure 5.5. IL-18 Assays in Urine: Fluorescence obtained from 5 μm-period, square-array ZnO NR platforms after carrying out IL-18 assays in urine. Panels (A) 140 x 140 μm, (B) 60 x 60 μm, and (C) 30 x 30 μm display fluorescence images obtained from a sandwich assay involving 20 fg/ml of IL-18 in urine. Brightness and contrast are adjusted in order to show the images more clearly. (D) After performing various assays on ZnO NR platforms, the measured fluorescence signals at various IL-18 concentrations in urine were normalized with respect to the fluorescence intensity detected when using 0.2 ng/ml of IL-18. The normalized fluorescence intensity is plotted against the logarithmic value of the IL-18 concentration. Assay results from two independent runs on ZnO NR platforms are displayed. (E) ELISA assay of urine containing the indicated amounts of IL-18. The measured absorbance signals at various IL-18 concentrations were normalized with respect to the absorbance detected using 1 ng/ml IL-18.
Figure 5.6. TNFα Assays in Urine: Fluorescence obtained from 5 μm-period, square-array ZnO NR platforms after carrying out TNFα assays in urine. Panels (A) 90 x 90 μm and (B) 45 x 45 μm show fluorescence images obtained from a sandwich assay involving 10 fg/ml of TNFα in urine. Brightness and contrast are adjusted in order to show the fluorescence images more clearly. (C and D) After performing direct (C) and sandwich (D) assays on ZnO NR platforms, the measured fluorescence signals at various TNFα concentrations in urine were normalized with respect to the fluorescence intensity detected when using 0.1 ng/ml of TNFα. The normalized fluorescence intensity is plotted against the logarithmic value of the TNFα concentration. Assay results from two independent runs on ZnO NR platforms are displayed. (E) ELISA assay of urine containing the indicated amounts of TNFα. The measured absorbance signals at various TNFα concentrations were normalized with respect to the absorbance detected using 1 ng/ml TNFα.
Chapter 6

Carbon Nanotube Cantilevers on Self-aligned Copper Silicide Nanobeams

6.1 Background

The excellent mechanical, thermal, and electrical properties of carbon nanotubes (CNTs) have so far been exploited in numerous important technological applications. The extraordinary mechanical properties of CNTs involving large Young’s modulus and high aspect ratio are utilized as megahertz flexural oscillators and atomic force microscopy (AFM) tips. The outstanding electrical and thermal conductivities of CNTs are employed in semiconductor devices, interconnects, and heat sinks.

Recent advances in CNT synthesis allow for CNTs to be grown vertically or laterally on predetermined sites of substrates with precise control of diameter and length. In some cases, spatial alignment of CNTs is also achieved by applying electrical or magnetic fields during their synthesis. Owing to these recent improvements in CNT synthesis, new potential applications of CNTs are still in the process of being mapped out. Despite the progress in the synthesis of CNTs, their assembly into functional devices still faces crucial challenges. Such hurdles include difficulties in the controlled assembly of large numbers of CNTs and in the effective integration of CNTs with other nanomaterials. For applications involving large numbers of CNTs, lithographical procedures are often used in order to achieve vertical or lateral assembly of many CNTs. Although these previous approaches are effective in improving the assembly of CNTs, the additional fabrication steps required by these methods can be costly and time consuming. For most applications, mechanically weak Van der Waals attraction is utilized as the main interaction force to connect CNTs to other materials. As many applications demand...
mechanically reliable CNT devices and probes,\textsuperscript{4,14-17} improvements in CNT assembly is highly desired to ensure strong mechanical attachment and stable chemical linkage of CNTs to their accompanying nanomaterials.

Herein, we report a straightforward \textit{in situ} growth route to fabricate CNT cantilevers suspended on catalytic copper silicide (Cu$_3$Si) nanobeams. We first create self-assembled nanoscale structures of Cu$_3$Si via chemical vapor deposition (CVD). We then demonstrate an approach based on CNT synthesis at the self-assembled catalytic Cu$_3$Si nanostructures to overcome the difficulties in CNT assembly outlined above. In our experimental scheme, Cu$_3$Si nanobeams are designed to play a role both in synthesis and application of CNTs. During CNT synthesis, self-assembled Cu$_3$Si nanobeams function as active catalysts for the successful growth of CNTs. The resulting structures exhibit laterally-grown individual CNT cantilevers firmly anchored on Cu$_3$Si nanobeams. As the interaction of the two nanomaterials, CNT and Cu$_3$Si, is no longer dependent on weak Van der Waals interaction in our approach, the Cu$_3$Si nanobeams can function as an integral part in the final device as mechanical or electrical contact pads to actuate CNTs. Our approach based on self-assembly can eliminate traditional lithographic steps and the use of any external fields while providing regularly assembled CNT-Cu$_3$Si structures over very large areas. In addition, our method can significantly simplify the fabrication process in the current CNT-based nanoelectromechanical applications by eliminating steps required for integrating CNTs with other component materials. Our efforts can pave the way for additional CNT applications where possible applications of our integrated individual CNT cantilevers on Cu$_3$Si nanobeams may include nanosized pipettes, actuators, balances, oscillators, resonators, mass-sensitive biosensors, and scanning probe tips.

Copper silicides are effective as diffusion barriers against Cu ions and as passivation layers against surface oxidation in semiconductor devices. In previous studies, copper silicide layers were formed by either exposing Cu thin films to a silane mixture or by thermally annealing
In this letter, we present a CVD method to produce self-assembled nanoscale structures of Cu$_3$Si. When compared to the prior methods used to produce two-dimensional copper silicide layers, our new synthetic method yields one-dimensional Cu$_3$Si structures of well-controlled size and shape on the nanometer scale.

6.2 Experimental Details

6.2.1 Self-assembled, Self-aligned Copper Silicide Nanobeams

A source boat containing a mixture of 0.9 g of CuO and 0.6 g of graphite, obtained from Alfa Aesar (Ward Hill, MA), was placed at the center of a horizontal resistance tube furnace. A target boat containing clean Si (100) wafer substrates, Silicon Inc. (Boise, Idaho), was kept 5 inches away from the source boat at the downstream side. The furnace was then heated to 900 °C under a constant Ar flow of 100 standard cubic centimeters per minute (sccm) for a varying period of growth time up to 1 h. CuO is reduced by the presence of graphite, leading to the formation of Cu and CO$_2$. These products travel downstream by the flow of the carrier gas. The Cu reacts with the Si substrate to produce Cu$_3$Si while the unreacted CO$_2$ is evacuated from the tube furnace. Subsequently, self-aligned beam-shaped nanostructures were yielded on the growth wafers without the use of any catalysts.
6.2.2 Carbon Nanotube Growth

For CNT growth, the substrates with Cu$_3$Si nanobeams were treated with a 1:10 mixture of NH$_3$F/HF: deionized (DI) water for 1 min. The samples were then rinsed with DI multiple times and blow dried under a stream of N$_2$. The substrates were further treated with 0.01 N HNO$_3$ for 20 min followed by rinsing with DI water and blow-drying. CNTs were then synthesized in a CVD reactor using 600 sccm of Ar, 400 sccm of H$_2$, and 1000 sccm of C$_2$H$_4$ for 1 h at 750 °C.

6.3 Characterization

As-grown copper silicide nanostructures were characterized by using a scanning electron microscope, FEI/Philips XL 20 SEM, operating at 10 kV. XRD measurements were made using a Philips X’Pert MPD using Cu K$_\alpha$ radiation under an accelerating voltage of 45 kV. AES spectra were obtained using a PHI 670 Scanning Auger Microscope with an electron beam energy of 10 keV. The base pressure of the AES chamber was kept as 5x10$^{-10}$ Torr. When necessary, sputtering of the sample surfaces was carried out using a 3 keV Ar ion beam with a sputter rate of 2 Å/sec. CNT samples were characterized by an AFM, Multimode Digital Instruments, 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. Samples were also characterized with a field emission SEM (FESEM), JEOL 6700F, operating at 3 kV.
6.4 Result and Discussion

Figure 6.1(a) displays typical SEM images of the beam-like nanostructures formed on the growth wafers parallel to the plane of Si (100) substrates. The resulting nanostructures exhibit the average aspect ratio of 1:7 where the average width and length are 160 nm and 1.12 μm, respectively, after 1h growth at 900 °C. When compared with respect to the known azimuthal orientation of the growth wafer, these nanostructures align themselves along the directions of Si [011] and [01-1]. The two preferential growth directions of Cu$_3$Si nanobeams are indicated in Figure 6.1(a)-2. The SEM images of varying magnification in Figure 6.1(a) clearly show that Cu$_3$Si nanobeams prefer to grow along [011] and [01-1] directions of the Si (100) growth substrates. This self-alignment effect is remarkably precise in assembling the nanobeam structures within several degrees relative to those preferred growth directions. At early stages of their growth, square- or rectangular-shaped crystallite structures form as shown in the inset of Figure 6.1(a)-4. During an extended reaction period, these crystallites grow along the preferential directions. If two crystallites are formed co-linearly, they can merge into a single beam where this merging event can transform small crystallites into longer beam structures. The width and length of these beam-shaped nanostructures can be effectively tuned by changing their growth time. The lowest temperature that the self-aligned nanobeams readily form on Si wafers is 700 °C, below this temperature no Cu$_3$Si nanostructures are observed regardless of growth time.

The chemical composition of the self-assembled nanobeam structures is determined as Cu$_3$Si by performing X-ray diffraction (XRD) and Auger electron spectroscopy (AES) measurements. Figure 6.1(b) displays the typical XRD spectra of the as-synthesized samples where the two peaks near $2\theta = 45^\circ$ correspond to the characteristic peaks of Cu$_3$Si (320) and Cu$_3$Si (312), respectively. A typical AES spectrum of our samples shown in Figure 6.1(c) further confirms the formation of Cu$_3$Si on the growth wafers. When these Cu$_3$Si samples were kept in
air, an oxide layer develops on top of the Si surface, similar to an earlier study carried out by using Cu$_2$Si thin films.\textsuperscript{20} The AES spectrum in Figure 6.1(c) was acquired after sputtering the sample with an Ar ion beam for 5 min in order to remove the oxide layer. The two peaks, \textit{a} and \textit{b}, correspond to Cu (M$_{23}$VV) and a combined contribution of Cu (M$_1$VV) and Si (LVV), respectively. The three peaks, \textit{c}, \textit{d}, and \textit{e}, belong to Cu (L$_3$M$_{23}$M$_{23}$), Cu (L$_3$M$_{23}$V), and a combination of Cu (L$_3$VV) and Cu (L$_2$VV), respectively.

Further, we assessed whether our Cu$_3$Si nanostructures can function as active catalysts for synthesizing CNTs. Although the catalytic effects of other metal silicides, such as nickel silicide, iron silicide, and cobalt silicide, on the promotion or inhibition of CNT growth have been previously reported,\textsuperscript{21-23} copper silicides have not yet been explored as CNT catalysts. If proven effective in catalyzing CNTs, Cu$_3$Si nanostructures could be extremely useful as CNT-interfacing and actuating materials owing to their self-aligning nature during synthesis, as presented in Figure 6.1. As illustrated by AFM images in Figure 6.2, Cu$_3$Si serves as an effective catalyst for the nucleation and growth of CNTs. Specifically, Cu$_3$Si nanobeams lead to the lateral growth of CNTs with an average diameter of 8.5 nm as determined by both AFM and FESEM. The length of the resulting cantilevered CNTs on Cu$_3$Si nanobeams under our growth conditions ranges between 2 and 3 $\mu$m. The catalytic Cu$_3$Si nanobeams are anchored to the silicon substrate. Therefore, CNT growth on Cu$_3$Si nanobeams has to be promoted by the base-growth mechanism where catalysts remain at the base of the growing CNT because no catalytic particles are found at the tip of the synthesized CNT. As the number of nucleation sites on each Cu$_3$Si nanobeam is directly related to the number of CNTs grown on each nanobeam, further study needs to be done in order to control precisely the number of nucleation sites.

Due to the much larger physical dimensions of Cu$_3$Si nanobeams as compared to those of the suspended CNT cantilevers, AFM is much more effective at imaging CNTs grown perpendicular, rather than parallel, to the long axis of Cu$_3$Si nanobeams. Therefore, AFM images
shown in Figure 6.2 present only CNT cantilevers perpendicular to the long axis of Cu$_3$Si nanobeams. Figure 6.3 displays FESEM images of CNT cantilevers grown on Cu$_3$Si nanobeams parallel to the long axis. FESEM measurements indicate that CNTs are anchored to the Cu$_3$Si nanobeams along the length of mutual contact and the CNTs’ extensions beyond the Cu$_3$Si are suspended freely above the Si substrates. This suspension is determined due to the fact that, under certain FESEM imaging conditions, the free ends of the CNT cantilevers vibrate which causes them to appear blurry in the image.

The unique geometry of as-synthesized CNT cantilevers suspended from the self-aligned Cu$_3$Si nanobeams can make them extremely useful for many technologically important CNT applications such as nanoscale resonance frequency detectors and mass-sensitive nanobiosensors. Key advantages of our approach, involving the CNT-Cu$_3$Si assembly via the \textit{in situ} growth route, lie in the ability to produce self-aligned Cu$_3$Si without the use of clean-room procedures and to yield strongly attached and fully integrated arrangements of suspended CNT cantilevers on the Cu$_3$Si nanobeams. No external electric or magnetic fields nor lithographic procedures are required to produce the self-aligned Cu$_3$Si nanobeam structures. The lateral alignment along [011] and [01-1] directions of these Cu$_3$Si nanostructures within the plane of the growth substrate is remarkably persistent over the entire area of the substrates. The strong attachment of CNT to Cu$_3$Si will be extremely helpful in enhancing the reliability and accuracy of CNTs used in nanoelectromechanical systems, especially for CNT applications relying on robust and durable mechanical interactions between CNT and other interfacing materials. Another advantage of our method consists of the possibility for multipurpose utilization of the self-aligned Cu$_3$Si nanostructures. In addition to serving as CNT-producing catalysts, Cu$_3$Si nanobeams can be utilized as an integral component in carrying out desired CNT-Cu$_3$Si functions such as relaying electrical, thermal, or mechanical signals. Such direct integration of catalysts as a working
component of final CNT applications eliminates the need for post synthetic CNT purification in order to remove unwanted catalysts.

6.5 Conclusion

We demonstrate a straightforward in situ growth route to fabricate CNT cantilevers anchored to catalytic Cu₃Si nanobeams. We show that self-assembled nanoscale structures of Cu₃Si can be produced via CVD and these Cu₃Si structures can effectively function as active catalysts for creating CNTs. Our experimental scheme is based on the self-assembly of catalytic Cu₃Si nanostructures where the self-aligning nature of Cu₃Si nanobeams on Si substrates can be exploited for dual purposes in CNT synthesis and application. Our approach eliminates traditional lithographic steps while providing regularly assembled nanostructures over very large areas without the use of external fields. In addition, our new method leads to integration of CNTs with other useful nanomaterials directly upon CNT synthesis. Such efforts can be extremely beneficial to many important technological applications of CNTs in nanoelectromechanical systems.
6.6 References


Figure 6.1. (a) SEM images of as-grown self-aligned Cu$_3$Si nanobeams. The SEM images are (1) 45 x 45 µm, (2) 15 x 15 µm, (3) 4 x 4 µm, (4) 1.5 x 1.5 µm, and (inset in 4) 1 x 1 µm in scan size. Upon synthesis, Cu$_3$Si nanobeams preferentially grow along [011] and [01-1] directions on the underlying silicon (100) substrates. These two preferential growth directions of Cu$_3$Si nanobeams are indicated in (2) with white arrows. The self-assembled Cu$_3$Si nanostructures form on the entire substrate surface. The aspect ratio of Cu$_3$Si nanobeams in these images is 1:7 in width: length. The exact dimension and aspect ratio of the Cu$_3$Si beams can be effectively controlled by changing their growth time. (b) X-ray diffraction spectrum shows two small peaks near 2θ = 45° which correspond to Cu$_3$Si (320) and Cu$_3$Si (312), respectively. (c) Auger spectrum of CVD grown, self-assembled Cu$_3$Si nanobeams after on a Si substrate after Ar ion sputtering.
Figure 6.2. (a) AFM images showing CNTs grown on Cu3Si nanobeams. The AFM images are (1) 10 x 10 µm and (2, 3, and 4) 6 x 6 µm in scan size. Images 1 and 2 were acquired in phase imaging mode where image 2 is the magnified image of the area marked in 1. Image 3 displays topographic scan of CNTs grown on Cu3Si nanobeams. Image 4 displays a phase scan of the same CNT-Cu3Si system as in Image 3. For clarification, CNTs grown on a Cu3Si nanobeam are marked with white arrows in images 1 and 3. These AFM images clearly show that Cu3Si nanobeams catalyze CNT growth in a CVD reactor. (b) Typical line profile measured across a CNT grown on a Cu3Si nanobeam shows an apparent full width of 30 nm. This full width corresponds to the sum of the AFM tip diameter of ~20 nm and the CNT diameter of ~9 nm. The black line in the 2.5 x 2.5 µm phase AFM image, shown on the left, depicts the line analysis profiled on the right.
Figure 6.3. FESEM images of CNT cantilevers on Cu₃Si nanobeams. The FESEM images are (a) 4 x 4 μm, (b) 2.5 X 2.5 μm, and (c and d) 700 x 700 nm in scan size. The magnified images (c) and (d) display individual CNT cantilevers anchored on the two Cu₃Si nanobeams shown in panel (b). For clarity, CNTs are highlighted with white arrows. The average diameter of cantilevered CNTs on Cu₃Si nanobeams is determined as 8.5 nm.
Appendix
Protein activity on PS-b-PMMA

A. 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 g/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
Omkar R Parajuli was born in Pokhara valley near the Himalayas in Nepal. He received his Master of Science (M.Sc) in Chemistry from Tribhuvan University, Kathmandu, Nepal. He came to the United States in 2003 as an immigrant. After some struggle to adjust in a new country, he joined the Department of Chemistry at The Pennsylvania State University; University Park in Fall 2005 to pursue Ph.D degree. He joined Dr Jong-in Hahm’s research group in the Chemical Engineering Department. His research includes the study of novel nanomaterial platforms for enhanced biomolecular detection. His research contributions were honored with following awards including a highly competitive innovation award for the highly unexpected, innovative and multidisciplinary finding of “carbon nanotube cantilever on self-assembled, self-aligned copper silicide nanobeams.”


- **Dalalian Fellowship Award** for outstanding research efforts and excellent academic performance, Chemistry, The Pennsylvania State University, 2008

- **Graduate Student Travel Awards.** Chemistry, The Pennsylvania State University, 2008, 2009

- **Geiger Fellowship Award** for outstanding research and academic excellence Chemistry, the Pennsylvania State University, 2007

- **Incoming Graduate Student Award** for outstanding graduate course work and teaching performance. Chemistry, The Pennsylvania State University, 2006

- **Braddock Fellowship Award** for academic achievements Chemistry, The Pennsylvania State University, 2005