FABRICATION OF NANO-SCALE DNA ARRAYS USING DIBLOCK COPOLYMERS

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ABSTRACT

The objective of this report is to develop a method for fabricating substrates which can be used for patterning DNA/oligonucleotide arrays at nanometer length scale. The development of these arrays can be of major significance in the field of biological sensing. The present methods used for creating these nanoscale arrays are based on photo or e-beam lithography and the use of these techniques is expensive in terms of cost and time involved in the fabrication. In this report a simple and easy method is presented which is based on the property of self assembly shown by the diblock copolymers. The property of these self assembled polymer templates to pattern metal nanoparticles is also used. Gold nanoparticles have been used and patterned on the thin films of diblock copolymers replicating the exact pattern at nanometer length scale and spacing as shown by the diblock copolymer thin films.

For the first case, thin films of polystyrene-b-polymethylmethacrylate (PS-b-PMMA) diblock copolymer were used for their phase separation property. In this case, the two blocks of PS and PMMA show phase separation and form PS-rich and PMMA-rich domains separated by spacing at nanometer scale. PS forms the matrix and PMMA forms the lying down cylinders parallel to substrate, giving a finger print pattern striped structure. Then the property of preferential attraction of gold nanoparticles to the PS rich domains was used to create gold nanoparticle arrays showing the same nanometer spacing and the striped pattern. These gold particle domains were then used to bind DNA/oligonucleotide molecules using the gold-thiol linkage chemistry and hence one dimensional nanoscale DNA/oligonucleotide arrays were synthesized.
For the second case, we used the micelle forming property of amphiphilic diblock copolymers in a solvent selective for one of the blocks. For this study polystyrene-b-poly(4-vinylpyridine) (PS-b-PVP) was dissolved in toluene which is a good solvent PS as compared to PVP. Gold nanoparticles deposited on these films were seen replicating the same two dimensional hexagonal closed packing of the micelles with nanometer length scale spacing. These gold particles were again used for binding DNA/ oligonucleotidide molecules hence creating a two dimensional array. The presence and stability of these biomolecules on the gold decorated substrates has also been investigated by using Ultra Violet-Visible spectroscopy technique.
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CHAPTER 1

INTRODUCTION

Fabrication of nanoscale arrays of biomolecules like proteins and DNA have attracted a lot of attention in the field of nanobiotechnology because of their biological and medical applications (1). These biomolecular arrays are also useful in immobilizing and detecting various biosystems like viruses (2), bacteria (3) and cells (4) on the substrates. The technology of DNA microarrays (also know as DNA chip or gene array) is based on the single stranded DNA’s tendency to bind itself to its exactly complementary strand to form a double stranded DNA. In this technique, the DNA fragment to be analyzed is labeled with a fluorescent tag and incubated on to a substrate on which a large number of nucleotides are immobilized. The sequence of the oligonucleotide or the complementary DNA (cDNA) at each binding location is already known and the signal intensity coming from each location is analyzed to determine the extent of hybridization and the sequence of the target molecule. This technology allows the analysis of large numbers of genes at a high resolution by the hybridization of the labeled DNA to a substrate immobilized with cDNAs or oligonucleotides. These arrays can be used for many different purposes and the most prevalent of them is to measure gene expression levels (messenger RNA abundance) for simultaneous study of thousands of genes which has been used to investigate many biological processes (5). The mRNA from the sample under investigation is reverse transcribed to the corresponding DNA sequence, which is then immobilized on the substrate. DNA microarrays have also attracted attention due to their use towards understanding of diseases like cancer (6).
There are two general types of microarrays (7, 8), the first usually involves cDNA probes (about 500-5000 bases long) immobilized on glass or nylon substrates using robotic techniques. The probes can be obtained from various sources like amplification of samples by Polymerase chain reaction (PCR). Arrays of cDNA work like the widely used dot blots where a relative hybridization between two labeled sample e.g. disease versus normal, reveal differential expression (9). These kinds of arrays have several advantages, including relatively low cost and high specificity as these arrays can be easily customized by the addition of new cDNAs. The down side of these arrays is the required maintenance of cDNAs in PCR product form and problems of cross hybridization (10).

In the second approach the probes are shorter, oligonucleotides approximately 25 bases long. These oligonucleotide arrays are fabricated either by in-situ combinatorial synthesis or by conventional synthesis followed by immobilization on the substrate. In this case a number of oligonucleotides are constructed which identify with a unique sequence in the targets. The advantages of this kind of arrays is that the PCR maintenance is not required, better defined arrays and these oligonucleotides can be designed against a particular region of interest of the cDNA which will give high sensitivity and the low chances of cross hybridization.

A lot of research is going on in the area of the scaling down the feature sizes of the arrays which will enhance the number of investigated molecules and also the efficiency of the detection. At present there are only a few methods to fabricate DNA arrays at a nanometer length scale and all of them are based on lithographic techniques which are discussed in detail in the next section. All of these techniques are serial in nature and time consuming to produce the arrays at large scale.
In this report, a bottom up approach to fabricate the DNA arrays with length scale of the order of tens of nanometers is investigated. The properties of block copolymers of phase separation and self assembly at nanometer length scale are exploited for this purpose. We have used polystyrene-b-polymethacrylate (PS-b-PMMA) films, which after phase separation gives domains rich in PS and PMMA respectively, arranged in a regular striped pattern with nanometer spacing. One of the domains is then decorated with gold particles, hence forming one dimensional array of gold particles after the removal of polymer film. Micelle forming polystyrene-b-poly(4-vinlypyridine) (PS-b-PVP) system was used for their self assembly property of forming hexagonally packed structures in thin films. The gold decoration resulted in a two dimensional gold particle array after the polymer removal. The thiol linkage between the functionalized oligonucleotide and gold was used to create arrays replicating the gold patterns which in turn replicate the phase separated finger print stripes pattern in the case of one dimensional arrays and hexagonally packed structures for two dimensional arrays.

DNA arrays fabricated by this method have the advantages of the bottom up approach as many arrays can be fabricated in a parallel fashion in an easier and cheaper way as compared to the expensive and serial nature of the lithographic techniques. The nanometer length scale of these arrays also ensures increased efficiency as larger number of molecules can be now investigated on a single chip as compared to the conventional arrays.
CHAPTER 2

BACKGROUND

2.1 Patterning of DNA arrays

Many approaches have been used for the fabrication of biomolecular nanoarrays using various top-down lithographic and bottom up self assembly nanoscale biopatterning techniques (11, 12). McGall et al (11) used the photolithographic approach to synthesize high-density arrays, using repeated cycles of illumination and nucleotide coupling to construct a two dimensional array of oligonucleotide sequences in a combinatorial manner. In this approach a photo-labile protecting group is selectively removed from growing oligonucleotide chains by exposure through photolithographic masks.

![Light directed nucleotide array synthesis](source: Reference 11)

They used a resolution of about 20 microns which corresponds to array density of the order of $10^5$ sequences/cm$^2$. The problem with this technique is that a high contrast is required between exposed and unexposed regions and this contrast can not be maintained as the size of the array is decreased, due to the diffraction limit imposed on the Ultra violet rays and hence higher resolution is not possible.
Demers et al have used Dip-Pen nanolithography (DPN) to pattern oligonucleotides on both metal and insulating surfaces (13). DPN is a scanning probe based lithography in which an AFM tip is used to deliver chemical reagents to the substrate and the array size that can be achieved is below 100 nm. It has become an attractive technique to pattern biomolecules on the surfaces, as the molecules can be immobilized either in inert or ambient atmospheres without being exposed to possibly harmful UV or e-beam radiations. DNA or oligonucleotides arrays can be fabricated on a gold surface by modifying the biomolecules by thiol group. Given all the advantages of this technique, it requires a very precise control of the ambient humidity, functionalization and inking of the AFM tips.

Microcontact printing (µCP) has been used as a technique to pattern protein molecules on a substrate (14, 15). Lange et al used the same technique to pattern DNA molecules (16). In this technique a modified poly-dimethylsiloxane (PDMS) stamp is inked with the desired biomolecule forming a mono layer. The stamp is then rinsed with a buffer and the biomolecules are printed on the substrate by putting the stamp in close contact with the substrate and applying some pressure. They have used the negative charge of the DNA...
phosphate backbone to electrostatically attach it to the positively charged modified PDMS. In this case, features of 1 µm size are obtained (Fig.3). The low elastic modulus of PDMS stamp does not allow the replication of the nanoscale patterns hence the generation of DNA arrays of smaller sizes is not possible using this method. Improving this technique, Li et al have used the PDMS stamp for nanoprinting, to pattern biomolecules at sub 50 nm scales (17). They used an AFM tip grating as the master and the V-shapes stamps were created using the e-beam lithography.

E-beam lithography has also been used to pattern DNA using the negative potential of the phosphate backbone. Klinov et al aligned DNA molecules using a combination of high resolution patterning using e-beam and then macroscopic deposition. A graphite substrate

Figure 3. Microcontact printing of DNA. (Source: Reference 16)
functionalized by positive amino group was exposed to e-beam in a certain pattern and that neutralizes the charge in the exposed area. DNA molecules were then deposited which patterned themselves in the neutralized region on the substrate (18).

2.2 Block Copolymers

Block copolymers have been an interesting area of research for a long time especially owing to their phase separation property to form regular domains of different blocks, having a length scale of the order of 10-100 nm depending on the molecular weight of the polymer. This phenomenon gives access to a length scale which is difficult or expensive to obtain using photolithography or e-beam lithography.

A block copolymer consists of two or more polymeric chains (block) which are chemically different and covalently attached to each other. The chemically distinct and thus immiscible blocks undergo phase separation but due to the covalent bonding between the blocks, the separation is not at macro-scale as in a simple case of oil and water mixture, rather the separation is at molecular level in which the copolymer chains organize themselves to put different block on opposite sides of an interface and hence after phase separation various ordered patterns of the corresponding blocks are formed depending on various parameters discussed in this section.

In simple case of a two polymer blend system A and B, the phase separation may be controlled by three experimental parameters: the degree of polymerization (N), the composition (f) and the A-B Flory-Huggins interaction parameter ($\chi$) (19).

The Flory-Huggins equation shows how these parameters affect the free energy per segment of the system:
\[
\frac{\Delta G_{\text{mix}}}{k_b T} = \frac{1}{N_a} \ln(f_a) + \frac{1}{N_b} \ln(f_b) + f_a f_b \chi
\]

The first two terms corresponds to the configurational entropy of the system and can be regulated by changing the relative lengths of the chains and fractions of A and B polymer. In the third term, \( \chi \) is associated with the A-B segmental repulsion and is a function of both the chemistry of the molecules and temperature (19).

In a general form,

\[\chi = \frac{a}{T} + b\]

\( a \) and \( b \) are constants determined experimentally. The important assumptions for the Flory-Huggins equation are that it is a mean field theory, hence neglects spatial fluctuations in composition and that there is no volume change of mixing in the system.

In block copolymers, the equilibrium structure after phase separation must minimize the interactions of the incompatible A and B blocks without over stretching the blocks (20). This segregation is opposed by associated decrease in the entropy that arises from the localization of block-block joints at the interfaces and the stretching of chains to maintain a uniform density (21). The entropy of diblock system can be scaled as \( S \sim 1/N \), and hence the product \( \chi N \) controls the segregations, and the strength of the segregation of the two blocks is proportional to \( \chi N \). For \( \chi N \ll 10 \), the entropic factors dominate and the systems remains disordered and in a homogenous state. When \( \chi N \) almost equal to 10, there is a delicate balance between the entropic and enthapic factors, and as this parameter is increased the system changes to an ordered state with the blocks getting separated across a sharp boundary (Fig.4). Walton et al have predicted that the domain spacing would scale as \( d \sim \chi^{1/6} N^{2/3} \) (22).
Figure 4. The evolution of phase separation shown with respect to the parameter $\chi_N$ for a symmetric diblock copolymer with $f = 0.5$. (Source: Reference 21).

The temperature corresponding to the value of $\chi$ where the ordering starts is called order-disorder temperature (ODT). Below the ODT when the volume fraction of A ($f_a$) is small it forms spheres in body-centric cubic (BCC) lattice surrounded by a matrix of B. As $f_a$ is increased towards 0.5, block-A first forms cylinders in a hexagonal lattice, then a bicontinuous double gyroid structure, and finally lamellae as can be see in figure 4. The size and periodicity of the nanodomains are also functions of segmental interactions ($\chi$) and polymer size (N).

Figure 5. Effect of composition on the phase symmetry in the polystyrene-polyisoprene (PS-PI) diblock copolymer and $f_s$ refers to the overall volume fraction of polystyrene. (Source: Reference 21)
Semenov et al suggest that the phase transitions are driven by the tendency to curve the interface as the diblocks become asymmetric in composition (23).

In thin films or near surfaces, some additional driving forces exist which affect the morphology of the phase separated equilibrium state (24, 25). The polymer block with the lower surface energy will accumulate at the air-polymer surface and the component with the lower interfacial energy will be attracted to the supporting substrate. The phase separated structure has to adjust to two boundary surfaces and a certain film thickness, which can be a non integer multiple of the natural bulk repetition length. Because of these two constraints, the thin film systems show a lot of complexity and produce more complex structures as compared to bulk system.

Knoll et al chose thin films of a cylinder forming polystyrene-block-polybutadiene-block-polystyrene (SBS) triblock copolymer (24). In the figure 6, they have shown how the phase separated structures depend and changes with the film thickness. The structures vary from cylinders oriented parallel to the surface, hexagonally perforated lamellae to cylinders oriented perpendicularly to the substrate. In thin films, the lamellae formed by symmetric block copolymer can orient either parallel or perpendicular to the substrate. There are a number of possible arrangements of the lamellae depending on the surface energies of the blocks, substrate and the confinement of the film (26).
In the case where one block prefers the polymer-substrate and the other one prefers the polymer-air interface i.e. asymmetric wetting, a uniform film has a thickness \((n+1/2)d\), where \(d\) is the repeat spacing in bulk and \(n\) is an integer. In case of symmetric wetting, a uniform film has thickness \(nd\). If the initial thickness is not equal to \((n+1/2)d\) or \(nd\), then islands or holes are formed each having the required quantized thickness and the height difference of \(d\) to conserve the volume (27). Asymmetric block copolymers which show hexagonal or cubic packed spherical morphologies in bulk, form parallel cylinders or perpendicular cylinders in two dimensions (26). The long-range alignment of the morphologies of phase separated structures can be achieved in several ways e.g. using an electric field (28), patterning of substrates (29) or through solvent evaporation (30). Perpendicular alignment of lamellae or cylinders is also possible using a substrate with a
surface energy that is neutral with respect to both the components. This is usually done by coating the substrate with a random copolymer containing appropriate percentage of both components. Bicontinuous structures cannot exist in two dimensions; hence the gyroid phase is non existent in thin films.

Due to this long range pattern formation at nanometer scale, block copolymers have been used in various applications. For example, high density hard drives can be manufactured by using polymeric nanodomains to pattern magnetic bits with a larger number of bits per unit area as compared to the current technique of using optical lithography (19). Park et al showed the use of block copolymer films as masks to transfer dots and stripe patterns into semiconductors (31). The method they used was based on selective ozonation of the minority block in the block copolymer system (which was either PI or PB in this case). Ozone cleaves the unsaturated bonds and then they can be etched away using a solvent, leaving behind a pattern of holes or stripes in a PS matrix, which is then transferred to the underlying semiconductor substrate using Reactive Ion Etching (RIE) using CF$_4$. Kumar et al used the PS-b-PMMA phase separated domains to create protein arrays (32). They showed that the protein molecules show a strong preference to the PS domains after deposition and faithfully follow the patterns formed by the PS-b-PMMA copolymers and self organize in nanometer length scale arrays. Thurn-Albrecht et al created perpendicular cylinders in a PS-b-PMMA thin film by applying an electric field across the film (33). The PMMA was then degraded using UV radiation which simultaneously cross-links the PS. The degraded PMMA was etched using acetic acid and the resulting arrays of nanopores were filled with cobalt by electrodeposition, hence forming an array of metal nanowires.
When a block copolymer is dissolved in a liquid that is a good solvent for one of the blocks and a precipitant for the other, the polymer chains associate themselves to form micelles. The core consists of the insoluble polymer block while the corona consists of the soluble block. Micellization occurs in dilute block copolymer solutions at a fixed temperature above a certain concentration, called the Critical Micelle Concentration (CMC), below which the copolymer is present in the solution just as unimers. These micelles are generally spherical with a narrow size distribution, but the shape and size depends on certain conditions like block size, solvent etc. (34).

Depending on the relative block length, either star or crew cut micelles are formed. A star micelle is characterized by a small core surrounded by a large corona, whereas crew-cut micelles consist of a large core and a thin corona. Star micelles are usually prepared by direct dissolution of the diblock polymer in a solvent selective for the longer block. This method is not used for crew-cut micelles since the insoluble block is the higher fraction of the block copolymer. In this case, first the copolymer is dissolved in a common solvent good for both blocks and then a precipitant for the long block is added, hence resulting in the aggregation of the longer block forming the micelles.

The morphology is determined by the free energy balance among the chain stretching in the core, the core-corona interfacial energy and the repulsion between corona chains (35). By varying the corona size, the morphology change can be seen from spherical to cylindrical to bilayered vesicles. This change is because, if the corona size is reduced i.e. block length of the soluble component is shortened, it permits a larger aggregation number leading to larger core spheres resulting in large entropy penalty from stretching the chains in the core domain. Hence the micelles adopt a cylindrical form to satisfy the
interfacial curvature requirement and on further reduction in corona size, it changes to bilayer structures (36).

These polymeric micelles have been investigated before as their application for drug delivery (37). Since in an aqueous media, the hydrophobic block will form the core domain, a hydrophobic drug can be accommodated in there with improved solubility and the hydrophilic shell stabilizes the micelles in the solution. These polymeric micelles provide many advantages as drug carriers for the reduced side effects and selective targeting (38).

2.3 Metal patterning on polymer surfaces

Apart from applications of the block copolymer system mentioned in the above section, there is one more important property of block copolymers which is the ability to pattern metal nanoparticles at nanometer length scale (39, 40). These metal particles embedded in polymer films are of interest because of their optical and magnetic properties (41). Lopes et al used the phase separated PS-b-PMMA thin films having the thickness equal to the one repeat spacing and having the composition so that PMMA parallel cylindrical domains are formed in the PS matrix, with a repeat spacing of 50 nm (39). They thermally evaporated several metals and found selective decoration of either of the PS or PMMA domains. Au and Ag were found to prefer the PS domain and In, Pb, Sn and Bi preferred the PMMA domain and form wire like structures in the preferred domains. It was also observed that if the metal deposited polymer is annealed above its glass transition temperature ($T_g$) and below the degradation temperature, the selectivity of the metal particles to the preferred domain increases up to 100%. Lopes further showed that
this preferential selection of evaporated metal particles works only up to a certain extent. When the quantity of the evaporated gold was increased it completely ignored the underlying substrate pattern and did not return to the previous aligned pattern even after annealing for long durations (42). It is also suggested that these metal particles which form nanowires by coalescing with each other in their preferred block are non-equilibrium structure as the surface energies of metals exceed those of copolymers, and hence the metal-metal bond should overwhelm the metal-copolymer bonds and except for the a very small metal concentration, the final shape of the deposited metal should be a spherical aggregate which completely ignores the copolymer patterns (39).

Jenson et al have shown that the maximum density of particles on a two dimensional substrate is inversely proportional to the diffusion constant to the power 2/7 (43). Lopes compared the density of evaporated silver particles on homopolymer PS and PMMA and it was seen that the density of silver was much larger than on PMMA (42). Also the peak of the nearest distribution is at a larger distance for PMMA than PS. This shows that the silver particles are highly mobile on PMMA surface and get collected on the PS domain of PS-b-PMMA surface and that could be the reason behind the preferential decoration. This analogy can also be extended to gold particles as well, as gold particles also prefer PS than PMMA. Apart from the physical adsorption, chemical interaction have also been exploited to generate the metal nanostructures on polymer scaffolds. Zehner et al patterned thiol-passivated gold nanocrystals on PS-b-PMMA thin films (44). The reason for this selective decoration is given as the nonpolar alkane coating on the nanocrystals have much more energetically favorable interactions with nonpolar PS than comparatively polar PMMA.
Amphiphilic polymer systems like PS-b-PVP, which form micelles in a particular solvent which is compatible with just one of the blocks, have also been used for patterning metal nanoparticles in a hexagonally packed pattern imitating the same hexagonal packing of the micelles. There are two main routes to do this; one is to load the micelles with the nanoparticle or the precursor in the solution and then deposit it on a solid substrate or directly patterning of the nanoparticles on the substrate after depositing the micelles on it. In the first case, the periodic patterns are only possible if the concentration of the micelles is significantly high. The latter method exploits the already present periodic patterns of the micelles.

Spatz et al used PS-b-PVP copolymer in toluene which preferentially dissolves PS, hence forming micelles with PVP core and a PS corona (45). They treated this solution with gold metal precursor, tetrachloroauric acid (HAuCl$_4$) and then AuCl$_4^-$ ions were bound as counterions in the polar core of the micelles by protonating the pyridine units. These metallic salt loaded micelles were then treated with anhydrous hydrazine and the gold ions were reduced to form elemental gold particles in each micelle. The polymeric matrix is removed afterwards by oxygen plasma cleaning. They did several tests to show the strength of the binding of these gold particles to the substrates. These gold particles could not be removed by washing with different solvents and even after ultrasonication. The structure of the gold arrays also did not show any changes when annealed up to 800 °C, there was no sign of any particle movement or coagulation in the SEM images that were taken. The cause of this stability is assumed to be the even size distribution of the gold particles which precludes Ostwald ripening and the effective binding of the particles to the substrate. This also shows that the gold particles deposited by this method of using
the adsorption from a liquid colloid solution should also be equally stable on the substrates.
CHAPTER 3

EXPERIMENTS

Asymmetric PS-b-PMMA with 71% PS by weight and polydispersity of 1.06 was obtained from Polymer Source Inc. (Montreal, Canada). Silicon wafers were obtained from Silicon Inc. (Boise, Idaho) and were used as substrates because of their atomically flat topography. Silicon substrates (1 x 1 cm\(^2\)) were cleaned with ethanol, acetone and toluene and then spin coated with 2% (w/v) PS-b-PMMA solution in toluene, at a speed of 3500 rpm for 1 min. This spin condition was chosen so as to keep the thickness of the polymer films at about 50 nm which is equal to the bulk repeat spacing of the polymer. With this composition and film thickness, the PS-b-PMMA thin films forms lying down cylinders of PMMA in PS matrix parallel to the substrate as shown in fig.7. These Si supported polymer films were kept for annealing for 6 hours at 240 °C with a ramp up rate of 5 °C / min and a cooling rate of 2 °C / min in an inert argon atmosphere. After annealing clear phase separation can be seen giving a finger print pattern with PMMA cylinders in PS matrix and annealing also helps in removing the defects which occur in these patterns of PS-b-PMMA (46).

Figure 7. The cross sectional image of the film morphology showing the parallel lying down cylinders of PMMA (lighter region) in the PS matrix (dark region). (Source: Reference 39)
On the Silicon surface formation, Silicon oxide takes place due to the oxidation in air, and PMMA favors the silicon oxide surface due to its lower wetting energy and on the air-polymer interface, we get parallel lying down cylinders of PMMA in PS matrix as shown in the figure above.

PS-b-PVP with composition of 70% PS was bought from Polymer Source Inc. (Montreal, Canada). Silicon substrates after cleaning with methanol, de-ionized water and acetone, were spin coated with 0.5% (w/v) PS-b-PVP solution in toluene at a speed of 3500 rpm for 1 min. Since toluene is a selective solvent for PS, the polymer formed micelle with PVP core and PS corona and these micelles arranged themselves in the hexagonally closed packed structures.

Gold colloidal solution with a particle size of 2 nm was obtained from Ted Pelle Inc. (Redding, CA). The solution was then deposited on PS-b-PMMA substrate and PS-b-PVP substrates for 2 hours. It was then rinsed away with de-ionized water and gently blown dry using nitrogen. The substrates were then put in for annealing at 100 °C for 2 h with a ramp up rate of 5 °C / min and cooling rate of 2 °C / min in argon flow of 100 standard cubic centimeter (sccm) providing an inert atmosphere. After annealing, the polymer coating was removed using oxygen plasma cleaning using a plasma cleaner (Harrick Plasma) at RF for 2 mins. The remaining polymer coating was removed after the substrates were rinsed thoroughly with ethanol and toluene and then dried up using nitrogen.

In the case of PS-b-PMMA, Au particles preferred the PS domain and aligned themselves imitating the finger print pattern of the phase separated domains of PS and PMMA. In
case of PS-b-PVP it is assumed that the gold particles diffuse down to the metal attracting PVP core during the annealing and hence the patterned gold particles are achieved.

For DNA/oligonucleotide deposition, λ-DNA and a 16-mer (5’-HS-AGT GCG CGA GGA GCCT-Biotin-3’) sequence oligonucleotide (bast) functionalized with a thiol group on one end for providing linkage to the gold particles present on the substrate and a biotin group on the other end, were used. λ-DNA and bast were obtained from VWR Scientific Inc. (West Chester, PA) and the dilutions were made using a Tris-EDTA (TE) buffer.

For one part of the experiments, the DNA behavior on the control samples of randomly immobilized gold particles on silicon substrates was checked. For this, Poly-L-lysine (PLL), obtained from VWR Scientific Inc. (West Chester, PA) of concentration 0.1% (w/v), was deposited on silicon substrates for 10 mins. Then the gold colloid solution, diluted in deionized water to 50% concentration (v/v), was deposited for 30 mins in a humidity chamber and then rinsed off thoroughly with de-ionized water and gently blown dry using nitrogen. PLL is a positively charged amino acid due to the extra amino group in its backbone and attracts the negatively charged citrate ions which act as the stabilizer for gold colloid particles and hence gold nanoparticles are also attracted to the PLL molecules and are immobilized on the silicon substrate. The substrates were annealed at 80 °C for 2 hours followed by plasma cleaning for 2 mins and rinsing with deionized water and ethanol. This last step was to ensure that there are no impurities left on the substrates.

To confirm the thiol terminated DNA/oligonucleotide molecules attaching themselves to the gold particles, the control substrates of randomly immobilized gold particles on Silicon substrates using the PLL solution were used. The 16-mer oligonucleotide
sequence (BAST) was then deposited on the samples for 2 hours, allowing its thiol
groups to attach with the gold particles.

To show the presence of the biomolecules, UV-Visible experiments were performed on
these samples. For these experiments, Streptavidin attached Horseradish peroxidase
(HRP) enzyme molecules were deposited on to the substrate for 1 h and then rinsed away
with phosphate buffer saline (PBS). The Streptavidin-HRP was purchased form VWP
Scientific Inc. (West Chester, PA) and 10 µg/ml concentration was used for this
deposition. The interaction between Streptavidin and Biotin is very strong and these
groups have exclusive attraction for each other and this property has been used here. The
BAST sequence attaches itself to the gold particles because of the thiol group, and to
Streptavidin-HRP because of the biotin group on the other end. The presence of HRP
molecules on the substrate as confirmed by the UV-Visible experiment will also confirm
the presence of the oligonucleotide. For this experiment, the sample was dipped in a
solution of 1.25 mM 3, 3’, 5, 5’- tetramethylbenzidine (TMB) and 2.21 mM H$_2$O$_2$. The
TMB-H$_2$O$_2$ solution was bought from VWR Scientific Inc. (West Chester, PA). In
presence of H$_2$O$_2$, the HRP enzyme oxidizes TMB and gives a blue color. The change in
the color of the solution from colorless to blue gives a clear qualitative indication of the
presence of HRP molecules on the substrate.

The behavior of $\lambda$-DNA was also checked on the Silicon substrates with randomly
immobilized gold particles on them as compared to $\lambda$-DNA deposited on clean Silicon.
Although the $\lambda$-DNA did not have any thiol group modification, still it was clearly seen
that there is high concentration of DNA strands on the Gold-Si surface while there were
no strands visible on clean Silicon.
For quantitative measurements, UV-Vis absorbance spectrum was recorded using a Hewlett-Packard 8452A Diode array spectrophotometer at different time intervals. The blue color of the solution darkened with time due to the enzymatic activity and the intensity was recorded in the spectrum ranging from wavelength 500 to 800 nm with the maxima at about 650 nm.

The presence of the HRP molecules and hence the oligonucleotides on the patterned gold substrates can also be checked using the same UV-Visible spectroscopy technique. But a more useful technique to resolve the DNA/oligonucleotide arrays and image them would be Near-field scanning optical microscopy (NSOM). In NSOM technique, a light source with a diameter smaller than the wavelength of light is used as a probe. This probe is scanned very close over the substrate with a distance of a few nanometers. Using this method images can be taken with a high resolution which is not possible in simple microscopy owing to the diffraction limit. However, this NSOM imaging is beyond the scope of this report and is being reported here as the possible future direction of this work.

Atomic Force Microscopy (AFM) was used for imaging the polymer samples and the gold colloid deposited on to the polymer films. AFM imaging was done using a Veeco Multimode Nanoscope IIIa, in tapping mode at a scan speed of 1 Hz. Si tips with resonant frequency of 60 kHz and a spring constant of 5 N/m were used for the imaging.
CHAPTER 4

RESULTS AND DISCUSSION

Figure 8. AFM images (2 x 2 µm$^2$) showing phase separated domains of the thin films of PS-b-PMMA diblock copolymer. The bright and dark regions in the phase (A) and topography (B) are the PMMA and PS regions respectively.
In the figures (Fig. 8(a) and Fig. 8(b)), the phase and topography images taken by using the AFM are shown. The bright domains are the lying down cylinders of PMMA parallel to the substrate. The dark region is the PS matrix. The average repeat spacing of these domains is about 45 nm which is same as the thickness of the film. We have used these periodically spaced nanometer length scale domains to generate patterns of gold nanoparticles replicating the pattern and spacing, which will eventually be used to generate one dimensional DNA arrays with the same spacing and pattern.

Figure 9 shows the AFM images of the hexagonally closed packing of the PS-b-PVP micelles on a thin film. This micelle formation takes place when PS-b-PVP is dissolved in Toluene as it is a selective solvent for PS. This solution was spin coated on a Silicon substrate to form a thin film of the micelles. This closely packed structure is exploited to produce gold nanoparticle array, replicating the same hexagonal pattern. These substrates were then used to fabricate DNA arrays in two dimensions with repeat spacing of nanometer length scale.
Figure 9. AFM (1 x 1 µm²) of the PS-b-PVP thin film clearly showing the 2-D hexagonally packed micelles, formed when the diblock copolymer is dissolved in toluene and subsequently spin coated on a silicon substrate. Images A shows the topography and image B is the phase images.
Figure 10. (A) Topographic AFM image (1 x 1 µm$^2$) of the PS-b-PVP diblock copolymer thin film. (B) Sectional analysis taken along the black line shown in image A. The diameter of a micelle is measured to be about 45 nm.
Figure 11. (A) Topographic AFM image (1 x 1 µm\(^2\)) of the PS-b-PVP diblock copolymer thin film. (B) The section analysis of the topography image of an AFM along the black line shown in image A. The two arrows suggest the position of the centre of two micelles and the measured distance between them is about 40 nm.
In this micellar structures, the PS block forms the corona or the shell of the micelle because of its favorable compatibility with the solvent i.e. toluene while the PVP block forms the core as it is insoluble in toluene. The diameter of a micelle as measure by the AFM analysis is found to be about 45 nm and the distance between the centers of the micelles is measured to be about 40 nm (Figs. 10, 11).

After getting the phase separated domains of PS-b-PMMA and the hexagonally packed micellar structures of PS-b-PVP, the next step was to find the suitable conditions to pattern gold nanoparticles on these films; so that they replicate the exact pattern as on the polymeric thin film. In some previous works (39), it has been shown that in the case of phase separated domains of different polymers, metal particles prefer one or the other domain depending upon its nature. In these works, the authors have chosen evaporation of the metal on the polymeric thin film as the deposition method. However, the evaporation method poses some problems, like the requirement for an expensive set up and the difficulty in controlling particle size. Hence we used metal colloidal solutions and for deposition we placed a small droplet of the solution on the polymeric thin film and after a certain period of time, rinsed away the solution and dried off the film. The ideal time period of deposition was found after many trials, so that the deposition is not so little as there is no visible pattern and not so much that the density of the metal particles gets so high that they completely ignore the polymer template and form a lump type of structure, as it is mentioned before that the metal-metal interaction energy is higher than the metal-polymer interaction energy.
Figure 12. AFM images showing the topography (A) and phase (B) images of the gold nanoparticles which were deposited on to the PS-b-PMMA thin films and the film was later on removed, leaving just the gold nanoparticles on silicon substrate arranged in a pattern replicating the PS-b-PMMA phase separated polymeric domains. The bright regions in both the images show the gold nanoparticles.
Figure 13. (A) Topographic AFM image (1 x 1 μm²) of the gold decorated PS-b-PMMA substrate after the polymer film was removed. (B) The section analysis of the topography image (A) along the marked line. The spacing between the gold domains is about 40 nm, as measured by the AFM. It is about the same as the spacing between the PS-b-PMMA polymer domains.
Figure 12 show the gold deposition on PS-b-PMMA copolymer thin film. It can be seen that the gold nanoparticles are faithfully following the fingerprint patterns formed by the diblock copolymer films even after removal of the polymer film by oxygen plasma cleaning and solvent etching. The gold nanoparticles arrange themselves in the PS rich domain and this likeness of gold to PS as compared to PMMA has been studied and confirmed in some previous works (39). This is due to the higher mobility of gold nanoparticles on PMMA and hence they migrate to PS and get settled there. The spacing between the gold domains of these gold decorated polymer substrate is the same as the spacing between the phase separated polymeric domains of PS and PMMA which is about 40-45 nm.

In case of PS-b-PVP, some work has been done to use it to pattern metal nanoparticles using the PVP core to attract the metal precursors and hence forming the metal nanoparticles in the core of the micelles using chemical reactions. In this study, physical adsorption of gold nanoparticles directly from a colloid to the polymer surface was used. As shown in Figs (14, 15), the gold nanoparticles arranged themselves on the micelles in the same regular fashion as the micelles themselves. The polymer film was later on removed by plasma cleaning and rinsed with toluene and ethanol giving just a layer of gold nanoparticles in a regular hexagonal array with the regular spacing same as that of the micelles i.e. 40-45 nm. These arrays of particles provide us a template for a two dimensional array for DNA where every gold particle could work as a binding site for a different DNA/ oligonucleotide molecule, though the process of placing a different molecule on a different spot with a nanometer spacing is yet to be developed and can be considered as a future direction of this work.
Figure 14. AFM images (3 x 3 µm²) showing the topography (A) and phase (B) images of gold decorated substrates. Gold nanoparticles were first deposited on PS-b-PVP polymeric thin film supported on silicon and later on the polymer was removed leaving gold particles on silicon substrate. Gold particles can be seen to follow the hexagonal packing of the PS-b-PVP spherical micelles.
Figure 15. High resolution (1 x 1 µm$^2$) topography (A) and phase (B) AFM images of gold nanoparticles, replicating the hexagonal packing of PS-b-PVP micelles on silicon substrate.
Figure 16. AFM topography images (1 x 1 µm²) showing the comparison between the behavior of λ DNA on gold immobilized silicon surface (A) and clean silicon surface (B).

Figure 16 shows the λ DNA behavior on substrates where 20 nm size gold particles are randomly distributed as can be seen in image (A). A lot of DNA strands are visible in this
image and even though the λ DNA is not modified with thiol group it can be seen that the DNA is attracted to this substrate. This comparison becomes clearer when λ DNA is deposited and imaged on clean silicon as shown in image (B). In this case, the substrate looks clean with no visible strand, as the DNA is not attracted to the surface and got rinsed away after deposition.

Figure 17. Absorbance spectra recorded at different time intervals i.e. 0 min, 5 mins, 15 mins, 25 mins, 1 h and 2 h. The absorbance curve maxima is at 650 nm and the intensity can be seen to increase with time showing the activity of HRP molecules bounded on the substrate.
Similar AFM imaging could not be done in case of thiol and biotin-modified bast sequence as the oligonucleotide is too small to be imaged by AFM and hence we used the indirect technique of UV-Visible spectroscopy to ascertain the presence of presence of oligonucleotides on the surface. Figure 17 shows the data gathered from the UV-Vis experiments showing the activity of HRP molecules bound by the streptavidin-biotin linkage on the randomly deposited gold nanoparticles samples treated with bast. It shows that the gold particles are getting attached to the thiol-modified oligonucleotide sequence and hence the gold arrays fabricated using the polymer films can actually be used for DNA array formation.
CHAPTER 5

CONCLUSION

In this report, we presented a novel method to fabricate nanoscale DNA arrays, using the self assembly of diblock copolymers and the immobilization of gold nanoparticles with regular spacing on the block copolymers. The gold-thiol bonding was utilized to bind the DNA/ oligonucleotide molecules functionalized with a thiol group to the gold nanoparticles present on the substrate thereby forming DNA/ oligonucleotide arrays. The arrays are one dimensional in case of PS-b-PMMA as stripes of gold nanoparticles are formed due to their preferential attraction to the PS domain with nanometer spacings and then the DNA/ oligonucleotides are attached onto these gold domains. Two dimensional arrays are formed using the PS-b-PVP amphiphilic block copolymers where the gold nanoparticles are arranged in a hexagonal packing with regular nanometer length scale spacing. Hence every gold nanoparticle can be used as a binder to the DNA/ oligonucleotide molecules. However, the technology to immobilize a different biomolecule to a different gold nanoparticles arranged in a nanometer length scale spacing, is yet to be studied and developed. The scope of this report is limited to show a simple and easy way to create substrates containing nanometer arrays of gold nanoparticles which can be used to create DNA/ oligonucleotide arrays, though the development of this technique would surely be a future direction in this work. AFM tips can be used by functionalizing it with the biomolecules to be deposited on the gold rich domains.

We deposited a single kind of DNA/ oligonucleotide molecules on these substrates and the attachment of these biomolecules to the gold particles present on the substrates was
confirmed by the UV-Vis imaging, though NSOM would be a better technique to directly image the DNA present on the gold arrays. However, this part of the imaging is beyond the scope of this work and hence would be an important future step to be taken in the imaging of these nanoscale arrays. So using UV-Visible spectroscopy we have shown that these substrates can actually be used to create arrays of different biomolecules in future and carry the potential to positively affect the ongoing research in developing novel nanoscale biomolecular sensors.
REFERENCES


