Highly Sensitive Nanoparticle-based Multifunctional Biosensor for Antigen Detection

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Abstract

Precise and selective positioning of nanoparticles gives rise to many applications where assembly of nano building blocks with different biological or chemical functionalization is necessary. One remarkable application is the simultaneous early detection of multiple biomarkers in the field of miniaturized multiplex biosensors. To enable multiplex detection of antigens, nanoparticles with various antibody coatings can be selectively assembled in trenches on different regions on a biochip so that they bind selectively to the specific antigen of interest. The presented work utilizes electric field assisted assembly techniques to assemble nanoparticles with various surface functionalization and coatings. Nanoparticles are assembled into pre-fabricated via and trench patterns generated on a PMMA coated gold surface, using electron-beam lithography. Two techniques have been developed for selective assembly of nanoparticles: sequential size-selective directed assembly and sequential site-selective assembly. Both selective assembly techniques provide fast and reproducible assembly over large areas while achieving high yield. The sequential size-selective assembly is a template-assisted technique where the selectivity is achieved by controlling the size of the nanopatterns and the size of the nanoparticles. The possibility of particle detachment and the factors affecting the sorting efficiency for this technique is studied. We show that a complete sorting can be achieved when the size of the vias is close to the diameter of the nanoparticles and the size distribution of the chosen nanoparticles do not overlap. In the site-selective assembly, the selectivity is achieved by having electrically isolated sites (regions) on the same chip. Electrophoresis is performed for each region in a step
by step process. Selective assembly results, for up to four nanoparticles with various coating/functionalization are presented using the site-selective assembly technique. We use the electrophoresis technique to assemble the cancer specific anti-PSA, mAb-2C5 and CEA coated nanoparticles to show that the nanoparticle-based biochip can successfully measure low concentrations of various antigen. The principle of operation of these biosensors is the fluorescence based ELISA. Testing results of the nanoparticle-based biochips indicate very high specificity and the detection limit 200 times smaller than the commercially available devices for antigen detection, laying the foundation for early detection of various diseases. The optimized assembly of antibody coated particles and selective assembly techniques introduced in this work provide the necessary tools for fabricating a miniaturized nanoparticle-based in-vivo multiplex biosensor. The antigen detection results show the great potential for early detection of various diseases using the fabricated in-vivo device.
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1

Introduction

In this chapter, a general overview of the thesis is given with appropriate references to existing stat-of-art literature. Section 1.1 introduces the research objective; section 1.2 describes the background and literature review; section 1.3 presents the overview of the thesis.

Following the introduction which gives an overview of the existing research in the relevant field, Chapter 2 presents the background, experimental techniques, results and analysis for the assembly of functionalized and coated nanoparticles; Chapter 3 describes the background, experimental approaches, results and analysis for the selective assembly of nanoparticles; Chapter 4 presents the background, experimental procedures, results and analysis for the in-vitro antigen detection; Finally, Chapter 5 summarizes the conclusions and provides suggestions for future work.
1. INTRODUCTION

1.1 Research Objectives

The objective of this research is to develop fast and reproducible techniques for selective assembly of nanoparticles with various antibody or drug coating for miniaturized biosensing application, to understand the roles of various factors (e.g. stability of mAb-coated nanoparticles, capillary forces, pattern depth, etc.) on the assembly process, to manufacture nanoparticle-based biosensing chips with various antibodies and to test the biosensors for the detection of various cancer antigens.

The study will demonstrate that functionalized and mAb-2C5, PSA, CEA and Insulin coated particles with different sizes can be selectively assembled inside nanopatterns of a single chip. We will also show that the resulting chip can successfully detect low concentrations of various antigens. We will propose that the presented selective assembly techniques can be promising for future miniaturized nanoparticle-based multiplex biosensing devices. The specific objectives are listed as follows:

- Investigate the electrophoretic assembly of plain, functionalized and antibody coated PSL nanoparticles by understanding and controlling various parameters that affect the assembly.

- Investigate the electrophoretic assembly of drug coated PSL nanoparticles for potential drug delivery applications.
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- Develop fast, effective and repeatable techniques for selective directed assembly of plain or coated nanoparticles.

- Understand and address various challenges associated with the electrophoretic assembly of the aforementioned particles such as particle agglomeration, and particles underneath the insulating layer.

- Study the effect of various factors on the stability of mAb coated nanoparticles and develop effective procedures to address the problem of instability of mAb-coated particles.

- Understand the effect of various forces throughout the electrophoretic assembly process and the capillary force during the insertion and removal of the template into the liquid suspension.

- Develop effective technique for the preventing the particle detachment after the assembly by countering the effect of removal forces.


- Test the biosensing chip with various cancer antibodies and study the limit of detection.

- Address the challenge of non-specific binding of the biomarkers.
1.2 Background and Literature Review

Nanoparticles are considered among the building blocks for high-performance electronic and optoelectronic devices and have emerged as a new kind of inspiring material and have found applications in a wide range of areas, such as electronics, catalysis(5, 6), biomodeling(7 8), biolabeling(9 10), sensing(11), photonics(12), and optoelectronics(13) due to their special characteristics. For example, excellent biological compatibility and large surface area of Gold nanoparticles has been extensively used in modification of various electrodes and fabrication of different kinds of biosensors, they are also used as optical labels can detect antigen-antibody complex formation(14 15 16), DNA hybridization(17 18 19) or nucleic acids(20 21). Silica nanoparticles are generated by chemical reactions and are used for biological markers(22) and lasers(23). Ion sputtered aluminum(24), evaporated gold(25) or indium(26) nanoparticles can be used to create single electron transistor. Metal nanoparticles have been extensively used as catalysts for growing nanowires(27 28 29). High density magnetic recording media(30) has been made using transition-metal nanoparticles. Nanoparticles can also be utilized as etch masks in nanolithographic processes to fabricate nanopillars(31).

Most of the above applications are possible if we can find efficient ways to manipulating nanoparticles and assembling them into desirable nanostructures.
1. INTRODUCTION

1.2.1 Nanoparticle Assembly Techniques

Ordered array of nanoparticles have attracted tremendous interest because of their potential applications in various emerging fields such as nanophotonic crystals, nanosensors and nanoelectronics. One of the fundamental issues one faces in using nanoparticles for fabrication is the ability to distribute nanoparticles uniformly onto surfaces with desirable degree of control. A conventional approach is single nanoelement manipulation, where single particles are manipulated using atomic force microscope (AFM) or dynamic force microscopy (DFM). In such approach, control softwares are applied to reduce instrument errors. This approach is useful in applications varying from high-density data storage to single electron electronics and also building new device architectures. However, for precise and reliable positioning of nanoelements on a surface, the system operation should be monitored in real-time, and the speed of the operation is significantly reduced. Therefore, such approach is not appealing for manufacturing of nanodevices, and alternative methods should be applied.

To avoid mentioned barriers, researchers have tried employing different methods to generate nanoparticle arrays on a given surface. In most of these methods nanoparticles suspended in a solution are assembled onto pre-fabricated patterns on a template. These methods can be broadly classified into two kinds (i) self
assembly\(^{(40, 41, 42, 43, 44, 45)}\) and (ii) directed assembly\(^{(46, 47)}\).

The idea of self-assembly is borrowed from biology and chemistry, where initially chemical approaches were used to control deposition of composite materials. Template Assisted Self Assembly (TASA) uses capillary interaction to assemble monodispersed particles into prefabricated patterns with various size and shapes. For example, by producing stripes and channels with alternating wettability, one can build up monolayers onto different substrates.\(^{(48)}\) On the other hand, physical self-assembly is based on self-organizing behavior of nanoscale materials either during or after deposition. There are various interesting examples of physical self-assembly such as symmetry-driven self-organization\(^{(49)}\), smart templates\(^{(50)}\) and colloidal self-assembly (nanoparticles aggregation into clusters).

Self-assembly requires finely balanced particle-particle or particle-template interactions for achieving desired assembly. Also, it only applies to a subset of nanoparticles that match the specific chemistry of the template surface.

In directed assembly process, the assembly of nanoparticles on the patterns is initiated by an external force to direct the particle to the desired position. After the assembly, van der Waals interaction between the template and the particle takes over once the driving force is no longer applied. One method of providing an external force is by applying an electrostatic field\(^{(51, 52)}\).

Dielectrophoresis utilizes the response of polarizable particles to an external non-uniform electric field. A tremendous amount of early and current research on
dielectrophoretic assembly are dedicated on the manipulation of DNA\cite{53, 54, 55} and live cells.\cite{56, 57, 58, 59, 60, 61, 62, 63, 64}. In particular, by controlling the frequency-Dielectrophoresis endent cell membrane polarizability, live and dead cells and also cells of different genotype can be separated in flow-through devices.\cite{65, 66, 67, 68}. The combined action of dielectrophoresis and chaining forces (dielectrophoresis induced by other nearby particles) can be implemented for assembly of particles. For example, alternating electric fields has been applied to the gap between planar electrodes for a fast and switchable assembly of colloidal crystals from polymer and silica microspheres.\cite{69, 70}. More importantly, such two-dimensional crystals are precisely oriented by the applied electric field without requiring prior templating by microlithography or micromolds.

Electrophoretic deposition In contrary utilizes the uniform electric field for assembling charged particles. Giersig and Mulvaney\cite{71} first reported the electrophoretic deposition of gold and latex particles in 1993. Since, several research groups have successfully used electrophoretic deposition to assemble nanoparticles on conductive electrodes.\cite{72, 72, 73, 74, 75}.

Electric field directed assembly has several advantaged over self assembly techniques. One advantage is the ability to precisely control the field-induced particle-particle interactions and the forces exerted on the particles by adjusting various parameters that control the electric field. Another advantage of using electric fields assisted assembly is the simplicity and availability of the experi-
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mental equipment.

1.2.2 Selective Assembly of Nanoparticles

Physical properties of micron size or nanoparticles strongly depend on their relative dimensions, therefore positioning nanoparticles according to their size is a major requirement for many applications.

Previous attempts to position nanoparticles on a template according to their size, have been limited to template-assisted self-assembly techniques by controlling the chemical properties \(^{(76, 77, 78, 79)}\) or topography \(^{(80, 81, 82)}\) of the template.

First group of studies, chemical templates have been utilized to self-assemble multiparticle patterns on surfaces. The nanoparticles has been directed to the patterns using specific interactions\(^{(77)}\), wettability\(^{(76)}\), or charge contrast\(^{(78, 79)}\). For example, Fan et al. \(^{(76)}\) have shown that nanoparticles of various diameters can be sorted accordingly by employing self-assembly assisted by lyophilic features of various dimensions that were created on a template.

The disadvantage of self assembly using chemical templates is that the technique only facilitate selective assembly of a limited subset of particles with specific surface chemistry matching the employed template surface.

Other attempts employed topographical patterns or traps on the surface for
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physical trapping of the particles based on their size. Varghese et al. \cite{81} showed that it is possible to sort and assemble particles with various diameters by using the centrifugal force generated during spinning to drive the particles onto topographically pre-patterned photoresist surface. Kuemin et al. \cite{82} recently employed TASA to trap 200nm, 350nm and 500 nm particles on carefully designed topographic features. Despite good yield, their suggested technique is slow, limiting the possibility of high rate manufacturing.

Electric field assisted assembly provides exact positioning of the particles by allowing the control of various parameters that affect the assembly process. Template assisted electrophoresis therefore is a promising unexplored method for efficient sorting of nanoparticles on surfaces.

1.2.3 Nanoparticle-Based Biosensors

Biosensor discovery dates back to the 1960’s\cite{83} with emphasis on detection and monitoring in medical applications. For diagnostics and regular therapeutic screening of various diseases, biosensors have become a very valuable tool in the medical industry. Currently, not only these sensors are used for detection of biological species, but also they can be used to monitor various developmental stages of a particular disease or a biological process\cite{84,85}.

Recently, there has been tremendous interest in converging of biotechnology...
and nanotechnology to develop hybrid nanomaterials. Such materials combine the exceptional electronic, photonic, and catalytic features of nanoparticles with the highly selective recognition, transport and catalytic properties of biomaterials, such as DNA and enzymes. Combining of nanomaterials such as nanoparticle, nanorods and carbon nanotubes with biomolecules has flourished an appealing area of research within nanobiotechnology(86, 87).

Because of their high surface area and size compatibility with biological elements such as enzymes, antigens and antibodies(2-20 nm), nanoparticles can carry on important functions such as the immobilization of biomolecules and the catalysis of electrochemical reactions. The combination of nanoparticle-biomolecule systems with surfaces could provide new functionalities by providing new generation of surface ordered architectures. One can use photonic properties of nanoparticles to probe biological recognition events on surfaces. For example, surface Plasmon resonance (SPR), surface enhanced Raman spectroscopy (SERS), and surface-enhanced fluorescence spectroscopic techniques can be implemented to probe biomaterial structures adsorbed on the surfaces of nanoparticles. Moreover, the chemical reactivity of biomaterial structures can be enhanced by electronic and catalytic properties of nanoparticles.

Trapping and localization of biological molecules can be obtained through different methods such as electrostatic binding, physical adsorption, covalent coupling and specific recognition(88, 89, 90). Such methods allow immobiliza-
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tion of numerous biological molecules ranging from enzymes, antibodies, antigens to DNA with various application including bioreactors, affinity separations, Biosensing and construction of biofuel cells. Moreover, the immobilization of biomolecules on transducers such as piezoelectric crystals, electrodes, or field-effect transistors is promising for the development of biosensors and bioelectronic devices. (91, 92, 93)

Current established methods for sensing proteins include enzyme-linked immunosorbent assays ELISA (94) and Western Blots (95), (94). Unlike the commercially available ELISA based sensors, it has been shown that uniformly distributed mAb on nanoparticles increases the orientation and distribution of antibodies (96) increasing the antibody-antigen binding events and hence the sensitivity of the sensor. This makes the nanoparticles a strong candidate for fabricating biosensing devices. Recent efforts have been channeled to increase the orientation and distribution of antibodies by employing uniformly distributed nanoparticles that are coated with antibodies on the sensor substrate (97, 98, 99, 100, 101, 102, 103, 104, 105).

Microbead based sensors can detect antigens in solution (103, 105) or on chip (101, 106, 107). Several methods have been employed for assembling the antibody on chip including micromanipulation (102), microfluidic trapping (104), evaporation (100), self-assembly on chemically-modified surfaces and electrostatic assembly (108).
Apart from the improved sensitivity due to the better orientation of the antibodies on the surface of nanoparticles, faster response, portability and small sample volumes, nanoparticle-based biosensors can facilitate multiplex detection in a small device.

1.2.4 Miniaturized Multiplex Biosensors

Simultaneous detection of multiple biomarkers has attracted tremendous interest in the field of micro-biosensors because of a need for the simultaneous monitoring of a variety of biomarkers in biological fluids to assess the progress of disease, toxicity, stress, etc. Recent strides in nanotechnology, advanced materials, biology and fluorescent spectroscopy have unlocked the potential for high sensitive and multiple detection of various diseases\cite{108,109,110,111,112}.

There are various approaches toward developing multiple-marker devices. One approach utilizes antibody arrays with fluorescence or surface plasma detection\cite{113,114}. Another approach uses liquid chromatography/mass spectroscopy\cite{115}. A different approach includes flow cytometry of coded nanoparticles coated with capture agents\cite{116}. Various groups have tried spotted protein and antibody microarrays for multiplex detection in small sample volumes\cite{117,118,119,120,121,122,123}.

The Lieber group at Harvard University demonstrated that a Silicon nanowire
1. INTRODUCTION

device can detect biological and chemical species\textsuperscript{(124, 125)}. They modified the
surface of two different nanowire sensor devices with antibody (Ab) capture agents
specific for different viruses. The charges on the viruses were shown to have
opposite signs leading to an increase and decrease in conductance when binding
to the specific Abs on the respective nanowire devices. The device therefore offers
simultaneous detection of up to two oppositely charged viruses\textsuperscript{(111)}.

The mentioned approaches for multiplex detection of biomarkers are not com-
patible with an implantable device and involves large and complicated instrument-
tations.

1.2.5 Keck Multiplex Nanoparticle-based Microbiosensor

The ability to construct nanoscale multi-analyte assay devices would clearly pro-
vide a powerful means of detection of biomarkers at the source namely in tissue or
blood. Thus the development of nanochips with multifunctional capability would
provide a powerful platform in the emerging field of in-vivo multiplex biomarker
diagnostics. Our main motivation for developing techniques for selective assembly
on nanoparticles was to build a miniaturized multifunctional in-vivo biosensor for
early detection of diseases. This micron scale device is assembled with arrays of
multiple biomarkers necessary for in-vivo early detection of diseases\textsuperscript{(11)}.

Figure\textsuperscript{1.1} shows a schematic of the fabricated device. Selective assembly of
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Figure 1.1: Schematic description of the in-vivo biosensor\textsuperscript{[1]}; (a) shows antibody coated nanoparticles assembled into nanotrenches, (b) in-vivo biosensor assembled onto the catheter (c) in-vivo biosensor advanced into the vein for monitoring.

nanoparticles coated with various biomarkers conducted on a single biosensor surface results in a biosensor capable of detecting multiple diseases simultaneously. With well oriented biomarkers (antibody coated nanoparticles) the sensitivity of detection of the biosensor increases 3-5 folds\textsuperscript{[99]} relative to the standard ELISA kit. Such a biosensor device may have significant impact for diagnosis of disease at early stage, whereby the antigen concentration is very low needing high blood volume for possible detection. Further, the use of such devices for multi-drug release in real time disease treatment is an important goal. The detection of
biomarkers in combination with controlled drug release represents an exciting long-term application of multifunctional nanosystems. Some of the advantages of the proposed nanoparticle-based biosensing device include:

- Enabling simultaneous detection of multiple biomarkers with one device.
- Very small size (as small as $100\mu m \times 100\mu m$).
- Higher sensitivity due to better coating of the antibodies on the surface of the nanoparticles.
- Can be made of all biocompatible material.
- Enabling in-vivo measurement.
- Small sample volumes.
- No issues with sample collection and storage.
- Low cost.
- Future development will lead to a device where drugs are released based on the detected antigen.

1.3 Overview

In Chapter 2, we introduce electrophoresis as a fast and reproducible approach for assembling nanoparticles with various surface functional groups and coating
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on the surface of a nanopatterned template. We describe in detail the experimental procedure and materials used for the assembly of nanoparticles with various functional groups and coatings. We present the assembly results for particles with Carboxylic and ChloroMethyl functional groups. We also present the results for assembling antibody and drug coated nanoparticles into PMMA nanotrenches. We describe the main challenges faced for assembling nanoparticles and present theoretical and experimental analysis which helped solving the problems. The discussed challenges include: assembly of particles underneath the PMMA layer and removal of assembled particles during the insertion and removal from the liquid suspension.

In Chapter 3 We introduce two different techniques developed for selective assembly of nanoparticles on desired regions of a single chip. On the first method size-selective assembly is achieved by sequential template-assisted electric field directed assembly. Nano vias with various sizes were written on aPMMA patterned template using e-beam lithography and electrophoresis was performed in different steps starting with the largest and ending with the smallest particles. Successful repeatable results for sequential size-selective assembly with various pairs of polystyrene latex (PSL) nanoparticles (diameters ranging from 200nm to 50nm) will be presented. The defects and sorting efficiency are introduced and studied for various factors. The second method, electric field-selective assembly is described and the results of assembling up to 4 various nanoparticles on a sin-
1. INTRODUCTION

gle chip is presented. Finally a comparison between the two selective assembly
techniques is presented.

In Chapter 4 the direct ELISA and tagged antigen detection techniques for
testing the detection capacity of the nanoparticle-based sensor chips will be de-
scribed. The anti-PSA IgG chips will be tested for the detection of PSA, mAb-2C5
chips will be tested for the detection of nucleosome and CEA chips will be tested
for the detection of CEA antigens versus the negative controls. The various block-
ing conditions for prevent the attachment of the antibody-antigens to the surface
of the PMMA/Au chips will be investigated. To insure that the antibodies are
specific to the detected antigen only the cross reactivity tests will be performed.

In Chapter 5 we conclude the research and provide insight for future research
on the field.
Assembly of Functionalized and Coated Nanoparticles

This chapter describes our studies on electric field directed assembly of nanoparticles with various functional groups or coating.

Experimental data have shown that a variety of parameters will affect the assembly results for regular polystyrene particles; these factors include assembly voltage, current, particle concentration, pH, conductivity, template design and surface property. Depending on the above parameters nanoparticle assembly range from a mat of coverage, partial coverage and full coverage. These parameters have been studied and optimized for various nanoparticles including PSL nanoparticles [46, 75, 126, 126].

For certain applications, such as nanoparticle-based biosensors it is neces-
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

necessary to assemble functionalized and coated nanoparticles. Although the electrophoretic assembly of nanoparticles is a well-known technique, assembling functionalized or protein-coated nanoparticles can bear unexplored challenges. In this chapter we study the assembly conditions for particles functionalized with COO$^-$ or NH$_2^-$ groups and coated with insulin and various cancer antibodies.

Precise control over the assembly of functionalized and protein coated nanoparticles is crucial in the emerging field of nanoparticle-based biosensors. The main goal of the work described in this chapter is to develop effective ways to assemble antibody and drug coated particles into nanopatterns generated on PMMA coated gold substrate for biosensor and drug delivery applications. This method produces particle arrangements with controlled density and uniformity, which can lead to better sensitivity for biosensing. Background, experimental procedures used, assembly results and challenges in assembling coated nanoparticles are presented as follows:

2.1 Theoretical Background
2.2 Experimental Approach
2.3 Assembly of PSL nanoparticle
2.4 Challenges
2.5 Assembly of functionalized PSL nanoparticle
2.6 Assembly of protein coated particle
2.7 Instability of mAb-Coated Nanoparticle Suspension
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

2.1 Theoretical Background

Electric field assisted assembly has been widely used for assembly of nanoparticles. There are two main advantages in using electric fields to manipulate and assemble particles on a chip: controllability and simplicity. One can precisely control the forces exerted on the particles by either the direct field itself or the field-induced particle-particle interactions. In electric field assisted assembly many variables can be controlled independently to change the characteristics of the applied electric field; these variables include magnitude, frequency, electrode distance, zeta potential, assembly time etc.. The controllability of the electric field can not be obtained with any other techniques using liquid flow, evaporation, sedimentation or mechanical manipulation. Another great advantage is the simplicity of application of the electric field and availability of the experimental equipment needed.

Electric field assisted assembly can be categorized into two main categories: Dielectrophoresis and Electrophoresis. Dielectrophoresis is a phenomenon in which an uncharged object placed in a non-homogeneous electric field experiences a force. The strength of the force is determined by different factors such as electrical properties of the medium and object, the object size and shape and the frequency of the electric field. Electrophoretic Deposition (EPD)\(^{[27]}\) is the migration of charged particles or elements dispersed in a suspension towards oppo-
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

sitely charged electrode under the influence of an applied uniform electric field. Dielectrophoresis is the proper method for assembling anisotropic objects that require alignment along the patterns. For assembling charged spherical particles with no alignment, electrophoresis is employed.

In electrophoresis the electric force exerted on charged particles can be altered by changing the electric properties of the liquid suspension. The electrostatic force on the particle is given by:

\[ F_e = q.E \tag{2.1} \]

where \( F_e \) is the electric field force and \( E \) is the electric field. \( q \) is the effective charge carried by the body which depends on the original charge of the body and electrical properties of the body and the liquid medium. This force causes the charged particle to experience acceleration toward the oppositely charged electrode.

Most colloidal particles acquire a surface electric charge by ionization and/or by the adsorption of ions when they are brought into contact with an aqueous medium. The charge on the surface is balanced by an equal oppositely charged layer in the adjacent liquid. Therefore, in the absence of thermal fluctuation, the colloidal particle can be completely neutralized. However, in the presence of finite thermal fluctuation can inhibit the formation of such compact electrical double
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

layer. Schematic diagram of the electrical double layer is shown in figure 2.1.

![Schematic of electrical double layer](image)

**Figure 2.1:** Schematic of electrical double layer

Close to the surface of the charged particle, the counter-ions are uniformly absorbed and distributed. However, the concentration of the counter-ions further away from the particle surface, drops off rapidly at first and then more slowly with increasing distance. This is because of the screening of the electric potential of the particles by counter ions. Eventually, the concentration of counter ions goes to zero at an infinite distance from the particle surface where the charge distribution becomes uniform.

The interplay between electrical forces and thermal agitation forms a diffusive layer of neutralizing counter-ions, which can be considered as an electrical double layer. The total charge on the sphere surface is given by (128):
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

\[ q = 4\pi R^2 \sigma' = 4\pi R\epsilon(1 + \kappa R)\zeta \]  
\hspace{3cm} (2.2)

where \( \zeta \) is the zeta potential and is defined as the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. \( R, \epsilon \) and \( \kappa \) are the radius of the particle, dielectric constant of the particle and the inverse Debye length, respectively. The inverse Debye length is defined as:

\[ \kappa = \sqrt{\frac{2N_Ae^2I}{\epsilon_0\epsilon_rk_BT}} \]  
\hspace{3cm} (2.3)

where \( \epsilon_0 \) is the vacuum permittivity, \( \epsilon_r \) is the dielectric constant, \( k_B \) is the Boltzmann’s constant, \( T \) is the absolute temperature, \( N_A \) is Avogadro’s number, \( e \) is the elementary charge and \( I \) is the ionic strength of the electrolyte which can be calculated as:

\[ I = \frac{1}{2} \sum_{B=1}^{n} c_B z_B^2 \]  
\hspace{3cm} (2.4)

where \( c_B \) is the molar concentration of ion \( B \) and \( z_B \) is the charge number of that ion.

PSL nanoparticles are negatively charged in the aqueous ionic solution. A uniform electric field is generated between the conductive electrode and the pat-
terned template when connected to a DC electric voltage in the solution. Under the influence of the applied field the negatively charged colloidal particles are directed to the positively charged template, and subsequently, they attach to the template surface by a combination of electrostatic attraction and van der Waals force. Note that when the distance between the particle and the template decreases, the van der Waals force dominates.

In an electric field assisted assembly process, the photoresist acts as a shielding film that will not only affect the electric field distribution, but also influence the current leakage. Schematic diagram of the electrophoretic deposition of nanoparticles is shown in figure 2.2.

![Figure 2.2: A Schematic of nanoscale electrophoretic deposition of nanoparticles onto the PMMA patterned anode substrate](image-url)
2.2 Experimental Approach

In this section we describe in detail the experimental procedure and materials used for the assembly of nanoparticles with various functional groups and coatings. The procedure include template fabrication, preparing the nanoparticle suspension for the assembly and finally the electrophoretic assembly process.

2.2.1 Facilities and Materials

We have performed the experiments in this work using the facilities in the George J. Kostas Nanoscale Technology and Manufacturing Research Center at Northeastern University. The Kostas center in the Egan Research Center features a 5000 square feet of class 10, 1000, and 10000 cleanroom space with capabilities for optical lithography, nanolithography, thin film deposition, furnace processes, wet chemical processes, ICP plasma etching, ion milling, and characterization, including surface topography, thin film characterization, optical microscopy, and particle characterization; non-cleanroom lab space with hoods, device testing areas with a full range of electrical test instruments, design areas, and an imaging lab with capabilities such as FESEM, e-beam writing and AFM. The fabrication facilities used in the experiments reported in this work are listed as follows:

Wet benches were used for standard pre-diffusion wafer clean; Bruce Furnace was used for dry and wet oxidation; RF and magnetron sputtering was used for
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

metal deposition using MRC-8667 and Perkin Elmer 2400; Quintel 4000 Optical Aligner was used for optical lithography; Supra-25 FESEM was used for surface imaging; Nano pattern Generation System (NPGS) was used to generated e-beam files and Raith Beam Blanker installed in Supra-25 FESEM was used for e-beam lithography.

Nikon Optiphot 200D Fluorescent Microscope (figure 3-3) was used for imaging fluorescent nanoparticles and the sensors after the ELISA and tagged antigen detection; NanoSpec was used to measure transparent film thickness on Silicon surface; SEO Wafer Surface Analyzer- Pioneer 300 was used to measure surface tension and static/dynamic contact angle for calculation of surface energy; Dry etch was done using ICP Plasma Therm 790 and Veco MicroEtch Ion Beam Milling System; Malvern Zetasizer was used to measure the size distribution and zeta potential of colloidal nanoparticles. For electrophoretic assembly and current measurements Keithley 487 picoammeter controlled by Labview 7.0 was used; Dip coater by KSV instruments was used to control the vertical speed of the removal and insertion of the chips during EP experiments.

Experimental materials: 3 inch silicon wafer; Piranha (H2SO4, H2O2) used for pre-cleaning of the wafers; Deionized (DI) water; Shipley 1813/1816, 1805, AZ2020, PMMA and NEB 31 photoresists for patterning; MF 319, AZ MIF 3000 and MIBK for development; Shipley 1165, Acetone and AZ 400k for photoresist stripping; PSL particles used for assembly purchased from Duke Scientific, Inc.;
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Ammonium hydroxide used to control the pH of the particles solution.

Functionalized particles: SPHEROTM Carboxyl Polystyrene Particles 5%(w/v); SPHEROTM Hydroxy Polystyrene Particles 5%(w/v); SPHEROTM Amino-Polystyrene Particles 2.5%(w/v) and SPHEROTM Streptavidin-Polystyrene Particles 1%(w/v) purchased from Spherotech Inc.; Surfactant-Free White Aldehyde/Sulfate Polystyrene Latex, 4.2%(w/v) purchased from Invitrogen, Molecular Probes Inc.; Cholromethyl Latex 4.2%(w/v) purchased from Interfacial Dynamics, Molecular Probes Inc.

Antibody-antigen proteins including: monoclonal Anti-body Purified 2C5, (3.29mg/ml) purchased from HBPS, HARLAN Laboratories; monoclonal Antibody to PSA, 1MG/VIAL purchased from BIODESIGN International; Insulin, Human Recombinant Zinc Solution (4mg/ml), 5ML purchased from GIBCO, Invitrogen Corporation; Nucleohistone, Calf Thymus (NHL), 1GM purchased from Worthington Biochemical Corporation; Calf-Thymus DNA Antibody purchased from Abbiotech, LLC.

Buffer solutions used for sensor testing: Phosphate Buffer Saline pH 7.4; Citrate Buffer Saline pH 5.5, 0.1 M MES Buffer pH 6.0; Tris Buffer Saline pH 7.4, HEPES Buffer Saline pH 7.4; Tris Buffer Saline with Tween 20, 0.01%(w/v); Tris Buffer Saline with Tween 20, 0.01%(w/v) containing Caseine (2mg/ml); 0.1 M Sodium Bicarbonate Buffer pH 8.3; 0.03 M Hydrochloric Acid pH adjusted to 8.5 by Sodium Hydroxide
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

2.2.2 Template Fabrication

6 nm Cr/40 nm Au was sputtered on a 380 μm thick silicon substrate with 150 nm of thermally grown $\text{SiO}_2$. Subsequently, 150 nm or 300 nm thick PolyMethylMethAcrylate (PMMA) film was spun coated followed by baking on a hot plate at a temperature of 100°C for 90 seconds.

The patterns were generated using electron beam lithography (EBL). For EBL, the PMMA coated template was exposed to electron beam to generate nanopatterns on the PMMA. The exposed PMMA film was developed in Methyl Iso-Butyl Ketone (MIBK):Iso-Propyl Alcohol (IPA) [1:3] solution mixture for 90 seconds followed by rinse in IPA and De-Ionized (DI) water for 30 seconds and 5 minutes respectively. The template design consists of arrays of trenches or vias with various sizes depending on the size of the chosen particles. The schematic of the template fabrication process is shown in figure 2.3.

![Figure 2.3: Schematic diagram of template fabrication.](image)

Figure 2.4(a) shows a picture of the Scanning Electron Microscope (SEM) used for writing electron beam patterns and high magnification surface imaging.
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

A sample of the nanopatterns written using the e-beam lithography technique is shown in figure 2.4(b).

![Figure 2.4:](image)

**Figure 2.4:** (a) Scanning electron Microscope used for EBL pattern generation and high magnification surface imaging. (b) SEM image of a via array generated using EBL. Via sizes vary from 50 nm to 400 nm and the via spacing is 1µm.

Alternatively, nanoimprint techniques could be employed for faster production of nanopatterned templates. Nanoimprint tool uses a nanotemplate mold to fabricate the nanotrenches on soft polymer substrate (e.g. PMMA). The template mold can be made by e-beam lithography.

2.2.3 Nanoparticle Suspension preparation

Plain, florescent, functionalized or coated Polystyrene latex (PSL) nanoparticle were used in various experiments. The particles from the original solution were first diluted in DI water. $NH_4OH$ was added to DI water-nanoparticle suspension in order to increase the pH and conductivity of the nanoparticle suspension while a dilute solution of $Na_2CO_3$ and $NaHCO_3$ salts were added to control the ionic
conductivity in some experiments.

PSL nanoparticles were used in the experiments because of their ability to have a stable negative zeta potential\(^{129}\) over a wide range of pH of the aqueous solution. The charge on such a particle is given by equation \(2.2\). Throughout the experiments, the pH and conductance of the nanoparticle solution were maintained at 10-11 and 400-450 \(\mu S\), respectively.

### 2.2.4 Assembly Process

To perform electrophoresis, a DC voltage was applied between the topographically patterned PMMA/gold template (anode) and a gold substrate (cathode) dipped in the nanoparticle suspension. A DC power source (Keithley 2400) was used to control the magnitude of applied voltage. The spacing between the electrodes (patterned template and gold substrate) was kept at 5mm during the assembly process. For the experimental consistency, we used a dip coater provided by KSV instruments to control the vertical speed of the template during the insertion and removal from the suspension. The vertical speed of the electrodes was maintained at 85 mm per minute. The electrostatic force acting on the negatively charged colloidal nanoparticles is directly proportional to the strength of the electric field between the electrodes and the particle charge. Our experiments show that nanoparticles assemble into the vias when a voltage of 2-3 V
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

was applied between the electrodes for 1-2 minutes. The experimental setup for the electrophoretic assembly is shown in figure 2.5.

![The electrophoretic assembly setup.](image)

**Figure 2.5:** The electrophoretic assembly setup.

2.3 Assembly of PSL Nanoparticles

It is possible to electrophoretically assemble nanoparticles with various sizes inside trenches with width and depth comparable to their size. To achieve the best assembly results for each nanoparticle type, size, or functional group in a certain pattern, assembly conditions such as the pH and conductivity of the solution, voltage of the assembly, time of the assembly, electrode spacing etc. must be optimized and carefully controlled.
2.3.1 Results

The pH of the solution is kept between 10-11 for optimum results and the zeta potential varies for particles with various size or functionalization/coating and is measured to be -40 mV to -90 mV for the PSL particles used in our experiments.

Figure 2.6 shows the results obtained from assembling 500 nm PSL, 330 nm PSL, and 200nm florescent PSL in 550 nm, 400nm and 250 nm wide trenches respectively. The trench depth is 300 nm for the 500 nm and 330 nm particles and 150nm for the 200 nm particles.

Assembly conditions for above particles were pH of 10.9, DC voltage of 2.4 V for 90 seconds for the 500 nm particles, pH of 10.8, DC voltage of 2.2 V for 100 seconds for the 330 nm particles and pH of 10.5, DC voltage of 2 V for 90 seconds for the 200 nm particles.

2.4 Challenges

In this section we will describe the main challenges faced for assembling nanoparticles and present theoretical and experimental analysis which helped solving the problems. These challenges include: assembly of particles underneath the PMMA layer and detachment of assembled particles during the insertion and removal from the liquid suspension.
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

Figure 2.6: Shown is the SEM micrograph of 500 nm PSL assembled in 550 nm wide and 300 nm deep trenches (top), 330 nm PSL particles assembled in 400 nm wide and 300 nm deep trenches (middle), and 200 nm fluorescent nanoparticles assembled in 250 nm wide and 150 nm deep trenches.

2.4.1 Particles Migration Under the PMMA Layer

One of the first challenges encountered was the problem of assembly between the nanopatterns and underneath the insulating layer. Figures 2.7(left) and 2.8(left) show examples of the observed phenomenon with 200 nm PSL and 100 nm Car-
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

boxyl functionalized PSL particles. Particles are assembled at voltage of 2V for 120 seconds and 90 seconds respectively.

Figure 2.7: SEM micrograph of 200 nm PSL particles assembled underneath the PMMA nanopatterns. Left: The PMMA covered chips were baked at 180°C. Particles were assembled underneath the PMMA. Right: The PMMA covered chips were baked at 100°C. Reducing the baking temperature solved the problem of assembly underneath the PMMA layer.

Figure 2.8: SEM micrograph of 100 nm COOH-PSL particles assembled in PMMA nanopatterns. Left: The PMMA covered chips were baked at 180°C. Particles were assembled underneath the PMMA. Right: The PMMA covered chips were baked at 100°C. Reducing the baking temperature solved the problem of assembly underneath the PMMA layer.

This problem was observed for a wide range of assembly conditions such as the applied voltage, time and the pH of the solution. Therefore, we have focused
our attention to the substrate itself. The most probable explanation was the poor adhesion of the PMMA layer to the Au layer underneath.

The standard lithography process required baking of the wafer at 180°C for 30 minutes inside the oven after spinning the PMMA layer. The glass transition temperature for PMMA is 105°C, which means our making process make the PMMA to undergo glass transition. We have modified the process by baking the PMMA covered substrate at 100°C for 30 minutes inside the oven. The result improved the adhesion of PMMA to the Au layer and successful assembly was achieved after modifying the PMMA baking condition (figures 2.7(right) and 2.8(right) ).

2.4.2 Particle Detachment

We notice that in the assembly process, when a template with assembled nanoparticles was removed from the liquid suspension (or inserted into another nanoparticle suspension as required for sequential assembly explained in chapter 3), many of the previously assembled nanoparticles were detached. Removing and inserting the substrate in and out of the aqueous solution exert an interfacial capillary force on the particles assembled inside nanopatterns. The moment acting on the nanoparticles due to the capillary force is in the direction that promotes particle detachment.
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

In this section we present a detailed analysis of the particle detachment and shows that there are 2 important contributing factors involved in particle detachment: (1) the applied voltage and (2) the particle size to pattern depth ratio. In the following, we show that for our experimental condition, applying the voltage causes an increase in the total moment exerted by the adhesion forces and therefore, the voltage can prevent the particle detachment when the particle radius is smaller than the depth of the patterns.

2.4.2.1 Effect of Applied Voltage

To demonstrate the effect of voltage, we conducted a single step assembly experiment where the voltage is kept on while the gold template is withdrawn from the suspension and the voltage is turned off when the template is half way out of the suspension. Figure 2.9 shows that assembled 200 nm particles are removed from the 1µm spaced vias in the bottom part of the template, where the voltage is turned off during removal.

At the instant when the voltage was turned off, the top half of the substrate (the region above the dashed lines) was already out of the suspension and therefore the particles have remained intact. The region between the lines is the interface of the liquid-air-substrate or the liquid level when the voltage was turned off.

In the interface region some particles where detached while some other re-
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

Figure 2.9: (a) The SEM micrograph shows a patterned substrate assembled with 200 nm particles. The voltage was turned off when the substrate was half way out from the particle suspension. (b) Schematic diagram of the moment when the voltage was turned off during the removal of the substrate from the liquid suspension.

mained assembled. The region below the dashed lines was submerged in the liquid suspension when the voltage was turned off. All the particles in this region where detached from vias upon removal of the substrate from the liquid suspension as a result of the moment exerted to the particles by the interfacial capillary force at the liquid-air-substrate interface.

Theoretical Analysis

During the electrophoretic assembly process, various forces act on the particles. These forces play the major role in driving the particles toward or away from the designated nanopatterns. Electrostatic force causes the negatively charged nanoparticles to migrate toward the positive electrode. Moreover, the van der Waals and the deformation induced adhesion forces act when the particle is in
contact with the surface and cause better adhesion. There is also the interfacial capillary force which is present at the interface of water and the particle during the removal and insertion of the template. The interfacial force causes a rotational moment that can lead to removal of particles from their assembled site.

In order to understand the detachment phenomenon and find practical methods for preventing it, we need to carefully analyze the effect of different dominant forces on the nanoparticles during various stages of the assembly.

**Forces on Particles:** Various forces are involved in an electrophoretic process during the substrate removal and insertion. The significant forces acting on the particles are introduced and calculated as follows:

**Electrostatic Force:** The electrostatic force exerted on the charged particle due to the electrodes electric field is given by equation 2.1. In tens of µm vicinity of fabricated nanostructures, the local electric field is as large as $10^6 - 10^7$ V/m. Therefore, the order of magnitude of the electrostatic force can be estimated as:

$$F_e \simeq 10^{-8} N \quad (2.5)$$

**Interfacial Capillary Force:** Interfacial capillary force is the result of the nanoparticle surface interaction with the liquid. For a spherical particle, assuming hydrophilic surfaces with wetting coefficient $\cos(\alpha)$, the capillary force is
approximately given by (130, 131):

\[ F_c = 4\pi \gamma R \cos(\alpha) \tag{2.6} \]

where \( R \) is the radius of nanoparticles, and \( \gamma \) is the surface tension of the liquid electrolyte. Therefore, the maximum capillary force exerted on a hydrophilic particle in contact with the flat surface is \( F_c \simeq 4\pi R \). For a 100 nm particle, in an aqueous solution at room temperature (\( \gamma < 0.072 \ \text{N/m} \)), we can estimate the maximum capillary force to be of the order of \( \simeq 10^{-8} \ \text{N} \).

**Adhesion Force**: The van der Waals force for a sphere near a plane surface is the sum of the interactions between the individual atoms making up the sphere and the plane. This force can be represented in terms of Hamaker constant as:

\[ F_{vdw} = \frac{A_{132} R}{6Z_0^2} \tag{2.7} \]

where \( A_{132} \) is the Hamaker constant for substances “1” and “2” in presence of medium “3”, \( R \) is the particle radius and \( Z_0 \) is the separation distance between the particle and the substrate. For a particle attached to a wall, \( Z_0 \) is about 4 to 10 Å. For smooth surfaces, \( Z_0 = 4\ \text{Å} \) is typically used.

When two dissimilar materials are in contact with each other, in the presence of a third media, the Hamaker constant may be estimated in term of Hamaker
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

<table>
<thead>
<tr>
<th>Materials</th>
<th>Air</th>
<th>Water</th>
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<tr>
<td>Polystyrene</td>
<td>6.6</td>
<td>1</td>
</tr>
<tr>
<td>Gold</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>PolyMethylMethacrylate</td>
<td>7.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Water</td>
<td>3.7</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.1: Values of Hamaker Constant for some materials [4], units are in $10^{-20} \text{A}/\text{J}$

constant of each material. That is [133]:

$$A_{132} = (\sqrt{A_{11}} - \sqrt{A_{33}})(\sqrt{A_{22}} - \sqrt{A_{33}})$$  \hspace{1cm} (2.8)

The calculated Hamaker constant values for our material are presented in table 2.1.

Therefore, for the Au/PSL contact in water, we have:

$$A_{132} = 3.5 \times 10^{-20} \text{joules}$$  \hspace{1cm} (2.9)

and for PSL/PMMA contact in water:

$$A_{132} = 0.53 \times 10^{-20} \text{joules}$$  \hspace{1cm} (2.10)

When a sphere and a flat substrate are in contact, the van der Waals force can deform the interface between them and form a circular contact area [134]. This deformation increase the adhesion force. The total adhesion force consists
of two additive components, the van der Waals forces before deformation and the force acting on the contact area due to deformation:

\[ F_a = F_{vdw} + F_{deformation} = \frac{A_{132} R}{6Z_0^2} \left( 1 + \frac{a^2}{RZ_0} \right) \]

(2.11)

where \( a \) is the contact radius that may result from adhesion-induced deformation. Previous measurement [134] of the contact radius has shown that \( a_{exp} \approx 0.1R \). Therefore the total adhesion force for a 100 nm PSL particle in contact with Gold and PMMA surface can be estimated as \( F_{a1} \approx 4.1 \times 10^{-9} \text{N} \) and \( F_{a2} \approx 6.2 \times 10^{-10} \text{N} \), respectively.

**Adhesion and Removal Moments:** In this section, we investigate the different moments due to various forces described in the previous section.

In particular, we want to find a condition for adhesion and removal of particles in various regimes. We consider nanoparticles of radius \( R \) that are assembled into nanopatterns of the width \( w \) and the height \( d \). The diameter of the nanoparticle is considered to be less than the width of nanopatterns, which is also the case in our experimental conditions. We also assume that the particle radius is smaller than the depth of the patterns: \( R + a < d \).

The moments about the axis of rotation \( O \), due to various forces acting on the nanoparticle, are illustrated in figure 2.10. We consider the situation where the template with assembled nanoparticles is being removed vertically from the
solution.

**Figure 2.10:** (a) Shown is a schematic diagram of the electrophoretic assembly setup being removed from the particle suspension; negatively charged nanoparticles are assembled on the positively charged patterned template. (b) Schematic diagram of the cross section of the patterned template showing various forces acting on the assembled nanoparticle during insertion and removal of the template into the nanoparticle suspension. $F_e$ is the force experienced by the particle due to the applied electric field, while $F_{a1}$ and $F_{a2}$ are the adhesion forces between the nanoparticle and gold and nanoparticle and PMMA, respectively. Here, $a$ is the contact radius as a result of the adhesion induced deformation.

We start by evaluating moments due to adhesion forces. We call adhesion moments due the vertical and horizontal walls, $M_{a1}$ and $M_{a2}$ that are due to adhesion forces $F_{a1}$ and $F_{a2}$, respectively. We have:

\[
M_{a1} = F_{a1} \sqrt{R^2 - a^2} \tag{2.12}
\]

\[
M_{a2} = F_{a2} a \tag{2.13}
\]
and

\[ M_c = F_c \sqrt{R^2 - a^2} \]  \hspace{1cm} (2.14)

The second contribution is due to the capillary forces. The moment exerted by the capillary force depends on the location where the interface touches the particle, as illustrated in figure 2.10. Therefore, the capillary moment can be derived as a function of the incident angle:

\[ \vec{M}_c = \vec{r} \times \vec{F}_c \]  \hspace{1cm} (2.15)

where the force and the distance of the interface from point \( O \) is given by:

\[ \vec{F}_c = F_{c,x} \hat{i} - F_{c,y} \hat{j} = -F_c \cos \theta \hat{i} - F_c \sin \theta \hat{j} \]  \hspace{1cm} (2.16)

and

\[ \vec{r} = R \sin \theta \hat{i} + \left( \sqrt{R^2 - a^2} - R \cos \theta \right) \hat{j} - a \hat{i} \]  \hspace{1cm} (2.17)

Therefore, the moment \( M_c \) can be written as:

\[ M_c = (R \sin \theta - a)(-F_c \sin \theta) + \left( \sqrt{R^2 - a^2} - R \cos \theta \right) (-F_c \cos \theta) \]  \hspace{1cm} (2.18)
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

\[ M_c = F_c R \left( 1 + \frac{a}{R} \sin \theta + \sqrt{1 - \frac{a^2}{R^2} \cos \theta} \right) \]  \hspace{1cm} (2.19)

and by introducing \( f(\theta) \) to represent the angle dependence, the capillary moment becomes: \( M_c = F_c R f(\theta) \). For \( a = 0.1R \) [See reference (134)], \( M_{c,\text{max}} \) can be estimated by maximizing \( f(\theta) \), as shown in figure 2.11.

![Figure 2.11:](image)

**Figure 2.11:** Capillary moment is shown to be a function of incident angle and it is maximized when the incident angle is equal to 120 degrees.

Using the above calculations, one can determine the effect of dominant forces during the assembly process. By controlling these forces, one can manipulate their effect in order to reach optimal assembly results. Putting all of the moment together, we can define the moment ratio as:

\[ MR = \frac{M_c}{M_{a1} + M_{a2} + M_e} \]  \hspace{1cm} (2.20)
such that when this quality is greater than one, the particle tends to roll out of
the trench and if it is less than one, the particle tends to stay inside the trench:

\[ MR > 1 \Rightarrow \text{particle detachment}; \quad MR < 1 \Rightarrow \text{particle adhered} \]

For a PSL nanoparticle with diameter 100 nm assembled into vias with a depth
\[ d > R + a, \]
the capillary force is on the order of \(10^{-8} \text{N} \) and for \( a_{\text{exp}} \sim 0.1R, \)
the maximum moment due to the capillary force during insertion is

\[ M_{c,\text{max}} = 5 \times 10^{-16} (J). \]

The moments due to adhesion of the PSL particle with diameter

100 nm to the gold and PMMA are approximately

\[ M_{a1} = 2.03 \times 10^{-16} (J) \] and \[ M_{a2} = 3.1 \times 10^{-18} (J) \] respectively. The deformation of a soft particle on hard
substrate increases the adhesion force and this deformation typically occurs a
few hours after the particles have been deposited\(^{135}\). In our process, since
various stages of the sequential assembly of nanoparticles are carried out within
minutes, the contribution of the deformation induced adhesion force would be
negligible and hence the actual total adhesion force would be less than \(10^{-9} \text{N}. \)

In the absence of the electric field, the moment ratio is \(2.4 > 1. \) This implies that
the particle will be detached.

For our experimental conditions the charge on a 100 nm particle calculates
from equation \[ 2.2 \] is \( q \sim 10^{-15} \text{ C} \) while the magnitude of the local electric field
applied in the vicinity of the fabricated vias is on the order of \(10^7 \text{ V/m} \) for an applied potential of \(2 \text{V} \) between the electrodes separated by a distance of
5\text{mm}. Therefore, the electrostatic force acting on the particle is on the order of
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10–8N and the moment due to this electrostatic force about $O$ is $4.95 \times 10^{-16}$ (J). When the electric field is applied, the moment ratio is $0.7 < 1$ and hence the particle remains adhered to the substrate. Therefore an electrostatic field must be applied while the template is inserted or removed into a suspension to prevent already assembled particles from being detached.

2.4.2.2 Effect of Trench Depth

When the template is removed from the aqueous solution after the assembly, a lot of the particles can be removed due to the interfacial capillary force acting on the assembled particles. In the previous section, we showed that in order to prevent particle detachment, the voltage must be applied while removing and inserting the patterned substrate containing the assembled particles into the liquid suspension. However we observed that sometimes nanoparticles are removed from the trenches even when the electric field is applied while removing the substrate. Figure 2.12 shows an example of the observed phenomena for 330 nm mAb-coated particles assembled in 150 nm deep trenches with various sizes.

In order to understand this, we need to recalculate the moments ratio by considering various moments acting on the particles for the case when the radius of the particles is larger than the trench depth.

For the case when $R > d$, the moments about the axis of rotation $O$ due to various forces acting on the nanoparticle are illustrated in figure 2.10. The
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

Figure 2.12: Shown is the SEM micrograph of 330 nm COOH-PSL particles assembled in 150 nm deep trenches and vias. Many particles are removed due to the interfacial capillary force during the removal of the substrate from the solution. The template is being removed vertically out of the nanoparticle suspension. \( F_e \) is the force experienced by the particle due the applied electric field while \( F_{a1} \) and \( F_{a2} \) are the adhesion forces between the nanoparticle and gold and nanoparticle and PMMA respectively. Here \( a \) is the contact radius as a result of the adhesion induced deformation.

The moments due to the adhesion and electric force can be calculated to be:

\[
M_{a1} = (F_{a1} + F_e) \sqrt{R^2 - (R - d)^2} \tag{2.21}
\]

The moment exerted by the capillary force depends on the location where the interface touches the particle and can therefore be derived as a function of the angle of incident:
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

![Figure 2.13](image)

Figure 2.13: Schematic diagram of the cross section of the patterned template showing various forces acting on the assembled nanoparticle during insertion and removal of the template into the nanoparticle suspension for the case when the particle radius is larger than the trench depth.

\[ M_c = F_c R(1 - \cos \theta) \]  

(2.22)

When the template is removed from the liquid suspension, the maximum torque due to the capillary force occurs when \( \theta = \pi \), therefore \( M_{c,max} = 2RF_c \)

The moment ratio for this case will be:

\[ MR = \frac{2RF_c}{(F_{a1} + F_c)\sqrt{R^2 - (R - d)^2}} \]  

(2.23)

It is easy to show that for our experimental values where \( F_e \sim 10^{-8} \) N and \( F_c \sim 10^{-8} \) N and \( F_{a1} \sim 1.4 \times 10^{-9} \) N, the moment ratio is always greater than
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

1. Therefore, for the case when the trench depth is smaller than the particle radius the capillary moment is always greater than the total adhesion moments and particles will be removed by the interfacial capillary force, even if the electric voltage is applied between the electrodes.

This analysis means that in order to solve the problem of particle detachment, we should increase the trench size to be greater than the particle radius. We fabricated 300 nm deep trenches for the assembly of 330 nm particles. The e-beam was performed to generate patterns on a template coated with 300 nm thick PMMA. The assembly was performed in the same condition used for figure 2.12.

The result is observed in figure 2.14.

![Figure 2.14](image.png)

**Figure 2.14**: The detachment of 330 nm COOH-PSL particles is prevented by assembling the particles in 300 nm deep trenches instead of using 150 nm deep trenches.

Figures 2.12 and 2.14 clearly show that when the pattern depth is smaller than the particle radius, the particles are removed from the patterns due to external forces during the removal from the suspension, while increasing the pattern depth...
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

prevents the particle detachment.

2.5 Assembly of Functionalized PSL Nanoparticles

Experimental data have shown that many parameters will affect the assembly results for regular polystyrene particles; these factors include assembly voltage, current, particle concentration, pH, conductivity, template design and surface energy. However, once the particles are functionalized they behave very differently. In this section, we show the results of assembling PSL particles with various functional groups. Later we discuss the challenges associated with assembling functionalized particles and report how the problems were overcome.

Fig 2.15 shows the results of 200nm Carboxyl functionalized PSL particles assembled in 250 nm and 500 nm vias, 200 nm, 250 nm and 400 nm wide, 1 $\mu$m long trenches, and vertical and horizontal 250 nm wide and 100 $\mu$m long trenches. For this assembly, a voltage of 2-3 V was applied between the electrodes for 90-180 seconds and the electrolyte pH and electrical conductance were 10.7-10.9 and 400-450 $\mu$S respectively.

Fig 2.16 shows the results of 100 nm Carboxyl functionalized PSL particles assembled in 100 nm and 250 nm vias.

Fig 2.17 shows the results of 330 nm Chloromethyl functionalized PSL part-
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

Figure 2.15: Shown is a SEM micrograph of 200nm Carboxyl functionalized PSL particles assembled in patterns with various shapes and sizes.

Figure 2.16: Shown is a SEM micrograph of 100 nm Carboxyl functionalized PSL particles assembled in vias with various sizes.

cles assembled in 350 nm and 500 nm vias.
2.6 Assembly of Protein Coated Particles

The main goal of this work is to develop effective ways to assemble antibody and drug coated particles into nanopatterns generated on PMMA coated gold substrate for biosensor and drug delivery applications.

2.6.1 Assembly of Insulin Coated Particles

Drug coated nanoparticles can be used in future drug delivery chips for in-vivo drug release. We used Insulin coated 450 nm CHO-PSL and 900 nm OH-PSL particles. Figure 2.18 shows the results of assembling 450 nm Insulin coated particles; the voltage of 2.3 V was applied for 70 seconds for this assembly.

We have also coated nanoparticles with fluorescent-tagged insulin in order to find out if the insulin coating remains intact during the course of electrophoretic assembly process. The result is shown in figure 2.19.
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

Figure 2.18: SEM micrograph of 450nm Insulin coated CHO-PSL particles assembled into 500nm wide trenches with 3 µm spacing.

Figure 2.19: Bright field (left) and fluorescent (right) images of 450nm Florescent Tagged Insulin coated CHO-PSL particles assembled into 500 nm wide trenches with 3 µm spacing.

As it is clear from figure 2.19, insulin remains attached to the surface of particles during the short time of assembly even though for the assembly the particles are diluted in a liquid suspension with pH as high as 11.
2.6.2 Assembly of Particles with Antibody Coating

Prostate Specific Antigen (PSA), IgG antibody and monoclonal antibody 2C5 (mAb-2C5), coated on 330 nm Carboxyl functionalized polystyrene particles were used in the experiments.

Figure 2.20 shows mAb-2C5 coated COOH-PSL particles assembled in 400 nm wide trenches. The assembly was performed at pH 10.7 with a voltage of 2.6 applied for 180 seconds.

![SEM micrograph of 350nm 2C5 coated Carboxylic functionalized PSL particles assembled into 400nm wide and 10 micron long trenches with 3 micron spacing.](image)

**Figure 2.20:** SEM image of 330 nm 2C5 coated COOH-PSL particles assembled into 400 nm wide and 10 µm long trenches with 3 µm spacing.

In order to study the possible detachment of antibodies during the assembly process we labeled the antibodies with florescent tag and coated the tagged antibody on the surface of functionalized nanoparticles. Coated nanoparticles were then assembled inside trenches and imaged with fluorescent microscope. The re-
result for COOH-PSL particles coated with fluorescent-tagged mAb-2C5 is shown in figure 2.21.

![Figure 2.21: Bright field (left) and fluorescent images of 330 nm Florescent Tagged mAb-2C5 coated COOH-PSL particles assembled into 500nm wide trenches with 3µm spacing.](image)

Strong fluorescent signal is figure 2.21(right) show that mAb-2C5 remains absorbed on the surface of nanoparticles during the assembly process.

Figure 2.22 shows the results of assembling 330 nm IgG coated COOH-PSL particles in 400 nm wide trenches. For this assembly, the pH was kept at 10.9 and the voltage of 2.2 was applied for 60 seconds.

Figure 2.23 shows the successful assembly results of 330 nm anti-PSA coated COOH-PSL particles assembled into 700 nm wide trenches.

We have reported the successful assembly results for COOH-PSL particles coated with various antibodies. In the next section we will discuss the challenges...
Figure 2.22: SEM image showing 330 nm IgG coated particles assembled into 400 nm wide, 300 nm deep PMMA trenches.

Figure 2.23: SEM image of 330 nm anti-PSA coated COOH-PSL particles assembled into 400 nm wide and 10 μm long trenches with 3 μm spacing.

we were faced working on electrophoretic assembly of antibody coated particles.
2.6.3 Instability of mAb-Coated Nanoparticle Suspension

The assembly of mAb-coated particles can be complicated and challenging. The antibody is coated on the surface of the functionalized particles either by absorption or by chemical binding. For the attachment of mAb to happen, exact amounts of functionalized particles and antibody molecules are incubated in the presence of a buffer solution. The coating process will be explained in chapter 4.

The number of antibody molecules on a sphere depends on the size of the molecule as well as the diameter of the spheres and the incubation condition such as time, temperature and the chemical characteristics of the solution and particle surface. Since the antibody molecules attach randomly to the surface of the spherical particles, in an electrolyte solution, the shape of the double layer and the charge distribution around the particles is no longer symmetric. This can give rise to complications in the assembly process. The main complexity with antibody-coated particles is their tendency to deposit on top of the patterns in large or small aggregates during the electrophoretic assembly process. The formation of these aggregates may be due to the instability of the coated nanoparticles prior to the electrophoresis process, or as a results of the presence of ions in the electrolyte solution or the external field.

For our experiments we have used PSL particles with two different functional
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

groups: Carboxyl (COOH) and Chloromethyl (\(CH_2Cl\)). Various antibodies including PSA, 2C5, IgG and CEA were attached to the surface of the functionalized PSL particles by absorption or chemical binding. Figures 2.24 and 2.25 show the mAb-coated COOH-PSL and mAb coated \(CH_2Cl\)-PSL particles agglomerated on the trench patterns instead of assembling inside the patterns.

**Figure 2.24:** SEM micrograph of 330 nm 2C5 coated COOH-PSL (left) and 430 nm PSA coated COOH-PSL particles (right) assembled in trench patterns.

**Figure 2.25:** SEM micrograph of 330 nm 2C5 coated \(CH_2Cl\)-PSL particles (left) and 330 nm anti-PSA coated \(CH_2Cl\)-PSL particles (right) assembled in various patterns.

Figures 2.24 and 2.25 show that electrophoresis resulted in large or small
particle clusters on top of the patterns irrespective of the functional groups and antibody coating.

If the coated nanoparticle batch prepared and stored in a buffer solution lacks the required stability conditions, the particles form aggregates inside the colloidal suspension. An unstable nanoparticle suspension will result in an unsuccessful assembly. The stability of the particle suspension was checked with the following methods:

**Imaging the Dried Droplet:** Before purchasing the Malvern Zetasizer instrument, we did not have a reliable instrument for high precision particle analyzing. Therefore we tried to study the particle solution by SEM imaging. We used a pipette to drop $5\mu l$ of the concentrated solution on a piranha cleaned hydrophilic Au surface and let it dry either in the room condition or inside a vacuum chamber. Figure 2.26 (a-b) shows the SEM images of droplets of high concentration 2C5-coated COOH-PSL and anti-PSA coated COOH-PSL particles dried on a clean hydrophilic Au surface.

Image 2.26 shows clusters of particles with no crystallized order or self-assembly on the surface. This shows that nanoparticles formed clusters in the liquid suspension.
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

![Figure 2.26: SEM micrograph shows a droplet of (a) 330 nm 2C5 coated COOH-PSL and (b) 430 nm anti-PSA coated COOH-PSL particles stored in pH 7 buffer solution dried on a hydrophilic flat Au surface.](image)

Measuring the Size Distribution of Particles: After purchasing Malvern zetasizer, we could measure the size distribution of each freshly coated batch of particles. The machine has the capability to measure the size distribution of mono-disperse and poly-disperse solutions of nanoparticles of various types with high precision. The particles are diluted in DI-water before the measurement. Figure 2.27 shows the size distribution of an unstable mAb-2C5 coated COOH-PSL particles stored in buffer solution diluted 1000 times in DI-water.

As it is shown in figure 2.27 for an unstable particle solution, the reported average size (z-average) is much larger than the size of individual particles. The quality report mentions the presence of large or sedimenting aggregated in the solution. Two peaks are observed in the intensity/size graph, smaller peak is the size of an individual particle while a larger peak is observed at ~700nm which
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

**Figure 2.27:** Size distribution of a non-stable 2C5 coated COOH-PSL particles. The average measured size is much larger than the size of individual particles. Can be an aggregate of up to 6 nanoparticles.

### 2.6.3.1 Addressing the Instability and Agglomeration

Antibody coated particles have low colloidal stability and this can cause many problems when the particles are being assembled or tested for disease detection. Researchers have tried various techniques to address the instability and aggregation of nanoparticles in a colloidal suspension.

One technique suggested by various researchers is the post-treatment of the particles in order to cover non-occupied parts of the nanoparticle surface with an inactive protein acting as a stabilizer. The inactive protein most recom-
mended for this purpose is Bovine Serum Albumin (BSA). BSA is a protein that is highly charged at physiological pH and can provide electrostatic stabilization to antibody-coated particles\cite{137, 138}. BSA treatment can be applied by 1) Sequential absorption or re-suspending the particles in BSA of a certain concentration\cite{139} or 2) competitive co-adsorption\cite{140} or simultaneous incubation with and the specific mAb. There are disadvantages associated with both approaches, in the former approach only antibody covered PSL with low antibody coverage can be stabilized, and co-absorption suggested in the latter method can cause displacement of the pre-adsorbed antibodies from the nanoparticles\cite{141}.

Another method suggested by researchers is the use of a detergent as stabilizer molecules. The disadvantage of this method is that detergent molecules can inhibit the immunological reaction required for the antigen detection; also detergents can cause desorption of previously absorbed molecules\cite{142, 143}.

The methods mentioned above for stabilizing the nanoparticle suspension will either reduce the number of antibody molecules on each particles or inhibit the detection by blocking the antibody molecules. Therefore, these methods may cause a significant decrease in the sensitivity of the nanoparticle-based sensing device. Since we are aiming for early detection and high sensitivity, we need to make sure we have the most binding sites available on the surface of each particle and therefore none of the explored techniques could be employed for stabilizing our mAb-coated nanoparticles. To address the problem of instability and particle
agglomeration we have followed the following steps:

- **Re-suspending in DIW:** The particles are prepared in a buffer solution with high concentration of salts. High salt concentration has a significant effect on the particle-particle interaction by reducing the Debye length or the double layer thickness of the colloidal particles. When the double layer thickness is small particles can get close to each other and if they get closer than some distance the van der Waals attraction forces will cause the particles to adhere to each other and form particle aggregates. Therefore, reducing the concentration of salts in the suspension should result in a more stable solution.

We re-suspended the particles in DI-water by centrifuging and removing the supernatant buffer. Figures 2.28 and 2.29 show the SEM image of dried droplets of mAb-2C5 and anti-PSA coated COOH-PSL nanoparticles re-suspended in DI water following the coating.

Self-crystallization of particles in figures 2.28 and 2.29 show better stability and less tendency for aggregation among the particles. We also tried analyzing the re-suspended particles with Malvern Zetasizer to find out the size distribution. The result for the anti-PSA coated particles is show in figure 2.30.

As it is shown in figure 2.30 for a stable particle solution, the reported
average size (z-average) is very close to the size of original particles prior to coating. The quality report is good. A single peak in the intensity/size graph indicates a monodisperse suspension where no particle aggregates exist.

- **Controlling the temperature**: We investigated the effect of temperature during the coating process on the stability of the mAb-coated nanoparticles.
2. ASSEMBLY OF FUNCTIONALIZED AND COATED NANOPARTICLES

The coating process includes an overnight incubation step performed in laboratory that may have variable temperature and humidity in different nights or seasons. We found out that if the room temperature was too high, the resulting batch was more probable to be unstable. The possible explanation for this experimental observation could be the possibility of chemical change or physical deformation of the antibody molecules at high temperatures. In order to keep their activity, antibody molecules must be stored in the fridge at a constant temperature of 4°C for up to two weeks. For long term storage, antibodies must be frozen at −20°C. Therefore the

![Figure 2.30: Size distribution of a stable anti-PSA coated COOH-PSL particles with the average size of 338 nm.](image)

<table>
<thead>
<tr>
<th>Z-Average (d.nm)</th>
<th>% Intensity</th>
<th>Width (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>323.6</td>
<td>100.0</td>
<td>56.76</td>
</tr>
<tr>
<td>PdF: 0.059</td>
<td>Peak 1: 338.3</td>
<td></td>
</tr>
<tr>
<td>Intercept: 0.996</td>
<td>Peak 2: 0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Result quality :</td>
<td>Peak 3: 0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

The coating process includes an overnight incubation step performed in laboratory that may have variable temperature and humidity in different nights or seasons. We found out that if the room temperature was too high, the resulting batch was more probable to be unstable. The possible explanation for this experimental observation could be the possibility of chemical change or physical deformation of the antibody molecules at high temperatures. In order to keep their activity, antibody molecules must be stored in the fridge at a constant temperature of 4°C for up to two weeks. For long term storage, antibodies must be frozen at −20°C. Therefore the
overnight incubation step in the coating process must take place in low
temperature.

- **Optimizing Electrophoretic Assembly Conditions** Stability of the
  nanoparticle suspension has a direct effect on the assembly outcome but it
  is not the only cause for agglomeration of the particles. We observed that a
  stable nanoparticle suspensions could also result in agglomerated assembly
  on the chips.

  For the assembly process, the particles are diluted in a water-based liquid
  with high pH and ionic concentration. Since the particles are not coated
  with surfactant, as discussed before high concentration of ions in the solu-
  tion will decrease the double layer thickness and may cause the particles to
  form aggregates. Reducing the ionic concentration will increase the dou-
  ble layer thickness which has a positive effect on preventing the particle
  aggregation in the suspension.

  Since the charge on the nanoparticles is directly proportional to the ionic
  strength, reducing the ionic strength of the particle suspension reduces the
  charge and hence the electrostatic force for the assembly. To compensate for
  the smaller surface charge, the assembly voltage must be increased to attract
  particles into patterns. Higher assembly voltage can increase the possibility
  of agglomeration of particles on the chips. Therefore it is crucial to find the
exact balance between the ionic strength and the assembly voltage in order to achieve successful assembly with no agglomeration.

In the other hand, since the nanoparticles from the original solution are resuspended in buffer and DI-water for coating and stabilization, the concentration of the final nanoparticle batch used for the assembly is unknown. Therefore the assembly condition will differ from batch to batch due to varied nanoparticle concentration.

Hence, in order to prevent the agglomeration caused by the EP assembly, the assembly condition must be optimized for each new batch of mAb-coated particles. Particle concentration, ionic strength of the suspension, assembly voltage and assembly time must be carefully balanced and controlled.

In summary, for assembling mAb-coated nanoparticles, we first need to assure that the nanoparticles are stable in DI Water. In order to keep the stability during the assembly a delicate balance between the attractive electrostatic force and repulsive particle-particle interaction energy must be achieved. In other words, every time a freshly mAb-coated particle batch is received for assembly, a series of experiments must be performed and various parameters involved in the assembly process must be optimized to prevent the particle aggregation and at the same time provide enough force to attract particles inside the nanopatterns.
Selective Directed Assembly of Nanoparticles

Selective assembly of nanoparticles has tremendous potential in many applications where particles with specific coating or functionalization needed to be directly assembled in known locations. One important application is in the field of micro-biosensors for simultaneous detection of multiple biomarkers.

This chapter describes the two techniques we have developed for selective assembly of nanoparticles on designed regions of a template. The obtained results and analysis are presented for each technique.

3.1 Background

3.2 Sequential Size-selective Directed Assembly

3.3 Sequential Site-selective Assembly
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

3.4 Comparison of the Two Techniques

3.1 Background

Selective positioning of micron size or nanoparticles is challenging. Previous attempts for organizing small particles with a reasonable control on a template according to their size or type, have been limited to template-assisted self-assembly techniques. In template-assisted self-assembly process, topographically patterned\cite{80, 81, 82} or chemically patterned\cite{76, 77, 78, 79} templates are used.

In chemically patterned templates the nanoparticles have been directed to the patterns with matching surface chemistry using specific interactions\cite{77}, wettability\cite{76}, or charge contrast\cite{78, 79}.

Fan et al. \cite{76} have shown that nanoparticles of various diameters can be sorted accordingly by employing self-assembly assisted by lyophilic features of various dimensions that were created on a template (see figure 3.1).

The self assembly using chemical templates only facilitate selective assembly of a limited subset of particles with specific surface chemistry matching the chemical properties of the template surface.

Other attempts employed topographical patterns or traps on the surface for physical trapping of the particles based on their size. Varghese et al. \cite{81} showed that it is possible to sort and assemble particles with various diameters by us-
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

Figure 3.1: (A) An optical micrograph of 2.1 μm and 810 nm particles deposited on a surface patterned with alternating rows of 5 μm and 25 μm squares. (B) An overlay of fluorescence images obtained by exciting fluorophores bound to 900 nm particles and 5.46 μm particles deposited on a surface with 5 μm and 25 μm square hydrophilic regions. (Fan et al. 2005)

...ing the centrifugal force generated during spinning to drive the particles onto topographically pre-patterned photoresist surface (see figure 3.2).

Although spin-coating is a fast and simple procedure, the sorting efficiency achieved using this technique was low especially when the diameters of the assembled particles varied by 100nm or less. Also, the technique was unable to prevent smaller nanoparticles from assembling in larger patterns.

Another topographic approach was developed recently by Kuemin et al. (82). They employed template-assisted self assembly (TASA) to trap 200nm, 350nm and 500 nm particles on carefully designed topographic features (see figure 3.3). They used a glass slide to move a drop of colloid on the topographical template at constant velocity of 0.5 – 1μm/s. The capillary force acted on particles as the meniscus moved over the patterns and guided the particles inside the fabri-
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

Figure 3.2: SEM images of polystyrene sphere patterned on identical SU8 template by spin coating a mixture of colloidal solution of diameters (a) 1.5 and 2 µm. (b) 1.5 and 3.1 µm, and (c) 1.5 and 4.78 µm, respectively. (d) Sorting efficiency versus difference in size of the microspheres comprising the coating solution. (Varghese et al. 2006)

cated traps. Despite good yield, their suggested technique is slow, limiting the possibility of high rate manufacturing.

In this chapter we introduce sequential size-selective directed assembly and sequential Site-selective assembly techniques as fast, high-yield and reproducible methods for selective assembly of nanoparticles. The first technique enables selective assembly of particles with various sizes and the second technique works for selective assembly of particles of different batches regardless of their size, shape and type. Both techniques employ electrophoresis in multiple steps and enable
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

Figure 3.3: Particles selectively placed at designated traps on dedicated assembly templates by capillary assembly: a) 200-nm particles in small traps, b) 350-nm particles in medium traps, c) 500-nm in large traps. Traps other than the designated ones remained empty. (Kuemmin et al. 2010)

assembling nanoparticles with various functionalization and/or surface coating.

3.2 Sequential Size-selective Directed Assembly

The first technique we developed for selective assembly of nanoparticles combines template-assisted assembly and electric field-directed assembly techniques. The developed selective assembly process includes electrophoretic assembly in various steps of the experiment. In electrophoresis patterned polymer substrate along with a reference electrode are immersed in a suspension of particles and a DC voltage is applied between the electrodes. Certain salts and bases are added to the nanoparticle suspension in order to increase the pH and ionic strength of the solution.

To achieve optimal selectivity in the assembly, various parameters such as applied voltage, pH and electric conductance of the solution, particle concentration, time, pattern shape and pattern size-particle size ratio have to be optimized for
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

In this section we will first describe the template fabrication and selective assembly process, then we show the assembly results and discuss the parameters that affect the sorting efficiency. Finally we address the effect of particle-particle interaction forces in clustering of particles.

3.2.1 Template Fabrication

6 nm Cr/40 nm Au was sputtered on a 380-micron thick silicon substrate with 150 nm of thermally grown SiO$_2$. Subsequently, 150 nm or 300 nm thick Poly-MethylMethAcrylate (PMMA) film was spun-coated followed by baking on a hot plate at a temperature of 100°C for 90 seconds. The sample was then exposed to electron beam to generate arrays of vias on the PMMA. The exposed PMMA film was developed in Methyl Iso-Butyl Ketone (MIBK):Iso-Propyl Alcohol (IPA) [1:3] solution mixture for 90 seconds followed by rinse in IPA and De-Ionized (DI) water for 30 seconds and 5 minutes respectively. The template design consists of arrays of vias with different diameters providing a platform for the selective assembly of different size particles. The schematic of the template fabrication process is shown in figure 3.4.
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3.2.2 Assembly Process

The assembly process employs electrophoresis in multiple steps. To perform electrophoresis, the topographically patterned PMMA/gold substrate (anode) and a gold substrate (cathode) were connected to a DC voltage and inserted vertically into the nanoparticle suspension. The pH of the solution and ionic conductivity were maintained at 11 and 450 $\mu$S respectively throughout the experiments to achieve optimal coverage. Our experimental results show that for these conditions a successful complete assembly can be achieved using a voltage of 2-3V when the electrodes are spaced over a distance of 5mm, while the time period of assembly is 2-3 minutes.

For the size-selective assembly of particles with two different sizes, we patterned the template with arrays of two different size vias. Each via size was designed to be equal or slightly larger than the corresponding nanoparticle size. The sequential assembly process for sorting two different particle sizes consists of two steps (figure 3.5). First, the larger particles were assembled into the vias.
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

that were equal or larger than the particle diameter. After the first step, the smaller vias remained empty since the larger particles did not fit in them. Then, the same template with already assembled larger particles was submerged into another suspension to assemble the smaller size nanoparticles in the smaller vias. This sequential assembly process can be used to sort many particle sizes by adding more via sizes and assembly steps.

Figure 3.5: Schematic diagram of the sequential electrophoretic assembly process.

A schematic diagram of the sequential assembly process is shown in figure 3.5. The process starts with assembling largest diameter particles first using electrophoresis. Larger nanoparticles will not assemble on patterns smaller than the particle diameter. The next step in the sequence is carrying out assembly for the for next smaller particle size. This sequence can be repeated for various nanoparticles starting with the largest size and ending with the smallest.
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3.2.3 Size-selective Assembly Results

To demonstrate the effectiveness of the assembly technique in sorting nanoparticles of two different sizes, we have performed size-selective assembly experiments with various size pairs including 200 nm-100 nm, 200 nm-50 nm and 100 nm-60 nm particles.

Figure [3.6] shows assembly results achieved after the first stage in the sequential assembly process with 200 nm particles. Note that particle clusters are formed when the dimensions of the patterns are much larger than that of the diameter of the particles while a single particle is assembled if the pattern dimensions are comparable to the particle diameter. For structures with dimensions less than the particle diameter no assembly is observed.

![Figure 3.6: Shown is the SEM micrograph after the first stage of the sequential assembly process in which 200 nm fluorescent PSL nanoparticles are assembled in nanopatterns arrays with dimensions 400 nm, 225 nm and 75 nm separated by 1µm.](image)

The next step in the sequence is carrying out assembly for the for next smaller...
particle size. The resulting assembly is shown in figure 3.7. Figure shows the sequential assembly of 200 nm and 100 nm PSL particles into the 210 nm and 110 nm via array. The 200 nm particles are first assembled into 210 nm vias, and then, the template with already assembled 200 nm particles is inserted into 100 nm particle suspension for the electrophoretic assembly. Since the 210 nm vias are all filled with 200 nm particles, 100 nm particles only assemble in the 110 nm vias as shown in figure 3.7.

Figure 3.7: Shown is a SEM micrograph of 200 nm and 100 nm PSL particles assembled onto patterns with dimensions 225 nm and 125 nm. The alignment of the assembled nanoparticle improves when the dimensions of the particle and that of the patterns are compatible.

Figure 3.8(a-b) shows the sequential assembly of 200 nm and 50 nm particles in the 225 nm-100 nm and 300 nm-150 nm vias, respectively. In contrast to non-defective sorting in figure 3.8(a), assembly of 50 nm particles in some of the 300
nm vias is observed in figure 3.8(b).

Figure 3.8: (a) The high resolution SEM micrograph after the second stage of the sequential assembly process in which 200 nm and 50 nm PSL nanoparticles are assembled in vias separated by 1µm with dimensions of 225 nm and 100 nm respectively. (b) The SEM micrograph of 200 nm and 50 nm PSL nanoparticles assembled in vias separated by 1µm with dimensions of 300 nm and 150 nm respectively. The arrows show the defects in the assembly.
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

Since the size of the vias in figure 3.8(b) is chosen to be much larger than the respective nanoparticle diameter, 50 nm particles get assembled in the empty space next to 200 nm particles. This leads to defects in the array decreasing the sorting efficiency.

We have also tried the size selective sequential assembly with functionalized particles. Figure 3.9 shows the assembled 330 nm COOH-PSL particles along with 100 nm PSL inside 150 nm deep vias using the sequential electrophoresis assembly.

![SEM micrograph of 330 nm Carboxylic functionalized PSL and 100 nm PSL particles assembled inside 800 nm and 250 nm patterns with 1.2 µm spacing.](image)

**Figure 3.9:** SEM micrograph of 330 nm Carboxylic functionalized PSL and 100 nm PSL particles assembled inside 800 nm and 250 nm patterns with 1.2µm spacing.

To explore the capability of sequential size-selective technique in separating particles with sizes close to each other, selective assembly of 100 nm and 60 nm particles was performed. The result is shown in figure 3.10.
Figure 3.10: Shown is a SEM micrograph demonstrating the size selectivity of particles when sizes differ by 40nm. 100nm and 60nm particles assembled in 110 nm and 70 nm vias separated by 500 nm.

Due to the large size variation in the 100 nm particle suspension, various particle sizes besides the 100 nm particles were assembled in the 110 nm vias after the first assembly step. As a result, defects were generated and the sorting efficiency was decreased. An analysis of the 60nm and 100 nm particle suspensions with Malvern zetasizer showed a large overlap between the two particle size distributions. Figure 3.10 shows the results and defects observed for the assembly of 100 nm and 60 nm particles into 110 nm and 70 nm vias. These types of defects were not observed during the sorting of 200 nm and 50 nm nanoparticles where
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there is no overlap between the size distributions of the pair.

3.2.4 Sorting Efficiency

In order to make quantitative analysis of the results obtained from the size selective assembly experiments with various parameters such as particle sizes and pattern sizes we introduce a new parameter called sorting efficiency, which represents the number of defects after the complete assembly process. A defect occurs when there is no particle assembled in a via or if a smaller particle assembles into a larger via. Smaller particles can assemble into the larger vias if the larger via is empty or if there is enough space left next to the assembled larger particle. The sorting efficiency is defined as the ratio of total number of vias minus defects to the total number of vias in the array. The sorting efficiency of each template is evaluated by considering 25 vias (5x5 array) in the middle of the array. To achieve an accurate estimation on sorting efficiency, the arithmetic average of 3 experiments is calculated.

Different factors affect the sorting efficiency. These factors are discussed below:

3.2.4.1 Effect of Size Distribution of Nanoparticles

We used Malvern Zetasizer instrument to measure the size distribution of the particles used in the experiments. Figure 3.11(a) and (b) show the size distribution
of the 100 nm-60 nm and 200 nm-50 nm particles, respectively. For the 100 nm-60 nm particle pair a large overlap of the two particle size distributions is observed. Hence the size selectivity for these particles becomes challenging and sorting efficiency drops. For example, 70 nm particles existing in the 100 nm suspension assemble in the 70 nm diameter vias in the first step preventing the assembly of 60 nm particles in those vias in the second assembly step. However, as shown in figure 3.11(a), this type of defects is not observed during the sorting of 200 nm and 50 nm nanoparticles since there is no overlap in the size distribution.

The results clearly show that in order to achieve highly efficient selective assembly of nanoparticles with 2 different sizes, they have to be chosen in a way that the size distributions of the pair have no overlap.

3.2.4.2 Effect of Particle Size-Via Size Ratio on Sorting Efficiency

Since nanoparticles are assembled into nanopatterns that match their size, if the pattern sizes are not compatible with particles sizes used in the experiments, defects can be generated and the sorting efficiency drops. This type of defects are shown in figure 3.8.

Table 4-1 summarizes the calculated sorting efficiency for the size-selective assembly experiments carried out with four different via size and particles sizes. The sorting efficiency of each template is calculated by considering 25 vias (5x5 array) in the middle of the array. To achieve an accurate estimation on sorting
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Figure 3.11: (a) Size distributions of 100 nm and 60 nm particles measured using Malvern Zetasizer, a large overlap is observed for 100nm and 60nm size. (b) Size distributions of 200 nm and 50 particles have no overlap.

efficiency, the arithmetic average of 3 experiments is presented for each of the four conditions.

The results in table 3.1 show that the sorting efficiency is higher when the particle diameter is closer to the via size. When the via size is not close to the respective particle diameter, there is enough space for the smaller particle to
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

Table 3.1: Sorting efficiency

<table>
<thead>
<tr>
<th>Particle sizes (nm)</th>
<th>Via sizes (nm)</th>
<th>Sorting Efficiency</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 - 100</td>
<td>210 - 110</td>
<td>100 %</td>
<td>0</td>
</tr>
<tr>
<td>200 - 50</td>
<td>225 - 100</td>
<td>98.7 %</td>
<td>2.31</td>
</tr>
<tr>
<td>200 - 50</td>
<td>300 - 150</td>
<td>81.3 %</td>
<td>12.2</td>
</tr>
<tr>
<td>100 - 60</td>
<td>110 - 70</td>
<td>&lt;56 %</td>
<td>-</td>
</tr>
</tbody>
</table>

assemble next to the larger ones and the sorting efficiency decreases. The size variation in the particle suspension also decreases the sorting efficiency when two particles that close in size are being sorted.

3.2.5 Particle Agglomeration

In a colloidal suspension of charged nanoparticles, since all the charges are of the same kind (negative or positive), the coulomb repulsion prevents the particles from getting close to each other. However, if a particle becomes immobilized on an assembled site, in the existence of competing attractive forces, suspended nanoparticles may be able to get close enough and finally attach to the assembled particles by van der Waals adhesive force. In that case agglomeration of particles on top of the assembled patterns is observed.

In order to estimate the condition under which the particles agglomerate, we have to take into account the kinetic energy ($E_k$) of the incoming particle, Coulomb ($E_c$) and van der Waals ($E_vdW$) interactions between the immobile
(assembled) and the incoming particle. If the initial kinetic energy of the moving particle is large enough to overcome the Coulomb potential and bring the particles into proximity of each other \( r \approx 2R \) then the van der Waals interaction can take over and stick the particles together.

Therefore, in order to evaluate the potential barrier for bringing the particles close to each other, we calculate the Coulomb potential between two spheres:

\[
E_c = \frac{q^2}{4\pi\epsilon_0 r^2} e^{-k(r-d)} \quad (3.1)
\]

Where \( r \) is the center to center separation distance and \( d \) is the diameter of the particles. (The exponential suppression comes from the screening potential.) The upper limit of the above expression is obtained when particles almost touch each other:

\[
E_c \approx \frac{q^2}{4\pi\epsilon_0 d^2} \quad (3.2)
\]

On the other hand, the kinetic energy to overcome such barrier should be provided by the applied electric field.

\[
E_k = \frac{1}{2}mV_t \quad (3.3)
\]

where \( V_t \) is the terminal velocity of the nanoparticle and is given by:
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

\[ V_t = \frac{F_E}{2\pi d \mu} \]  

(3.4)

Where \( \mu \) is the viscosity constants and \( FE \) is the applied electric force. Putting above expressions together, one can obtain the condition under which particles to stick to each other \( (E_k > E_c) \).

Figure 3.12 shows by increasing the applied electric field, the kinetic energy of the incoming particle becomes so large that it can bypass the Coulomb barrier and lead to adhesion of the particles together.

![Figure 3.12](image.png)

**Figure 3.12:** The comparison of kinetic energy of nanoparticles in fluid suspension and coulomb repulsion between the particles versus the applied electric force.

In figure 3.12, we have assumed that particles are of the diameter 100nm. Figure 3.13 shows that for a fixed applied electric field, depending on the size of the particle; one can have different regimes for sticking of the particle. For small particle, kinetic energy is not enough to overcome the barrier while for large
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

particles, the kinetic energy is large enough to overcome the Coulomb potential and stick the particles together.

![Figure 3.13](image.png)

**Figure 3.13:** The comparison of kinetic energy of nanoparticle in fluid suspension and coulomb repulsion as a function of the diameter of the particles.

A better estimation, should take into account the deformation of the double layers when particles approach each other.

This analysis show that it is possible to prevent certain types of defects created when the pattern sizes are comparably larger than particle sizes (e.g. defects in figure 3.8(b)). In order to prevent those defects, one should adjust the variables such as electric voltage, charge on the particles etc. so that the resulting kinetic energy felt by the particles is less than the potential barrier between the particles.
3.3 Sequential Site-selective Assembly

Sequential Site-selective assembly was developed as an alternative method to size-selective assembly for selective assembly of particles which do not necessarily have different sizes. The movement and deposition of particles in this technique purely guided by the template. Electrophoresis is performed in multiple steps and in each step, a specific region on the template is activated by the electric field and hence other regions do not attract any particles and the particles of each step assemble on the desired region only. Using this technique we have achieved the assembly of up to four different particles on a single chip. The assembly procedure and results are presented in this section.

3.3.1 Template Fabrication

Template fabrication for Electric field directed assembly is very similar to that of size selective assembly but before spinning and patterning PMMA, one extra step was added to generate lines for separating the chip into 2, 4 or more regions using optical lithography. The optical lines on the photoresist were then developed and the conductive Au and Cr layers below the PMMA were removed using wet etch. The template fabrication process is shown in figure 3.14.
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

3.3.2 E-Field-selective Assembly Technique

For electric field directed selective assembly, we perform sequential electrophoretic experiments on each electrically separated region of the substrate. As the first step, one of the regions is connected to the positive voltage and the substrate is dipped into the suspension of first particle size/coating along with the counter electrode and electrophoresis is performed. Electric field drives nanoparticles toward the nanopatterns in the region that is connected to the DC voltage and assembles them into the nanopatterns in that region while patterns in other regions remain empty. In the next assembly sequence, the next region of the substrate is connected to DC voltage and the substrate is inserted into the nanoparticle suspension of the next coating/size and electrophoresis is performed. This process is repeated until nanopatterns in each separate region of the substrate are filled. A

Figure 3.14: Schematic diagram of template manufacturing process for Electric Field directed sequential assembly.
schematic diagram of the Electric Field directed sequential electrophoresis process for a 2-region substrate is shown in figure 3.15.

![Figure 3.15](image_url)

**Figure 3.15:** Schematic diagram of the sequential electric field selective assembly of a template consisting two electrically separated regions.

The electric field selective assembly can be carried out for a potential biosensor chip with four or more electrically isolated regions. Particles with various antibody coating can be assembled on different regions of the biosensor and various target proteins can be detected simultaneously.

Figure 3.16 shows the schematic diagram of a chip with four electrically isolated areas assembled with particles with different size or different antibody coating on each region.

### 3.3.3 Results

Figure 3.17 shows the SEM micrograph of 100nm and 500nm PSL particle assembled into 650nm wide trenches on two electrically separated regions of the same chip using electric field directed sequential electrophoresis.

We also performed selective assembly of 320 nm CEA antibody coated PSL...
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

**Figure 3.16:** Schematic diagram of a chip with four electrically isolated areas assembled with particles with different size or different antibody coating on each region.

**Figure 3.17:** SEM micrograph of 100nm PSL (Left) and 500nm PSL (Right) assembled into 650nm wide trenches on two electrically separated regions of the same chip.
and 100 nm PSL nanoparticles, as well as 100 nm and 60 nm particles. The result is shown in figures 3.18 and 3.19.

**Figure 3.18:** SEM micrograph of 320 nm CEA antibody coated PSL (Left) and 100 nm PSL (Right) assembled into 650 nm wide trenches on two electrically separated regions of the same chip.

**Figure 3.19:** SEM micrograph of 100 nm PSL (Left) and 60nm PSL (Right) assembled into 650 nm wide trenches on 2 electrically separated regions of the same chip.

Templates can be prepared to have as many electrically separated regions as required. We have prepared a template with four electrically separated regions and performed electrophoresis on three of the region with three different particles.
including antibody coated PSL particles. 320 nm CEA coated PSL; 60nm PSL and 100 nm PSL nanoparticles were assembled into nanopatterns in three of the four electrically separated regions of a single chip as shown in figure 3.20.

![Image](image_url)

**Figure 3.20:** Shown is the SEM micrograph of 320 nm CEA coated PSL; 60nm PSL and 100 nm PSL nanoparticles assembled in 650nm trenches on 4 electrically separated regions of a single chip. No assembly is observed in region 4 that is not connected to DC voltage.

As it is clear from figure 3.20 on region 4 that was not connected to DC voltage, no assembly is observed. Also there is no mixing between particles of various regions and the selectivity is 100 percent accurate. Therefore, electric field directed sequential electrophoresis is an excellent technique for assembling particles coated with different antibodies on different region of the same chip.

The accuracy and convenience of this technique makes it our preferred selec-
3. SELECTIVE DIRECTED ASSEMBLY OF NANOPARTICLES

tive assembly technique for the nanoparticle-based multifunctional microbiosensor device fabrication.

3.4 Comparison of the Two Techniques

Electric-field selective assembly technique has several advantages to template-assisted size-selective assembly technique. First of all, this technique can be used to assemble same size particles with different coating on various regions on the same chip. Also there is no limitation in the size and shape of the patterns. We can assemble different nanoparticles on patterns with identical shape and size. Unlike size selective assembly, the selectivity does not depend on factors such as size distributions of each nanoparticles size, size difference between the particles and also factors that control the particle-particle interaction forces in electrophoresis. For example, Debye length and ionic concentration of nanoparticle suspensions have to be carefully controlled in order to prevent the assembly of smaller particles next to larger ones in size-selective assembly while these factors have no effect in electric field directed assembly.
Biosensing Application

In the previous chapter, we showed assembly of nanoparticles with various antibody coatings on designed regions of a chip. There, the primary goal for assembling nanoparticles was to fabricate a nanoparticle-based biosensor for detection of diseases such as cancer. In this chapter, we show that the fabricated biochip can successfully detect low concentrations of cancer biomarkers. In the following, we first describe the experimental procedure that includes coating, antigen detection and florescent imaging. Then, we present in-vitro antigen detection results:

5.1 Background

5.2 Experimental Procedures

5.3 In-Vitro Antigen Detection Results
4. BIOSENSING APPLICATION

4.1 Background

The discovery of biomarkers and recent advances in nanotechnology and biosensors have created a new era of early stage cancer detection and precise drug delivery. A biomarker indicates a specific state of the disease and can be used to target disease, study cellular processes and monitor or recognize any alterations in the cellular processes of specific cells.

Detecting cancer biomarkers verifies the existence of a specific cancer. With the development of proteomic technologies and DNA methylation analysis many protein biomarkers as well as DNA biomarkers have been discovered for different types of cancer\cite{144},\cite{145}.

Current established methods for sensing proteins include enzyme-linked immunosorbent assays ELISA\cite{94} and Western Blots\cite{95},\cite{94}. In ELISA, an antibody with specificity for a particular antigen is used to immobilize an unknown amount of antigen to a surface. Then the surface is incubated with a specific secondary (capture) antibody that can bind to the antigen. The capture antibody can be tagged with fluorescent or linked to an enzyme, which can convert to some detectable signal. Western blot uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide or the 3-D structure of the protein. After the separation, the proteins are transferred to a membrane for detection by antibodies.
4. BIOSENSING APPLICATION

Because of their high surface area and size compatibility with biological elements such as enzymes, antigens and antibodies (2-20 nm), nanoparticles can carry on important functions such as the immobilization of biomolecules and the catalysis of electrochemical reactions. Biological elements could be probed by photonic properties of nanoparticles. For example, surface Plasmon resonance (SPR), surface enhanced Raman spectroscopy (SERS), and surface-enhanced fluorescence spectroscopic techniques can be implemented to probe biomaterial structures adsorbed on the surfaces of nanoparticles.

Unlike the commercially available ELISA based sensors, it has been shown that employing uniformly distributed mAb-coated nanoparticles on the sensor surface increases the orientation and distribution of antibodies\cite{96, 97, 98, 99} increasing the antibody-antigen binding events and hence the sensitivity of the sensor. Moreover, the chemical reactivity of biomaterial structures can be enhanced by electronic and catalytic properties of nanoparticles. This makes the nanoparticles a strong candidate for fabricating biosensing devices.

Apart from the faster response and improved sensitivity due to the better orientation of the antibodies on the surface of nanoparticles, nanoparticle-based biosensors can facilitate multiplex detection in a small device.

In this chapter, we test the nanoparticle-based sensors for the detection of various cancer antigens.
4. BIOSENSING APPLICATION

4.2 Experimental Procedures

In this section, we describe the experimental procedures to fabricate nanoparticle-based biosensors. Following the coating, mAb-coated nanoparticles were assembled inside nanopatterns on the biochip and the fabricated chip was utilized for the detection of the antigen specific to the mAb used. One common technique for quantifying the collected antigen is using the fluorescent tags. Fluorescent tag can either be conjugated with the antigen to check the detection limit of the chip, or with a secondary antibody (detection antibody) that has specificity towards the antigen. The former is the tagged antigen technique and the latter is the standard ELISA performed on the chips. The experimental procedure for each technique is explained in this section.

4.2.1 Coating Nanoparticles

Various cancer antibodies were chosen for antigen detection with our nanoparticle-based biosensor chips. Mouse mAb-2C5 showing specificity towards Nucleohistones (NHS), mouse anti-CEA showing selective binding towards Carcinoembryonic (CEA) and anti-PSA showing selectivity towards Prostate Specific Antigen (PSA) were among them. Each specific antibody must be coated on the nanoparticles before the electrophoretic assembly of particles on the biochip. Coating Procedures have been developed in Prof. Vladimir Torchilin laboratory.
Coating nanoparticles with Antibodies: Carboxylic functionalized polystyrene latex (COOH-PSL) particles were used for their ability to passively absorb various biological molecules to their surface. IgG, monoclonal antibody (mAb) 2C5, CEA and PSA were coated to the surface of 330 nm COOH-PSL with the following procedure: 10 mg of 1\%wt COOH-PSL, 1.56 mg antibody and PBS buffer in total volume of 1 ml were incubated overnight at room temperature (25°C). Following the incubation the suspension was centrifuged at 1200 rpm for 15 minutes and the supernatant containing the free non-adsorbed mAb was withdrawn and fully characterized. Then the mAb-coated particles were re-suspended in DI-water for assembly and antigen detection experiments. If needed the mAb-coated particles could be stored for a short term in the fridge at 4°C.

The schematic diagram of the mAb coating process is shown in figure 4.1.

**Figure 4.1:** Antibodies (PSA, 2C5, CEA and IgG) were passively adsorbed onto Polystyrene (PSL) particles as shown above.
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4.2.2 Florescent-tagged Antigen Detection

Antigen of interest can be conjugated with fluorescent tag. Tagged antigen binds to the specific biomarkers on the surface of the nanoparticle-based biochip. Once detected, the chip can be imaged with a fluorescent microscope and the intensity of the fluorescent signal would be proportional to the antigen concentration detected by the chip. The schematic diagram of the tagged antigen detection procedure is shown in figure 4.2.

![Diagram of the tagged antigen detection procedure.](image)

**Figure 4.2:** Schematic diagram of the tagged antigen detection procedure.

The procedure is as follows: Antigen is labeled with a *fluorophore* such as FITC. Then the chips are blocked overnight with Bovine Serum Albumin in D.I water. Later, antibody coated chips are incubated with different concentrations of fluorescent-Labeled antigen in HBS pH 7.4 at 4°C. IgG antibodies are generic antibody that show no specificity towards any antigen and are used as control in
4. BIOSENSING APPLICATION

experiments.

A similar procedure can be used to detect various concentrations of labeled antigen in Blood. Approximately, 1-2 ml of Blood is obtained from Balb/c female mice. The Blood is spiked with various concentration of labeled antigen. After the blocking, mAb biochips are incubated with blood samples spiked with labeled antigen for a period of one hour, then washed and Imaged.

4.2.3 Antigen Detection with Sandwich ELISA

Antigens targeted in the natural biological condition are not conjugated with fluorescent tags. Florescent tagged antigen detection is useful for investigating the detection limits of the device but is not practical for real disease detection. Sandwich ELISA is a common technique for measuring the antigen detected from a biological sample with an unknown antigen concentration. In ELISA, an antibody with specificity for a particular antigen is used to immobilize the unknown amount of antigen to a surface. Then the surface is incubated with a specific secondary (capture) antibody that can bind to the antigen. The capture antibody can be tagged with florescent or linked to an enzyme, which can convert to some detectable signal. Schematic diagram of the various steps of the sandwich ELISA is demonstrated in figure 4.3.

In order to prevent non-specific binding, a blocking is performed for each chip
prior to the antigen incubation. Blocking consists of an overnight incubation with Bovine Serum Albumin (BSA) at 4°C. Blocked chips are rinsed with PBS buffer, followed by a 40 minutes - 2 hours incubation with different concentrations of antigen. Antigen incubation can be performed in different media including HBS buffer pH 7.4, fetal bovine serum (FBS) and 50% murine blood. For the control experiment, no antigen is used in the antigen incubation step of the experiment. Then the chips are washed with PBS, followed by an incubation with fluorescent-labeled detection mAb for 40 minutes - 2 hours. As a last step, the chips are rewashed with PBS and DI-water. Finally, the chips are imaged with florescent...
microscope and the images are analyzed using ImageJ software.

4.2.4 Florescent Imaging

Fluorescence is the phenomenon in which a florescent molecule absorbs the light of a given wavelength and emits light at longer wavelengths. The florescent molecule is often termed as fluorophore or dye. The fluorophore absorption intensity peak usually has a smaller wavelength and magnitude than the emission peak. Figure 4.4 illustrates the absorption and emission profile for a typical florescent dye, Alexa Fluor 555.

![Fluorophore Absorption and Emission Profiles](image)

**Figure 4.4:** A sample absorption/emission profile.

Florescent detection has several advantages over regular imaging techniques. One advantage is higher sensitivity and higher imaging speed. The florescent signal detected is proportional to the density of the substance being measured.
Another advantage is that florescent imaging is a safe process which does not include any hazardous material. Living tissues can be investigated with no adverse effects on their natural physiological behavior and the sample will not be destroyed.

Florescent microscope has the ability to irradiate the specimen with the desired band of wavelength and separate the weak emitted florescent from the light used for excitation. The detection is limited by the darkness of the background. Most fluorescence microscopes operate in the Epi-illumination mode (illumination and detection from one side of the sample) to further decrease the amount of excitation light entering the detector.

We used a Nikon Optiphot 200D florescent microscope for imaging our florescent samples. A picture of the microscope setup is shown in figure 4.5.

Figure 4.5: Nikon’s Optiphot 200 optical microscope.
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High quality filters are used to block the excitation light from reaching the detector. These filters usually consist of an excitation filter that selects the range of excitation wavelengths, a dichroic mirror, and an emission filter that blocks the excitation light. The resulting image superimposes the fluorescent structures with high contrast on a dark background. A schematic diagram of a fluorescent filter and a sample absorption/emission profile are shown in figure 4.6.

Figure 4.6: A sample absorption/emission profile(3).

We used various fluorescent dyes in our antigen detection and ELISA experiments and utilized specific filter sets(146) assembled in Nikon Optiphot 200D optical microscope matching the fluorescent dye in use. Some of the fluorescent dyes and the filter sets used in our experiments are listed below:

- **Alexa fluor 488**: Cyan green, excitation 495 nm, emission 519 nm.
  
  Filter: Nikon blue excitation filter B-2A.

- **Fluorescein isothiocyanate (FITC)**: Green, excitation 495 nm, emission
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521nm

Filter: Nikon blue excitation filter B-2A.

Blue Excitation Filter Block B-2A specifications:

Excitation Filter Wavelengths: 450-490 nanometers (bandpass, 470 CWL)
Dichromatic Mirror Cut-on Wavelength: 500 nanometers (longpass, LP)
Barrier Filter Wavelengths: 515 nanometer cut-on (longpass, LP)

The imaging conditions were as follows:

Magnifying objective lenses used: 50X and 100 X
Exposure time: 5 seconds to 30 seconds
Gain: 0.6

A Xenon arc lamp is used for fluorescent imaging. It uses ionized Xenon gas to produce a bright white light close to natural daylight. The intense light emitted from xenon arc lamp has a color temperature of around 5500K. The plasma ball in a Xenon arc lamp is closer to the anode making it about 5 times brighter than the surrounding cathode. The Xenon arc must be aligned and focused before each experiment to assure homogeneity in the field of visualization. We have used a Nikon HMX-4 Mercury/Xenon lamphouse for illuminating our samples.
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A schematic image of the Xenon arc lamp used in our experiments is shown in figure 4.7.

![Nikon HMX-4 Mercury/Xenon Lamphouse](image)

**Figure 4.7**: A Xenon lamphouse assembly used for fluorescent imaging.

4.2.5 Image Analysis

Following the fluorescent imaging, the images are fed into ImageJ, a java-based image processing software developed at the National Institutes of Health for analysis. ImageJ software allow us to quantify the fluorescent signal detected from the chips. The program enables many image analysis functions such as separating various color channels, removing the background light, filtering the detected light and measuring the light intensity at the desired regions of the image.
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For quantifying the florescent light on the biosensor chips, the florescent image from each chip is fed into the software. Then color channels are separated, the light color of the desired wavelength is chosen and the filtered image is saved in a new file. The new file is then measured using ImageJ at a chosen square box with a specific area. The chosen box is usually a $20 \mu m \times 20 \mu m$ area on a nondefective region of the patterns.

4.3 In-Vitro Antigen Detection Results

In chapter 2, we demonstrated that functionalized and mAb coated nanoparticles with various sizes can be assembled electrophoretically inside trenches. In this section we show that the resulting chip can be employed as a biosensing device for in-vitro detection of biomarkers.

Different antibodies were tested with the in-vitro chip to detect various cancer antigen proteins in spiked serum, spiked murine blood or tumor infected mice in different stages of the disease. The factors considered for choosing the antibodies were the availability of the antibody, antigen and the secondary antibody in the market and specificity of the antibody towards the desired antigen. The chosen antibodies must have been specific so that issues such as binding to the chip surface and cross-reactivity with control antibodies could be avoided.

Prostate Specific antigen (PSA), Nucleohistones (NHS) and Carcinoembryonic
antigen (CEA) are among the many biomarkers that are pathologically indicated in cancerous condition. PSA is the biomarker for the prostatic cancer; NHS is a classic biomarker that is released by most of carcinomas and is not associated to a particular type of cancer; and CEA is released in the blood in 7 different cancerous conditions including colorectal, gastric, pancreatic, lung, breast, carcinomas and modularly thyroid.

Mouse anti-PSA-IgG shows specificity towards PSA; mouse anti-tumor monoclonal antibody 2C5 with the nucleosome-restricted specificity shows specificity towards NHS cancerous antigens in the blood; and mouse Carcinoembryonic antibody (CEA) shows selective binding towards CEA antigen. The use of above mentioned antibodies give us the ability to prepare formulations that are specific to biomarkers PSA, NHS and CEA.

Immunoglobulin G (IgG) with two antigen binding sites are generic antibodies that show no specificity towards any antigen and are used as control in experiments.

4.3.1 PSA Detection Results

ELISA based detection of PSA was performed for the chips assembled with anti-PSA coated nanoparticles as follows: an overnight blocking was performed by incubating the chips with 1% Bovine Serum Albumin (BSA) at 4°C to prevent
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non-specific binding. Blocked chips were rinsed 6 times with PBS buffer followed by a 40 minutes incubation with different concentration of PSA in mouse serum or blood at 37°C. Then the chips were washed 6 times with PBS followed by an incubation with fluorescent labeled detection mAb for 40 minutes at 37°C. As a last step, the chips were washed 4 times with PBS and 4 times with water.

Following ELISA the chips were imaged with the florescent microscope and the images were analyzed using ImageJ software. Figure 4.8 shows the florescent intensity detected for various concentration of PSA in spiked serum and murine blood. The graph shows similar increasing trends in the signal by increasing the PSA concentration for the detection in serum and murine blood.

![PSA detection in spiked murine blood](image)

**Figure 4.8:** Graph shows the intensity of the detected florescent for various concentrations of PSA detected in murine blood and serum.

Although ELISA experiments using PSA were successful, tagged detection
antibody showed nonspecific binding towards the PMMA surface of the chips. Therefore the detection limited was influenced by the strong noise coming from the background.

4.3.2 Nucleosome Detection Results

The second cancer antibody tested in the nanoparticle-based sensors was mAb-2C5. Prior to performing complete sandwich ELISA, we used fluorescent-tagged antigens in detection tests to check the specificity of the 2C5 towards nucleosome on a nanoparticle-based chip.

4.3.2.1 Tagged Antigens

**Detection in Serum:** As a first step the activity of the mAb-2C5 coated PSL beads were tested compare to free mAb-2C5 and non-specific IgG against NS antigen. Results presented in figure 4.9 show that the mAb-2C5 coated particles showed good reactivity towards the nucleosome and the detected fluorescent signal was similar to the free mAb-2C5 antibody. The control IgG antibody did not react with the nucleosome antigen.

NHS concentration as low as 1µg/ml was detected in the indirect detection method presented in figure 4.9.

As a next step mAb-2C5 coated particles were assembled inside trenches on the surface of the biosensor chip. The chips were then tested for the detection of
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Figure 4.9: Indirect ELISA testing of the activity of the mAb-2C5 coated PSL beads compare to free mAb-2C5 and non-specific IgG against nucleosome antigen.

NHS antigen labeled with FITC fluorophore.

Similar to PSA detection, the first step was to block the chips overnight by incubating with 2% BSA in DI-water at room temperature. The chips were then incubated with different concentrations of labeled NHS, pH 7.4 at 4°C. Chips containing IgG-coated particles were used as negative control. The chips were then imaged with a fluorescent microscope to measure the intensity of the fluorescent signal. The results showed that the intensity of the fluorescent detected correlates with the NHS concentration used in the experiment.

Figure 4.10 shows a fluorescent microscope image of chips containing 2C5 coated nanoparticles (left) and IgG coated nanoparticles (right) incubated with 125pg/ml concentration of labeled Nucleosome.
As shown in figure 4.10, the fluorescent signal collected from the control IgG chips was very low compared to the 2C5 chip. This shows the specificity of mouse mAb-2C5 towards nucleosome.

**Detection in Blood:** Similar experiment with tagged antigens was performed in blood. The chips were incubated with 1-2 ml of murine blood obtained from Balb/c female mice spiked with various concentrations of labeled Nucleosome. Following the 1 hour detection incubation, the chips were washed and imaged. Figure 4.11 shows bright field and fluorescent images of 2C5 microchips incubated with various concentration of tagged antigen.

The detected signal from the chips incubated with 200µg/ml, 100µg/ml and 50µg/ml of labeled nucleosome was visibly proportional to the antigen concentration as shown in figure 4.11. Certain artifacts observed in the image do not contribute to the analysis of the results.
4.3.2.2 Indirect Sandwich ELISA

Antigen proteins produced by diseases do not have any fluorescent tag to enable visualization once detected. Once collected by antibody, the chips must be incubated with a tagged secondary antibody that has specificity towards the detected antigen. In order to perform a sandwich ELISA with 2C5 antibody specific to nucleosome antigen, we used anti-DNA antibody as a secondary antibody.

We began investigating the activity of the secondary antibody by Indirect ELISA before performing sandwich assay on the chips. Indirect ELISA was performed on a 96-well plate with 2C5, IgG control and anti-DNA (as detection antibody) using TBST-Casein (Tween 20 0.01% and 2mg/ml Casein) as blocking buffer. The result is shown in figure 4.12(b).

As seen in figure 4.12(b), 2C5 showed good reactivity against nucleosome but...
anti-DNA did not show any reactivity. Since the blocking buffer used might have blocked the reaction between the anti-DNA and nucleosome, we decided to repeat the experiment using BSA as blocking agent instead of Casein. The result is shown in figure 4.12(a).

**Figure 4.12:** Indirect ELISA using different blocking buffers (a) Indirect ELISA performed with Standard 2C5, Anti-Histone antibody and control IgG using BSA blocking buffer (b) Indirect ELISA performed with Standard 2C5, Anti-Histone antibody and control IgG using casein blocking buffer.

Figure 4.12(a) shows that using BSA blocking agent instead of Casein increased the reactivity of anti-DNA against nucleosome in the 96-well plates.

The same concentration of Blocking agent and anti-DNA was used in the sandwich ELISA performed on the nanoparticle-based biochips for the detection of nucleosome; however no antigen detection was observed. The result is shown in figure 4.13(a-b).

Although the anti-DNA antibody showed a slight activity towards the nucleosome in 96-well plate, sandwich ELISA performed on 2C5 chips showed no
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Figure 4.13: Fluorescence images of (a) 2C5 chips and (b) IgG control chips

fluorescent signal as seen in figure 4.13(a-b).

We tried several other secondary antibodies for the ELISA process. Only the anti-DNA purchased from a different company (abbiotec) was active towards nucleosome, however the new anti-DNA showed non-specific binding to the PMMA chips.

Attempts to develop successful sandwich ELISA for the detection of nucleosome were unsuccessful using various secondary antibodies. The anti-DNA antibody purchased from abbiotec showed successful indirect ELISA in 96-well plates and specificity toward nucleosome. Therefore, this anti-DNA antibody was used to develop sandwich ELISA on chips containing mAb-2C5 coated particles.

The chips were blocked with 2% Bovine Serum Albumin in DI Water and later on incubated with various concentrations of nucleosome and finally incubated with FITC-conjugated anti-DNA antibody. The results are shown in figure 4.14.

Figure 4.14 shows a strong fluorescent signal detected from the PMMA surface of the 2C5 chips after the ELISA process.
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Figure 4.14: fluorescent microscope image of the Sandwich ELISA performed on 2C5 chips (left) and IgG chips (right). Chips were incubated with nucleosome antigen followed by incubation with FITC-labeled anti-DNA.

We performed an experiment to check the interaction of the antigen and the secondary antibody with the PMMA surface. After blocking with BSA, we incubated PMMA coated chips with tagged nucleosome, and tagged detection antibody (anti-DNA) in buffer solution. The results are shown in figure 4.15.

Figure 4.15: PMMA Chips showing cross reactivity with FITC-labeled anti-DNA.

Figure 4.15 shows that the FITC-labeled secondary anti-DNA used in the sandwich assay binds to the PMMA surface of the chips whilelabeled nucleosome showed no florescent signal and no affinity to bind to PMMA. The nonspecific binding to PMMA problem is discussed in the next section.
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4.3.3 Nonspecific Binding to PMMA

The problem of nonspecific binding to PMMA was observed in the ELISA experiments performed for the detection of nucleosome and PSA antigen. In order to achieve successful detection with high sensitivity, we need to address this problem. In this section we discuss our various experimental approaches to overcome this issue.

4.3.3.1 Blocking Buffer

We tried changing the concentration of the BSA blocking buffer in order to optimize the surface blocking and reduce the PMMA-protein binding incidents. BSA with concentrations of 1%, 2% and 5% was used. 5% BSA blocked all the sites including the available sites on the antibodies and inhibited the antigen detection. 1% BSA showed stronger nonspecific binding to the PMMA, therefore 2% BSA was the optimal concentration for blocking the chips.

4.3.3.2 Incubation Times

Another variable which could be optimized in order to minimize the PMMA-protein binding, is the incubation time. Numerous experiments were performed for various conditions in order to find the optimal incubation time and detection process for preventing the nonspecific binding to the PMMA surface. The various experimental conditions and results are shown in a diagram in figure 4.16.
Figure 4.16: The diagram shows various experimental conditions designed for preventing the nonspecific binding to the PMMA. The highlighted green color below the processes indicates the fluorescent light detected from the chips. No nonspecific binding were observed for the chips not highlighted in the diagram.

As seen in figure 4.16 no nonspecific binding with PMMA is observed for the tagged antigen and control experiments. One-step sandwich ELISA experiments with FITC-labeled anti-DNA showed strong signal on the PMMA regardless of the incubation time. However, two step ELISA process with FITC-labeled IgG as a secondary antibody showed no signal for 25 minutes incubation. A fluorescent microscope image of the two-step ELISA results are shown in figure 4.17.

Results show that it is possible to prevent non-specific binding by doing a two-step ELISA process. However, other techniques might be tried to prevent the binding to PMMA.
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4.3.3.3 Hydrophobic Surface Coating

The nonspecific binding to PMMA was observed for most of the incubation conditions shown in the chart of figure 4.17. For the biosensor detection, we will be performing one step ELISA (the left branch of the chart) and therefore the nonspecific binding problem observed in these series of experiments must be prevented.

In order to study whether a more hydrophobic PMMA surface would prevent the nonspecific binding to the PMMA, the PMMA covered chips were coated by a layer of extremely hydrophobic FOTS using a Thiol based-linker. The Thiol and FOTS coating processes are explained below:

**Thiol coating Process:** In order to prepare a 1 mM Thiol solution, 14.42 mg of 16-Mercaptohexadecanoic acid is dissolved in 50 ml Ethanol. The chips are incubated in the 1 mM Thiol solution for 3 hours, then washed with a 1 v% of...
HCL/Ethanol for 15 seconds and subsequently rinsed in Ethanol for 20 seconds.

**FOTS coating process:** In order to coat the chips with FOTS, they are placed in a large glass petri dish covered with a large beaker. A 0.1 ml of Trichloro(1H,1H,2H,2H-perfluorooctyl)silane is placed on the further side of the petri dish. The system is placed inside the vacuum oven and heated at 150 degrees for 30 minutes.

Figure 4.18 compares the contact angle obtained on the surface of the normally washed or Piranha cleaned Au chips coated with PMMA, Thiol and FOTS. As it is clear, the FOTS coating makes the surface more hydrophobic.

![Figure 4.18: The contact angle measured on the Au surface with various coatings.](image)

Finally the PMMA, Thiol and FOTS coated chips were tested in an ELISA process in order to compare the nonspecific binding on each surface. As a first blocking step, the chips were incubated overnight in a 2% solution of BSA. They
were then incubated for 1 hour with 12.5 ng/ml solution of Nucleosome followed by a one hour incubation with 1µg/ml FITC-anti-DNA. FITC-tagged anti-DNA purchased from abbiotec was used as a secondary antibody to be able to compare the results with the one-step ELISA results in figure 4.17. Figure 4.19 shows the microscope image of the FOTS coated chips after the ELISA process. Figure 4.19 (a-b) are the chips containing 2C5 coated particles and 4.19 (c-d) are chips with no trenches. As we can see clearly from the image, the fluorescent signal is only detected from the antibody coated particles where the antigen has been detected and no non-specific binding has been detected on the PMMA.

Figure 4.19 shows no non-specific binding on the surface of FOTS-chips when one-step ELISA is performed with FITC-tagged anti-DNA secondary antibody. In order to check if this is due to the hydrophobic surface coating on the PMMA we have performed the one-step ELISA experiments on the PMMA chips with no coating. The results were rather surprising as no non-specific binding was observed on the PMMA chips in these series of experiments.

In the previous experiments, shown in figure 4.17, the anti-DNA was purchased from abbiotec and tagged with FITC in Prof. Torchilin’s lab. In the new experiments however, we have purchased anti-DNA that was already conjugated with FITC. Therefore, the non-specific binding in the earlier experiments could be caused by an inefficient tagging process which resulted in the excess of free...
mAb-2C5-PSL assembled in trenches on FOTS coated PMMA chips

**Figure 4.19**: Florescent microscope image of the FOTS coated PMMA chips after the ELISA process.

dye in the antibody suspension. The problem of non-specific binding in that case could be simply prevented by purchasing the florescent-conjugated antibody instead of performing the conjugation in the lab.
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4.3.4 CEA Detection Results

CEA antigen was next to be detected using nanoparticle-based biochips. This antigen is released in 7 different cancer conditions. The mAb-CEA coated particles were assembled inside trenches on the surface of a chip in order to be tested for the detection of CEA antigen. The developed detection was similar to standard 96-well plates ELISA. In our experiments the secondary antibody was conjugated with FITC to allow the detection using florescent microscopy.

The blocking was performed overnight by incubating the chips with 2% bovine serum albumin (BSA) in DI-water. The chips were then incubated for 2 hours with CEA with concentrations ranging from 1.25 ng/ml down to 15.6 pg/ml to test the highest detection sensitivity of the chips. Antigen incubation was performed in different media including HBS buffer pH 7.4, fetal bovine serum (FBS) and 50% murine blood. As negative control, zero concentration of CEA antigen was used in the ELISA process.

The chips were then imaged using the florescent microscope and subsequently the images were analyzed by ImageJ software. The florescence was measured on a known area of each pattern. The fluorescence detected from the control chips was subtracted from each sample.
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4.3.4.1 CEA Antigen Detection in Buffer, Serum and Blood

Figure 4.20 shows the florescent microscope image of chips containing mAb-CEA coated particles incubated with 0, 15.6 pg/ml and 21.25 pg/ml of CEA antigen in 50% murine blood followed by incubation with a secondary FITC-labeled antibody.

![Florescent microscope image of CEA chips after the ELISA process.](image)

**Figure 4.20:** Florescent microscope image of CEA chips after the ELISA process.

As it is clear from image 4.20, the fluorescence intensity is increasing with the CEA concentration. CEA antigen as low as 15 pg/ml was detectable using the nanoparticle-based sensors.

Figure 4.21 shows the detection results obtained from the incubation of CEA chips with known concentration of CEA antigen in pH 7.4 buffer, fetal bovine serum (FBS) and 50% murine blood.

The graph in figure 4.21 shows that the biosensor chip provides very high sensitivity detecting CEA concentrations as low as 31.2 pg/ml in buffer and FBS media, and 15.6 pg/ml in 50% murine blood compared to commercially available

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Figure 4.21: The fluorescent intensity detected from the incubation of known concentration of CEA antigen in buffer at pH 7.4, fetal bovine serum (FBS) and 50% murine blood.

CEA antigen detection kits that can detect a minimum concentration of 3 ng/ml.

Control experiments was performed on chips assembled with 330nm COOH-PSL particles and 200 nm PSL particles against CEA chips. The results are shown in figure 4.22.

Figure 4.22 clearly shows that the CEA antigen only reacts with CEA chips; chips containing Carboxyl functionalized and plain PSL particles emit no fluorescent light.
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Figure 4.22: Bright filed (top), and florescent (bottom) images of the ELISA performed on chips containing PSL, Carboxyl PSL and CEA-coated PSL particles.

4.3.4.2 CEA Antigen Detection in Tumor Bearing Mice

Confirming the specificity and sensitivity of our nanoparticle-based biosensor chips, we took one step further and performed in-vitro CEA detection tests on our microchips with the blood extracted from tumor infected mice in different stages of the cancer disease.

Female Balb/c mice were inoculated with 100 µl of 4T1 breast cancer cells suspended in PBS pH 7.4 (100,000 cells/mice). Blood was withdrawn by cardiac puncture from anesthetize mice at days 1, 3 and 7 after the inoculation. The blood samples, 0.3 to 0.7 ml in volume, contained 50 µl of 1% sodium citrate to
prevent coagulation. Blood samples were stored at 4°C if required.

Biosensor chips containing CEA coated particles were blocked overnight with 2% Bovine Serum Albumin at 4°C followed by 3 times wash in DI-water. Subsequently the chips were incubated with blood samples for one hour at 37°C, then washed and incubated with a FITC-labeled secondary antibody for two hours at room temperature (26 – 28°C). The chips were rinsed with DI-water and imaged within 1-2 hours from the completion of the ELISA process.

Figure 4.23 shows the florescent intensity detected by analyzing the images obtained from ELISA experiments at day 1, 3 and 7.

![Figure 4.23: Fluorescence detected from the CEA chips treated with blood samples extracted from tumor bearing and non-tumor bearing mice.](image)

As the tumor developed from day 1 to day 7, an increase in the levels of CEA antigen was observed while the CEA level remained constant for non-tumor bearing mice tested as negative control. Initially, the CEA level is low (comparable to the non-tumor bearing level) and the increase in CEA indicates the progress...
in the tumor growth releasing more CEA antigen into the blood stream as time progress. Day 1 and day 3 mice showed no sign of a tumor’s presence. Day 7 mice had clearly detectable tumors with apparent blood vessels of 0.62 and 0.33 mg weight.

In order to compare our results with commercially available detection techniques, we used BIOQUANT CEA detection kit to detect the antigen from the blood obtained from tumor bearing mice. The standard CEA ELISA kit has a limit of detection of 3ng/ml. The blood samples were withdrawn from mice 26 days post inoculation and tested with the CEA kit. The results are presented in figure 4.24.

![CEA antigen detection by ELISA KIT](image)

**Figure 4.24:** Absorbance of CEA Standards and Blood sample using the ELISA detection KIT.

Blood samples taken from tumor bearing mice at day 26 post inoculation analyzed with the KIT showed no signal as seen in figure 4.24.
4. BIOSENSING APPLICATION

based biosensor chips were sensitive enough to detect CEA antigen at very early stage of the disease progress, promising great potential for early detection of cancer using the nanoparticle-based biosensor chips.

4.3.4.3 Cross Reactivity Test

In order to demonstrate the specificity of mAb-CEA to CEA antigen we performed an experiment to check the cross reactivity against a different antigen. We have incubated the CEA chips with FITC-labeled nucleosome antigen with concentration of 12.5 ng/ml in buffer for 2 hours at room temperature. Figure 4.25 shows the fluorescent microscope image of CEA chip after the cross-reactivity experiment.

Figure 4.25: Florescent microscope image of a CEA biochip incubated with 12.5 ng/ml of FITC-NHS antigen for 2 hours.
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No significant fluorescent signal was detected confirming the high specificity of the mAb CEA antibody against CEA antigen and no cross reactivity of mAb-CEA against nucleosome.

Similar cross reactivity tests were performed in serum and 50% murine blood and showed no cross-reactivity of the CEA with 2C5.
Conclusion and Future Work

In this chapter, we conclude the presented work and present suggestions for future research:

5.1 Conclusions

5.2 Future Work

5.1 Conclusions

We have shown that molecules such as proteins (e.g., antibodies) can be attached to the surface of functionalized particles by chemical or physical bonds. For certain applications, such as nanoparticle-based biosensors, it is necessary to assemble functionalized and coated nanoparticles. We have developed effective ways to assemble antibody and drug coated particles into nanopatterns generated...
on PMMA coated gold substrate for biosensor and drug delivery applications. We presented experimental results which proves the feasibility of assembling coated nanoparticles using the EP technique. This method produces particle arrangements with controlled density and uniformity, which can lead to better sensitivity for biosensing.

We have illustrated that to achieve successful assembly and detection, a delicate balance between the attractive and repulsive forces must be achieved by manipulation of pH and ionic strength of the reaction medium during the electrophoretic assembly, as well as the antigen detection. Repulsive forces are required to maintain the stability of the nanoparticle suspension for successful assembly results while making sure the repulsive energy is less than the antigen-antibody bonding energy.

We have developed two novel techniques for precise selective assembly of nanoparticles on a large scale and in a short time. Our developed processes allow selective positioning of nanoparticles in designated nanopatterns on different regions of a single chip. Nanoparticles coated with specific antibodies can be assembled on each region. The developed techniques are simple, fast and reproducible and can have tremendous applications in multiplexing the nanoparticle-based biosensors.

The first developed technique, sequential size-selective assembly, combines the electric field directed assembly and the template assisted assembly to as-
semble particles with specific sizes into nanopatterns that match their size. In size-selective assembly large particles are assembled first with smaller particles assembled afterwards. Polystyrene latex (PSL) nanoparticles with diameters ranging from 200nm to 50nm have been selectively assembled on different regions on the same template using sequential size-selective assembly process.

For this directed assembly technique, the sorting efficiency depends on the particle size via size ratio and also the size distribution of the particles when the chosen nanoparticles are close in size. We showed that high sorting efficiency is achieved when the nanoparticle are assembled in vias close to their size. If the pattern sizes are larger than the particle sizes, particles with the smaller size can assemble next to the larger particles and form clusters. We showed that the clustering or agglomeration of nanoparticles can be prevented by adjusting variables that control the electrostatic force on particles and the particle-particle interaction. Moreover, in order to have distinguishable particles, the size difference between the larger and the smaller particles in the assembly sequence should be larger than the width of the size distribution of the particles.

The second technique developed for selective assembly is abased on sequential electric field-selective assembly. In EField-selective assembly, electrophoretic is used in multiple steps to activate various regions of the substrate for each assembly step. Our results proves successful selective assembly of particles, with various size and coating, in up to four electrically isolated regions of the chip.
5. CONCLUSION AND FUTURE WORK

In addressing different challenges, we have shown that in order to increase the adhesion of the PMMA layer to the Au layer underneath, the PMMA must be baked at a temperature below its glass transition temperature. Moreover, we have presented a detailed analysis of the detachment of assembled particles during the removal and insertion into the liquid suspension and showed that there are two important contributing factors involved in the particle detachment: (1) the applied voltage and (2) the ratio between particle size and pattern depth. We showed that for our experimental condition, applying the voltage causes an increase in the total moment exerted by the adhesion forces and therefore can prevent the particle removal when the particle radius is smaller than the depth of the patterns. However for the case when the trench depth is smaller than the particle radius the capillary moment is always greater than the total adhesion moments and particles will be removed by the interfacial capillary force, even if the electric voltage is applied between the electrodes. We concluded that in order to solve the problem of particle detachment: (1) Certain voltage should be applied during the removal and insertion of the template into the liquid suspension, (2) the trench size should be greater than the particle radius.

The antibody coated nanoparticles assembled on the chips were used as a single-analyte nanoparticle-based biosensor and were successfully tested for the detection of cancer specific PSA, Nucleosome and CEA.

We showed that the chips can detect CEA antigen obtained from mice bearing...
mammary tumors at very early stage, compared to commercially available CEA
detection kits (BIOQUANT) with a limit of detection of down to 3ng/ml.

5.2 Future Work

The ability to perform selective assembly of nanoparticles with various antibody
coatings can pave the way for fabricating nanoparticle-based biosensors for the si-
multaneous detection of multiple biomarkers. The selective assembly and antigen
testing results, presented in this work, show the potential for further development
of this nanoparticle-based chips into miniaturized multi-analyte sensing devices
for early detection of diseases such as cancer. Some suggestions for the future
research are as follows:

- Performing experiments with tumor bearing mice for a longer time to ensure
  repeatability and to reach the saturation curve.

- Developing successful on-chip ELISA for antigens other than CEA.

- Reach low detection limits on ELISA performed with antigens other than
  CEA.

- Assembling multiple antibody-coated particles in one chip and performing
  simultaneous detection of antigens.

- Performing in-vivo detection tests.
• Adding the drug delivery capability.
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