GUPTA, SHALINI. On-chip Assembly of Electrically Functional Structures from Biological and Colloidal Particles. (Under the direction of Orlin D. Velev and Peter K. Kilpatrick.)

We study the assembly of electrically functional and miniaturized biosensors from colloidal systems ranging from synthetic organic/inorganic micro- and nanoparticles to naturally existing live cells. The unique combination of highly specific biological interactions and different assembly schemes – 1) controlled manipulation in externally applied electric fields, and 2) free diffusion in bulk suspensions are investigated as a versatile tool for fabricating simple, rapid and inexpensive sensing devices that can potentially be interfaced with electrical circuits on a chip. For each system, a detailed modeling is carried out with the aim to acquire fundamental understanding of the operating parameters involved in the assembly process.

Gold nanoparticle-tagged silver enhanced immunoassays are designed for detecting low concentrations of target antigens and their performance is optimized by developing a simple adsorption-controlled kinetic model. Antigens in solution are allowed to diffuse toward and bind with primary antibodies immobilized on a glass substrate. Secondary labeling of antigens with gold nanoparticles and their subsequent silver enhancement enables visual detection of the antigens with bare eyes. The results are quantified by measuring the darkness of the spots using densitometry technique. In order to systematically probe the influence of the operating parameters on the assay performance, the concentration and incubation time of each reagent are varied individually and the role of mass transfer is explored in each case. Since the electroless deposition of silver metal on the bound
nanoparticles makes a conductive patch on the substrate, these assays could allow direct interfacing to on-chip electrodes for electrical readout of the results.

The co-assembly of live cells and synthetic microparticles on electrically controlled chips is demonstrated as a simple and facile route to making novel biocomposite materials, in which the biological functionality of the cells is augmented by the physical functionality of the particles. One-dimensional chains and planar membranes from live cells and colloidal particles are assembled by dielectrophoresis. The experimental results and numerical simulations of the assembly dynamics indicate that the electric field draws the particles into the interstitial junctions between the cells, where the field strength is greatest. This allows using the particles as binding elements. Permanent cell-particle chains and membranes are assembled dielectrophoretically by biospecifically binding yeast and mouse fibroblast cells with lectin-conjugated magnetic microparticles. Such biomagnetic cell-particle assemblies may find application in sensors, microassays, microsurgery, or as responsive biomaterials.

A novel method for direct electrical detection of antigens is described in which the results of latex agglutination test are measured using electrochemical impedance spectroscopy (EIS). Antibody-conjugated latex microspheres are agglutinated in the presence of complementary antigens above interdigitated microelectrodes. As the particles sediment on top of the electrodes, a reproducible impedance spectrum is generated in the 0.1 – 1 MHz frequency range in 10 min that depends strongly on the morphology and the sedimentation rate of the agglutinates. The performance of the sensor is interpreted by fitting the experimental data to an equivalent circuit model. The effects of the various operating parameters are also characterized in detail.
On-chip Assembly of Electrically Functional Structures from Biological and Colloidal Particles

by

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DEDICATION

this dissertation is dedicated to my parents

Vijay and Bhagwan Das Gupta

and to my husband Ashish

for their unconditional love, support and sacrifices
BIOGRAPHY

Shalini Gupta was born on March 5, 1980 in the city of Roorkee nestled on the banks of river Ganges in the north Indian state of Uttarakhand. She was, however, raised in Kanpur (formerly known as Cawnpore) - a populous and historic town that not only stands proud as one of north India’s major centers for leather and textile industries but is also host to several premiere educational institutions including the famous Indian Institute of Technology (I.I.T.). The academically charged milieu of I.I.T., where her father is a professor, played a crucial role in kindling in Shalini a curiosity for science and engineering at an early age. Consequently in 1998, she secured a rank in the All India Joint Entrance Examination (AI-JEE). This selection and a disclosed desire to experience life away from home lead to the subsequent choice of studying Chemical Engineering at the Institute of Technology–BHU, Varanasi, India.

On one hot afternoon in the year 2000, she got her first glimpse into the wonders of nanotechnology during a class presentation made by her fellow undergraduate chemical engineer. The fascination for the word *nano* culminated into her joining the PhD program at North Carolina State University immediately after receiving a B.Tech. degree in Chemical Engineering in 2002. Since then, she has been pursuing graduate level research in the field of colloid science and nanoscale surface interactions under the guidance of Dr. Orlin Velev and Dr. Peter Kilpatrick.
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Chapter 1

Introduction and Dissertation Goals*

*Partially based on Velev and Gupta, Review article to be submitted to *Adv. Mater.*
1.1 Introduction

The organization of matter in nanomaterials and nanodevices can be accomplished by top-down miniaturization techniques or bottom-up methods typically based on self-assembly or directed assembly.\textsuperscript{1,2} Colloid micro and nanoparticles are one of the most widely used classes of objects in bottom-up assembly. A large research effort is directed to the fabrication of materials from highly organized particles with advanced functionality and specific applications in areas such as photonics, electronics, sensing, and catalysis.\textsuperscript{3-15} We begin this Dissertation with an overview of some of the basic types of particle assemblies reported in the literature and the strategies for their fabrication, classified on the basis of their dimensionality. Various applications of the structures assembled from particles are surveyed.

1.2 Methods and Strategies for Assembly of Structures from Micro and Nanoparticles

A convenient way to classify the majority of the colloidal assemblies could be based on their dimensionality and degree of ordering - three, two, one-dimensional, or independent aggregates. In order to evaluate the ease and precision of the controlled fabrication of such materials, we also need to consider in each case the method by which a certain structure is assembled. The assembly can be accomplished by an extensive array of techniques using various physical mechanisms. A survey of a few major groups of methods that have found use in the controlled assembly of particles into materials with well-organized and defined microstructure is presented in Table 1.1. The goal of all these techniques is to collect the particles, organize them and bind the structure formed into a permanent material. The collection can be achieved by methods as simple as sedimentation,\textsuperscript{16-18} but can also be
directed by capillarity\textsuperscript{19-22} or driven by external fields.\textsuperscript{23-34} The organization can be a result of packing the particles to initiate crystallization,\textsuperscript{35-37} or templating them on surfaces and objects.\textsuperscript{38-41} The binding can be achieved with methods as simple as adding electrolytes of opposite charge\textsuperscript{42, 43} or as complex as directed key-and-lock recognition of proteins or DNA molecules.\textsuperscript{44-49} Following are a few examples of the major classes of particle structures based on their dimensionality and some of the potential applications of such particle assemblies.

1.2.1 One-Dimensional Structures

These assemblies are restricted in size and structure in two directions, while extending in one direction. The simplest prototype of such a material is an aggregated chain of particles.\textsuperscript{50-52} The dipolar attraction induced by magnetic or electric fields is one of the most efficient ways to align the particles in chains (Figure 1.1a - c). The chains of particles aligned in the directions of the field can be conveniently rotated\textsuperscript{53-58} and if the interactions induced by strong fields are large enough, the chains can also assemble into in 2- and 3-dimensional crystals by lateral attraction and alignment.\textsuperscript{59}

AC electric fields can also be used to assemble 1D structures from metallic nanoparticles as well (Figure 1.1b).\textsuperscript{60, 61} The nanoparticles in suspension are drawn into "microwires" growing in the direction of the field. The main effect driving the particle collection during the microwire growth is the dielectrophoresis.\textsuperscript{62, 63} These microwire assemblies are many particles thick and permanently aggregated because of the strong irreversible aggregation of the particles collected by the field. An alternative method for assembling particles in alternating chains of strictly defined structure is the use of templates
where the particles are deposited inside precisely etched grooves and pits on the surface of micropatterned wafers (Figure 1.1d). The particles collected in the groves can be bound together, resulting in chains of varying "bond angles" between the particles. The chain-like assemblies can also be produced in microfluidic devices. On a larger size scale, particles and particle-based materials can be assembled in the form of uniform cylinders by crystallization inside capillaries.

1.2.2 Two-Dimensional Films and Crystals

The organization of particles in films with a controlled structure and thickness opens many possibilities for making products and devices and is presently one of the most intensively investigated research topics. The simplest way to deposit nanoparticle films on surfaces is adsorption from suspension onto oppositely charged or otherwise "sticky" surfaces (Figure 1.2a). The process of Random Sequential Adsorption (RSA) can result in coverage of up to ~ 55% of the surface with monodisperse spherical particles in a relatively dense structure without long-range ordering. The adsorption can be made strong and irreversible by covering the surface with a layer of polyelectrolyte of charge opposite to the one of the particles. The processes of adsorption of the polyelectrolytes over the particles can be repeated to bind them from the top and restore the surface to its original functionality. The subsequent "layer by layer" (LBL) particle and polyelectrolyte adsorption can be repeated as many times as necessary, resulting in polyelectrolyte-particle films with well controlled thickness and number of layers. These films can be used to impart to the surfaces desired properties such as wettability and antireflectance and form devices such as diodes and solar
cells.\textsuperscript{76} Formation of free-standing elastic films containing nanoparticles and polyelectrolytes by similar LBL methods has also been reported.\textsuperscript{77, 78}

An additional level of functionality of the films of particles can be achieved by arranging them into large-scale arrays, or 2-Dimensional (2D) crystals. This can not be achieved by direct random sequential adsorption. One of the commonly used processes is the convective assembly.\textsuperscript{19, 79, 80} The 2D crystals in this method are assembled in the moving meniscus of the drying particle suspension. The crystallization occurs when the particles carried by the flux of liquid towards the drying front are concentrated and incorporated in the transition region between the meniscus and the drying crystal (the process can be compared to filtering of the suspension on the moving crystal front). Various implementations of the convective assembly aimed at making the process simple and inexpensive or highly controlled have been reported. The deposition can be speeded up by using suspensions of particles of high volume fraction or volatile organic solvents (Figure 1.2b - e),\textsuperscript{81-84} yet the relative slowness of the process remains a problem. Alternative techniques for the assembly and deposition of 2D particle crystals have been based on the ability of the particles adsorbed onto free liquid interfaces to form two dimensional crystals, either by compression\textsuperscript{85} (akin to Langmuir-Blodgett molecular self-assembly) or due to capillary forces.\textsuperscript{86} The organized films of adsorbed particles are then transferred and immobilized onto solid surfaces.

Processes of direct particle self-assembly onto plain surfaces currently lack the ability to deposit single-domain crystals of specific orientation, due to multiple nucleation sites in the drying contact line.\textsuperscript{87, 88} One of the promising routes to organizing particles into large crystals and arrays with specific orientation is the application of external electric fields across
the suspension. In one of the most studied configurations, the fields can be applied normal to
the plane of the crystal (residing onto one of the electrodes). The particles can be assembled
into closely packed crystals by the electrohydrodynamic flows pulling them together or
into a long-ranged lattice by the repulsion of the parallel induced dipoles. The particles can
also be organized into very long-ranged 2D crystals by alternating (AC) fields applied in the
direction of the crystal plane (Figure 1.2f). The forces involved include induced dipole
chaining and dielectrophoresis, the mobility of particles along the direction of the gradient of
the field. The use of dielectrophoresis facilitated the assembly of single-domain 2D crystals
with centimeter size and excellent, "clean" diffraction patterns. This can be used for the
formation of new types of materials with strong anisotropy. It is still necessary, however,
to develop processes for the immobilization and extraction of such crystals assembled in
liquid chambers.

1.2.3 Three-Dimensional Materials

The simplest and fastest way to obtain such materials is aggregation of suspended particles
resulting in a gel or solid phase. Materials formed by such processes usually have
disordered, fractal-like microstructure. This "assembly" process is very simple, inexpensive
and scalable and works with many classes of particles without need for uniformity in size or
shape. It is routinely used in the fabrication of ceramics, catalysts and a large variety of sol-
gel materials. While the focus of this review are materials with much better defined
structure and more complex properties it should be noted that the disorganized 3D particle
aggregates are possibly the only type of "assemblies" that are presently produced on an industrial scale.

A class of particle-derived materials with much higher degree of organization are the three-dimensional colloidal crystals. The interest in the physical origin of the particle crystallization in bulk suspensions predates the present day thrust in advanced materials fabrication. The original methods of crystallization by deep deionization and long-ranged electrostatic repulsionm$^{98}$ are not very practical for materials fabrication. Three-dimensional crystals, however, can be easily fabricated by concentration of monodisperse spherical particles thorough sedimentation.$^{16-18}$ The process can be speeded up by centrifugation, electrophoretic collection, filtration and other methods.$^{99-102}$ The resulting materials possess uniform porosity, higher mechanical stability, well-defined internal transport characteristics and extraordinary optical properties. These materials could find applications in catalysis, separations, substrates for biomaterials and templates for the formation of "inverse opal" structures.$^{4,99}$

One of the major challenges in the formation of three dimensional crystals is shaping them into a desired shape and controlling the symmetry and orientation of the particle lattice. The overall shape can be templated by the assembly vessel or, in more straightforward implementations of the technique, - by filtering the suspension inside specially designed thin cells.$^{101}$ The control of the particle organization is much more difficult, as the particles in confined geometries typically self-assemble into a trivial symmetry of randomly stacked hexagonal planes (rhcp).$^{103}$ The symmetry and the orientation of 3D crystals from particles
can be controlled by the application of external electrical fields, but extraction of the obtained body-centered tetragonal crystals has not been developed as a routine process.

The assembly of nanoparticles in crystalline arrays has been another active area of colloid assembly. The forces involved in this case are more complex because the size of the particles is comparable to the range of the intermolecular and surface forces. It has been recognized that nanoparticles can be crystallized by slow concentration similarly to the larger microspheres. Binary mixtures of nanoparticles of different sizes form a variety of crystalline phases. Dense nanoparticle phases could have very interesting electronic and optical properties.

The crystallization of particles by restriction of the free volume requires repulsive electrostatic interactions to allow for their rearrangement and organization with minimal friction. Strongly attractive interactions in particulate suspensions lead to aggregation without long-range ordering. It has been realized recently that weak, precisely balanced attractions between oppositely charged spheres can lead to crystallization broadly similar to the formation of ionic crystals by cations and anions. The concept of "ionic colloidal crystals" has now been proven both for oppositely charged particles in the micron and nanometer size ranges. It is expected that electrostatic binary crystal assembly could lead to crystals of new symmetry and composition that might find applications in photonics and electronics. Finally, the nanoparticles can be crystallized by inducing attractive dipolar forces photochemically.

The two-dimensional and one-dimensional assemblies are the common precursors and components in the fabrication of photonic and electronic devices, sensors and
biologically active structures.\textsuperscript{111-114} For example, semiconductor particles can be layered with polyelectrolytes to form electronic components such as rectifying and light-emitting diodes and solar cells.\textsuperscript{115, 116} Quantum confinement in arrays of metallic particles could be used in memories and nanoelectronic circuits.\textsuperscript{64} Thin films of colloidal crystals have been studied as potential precursors to photonic devices.\textsuperscript{38} Methods for patterning of the crystals in 3D have been developed, even though no true photonic circuits have been demonstrated to date.

1.2.4 Supraparticles, Colloidosomes and Colloidal Molecules

The particles in these systems are assembled into small self-contained clusters with well-defined structure and size. A simple, large-scale process to fabricate assemblies of large and small particles is to adsorb the small particles around larger particles of opposite charge. This process results in raspberry-type assemblies.\textsuperscript{117} A more elaborate method for assembling clusters from a single type of particle components is to use liquid emulsion droplets as templates that can be dissolved after the assembly and immobilization of the particles. This method has first been demonstrated with assemblies of particles both around and inside droplets (Figure 1.3a, e, f).\textsuperscript{118-122} Subsequently, the empty ordered shells of particles assembled around droplets have been named "colloidosomes" (Figure 1.3b, d).\textsuperscript{123-125} The assemblies of small clusters of particles compressed inside droplets have been called "colloidal molecules" due to their resemblance to the ball-and-stick molecular models (Figure 1.3c).\textsuperscript{126, 127} The colloidosomes can find applications as semipermeable capsules for drug delivery and biotechnology as well as in pharmaceutical, cosmetic and food products with advanced structure. The colloidal molecules have potential as precursors for making of
colloidal and photonic crystals of unusual symmetry, even though the fabrication of large quantities of monosized assemblies still appears to be a challenge.

1.3 Advanced Materials with Biological Functionality

Many of the potential applications of the particle assemblies are connected to some biological or medical uses in order to fulfill the growing needs of health care, drug and gene delivery, diagnostics, and tissue regeneration. Particle assemblies have formed the basis of many types of biosensors including those for protein, bacteria and DNA detection. However, the biological functionality of these assemblies is in many cases limited by the relatively simple chemistry and properties of the particles involved. One of the challenging milestones on the pathway to nanomedicine (cross between the disciplines of nanotechnology and medicine) is to manufacture active hierarchical nanostructures which have advanced features for performing complex biological functions. A significant portion of this Dissertation describes our efforts towards reaching this aim.

We developed methodologies that allow us to construct miniaturized sensing devices which can be interfaced with electrical circuits to achieve simple and rapid biodetection. Our approach involves the organization of “tiny” building blocks like nanoparticles, microparticles, proteins and cells into useful structures made via either biological interaction-mediated self-assembly or electric field-induced dipolar forces. The assembly process not only allows creation of potentially viable hybrid structures but also facilitates understanding the physics governing the interaction between the synthetic particles and biological species.
This knowledge could be valuable in a wide variety of other applications (protein adhesion to scaffolds, cell migration studies, stem cell research, etc.).

1.4 Layout of this Dissertation

We start with the description of our work in the assembly and characterization of particle-based immunoassays used for detecting analyte in dilute suspensions. Specifically, we report on a sandwich immunoassay in which a direct optical readout was accomplished by secondary labeling with antibody-conjugated nanoparticles and their enhancement by silver nucleation. By varying the effects of the operating parameters, a semiquantitative kinetic model was developed that not only provided better insight of the binding process but also served as a guideline to optimize the assay performance. In Chapter 3 of the Dissertation, we portray a novel approach to increase the functionality of the particle assemblies by using live cells as "particles" that could be assembled by electric fields on chips. The cell structures were made permanent by using functionalized particles as binding units. Control over the cell-particle architectures was achieved by tuning the experimental parameters. These biocomposites have a variety of potential applications in micro bioassays, sensors, artificial tissues, and drug delivery systems. Chapter 4 details the use of electrochemical impedance spectroscopy as a tool to measure results of latex agglutination tests that are performed inside custom-designed thin chambers above micropatterned interdigitated electrodes. The digital yes/no type detection response renders these immunosensors potentially useful in components of micro total analysis systems (μTAS).
Finally, we summarize our findings in Chapter 5 and provide an outlook on why the potential of particle structures is not yet realized to a large extent in technology practice. The problems to a large extent could be solved by assembly processes that are scalable, controllable, rapid and inexpensive enough. We discuss some of these challenges and their impact on the future research in this area.
Table 1.1 Strategies and limitations for assembly of colloidal structures.

<table>
<thead>
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<th>Methods of Assembly</th>
<th>Schematics</th>
<th>Types of Assembly</th>
<th>Complexity/Cost</th>
<th>Scalability</th>
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<td>3D</td>
<td>Small</td>
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<td>Evaporation</td>
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<td>2D, 3D</td>
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Figure 1.1 Examples of one-dimensional structures. Colloidal assembly in external AC electric fields: (a) Permanent chains from live yeast cells and lectin functionalized microparticles,\textsuperscript{55} (b) Self-repairing microwires from gold nanoparticles,\textsuperscript{60,61} and (c) Zigzag chains from anisotropic microspheres;\textsuperscript{53} (d) Zigzag chains formed via templated assembly.\textsuperscript{39}
Figure 1.2 Examples of two-dimensional assemblies. Structures fabricated via convective assembly: (a) Electrostatic deposition of gold nanoparticles on a self-assembled monolayer (SAM) of APTES,\textsuperscript{69} (b) Unordered assembly from a binary mixture of particles,\textsuperscript{83} (c) Organization of live yeast cells into arrays,\textsuperscript{84} (d) Assembly of Tobacco Mosaic Virus (TMV) into arrays,\textsuperscript{82} and (e) Highly ordered colloidal crystal.\textsuperscript{81} (f) 2D crystallization of latex microspheres using AC electric field.\textsuperscript{59}
Figure 1.3 Examples of self-contained particles. (a) Particle collection around emulsion droplets;\textsuperscript{118} (b) Semi-permeable colloidosomes;\textsuperscript{123} (c) Colloidal molecules;\textsuperscript{126} (d) Microrod-stabilized “hairy” colloidosomes;\textsuperscript{124} (e) Anisotropic “eyeball” supraparticles;\textsuperscript{122} (f) Doughnut-like assemblies.\textsuperscript{121}
1.5 References

(1) http://www.zyvex.com/nanotech/feynman.html


(84) Jerrim, L. B.; Velev, O. D. *To be submitted to Langmuir* **2007**.


Chapter 2

Silver Enhanced Gold Nanoparticle Immunoassays*

2.1 Abstract

Silver-enhanced nanoparticle-labeled immunoassays provide a simple, low-cost, and effective way of detecting antigens in dilute solutions. The physical mechanisms behind their operation, however, have not been fully investigated. In this chapter, we outline a semiquantitative approach for optimizing sandwich nanoparticle immunoassays using an adsorption-controlled kinetic model. Primary antibodies were immobilized on a solid substrate to bind the target antigens in solution. An optical signal was measured by secondary labeling of antigens with gold nanoparticles and their enhancement by silver nucleation. The opacity of the silver-enhanced spots was quantified by densitometry. The selectivity of the sandwich immunoassays was adequately high, and antigen concentrations as low as 0.1 µg cm\(^{-3}\) (4 ng total) were detected reproducibly. The role of mass transfer was investigated, and a model was developed to optimize the performance of immunoassays by correlating the opacities of silver spots to the concentration and incubation times of antigens and gold nanoparticles. The results could allow the development of more rapid and reliable nanoparticle immunoassays.
2.2 Introduction

Diagnostic immunoassays are routinely used for biomolecular detection because they are simple, sensitive, and allow parallelization. Antigens such as proteins, antibodies, viruses, drugs, and other molecules, generally referred to as the analyte in an immunoassay, are selectively bound by their complementary immunoglobulins either in solution (agglutination assay) or on a solid surface (direct or sandwich assay). The bound antigens are detected with a reporter, typically an antibody that is labeled with a probe, and the intensity of the signal corresponding to the number of antigen-antibody complexes formed is measured.\(^1,2\)

The immunoassay formats differ in the type of probe and signal amplification techniques that are used in the detection process. Enzyme-linked immunosorbent assay (ELISA) is one of the oldest (ca. 1960) and most widely used laboratory method. It yields a colorimetric signal upon enzymatic cleavage of chemiluminescent substrate\(^3-5\) and has limits of detection in the picomolar analyte range. Fluorescence immunoassays, which are frequently used in cell biology, are based on an alternative detection strategy where antibodies labeled with fluorophores (Rhodamine, Alexa fluor, FITC, etc.) are used as markers that provide high optical contrast and do not require an additional processing step\(^6,7\). Extra care is, however, needed to avoid photobleaching of the fluorophores, which may lead to reduced accuracy. Both ELISA and fluorescence immunoassays are routinely performed in a microarray format for higher throughput and greater selectivity. A few alternative immunoassay techniques make use of evanescent wave fiber optics\(^8\), immunochromatography\(^9,10\), gel electrophoresis\(^11\), radioisotope (\(^{125}\text{I}, ^3\text{H}\)) labeling\(^5,12\), electrochemical sensing\(^13\), scanometry\(^14\), and surface enhanced Raman spectroscopy\(^15,16\).
Latex agglutination tests (LATs) have been used since the late 1950s. In LATs, antibody-coated microspheres are agglutinated and precipitated from suspension in the presence of antigens.\textsuperscript{17, 18} LATs have contributed greatly to diagnostic immunology because they are inexpensive, portable, and yield results rapidly. The detection is typically carried out by light scattering or by visual inspection. One popular modification of LATs is the strip tests where the agglutination event is made visible by the colored bands of antibody-conjugated particles bound by the antigen to the surface. More recent advances have been made using nanoparticles as detection probes, because their nanometer size gives rise to high diffusion coefficients and their optoelectrical properties can be tailored chemically or physically and used in the detection procedures.\textsuperscript{19} The nanoparticle-based surface immunoassays require only a few microliters of the sample and may be miniaturized.\textsuperscript{20-22}

Detection based on the nanoparticle color may not be sensitive enough due to the tiny amount of material present unless the particles are in proximity.\textsuperscript{23} The sensitivity and ease of detection in the case of gold or silver nanoparticle probes can be increased drastically by using “silver enhancement”,\textsuperscript{24-27} a procedure for electroless silver deposition where the nanoparticles act as nuclei. In this technique, colloidal metal particles act as catalysts to reduce silver ions (I) to metallic silver in the presence of a reducing agent (such as hydroquinone) and are enlarged in the process by up to 5 orders of magnitude. The amount of metallic silver deposited is typically quantified by electrochemical measurements,\textsuperscript{28} anodic stripping analysis,\textsuperscript{29} or colorimetry\textsuperscript{30, 31} among other methods. A few examples of immunoassays using colloidal gold nanoparticles combined with silver enhancement have
been demonstrated on microarrayed substrates\textsuperscript{32} between coplanar electrodes\textsuperscript{33-35} and through microfluidic channels.\textsuperscript{36}

The nanoparticle immunoassay techniques and especially the complex sandwich assays are poorly characterized in terms of the relation between mass transfer and performance. For example, the incubation time required for the saturation of the receptor sites with antigens or nanoparticle labels is commonly established either empirically or by operator judgment. Antibodies conjugated to gold nanoparticles are typically incubated for a longer period of time than molecular antigens owing to their larger size. Good understanding of the fundamentals of mass transfer is required to optimize the overall performance of a nanoparticle sandwich immunoassay.\textsuperscript{37, 38}

In this chapter, we report the use of silver enhancement as a semiquantitative means of determining the concentration of gold nanoparticles on a surface. We also illustrate that the optically measured darkness of the enhanced spots could be a simple tool to compare and characterize the rate of mass transfer of antigens and gold nanoparticles in the system. The aim of our work is to (i) provide a simple procedure to assemble a compact, rapid, and low-cost sandwich immunoassay using a combination of gold nanoparticle labels and silver amplification technique, (ii) investigate to what extent the nanoparticle adsorption can be quantified using silver enhancement, (iii) characterize the immunoassay’s selectivity and sensitivity via optical densitometry, (iv) match the adsorption kinetics against a simple mass-transfer model, and (v) formulate on the basis of the analysis a general procedure for optimizing the operation time of the assay by studying the adsorption kinetics of each reagent individually.
2.2.1 Kinetics of Analyte Adsorption

We used a simple unified model for the adsorption kinetics of the species onto the binding sites on the surface. During the adsorption process in surface immunoassays, the antigen molecules in the buffer freely diffuse toward the solid substrate (semi-infinite diffusion to a plane), where they may either attach with high affinity to the binding sites on the antibodies or adsorb nonspecifically to the surface. At steady state, assuming negligible convection in the very thin chamber used, the relation between the rate of change of surface coverage by an adsorbing species and the concentration gradient adjacent to the surface follows from a simple mass balance at the surface.39

\[
\frac{d \Gamma}{dt} = D \left( \frac{\partial c}{\partial x} \right)_{x=0}
\]  

(1)

In the above expression, \( D \) is the diffusion coefficient (cm² s⁻¹) and \( \Gamma \) is the surface coverage (molecules cm⁻²). The kinetic equation (1) may be related to the rate of diffusion in solution (\( J_D \)) or to a simple Langmuir-type first-order rate of adsorption (for low coverage) at the surface (\( J_R \)),

\[
J_D = D \left( c_b - c_s \right) / L
\]  

(2)

\[
J_R = k_{ads} c_s \left( \Gamma_{max} - \Gamma \right)
\]  

(3)

where \( c_b \) is the bulk concentration of the adsorbing species (molecules cm⁻³), \( c_s \) is the concentration of the adsorbing species in the vicinity of the surface, \( L \) is the diffusion path
length (cm), $k_{ads}$ is the adsorption rate constant (cm$^3$ molecules$^{-1}$ s$^{-1}$), and $\Gamma_{\text{max}}$ is the total number of free sites available per unit surface area. At steady state, $J_D = J_R$ and a simple relation follows from eqs 2 and 3,

$$c_s = \frac{c_b}{1 + k_{ads} L (\Gamma_{\text{max}} - \Gamma) / D} = \frac{c_b}{1 + \Theta}$$

(4)

Here, the dimensionless parameter $\Theta = k_{ads} L (\Gamma_{\text{max}} - \Gamma) / D$ is known as the Thiele modulus. For $\Theta \gg 1$, $c_s$ approaches zero. In other words, as soon as a diffusing molecule reaches the surface it is adsorbed and the rate of surface coverage is determined by the rate of diffusion in solution ($J_D = D c_b / L$). For $\Theta \ll 1$, $c_s$ approaches the bulk concentration ($c_b$); i.e., the diffusion in the solution is much faster than adsorption and the kinetics of the process is governed by the rate of adsorption at the surface, $J_R = k_{ads} c_b (\Gamma_{\text{max}} - \Gamma)$. In all other nonlimiting cases, a numerical procedure can be used to determine the concentration gradient at the surface.

2.3 Materials and Methods

2.3.1 Immunoassay Principles

The main steps of the immunoassay that we developed are schematically outlined in Figure 2.1. The assay was performed on microscope glass slides, which were activated with aldehyde-terminated silanes using standard coupling chemistry. The silane ends of the molecules attach to the glass substrate leaving the aldehyde groups available to react with
amine groups on the proteins. Primary antibodies were irreversibly attached to the aldehydes on the substrate via formation of Schiff’s base links (step I). The glass slide was washed off to remove any unbound antibodies, and the unreacted aldehydes were reacted with bovine serum albumin (BSA), which was used as a blocking agent to prevent nonspecific adsorption of the antigens in the next step. BSA and nonionic surfactant (Tween-20) were also added to the antigen and nanoparticle solutions to minimize nonspecific binding and aggregation.

A few microliters of dilute antigen solution were incubated over the glass substrate to allow the binding of antigens to the primary antibodies in the immobilized spot (step II). Unbound antigens were removed from the glass slide by washing with a mixture of BSA and surfactant. The bound antigens were sequentially tagged with gold nanoparticles by incubating the assay chamber with a dilute suspension of secondary antibodies conjugated to colloidal gold (step III). The attached gold nanoparticles were enlarged by silver metal deposition (step IV). Silver enhancement proceeds as an autocatalytic reaction: the gold nanoparticles serve as nucleation sites to catalyze the reduction of silver ions to metallic silver. The enhanced plates were washed, dried, and characterized by microscopy and optical densitometry.

2.3.2 Antibodies and Reagents

The following immunoglobulins (IgGs) were used in the experiments: monoclonal goat anti-mouse (GAM IgG) and goat anti-rabbit (GAR IgG) antibodies (Jackson ImmunoResearch Lab Inc.; West Grove, PA); mouse (M IgG) and rabbit (R IgG) antibodies acting as their antigens (Calbiochem; San Diego, CA); 5-nm colloidal gold conjugated to goat anti-mouse
(GAMg IgG) and goat anti-rabbit (GARg IgG) antibodies (Ted Pella, Inc.; Redding, CA). Other reagents used include the following: 11-(triethoxysilyl)undecanaldehyde (Gelest, Inc.; Morrisville, PA); phosphate buffer saline, BSA, ≥ 99% acetone, (3-aminopropyl)triethoxysilane (APTES), and anhydrous toluene (Sigma; St. Louis, MO); Tween-20 and light microscopy silver enhancement kit (Ted Pella, Inc.). All reagents were used as received.

2.3.3 Substrate Preparation and Experimental Setup

Microscope glass slides (Fisher Scientific, PA) were immersed in a strong oxidizing solution (N ochromix) overnight, washed with deionized (DI) Millipore water (resistivity ~ 18 MΩ cm), and oven-dried for ~ 1 h. A 2% solution of 11-(triethoxysilyl)undecanaldehyde was freshly prepared in dry acetone and a 10 µL droplet of it was placed on the clean glass slides for 5 min. The silane reagent was rinsed off using DI water, and the slide was air-dried.

The immunoassay was performed in a transparent 18 µL flow cell (HBW13, Grace BioLabs; Bend, OR) as shown in Figure 2.2a. The cell contained two openings through one of which the reagents were manually injected using a micropipet and through the other withdrawn controllably using a micropump. Importantly, the Teflon tubing connected to the withdrawal pump was of diameter smaller than the cell opening into which it was inserted. This allowed for the level of the liquid in the chamber to be maintained even though the pump could be kept running continuously. When new liquid was added through the inlet opening, it replaced the contents of the microchamber by laminar flow and was sucked by the end of the tubing connected to the pump. After the excess liquid in the cell was sucked in the
tubing, however, the capillary meniscus between the end of the tube and the opening of the cell broke, leaving the cell full of liquid. Thus, the contents of the cell were rapidly and easily displaced at the end of each step of the protocol without ever exposing the functionalized surface to air.

2.3.4 Immunoassay Protocol

The samples containing IgGs, BSA, and Tween-20 were prepared in 10 mM phosphate buffer saline (PBS) at pH 7.4. The range of concentrations of reagents and the incubation times used in the experimental steps are listed in Table 2.1. All experiments were performed at room temperature.

The assay was initiated when 0.2 µL of buffer containing 50 µg cm⁻³ GAM IgG was carefully placed on the aldehyde-functionalized glass slide for 5 min and sealed with the flow chamber. Drying of the solution was minimized to avoid denaturation of the IgGs on the substrate. The chamber was then filled and incubated for 15 min with BSA followed by 15-min incubation with a mixture of BSA and Tween-20. The contents of the chamber were replaced with varying concentrations of M IgG (antigen) and incubated for different periods of time (ranges shown in Table 2.1). This step was followed by 10-min incubation with the BSA and Tween-20 mixture. Dilute suspensions of GAMg IgGs (nanoparticle labels) were incubated in the chamber for varying periods of time. The excess colloidal gold was washed off with the BSA and Tween-20 mixture. The chamber was rinsed thoroughly with DI Millipore water. The washing step was essential to remove all free ions, which could result in background noise in the consecutive enhancement step. The gold nanoparticles were silver
enhanced for 10 min with a mixture of equal volumes of the initiator and enhancer provided in the light microscopy silver enhancement kit. The silver-enhanced spot was washed extensively with DI water to remove the unreacted silver ions. The opacity of the spot was quantified (after air-drying) by densitometry analysis. Each experiment was repeated twice before data analysis.

2.3.5 Optical Densitometry Analysis

The silver-enhanced spots were viewed in transmitted light mode using an Olympus BX61 optical microscope. The dark black (or brown) silver deposit had good contrast against the transparent glass background. Typical optical images of ~1-mm-sized silver-labeled spots are shown in Figure 2.2b and c. The images were captured in gray scale (8 bits/channel) at a fixed exposure time of 1/1200 s using an Olympus DP70 CCD digital camera. The total area of the silver-enhanced spot in each image was selected using the lasso tool in the Adobe Photoshop software (version 7.0.1). The darkness ($I_s$) of each spot was quantified by measuring its average opacity using the luminosity histogram tool in Photoshop. The data were normalized by the opacity of the background ($I_b$), which was calculated by selecting a clean area from the sector surrounding the spot. The normalization procedure was necessary to correct for the background scattering, which could vary slightly from one experiment to another. The final signal was recorded as an “optical darkness ratio” ($ODR$) of the relative intensities of the enhanced spot ($I_s$) and the background ($I_b$).

$$ODR = \frac{(I_b - I_s)}{I_b}$$

(5)
The final $ODR$ value was plotted as an average of the two data points obtained by repeating each experiment. The $ODR$ values of the two data points were indicated as error bars. Please note that $ODR$ is different from the spectroscopic optical density, which is defined as a logarithm of the corresponding intensity.

### 2.3.6 Formation of Gold Gradients via APTES Monolayer Self-Assembly

In order to characterize the silver-enhancing procedure, a cycle of silver enhancement experiments were performed with gold nanoparticles deposited in a gradient of changing surface concentration. The procedures used in making and studying well-defined nanoparticle gradient substrates are as follows.

(i) **Substrate Treatment.** A microscope glass slide was cut into 7.5 cm × 1.25 cm rectangular pieces and dipped in Nochromix overnight. The slide was washed thoroughly with DI water prior to the experiment and air-dried. It was then cleaned and hydrophilized by ultraviolet/ozone (UVO) treatment for 30 min.

(ii) **APTES Monolayer Self-Assembly.** A 1 wt % solution of APTES was prepared in anhydrous toluene and enclosed in a container. The solution was heated to 60 °C in a water bath, and the UVO-treated glass slide was immersed in it. The slide was taken out of the container after 6 min and immediately dipped into toluene. The slide was sonicated in toluene and ethanol for 5 min each to remove the physiosorbed silane molecules, washed with DI water, and completely dried in air.

(iii) **Deposition of Gold Nanoparticle Gradients.** An aqueous suspension of ~ 12-nm gold particles was synthesized by citrate reduction of HAuCl4 (ACS reagent, Aldrich; St.
Louis, MO). One end (~1 cm) of the APTES-coated substrate was dipped in the gold suspension for 2-3 h to ensure complete saturation of nanoparticles on the surface. The chamber was then slowly filled with the gold suspension at a rate of ~0.83 mm min⁻¹ until the other end of the substrate was also completely submerged in the colloidal solution. The varying exposure time lead to decreasing Au nanoparticle adsorption along the height of the substrate. The substrate was withdrawn from the gold suspension, washed with DI water, and air-dried.

(iv) Surface Characterization. To determine the number density of gold nanoparticles, 1 µm x 1 µm tapping mode scans were taken with a digital Nanoscope IIIa controller atomic force microscope (AFM; Veeco; Santa Barbara, CA) at various positions on the substrate along the direction of the gradient (x-dir). The particle densities were obtained by manually counting the particles in the AFM micrographs. The results were averaged by taking two transverse measurements (in the y-dir) for each position.

The gradient substrates were silver enhanced for 10 min following the standard procedure, washed with DI water, and air-dried. The slides were imaged in the optical microscope at the same positions as the previous AFM measurements. We also scratched off a portion of silver at these positions to obtain ~1-mm silver islands similar in shape to the typical immunoassay spots (Figure 2.2b and c). These spots were also imaged in the optical microscope. All images were analyzed by densitometry with the same microscope and camera settings as for the immunoassay experiments. We obtained similar results in both cases, which indicated that the densitometry method is independent of the spot size and that there is little effect of scattering from the edges of the silver deposits. The topography of gold
nanoparticles, before and after silver enhancement, was characterized using a JEOL 6400F field emission scanning electron microscope.

2.4 Results and Discussion

2.4.1 Assay Selectivity

Our initial experiments evaluated the selectivity of the assay in distinguishing two types of polyclonal immunoglobulins. Antigens may bind nonspecifically (via hydrophobic or electrostatic interactions) to the substrate surface or to the immobilized antibodies to yield false positive results. On the other hand, specific binding of the antigens or the nanoparticle labels may be too weak due to surface hindrance or immunoglobulin degradation, leading to false negative results. These false positives or false negatives must be minimized for accurate detection. Thus, the first series of experiments were aimed to demonstrate the viability of our immunoassay and to gauge its selectivity. Four types of immunoglobulins were used, namely, goat anti-mouse, mouse, goat anti-rabbit, and rabbit IgGs. Direct and sandwich assays were performed using the standard experimental procedures.

In direct assays, antigens (M and R IgGs) were immobilized on an aldehyde-functionalized glass surface and incubated with antibody-conjugated gold nanoparticles (GAMg and GARg IgGs) in solution. This is not a practical assay, but it allows estimating the selectivity of each single binding event. Immobilized antigens were also incubated first with antigens (M and R IgGs) in solution and then with antibody nanoparticle conjugates. This first incubation step is of little practical value because the final binding events occur directly between the immunoglobulins on the surface and the ones on the gold nanoparticles.
(without any role of the intermediate immunoglobulin), but it allows estimating the impact of nonselective binding events taking place in the system. In the realistic sandwich assays, primary antibodies (GAM and GAR IgGs) were immobilized on the aldehyde-functionalized glass and the remaining steps were as shown in Figure 2.1. The outcomes of all possible combinations in the cross-reactivity experiments are presented in Table 2.2.

No false negatives were recorded in any experiment. All of the sandwich assays performed as expected and were selective to the type of immunoglobulin. Three false positive results were, however, observed in direct assays, which seem to have been a result of nonspecific interaction between R IgG and GAMg IgG. The lack of false positives in the sandwich assays probably results from the two-step process where R IgG binds first with GAM IgGs immobilized on the surface and second with GAMg IgGs in solution. The probability of two nonselective binding events occurring for a particular antigen is expected to be much smaller compared to a single attachment step in direct assays.\textsuperscript{20, 21} If necessary, nonspecific binding can be further reduced by increasing the number of washing steps, using higher concentrations of BSA and Tween-20, adjusting the pH, adding glycerol (to reduce hydrophobic interactions), increasing the salt concentration (up to 500 mM NaCl), and binding of the antibodies to the surface in an oriented fashion to minimize heterogeneities\textsuperscript{42, 43} however, that was not necessary for the sandwich format. Having proven that the actual sandwich immunoassays yield accurate results, we proceeded with the sandwich configuration in all of the following immunoassay experiments.
2.4.2 Estimation of Thiele Modulus for the Kinetic Model Based on the System Parameters

To identify the limiting kinetics of our process, we estimated the Thiele modulus based on the known assay parameters. The random sequential adsorption model for monodispersed charged spheres predicts that the jamming limit beyond which no more antibody-conjugated gold nanoparticles can be placed on a homogeneous surface is $\sim 5 \times 10^{11}$ particles cm$^{-2}$.

Previous studies have also shown that the specific binding capacity of randomly oriented antibodies covalently attached to a solid substrate is $\sim 25$ ng cm$^{-2}$.

This yields an antigen packing density of $10^{11}$ molecules cm$^{-2}$. In our sandwich assays, the antigens are further bound by secondary antibodies conjugated to bulky gold nanoparticles. The surface density of gold nanoparticles is therefore expected to be lower (by almost 1 order of magnitude) than the concentration of antigens on the surface. We used $\Gamma_{\text{max}} = 10^{10}$ gold nanoparticles cm$^{-2}$ for our Thiele modulus calculations. This value corresponds to the maximal particle surface density estimated by comparing the nanoparticle gradient and real assay experiments described in the next section.

The value of the diffusion coefficient, $D = 5 \times 10^{-7}$ cm$^2$ s$^{-1}$, was estimated using the Stokes-Einstein equation ($D = k_B T / 6 \pi \eta r$). The height of the flow cell was $L = 0.015$ cm.

The order of magnitude of the adsorption rate constant, $k_{\text{ads}} \approx 10^{-16}$ cm$^3$ molecules$^{-1}$ s$^{-1}$, was estimated based on values reported in the literature.

Based on the above parameter values, Thiele modulus was estimated to be $\ll 1$ and eq 1 reduced to a simple adsorption-controlled kinetic relation.

$$d\Gamma/dt = k_{\text{ads}} c_b (\Gamma_{\text{max}} - \Gamma)$$  \hspace{1cm} (6)
All experiments were conducted at constant temperature so \( k_{ads} \) remains constant. Equation 6 can be analytically integrated using the initial condition \( \Gamma(0) = 0 \) to give the coverage with time,

\[
\frac{\Gamma}{\Gamma_{\text{max}}} = 1 - \exp\left(-k_{ads}c_b t\right)
\]

As will become clear in the forthcoming discussion, the adsorption rate constant \( k_{ads} \) of antibody-conjugated gold nanoparticles also depends on the surface coverage, which is related to the orientation and density of the adsorbing proteins, as well as the surface transport of the labels as they attempt to find open binding sites.

2.4.3 Relationship between Optical Darkness Ratio (ODR) and Surface Coverage (\( \Gamma \))

In order to fit the kinetic model to the experimental data, we needed to establish the relationship between normalized \( ODR \) and the surface coverage with nanoparticles (\( \Gamma \)). Silver enhancement is widely used to detect nanoparticle binding in assays and microscopy\(^{24-27} \) yet the correlation between the surface concentration of nanoparticles and the degree of enhancement has not been quantified adequately. In the experimental characterization of this relation, we used microscope glass slides that were uniformly functionalized with amino (NH\(_2\)) groups using APTES self-assembly\(^{48} \). Negatively charged gold nanoparticles were electrostatically deposited on the glass slide such that their number density varied in a continuous gradient from one end of the substrate to the other.
The density of gold nanoparticles at various positions was determined by AFM prior to the silver enhancement. This allowed correlating the results of the densitometry analysis after enhancement to the particle surface density (Figure 2.3). Scanning electron microscopy (SEM) micrographs were also taken before and after silver enhancement to observe how selectively the silver metal is deposited on the gold nanoparticles and how significantly it increases their size (Figure 2.4). The SEM was performed on a silicon wafer (instead of glass) in order to improve the resolution of the images.

The $ODR$ data in Figure 2.3 increased approximately linearly for nanoparticle densities below $1.5 \times 10^{10}$ particles cm$^{-2}$ and then gradually leveled off. The dispersion in the data in regions a and b could result from difficult to control effects on the rate of silver nucleation for experiments performed on different days such as slight variations in the temperature, degree of premixing of the solutions and others. As we estimate the packing density of gold nanoparticles in the sandwich immunoassays to be to be $10^{10}$ particles cm$^{-2}$, we assume that $ODR$ increases linearly with $\Gamma$ and eq 7 can be rewritten as

$$\Gamma/\Gamma_{\text{max}} = ODR/ODR_{\text{max}} = 1 - \exp(-k_{\text{ads}} c_b t)$$

This simplified adsorption rate eq 8 was used for modeling all of our experimental data. The time constant of this equation is defined as $\tau = \frac{1}{k_{\text{ads}} c_b}$ (s). Like most other first-order linear time invariant systems, the signal was assumed to reach saturation at the end of $3 \tau$, which corresponds to 95% of the $ODR_{\text{max}}$ value.
2.4.4 Assay Sensitivity: Effect of Major Parameters

Typical optical images of two silver-enhanced immunoassay spots for sandwich assays are shown in Figure 2.2b and c. The optical darkness depends both on the concentration of the reagents used and on their time of incubation. For example, spot b, which appears darker than spot c, was obtained by conducting the experiment with a higher concentration of antigens but for a smaller incubation time of gold suspension than (c). Both spots appear slightly darker at the periphery because of the higher concentration of antibodies due to evaporation-driven convective transport within the droplet during the surface immobilization process. This phenomenon is known as the “doughnut effect” in biological systems\(^49\) (or the “coffee stain effect” in colloid science\(^50,51\)) and persisted despite our efforts to minimize evaporation. To determine the sensitivity of the assays, the concentration and incubation time of antigens and antibody-conjugated nanoparticles were varied systematically.

(i) Effect of Antigen Concentration and Incubation Time. In one series of experiments, we incubated different concentrations of the analyte mouse IgG for a fixed time in order to determine the minimum concentration of antigens required to saturate the binding receptor sites present on the surface and to obtain a clearly discernible signal. This set of experiments simulates assays where sample concentration or availability is limited, so it is important to minimize the amount of antigen required for detection. In a second set of experiments, we incubated a fixed concentration of mouse IgG (50 µg cm\(^{-3}\)) for varying time intervals. The concentration of anti-mouse conjugated gold nanoparticles was held constant at \(6.8 \times 10^{13}\) particles cm\(^{-3}\), and their incubation time was 45 min in all experiments.
One important feature of eq 8 is that the adsorption depends on the product $c_b t$. Thus, we can collapse the data from experiments at varying antigen concentration and varying incubation time on a single master curve. The $ODR$ signals obtained from these two sets of experiments plotted together as a function of the product of antigen concentration and incubation time are shown in Figure 2.5. The data were fitted with eq 8 by performing a Levenberg-Marquardt nonlinear regression of $k_{ads}$ and $ODR_{max}$ (OriginLab Corp.) until $\chi^2$ error was minimized. An adsorption rate constant of $(1.0 \pm 0.2) \times 10^{-16}$ cm$^3$ molecules$^{-1}$ s$^{-1}$ was estimated by the model, which compares well with values reported in the literature.$^{37,43,46,47}$ The results prove that the experimental data acquired independently by varying the antigen concentrations and incubation times collapse to a single master curve, which, as explained further down, can be a powerful tool in assay optimization.

The scatter in the experimental data in Figure 2.5 was likely due to the nonuniform darkness of the spots at the edges due to coffee ring effects or drying of the immunoglobulins on the surface. This effect is hard to quantify during the densitometry analysis. More uniform spots may be attained by controlling the humidity in the chamber, performing the assay on a hydrophobic substrate, or convective speeding up of the adsorption process.$^{20}$ Additionally, the silver enhancement procedure is complex and the response may become nonlinear if the adsorption of the nanoparticles is uneven and too high. The analysis of the data in Figure 2.5 shows that the $ODR$ signal saturated at $3.75$ µg cm$^{-3}$ (at the end of $3 \tau$). The minimum concentration of mouse IgG detected in 20-min incubation time was $0.1$ µg cm$^{-3}$ (0.67 nM). The $ODR$s of the silver-enhanced spots below this concentration were $<0.001$ so $0.1$ µg cm$^{-3}$ was assumed to be the lowest limit of detection (LOD) of the immunoassay. The sensitivity
limit lies within the range of a typical natural immune response in a human body\textsuperscript{52, 53} and also compares well with some previously reported silver enhanced and alternative assay techniques.\textsuperscript{10, 13, 36, 54} Some authors report silver-enhanced assays for analyzing antibodies,\textsuperscript{32} proteins,\textsuperscript{55} viruses,\textsuperscript{56} and DNA\textsuperscript{31, 57} that have a lower LOD, but our method offers miniaturization and the total amount of sample used in the process is very small (<4 ng total).

One key advantage of using silver enhancement detection is that we obtain a high-intensity signal within the first few minutes of antigen incubation. The kinetic data in Figure 2.5 indicate that the signal intensity was saturated even before the first reading was recorded, i.e., within 1 min of incubation with antigens. This strong rapid response of the immunoassay, visible to the naked eye, could be useful for many diagnostic applications where a rapid yes/no type detection is desired.

(ii) Effect of Concentration and Incubation Time of Gold Nanoparticle Suspension. The bound antigens in the colloidal sandwich immunoassay were detected by binding with antibody-conjugated gold nanoparticles. The diffusion rates of the nanoparticle labels are smaller than that of the free antibody. The antibody-conjugated particle binding could be the time-limiting step in the assay unless a high concentration of nanoparticles is used. The gold nanoparticle conjugates are, however, expensive and their excessive use increases the overall cost of the immunoassay. To determine the minimum concentration of gold nanoparticles required for generating a clearly detectable signal within a certain amount of time, we incubated different concentrations of the gold nanoparticle suspensions for a fixed time and collected the ODR data (Figure 2.6). The concentration and incubation time of the analyte mouse IgG was 50 µg cm\textsuperscript{-3} and 20 min, respectively, in every experiment. In a
related experiment, we held the concentration of the gold suspension fixed at $6.8 \times 10^{13}$ particles cm$^{-3}$ and incubated it for different periods of time. These experiments were performed with four mouse IgG concentrations: 50, 30, 20, and 5 $\mu$g cm$^{-3}$ (Figure 2.7) incubated for 20 min in every experiment.

The experimental data in Figure 2.6 and 2.7 could not be fitted globally with a single adsorption rate constant. The results of the fitting procedure indicated that the concentration of the antigens on the surface strongly modulated the kinetics of capture of the nanoparticle conjugates in suspension, such that the gold nanoparticles were adsorbed much faster at low antigen concentrations as compared to high ones. It was possible to fit the kinetic data collected for 5-30 $\mu$g cm$^{-3}$ mouse IgG concentrations (Figure 2.7b-d) using a single rate constant, the value of which was estimated to be $k_{adv} = 2.2 \pm 0.5 \times 10^{-16}$ cm$^3$ molecules$^{-1}$ s$^{-1}$ by the model. The $ODR_{max}$ values increased proportionally with the antigen concentrations. The kinetic data for 50 $\mu$g cm$^{-3}$ mouse IgG concentration (Figures 2.6 and 2.7a), however, yielded a much smaller rate constant, $k_{adv} = 2.4 \pm 0.6 \times 10^{-17}$ cm$^3$ molecules$^{-1}$ s$^{-1}$.

The data fitting results suggest that when the analyte concentration is low (below 30 $\mu$g cm$^{-3}$), resulting in a small number of antigen receptors on the surface, the adsorption rate constant of antibody-conjugated nanoparticles is comparable to that of the antigens (see Figure 2.5). When the analyte concentration is increased to 50 $\mu$g cm$^{-3}$, so that the antigen receptors form a denser layer on the surface, the rate constant of the nanoparticle conjugates decreases by almost 1 order of magnitude. We hypothesize that the rate of adsorption gets reduced because of steric hindrance between the very densely packed binding sites on the antigen receptors. This causes the adsorbing antibody-conjugated gold nanoparticles to
reorient themselves or diffuse laterally in order to reach an available binding site and attain a favorable position. These effects may contribute significantly to longer adsorption times. Analogous two-dimensional steric effects, which slow down the rate of or prevent adsorption at high coverage, have been reported in the literature previously.\textsuperscript{58,59}

In Figure 2.6, the bulk concentration of gold nanoparticles for which the surface reached saturation was $4.5 \times 10^{13}$ particles cm\textsuperscript{-3} and no signal was registered below a concentration of $2 \times 10^{12}$ particles cm\textsuperscript{-3} (3.32 nM). The dispersion of the data at higher concentrations of gold nanoparticles increased dramatically (Figure 2.6). This may again be attributed to variation and uneven packing of the particles at high surface coverage that can lead to a nonlinear increase in silver enhancement and as a result to a high scattering in the ODR signal. The receptor sites for 30 $\mu$g cm\textsuperscript{-3} mouse IgG concentration saturated within 5 min, whereas the time required was ~ 30 min for 50 $\mu$g cm\textsuperscript{-3}. The metal spots, however, were lighter (albeit clearly distinguishable by the naked eye) in the former case. The rapid increase in metal surface coverage with time for silver-enhanced immunoassays developed with 30 $\mu$g cm\textsuperscript{-3} mouse IgG is shown in Figure 2.8.

2.4.5 Formulation of General Procedure for Assay Optimization

Development of assays similar to the one studied here is commonly done by empirical optimization until a protocol achieving the desired sensitivity and selectivity is formulated. These protocols include relatively long times for incubation and adsorption of the antigens and labels. Few studies have ever considered the exact time required for achieving the desired degree of surface coverage in each incubation step. The model that we have
developed and tested here could allow formulation of such a procedure. We have determined the minimum incubation times required to reach saturation (95% of the $ODR_{\text{max}}$ value at the end of $3\tau$) for several concentrations of antigens and antibody-conjugated nanoparticles (see Table 2.3). The analysis of these results allows us to design a general algorithm to minimize the incubation time for a narrow range of analyte concentrations. This algorithm applies to any antibody-analyte binding that follows adsorption-limited kinetics similar to our assay system. It makes use of our finding that the adsorption is proportional to the product of the incubation time and analyte concentration; thus, this product will remain constant at the optimal adsorption. Once the optimal time for one analyte or label concentration is determined, the smallest time for lower or higher concentrations could be established by the relation $t_1 c_1 = c_2 t_2$, or the smallest label concentration for a certain target incubation time can be calculated. The data reported in Table 2.3 confirm that the product of the incubation time and analyte concentration ($c_b t$) always remains constant for both the analyte and gold labels. In addition to Table 2.3, Figure 2.6 also proves that the product $c_b t$ is a suitable parameter group for collapsing the change in the optical signal on a single master curve.

We propose a procedure for assay optimization that could operate as follows: First, an analyte or label of known concentration ($c_1$) is incubated for different periods of time while all other parameters are held constant. Second, the $ODR$ values of the developed silver-enhanced spots are measured and plotted as a function of time. Third, the experimental data are fitted with the adsorption-limited rate eq 8 by varying $k_{\text{ads}}$ and $ODR_{\text{max}}$ parameters until the least $\chi^2$ error between the data and the model is minimized. Fourth, the value of time
constant \( \tau = \frac{1}{k_{\text{ads}} c_i} \) is determined for the assay process and a certain criterion for completeness is established (e.g., \( t_1 = 3 \tau \), where 95% of adsorption will be complete). Finally, the relation \( 3 \tau c_1 = c_2 t_2 \) is used to calculate the smallest incubation time \( t_2 \) for any other known concentration \( c_2 \) of the same solute or to estimate the required label concentration for a target incubation time \( t_2 \).

**2.5 Conclusions**

We characterized the performance of a compact and low-cost nanoparticle sandwich immunoassay and proposed a general semiquantitative scheme to optimize its overall performance using a mass-transfer model. The results point out that the mass transfer of the antigens and gold nanoparticle labels in the immunoassay was controlled by the rate of their adsorption at the surface. A carefully designed experiment proved that the opacity of the silver-enhanced spots increased linearly with the surface density of gold nanoparticles. A simple kinetic rate equation was used to fit all our data. The binding rate constant of antibody-conjugated gold nanoparticles was a function of the density of the antigen receptors on the surface. It was 1 order of magnitude higher for low coverages. The selectivity achieved with our immunoassay was adequate in the sandwich format although false positives occurred in direct assays. The lower limit of antigen detection of this method was 0.1 \( \mu g \text{ cm}^{-3} \) (total of 4 ng of antigen). This LOD value is not extraordinarily high but is comparable to some of other state-of-the-art detection techniques. The developed
immunoassays produce a signal that can be visualized by naked eye. The silver-enhanced plates also have the advantage of being stable and portable.

2.6 Directions for Future Research

Although the focus of the research presented here was on the characterization and optimization of gold nanoparticle immunoassays, the key utilization of particle-based silver enhanced assays lies in the advancement of next generation on-chip microarrays and microsensors thanks to their high potential for miniaturization and easy interfacing with electronic circuits. Our preliminary conductivity measurements of the silver enhanced spot look promising in this regard (Figure 2.9). The current-voltage characteristics of the silver enhanced spots were measured at room temperature by using a computer-controlled sourcemeter (Keithley 2602, Keithley Instruments, Inc., OH) and a two-probe station (Janis Research Company Inc., MA). The distance between the probes was varied from 0.5–2.2 mm. The $I-V$ dependence was highly linear ($r^2 = 0.999$) in all cases which shows that the conductivity behavior of the spot is ohmic. The resistance, however, increased linearly only upto a probe distance of 1.5 mm (in accordance with the linear scaling law, $R = \frac{\rho l}{A}$) after which it decreased gradually.

Clearly, the reason for this drop is due the coffee-ring effect that has been alluded to in section 2.4.4. Since the amount of silver deposited is higher on the spot periphery, the current increases even though there is larger distance between the electrode probes. These results although preliminary nicely demonstrate that the silver enhanced spots are conductive and therefore, ideal for electronic integration. In future, the nanoparticle labeling may
directly be performed between a pair of electrodes, which will be short-circuited by the metallic silver, only if a specific analyte is detected in the sample solution.\textsuperscript{33-35} Outcomes of biospecific interactions could then be monitored directly by electrical measurements in real time. The electric readout could allow parallel processing of a series of immunoassays on a single chip, each of which can be miniaturized below the size restrictions of conventional optical detection.

\textbf{2.7 Acknowledgements}

This study was supported by grants from the National Science Foundation (NSF) and the U.S. Army Research Office (ARO). We are grateful to Rajendra Bhat for APTES gold gradients, Jeong-Seok Na and Greg Parsons for AFM measurements and, Brian Prevo for assistance with the SEM observations.
Figure 2.1 Schematic of the sandwich immunoassay depicting the sequential steps followed during the experiment. The figure is not drawn to scale.
Figure 2.2 (a) Experimental setup for performing the immunoassay. (b, c) Examples of two silver-enhanced spots. The spot in (b) was obtained with a mouse IgG concentration of 30 µg cm\(^{-3}\) incubated for 30 min with gold nanoparticle suspension, and (c) was obtained with a mouse IgG concentration of 1 µg cm\(^{-3}\) incubated for 45 min with gold nanoparticle suspension. All other conditions were maintained constant.
Table 2.1 Range of concentrations and incubation times of all solutions used in developing the immunoassays. All solutions except the ones for silver enhancement were prepared in 10 mM PBS at pH 7.4.

<table>
<thead>
<tr>
<th>Type of Solution</th>
<th>Concentration</th>
<th>Time of Incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-mouse IgG</td>
<td>50 µg cm$^{-3}$</td>
<td>5</td>
</tr>
<tr>
<td>BSA</td>
<td>0.3 wt %</td>
<td>15</td>
</tr>
<tr>
<td>BSA and Tween-20 mixture</td>
<td>0.15 wt % (BSA) and 0.25 wt % (Tween-20)</td>
<td>10 and 15</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>1 - 50 µg cm$^{-3}$</td>
<td>0.2 - 80</td>
</tr>
<tr>
<td>Goat anti-mouse IgG conjugated gold nanoparticles</td>
<td>$2 \times 10^{11}$ - $9 \times 10^{13}$ particles cm$^{-3}$</td>
<td>0.5 - 300</td>
</tr>
<tr>
<td>Silver initiator and enhancer</td>
<td>1:1 vol %</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 2.2 Selectivity control table. The symbols represent enhancement (√), no enhancement (x) and false positive (√(FP)).

<table>
<thead>
<tr>
<th>Immunoglobulins on the Surface</th>
<th>Immunoglobulins Incubated in the Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GAMg</td>
</tr>
<tr>
<td>Direct Assays</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>√</td>
</tr>
<tr>
<td>R</td>
<td>√(FP)</td>
</tr>
<tr>
<td>Sandwich Assays</td>
<td></td>
</tr>
<tr>
<td>GAM</td>
<td>x</td>
</tr>
<tr>
<td>GAR</td>
<td>x</td>
</tr>
</tbody>
</table>
Figure 2.3 Top: AFM micrographs of varying densities of ~ 12-nm gold nanoparticles adsorbed on a glass substrate. Optical images corresponding to these particle densities taken after silver enhancement are shown in the insets. Bottom: The graph shows an approximately linear relationship between $ODR$ and gold nanoparticle density at low coverage. The curves are to guide the eye; the densities corresponding to the frames above are labeled with arrows.
Figure 2.4 SEM images of samples containing ~12-nm gold particles deposited on an APTES-functionalized silicon wafer. (a, c) Areas of low coverage before and after silver enhancement. (b, d) Areas of high coverage before and after silver enhancement. (e, f) Silver layer deposits imaged at high and low magnification.
Figure 2.5 Optical darkness of silver-enhanced spots as a function of the product of antigen concentration in solution and incubation time \((ct)\). (▼) various concentrations of mouse IgG antigen incubated for 20 min each and (●) 50 µg cm\(^{-3}\) mouse IgG solution incubated for varying time intervals. The concentration and incubation time of gold nanoparticle suspension were \(6.8 \times 10^{13}\) particles cm\(^{-3}\) and 45 min in every experiment. The adsorption rate constant of the fitted curve is \((1.0 \pm 0.2) \times 10^{-16}\) cm\(^3\) molecules\(^{-1}\) s\(^{-1}\).
Figure 2.6 Optical darkness of silver-enhanced spots vs concentration of gold nanoparticle suspension incubated for 45 min. The concentration and incubation time of M IgG antigens were 50 µg cm\(^{-3}\) and 20 min in every experiment. The adsorption rate constant of the fitted curve is \((2.4 \pm 0.6) \times 10^{-17}\) cm\(^3\) molecules\(^{-1}\) s\(^{-1}\).
Figure 2.7 Optical darkness of silver-enhanced spots vs incubation time of $6.8 \times 10^{13}$ particles cm$^{-3}$ concentrated gold nanoparticle suspension. The plots are for different concentrations of M IgG antigens: (a) 50, (b) 30, (c) 20, and (d) 5 µg cm$^{-3}$. The incubation time of antigens was 20 min in every experiment. The adsorption rate constants of the fitted curves are as follows: (a) $(2.4 \pm 0.6) \times 10^{-17}$ and (b-d) $(2.2 \pm 0.5) \times 10^{-16}$ cm$^3$ molecules$^{-1}$ s$^{-1}$. 
Figure 2.8 SEM images of silver-enhanced immunoassays after (a) 1.5 and (b) 10 min of incubation with gold nanoparticle suspension. The concentration and incubation time of M IgG antigens were 30 µg cm\(^{-3}\) and 20 min in both experiments. The scale bar represents 30 µm.
Table 2.3 Smallest incubation times required to reach saturation for different concentrations of analyte and nanoparticle conjugates.

<table>
<thead>
<tr>
<th>Type of Solution</th>
<th>Mouse IgGs</th>
<th>Goat anti-mouse IgGs conjugated to gold particles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. $c_b$ (µg cm$^{-3}$)</td>
<td>Incubation time $t$ (min)</td>
</tr>
<tr>
<td>I</td>
<td>50</td>
<td>1.5</td>
</tr>
<tr>
<td>II</td>
<td>3.75</td>
<td>20</td>
</tr>
</tbody>
</table>
**Figure 2.9** I-V measurements of silver enhanced spots: (a) Schematic illustration of the experimental setup used. The sweep voltage was 0 to 10 V. (b) Silver enhanced spot on which the measurements were performed. The concentration and incubation time of the different reagents used were as follows: (i) M IgG antigens - 50 µg cm$^{-3}$ and 20 min, (ii) Gold nanoparticles: $1.36 \times 10^{14}$ particles cm$^{-3}$ and 1 h, (iii) Silver enhancement: 10 min incubation followed by washing and incubation with new silver solution for another 10 min. (c) Current-voltage (I-V) data taken for varying distances between the electrode probes. The results show a strong ohmic dependence in all cases. (d) The resistance of the spot has a peak at some intermediate probe distance due to the coffee ring effect.
2.8 References


Quinn, C. P.; Semenova, V. A.; Elie, C. M.; Romero-Steiner, S.; Greene, C.; Li, H.; Stamey, K.; Steward-Clark, E.; Schmidt, D. S.; Mothershed, E.; Pruckler, J.;


Chapter 3

Novel Structures from Cells and Particles using Dielectrophoresis
3.1 Abstract

We report the co-assembly of live cells and functionalized colloidal particles into a variety of freely suspended bioactive structures using dielectrophoresis on a chip. Alternating electric fields were applied to dilute suspensions of yeast (\textit{S. cerevisiae}) and NIH/3T3 mouse fibroblast cells to yield one-dimensional (1D) chains and 2D arrays. The cell structures were made permanent by binding with different types of functionalized synthetic particles and ligands that attached to the cells through biospecific or electrostatic interactions. The effects of voltage, frequency, pH, electrolyte concentration, cell concentration, and particle size on the assembly process were investigated in detail. We also performed Monte Carlo type numerical simulations to capture the dynamics of cell-cell and cell-particle assembly. The simulation results illustrate that the electric field draws the functionalized synthetic particles between the cells and enables the formation of permanent chains and monolayer membranes comprised of alternating cells and particles. This technique allowed the fabrication of magnetically responsive biomaterials that could be manipulated and transported in and out of the microchambers in which they were formed. These novel functional biocomposites have a variety of potential applications in sensors, prosthetics and drug delivery systems.
3.2 Introduction

Biomaterials incorporating live cells offer unique advantages for biosensing in comparison to the traditionally existing strategies using biomolecules (such as, proteins, DNA, phospholipids etc.). A large number of previous studies report that live cellular arrays can be assembled on two-dimensional (2D) platforms that have been templated using photolithographic microfabrication techniques or by controlled microcontact printing of oligopeptides. Cells from one or more different populations can also be patterned in stripes using laminar flow patterning in microfluidic devices. Other typical methodologies for preparing biomaterials include dip-pen nanolithography, cell adhesion and infusion to porous polymeric scaffolds and polyelectrolyte multilayers, and encapsulation of cells in poly(ethylene glycol) (PEG)-based hydrogels. However, we are not aware of studies describing assembly techniques that offer a combination of speed, low cost, simplicity of single-step fabrication procedure and flexibility of preparing freely suspended structures from cells, such as chains or membranes. The handful of free floating permanent assemblies that do exist are composed of only synthetic particles and this restricts the range of biorelated applications for which these materials can be used.

One alternative technique, which relates to the well established field of colloidal assembly in electric fields for synthetic micro- and nanoparticles, is the rapid assembly of cells in suspensions by using dielectrophoresis (DEP) in alternating electric fields. DEP is the mobility of virtually any type of dielectric particle in any type of media imparted by a spatially non-uniform electric field. The use of alternating current (AC) electric fields in DEP, in particular, allows precise and controlled organization of particles without causing
water electrolysis and electroosmotic effects. When an AC field with frequency \( \omega \) is applied across a colloidal suspension, it leads to the polarization of particles. The induced dipoles in the particles interact with the applied electric field and the magnitude of the dielectrophoretic force is proportional to the gradient of the electric field intensity squared.

\[
F_{\text{DEP}} = 2\pi \varepsilon_m R^3 \text{Re}\left[K(\omega)\right]|\nabla E_{\text{rms}}|^2
\]  \hspace{1cm} (1)

Here, \( \varepsilon_m \) is the dielectric permittivity of the medium, \( R \) is the particle radius, \( E \) is the electric field intensity and \( K \) is the Clausius-Mossotti function – the effective polarizability of the particle in media. The real-part of \( K \) is given by,

\[
\text{Re}\left[K(\omega)\right] = \frac{\varepsilon_m - \varepsilon_p}{\varepsilon_m + 2\varepsilon_p} + \frac{3(\varepsilon_p \sigma_m - \varepsilon_m \sigma_p)}{\tau_{MW}(\sigma_m + 2\sigma_p)(1 + \omega^2 \tau_{MW}^2)}
\]  \hspace{1cm} (2)

where, \( \varepsilon_p \) is the dielectric permittivity of the particle and, \( \sigma_{m,p} \) are the conductivities of the media and particles, respectively. The \( \text{Re}\left[K(\omega)\right] \) function changes sign at a crossover frequency, \( \omega_c = \tau_{MW}^{-1} \), where \( \tau_{MW} = \frac{\varepsilon_m + \varepsilon_p}{\sigma_m + 2\sigma_p} \) is the Maxwell-Wagner charge relaxation time. When \( \text{Re}\left[K(\omega)\right] > 1 \), the particles are attracted to the region of maximum field intensity and the phenomenon is known as positive DEP. Otherwise, they are repelled by negative DEP.
The dipoles induced in the particles by the AC field also exert attractive forces between particles and cause them to align into chains. The magnitude of this particle chaining force is proportional to the square of the field intensity and is given by,

\[ F_{\text{chain}} = -C \pi \varepsilon_0 R^2 \text{Re} |K(\omega)|^2 E^2, \quad 3 < C < 10^3 \]  

where, the coefficient \( C \) depends on the distance between the particles and the length of the particle chain. The combination of DEP and particle chaining forces can be used to speed up the colloidal assembly process and drive the formation of microscopic functional structures. A few examples include miniaturized biosensors,\(^{35, 36}\) electrically conductive microwires from gold nanoparticles\(^{37-39}\) and switchable particle crystals for photonic applications.\(^{40, 41}\)

In this chapter, we describe a method in which the fundamental principles of directed on-chip colloidal assembly are transcribed into the biological domain by effectively treating the live cells as “smart” or functional particles. Dilute suspensions of live cells are subjected to non-uniform AC electric fields on a chip and assembled into chains or 2D arrays using DEP. The mere assembly of cells between the electrodes is however, not enough to form a functional material or device component as the cells are electrostatically charged and the inherent cell-cell interactions are weak and so, the structures disassemble when the electric field is switched off. We demonstrate both experimentally and by simulations that the permanent binding of freely suspended cell structures can be accomplished by using surface-functionalized microparticles that get drawn between the cells by electric fields and attach to the cell’s surface via electrostatic and/or biospecific interactions. The wide range of cell
structures that we assemble is schematically outlined in Figure 3.1 in the increasing order of complexity. By using lectin-functionalized paramagnetic microparticles, we also illustrate how the biological functionality of the cell assemblies can be augmented by the physical functionality of the binder particles for making novel and responsive biomaterials. The effects of various operating parameters on the cell assembly process have been studied in detail.

3.3 Experimental Section

3.3.1 Materials and Sample Preparation

Dried, active brewer’s yeast (*Saccharomyces cerevisiae*) was purchased from MP Biomedicals, Inc. (OH) and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich (MO). A PBS solution containing 1.37 mM NaCl, 0.027 mM KCl, and 0.1 mM phosphate buffer was prepared by dissolving one PBS tablet in 20 L of deionized water (resistivity ~ 18.2 MΩ cm) that was obtained from Millipore RiOs purification system (MA). The yeast cells were suspended in PBS to obtain final concentrations between 0.05 – 0.2 % w/v at pH 6.3. NIH/3T3 mouse fibroblast cells were cultured in standard Dulbecco’s modified eagle’s medium (Gibco/Invitrogen, CA) for 2 days. The cells were trypsinized and resuspended in an isotonic solution of 0.45 M dextrose (anhydrous, ACS certified; Fisher Scientific, PA) in DI water containing 1 % v/v calf serum (Gibco/Invitrogen, CA) at pH 7.2.

The following types of monodisperse colloidal particles were used in the experiments: white sulfate-stabilized polystyrene latex spheres of 1.0 µm and 5.0 µm diameters (Interfacial Dynamics Corp., OR), fluorescent aldehyde/sulfate latex beads of 1.0
µm diameter (Molecular Probes, OR), amine-terminated magnetic iron oxide particles of 1.8 µm diameter (Bangs Laboratory, IN), and Concanavalin A lectin-coated paramagnetic particles of 0.95 µm diameter (Spherotech, Inc., IL). The particles were centrifuged and washed twice with PBS using a Marathon micro-A centrifuge (Fisher Scientific) to remove any surfactants, electrolytes, or preservatives from the media. For some experiments, the following microparticles were also conjugated with proteins using the coupling protocols described in Appendix A: the 1.0 µm aldehyde/sulfate latex beads and 1.8 µm amine-terminated magnetic particles were functionalized with fluorescein isothiocyanate (FITC)-labeled Concanavalin A lectin (Sigma-Aldrich); the 1.0 µm sulfate-stabilized latex spheres were coated with fibronectin (BD Biosciences, NJ).

The microspheres were added to the cell suspensions to obtain samples with final particle concentrations between 0.01 – 0.1 % w/v. An extra pure 96 % CaCl₂ (Acros Organics, New Jersey) was added to the cell-particle mixtures in small amounts of 0.15 mM to facilitate the binding of the lectins to polysaccharides. Bovine serum albumin (Sigma-Aldrich) and non-ionic surfactant Tween 20 (Ted Pella, Inc., CA) were each included in approximate quantities of 0.1 % w/v to prevent non-specific aggregation or adhesion of cells or particles. Other reagents used were - protein sequencing grade 20.2 % hydrochloric acid, cell culture tested 1.0 N sodium hydroxide (Sigma-Aldrich); D-mannose (Alfa Aesar, MA); low molecular weight poly(dimethyl aminoethyl methacrylate) (PDMAEMA) (Synthesized in lab).
3.3.2 Setup

The chaining experiments were performed between two coplanar gold electrodes with c.a. 3 mm gap (Figure 3.2a) that were vapor deposited on 25 × 75 mm plain microscope glass slides (Fisher Scientific) as described previously. The gold-coated slides were cleaned by immersing in a strong oxidizing solution (Nochromix) overnight and then they were washed with deionized water followed by air-drying. The electrodes were encapsulated in a 0.15 mm thin, transparent 18 μL flow-chamber (Grace Bio Labs, OR). The setup for assembling 2D arrays consisted of four brass needle electrodes orthogonally arranged around the sides of a 2 mm thick, transparent 560 μL flow-chamber (Grace Bio Labs) that was sealed on a glass slide as shown in Figure 3.2b.

The inner surfaces of the chips and microfluidic chambers were treated with 0.5 % F-127 Pluronic surfactant (Molecular Probes) for 45 min prior to performing the experiments. This treatment was necessary to hydrophilize the surface in order to minimize the non-specific interactions of the cells and particles with the substrate. Cell-particle suspensions were injected into the microfluidic flow-chambers and the electrodes were connected to a 33120A 15 MHz square wave field function generator (Agilent Technologies, CO) providing AC signal 2 - 10 V peak to peak in magnitude. The generated signal was amplified to a working voltage range of 15 - 200 V using an RG-91 voltage amplifier (Burleigh Instruments, NY). A 1 μF capacitor was connected in series to filter out any direct current (DC) component of the signal. The strength of the applied field was measured using a multimeter (Instek, CA) included in the circuit. Electric fields of intensities between 5 – 20 V mm⁻¹ and frequencies between 30 Hz – 5 kHz were applied to the samples at room temperature.
3.3.3 Optical Microscopy and Image Analysis

The assembly process was continuously monitored (top-down) using an Olympus BX-61 optical microscope equipped with transmitted-mode and fluorescence-mode microscopy. The images were recorded using Olympus DP-70 digital CCD camera. To characterize the effects of the various operating parameters, the electric field was applied for 2 min at each experimental condition. At least five representative high-magnification optical microscopy images (typically with 50x objective) were collected from near the middle areas of the chamber away from the electrodes. The results at each data point were reported as an average of these images.

3.3.4 Cell Viability Tests

The fluorescent tests for yeast cell viability were performed using the FUN-1 cell stain purchased from Molecular Probes. The cell stain was brought to room temperature from -20 °C (storage temperature) and then centrifuged at 975 g for 5 min. Approximately 0.2 µL of the 10 mM dimethylsulfoxide based dye was homogeneously mixed with 200 µL of 0.1 % w/v yeast cells in PBS. The mixture was kept in the dark for 30 min and then the cells were observed using fluorescence microscopy in the FITC-mode. In the case of NIH/3T3 mouse fibroblast cells, the viability experiments were performed using the Trypan Blue cell stain purchased from Sigma. An approx. 0.4 % w/v solution of Trypan Blue dye was prepared in PBS. It was slowly added and mixed with the fibroblast cell suspension to obtain a clear light-blue colored suspension. The cells were viewed after 5 min using transmitted-mode optical microscopy.
3.4 Results and Discussion

The freely suspended cells in the buffer began aligning into chains within 30 s of applying the AC electric field to the coplanar electrodes (Figure 3.3). The cells assembled in the direction of the applied electric field due to the attractive axial dipolar interactions and most cells were captured into chains in 10 – 15 min. This pattern of chain formation in electric fields is typical for colloidal suspensions in many different systems. The process of cell chaining was accompanied by slow sedimentation of the cells toward the bottom of the chip. The chains near the electrodes were also pulled into the region of maximum field intensity at the electrode edges by positive DEP and AC electrohydrodynamics. The assembly process was reversible and the cell chains came apart when the field was turned off. The cells then slowly redistributed due to Brownian motion but remained at the bottom of the chip due to gravity (Peclet number for yeast cells, $Pe \approx 40$).

3.4.1 Cell Viability under the Influence of Electric Fields

Our first goal was to check whether or not the viability of cells was preserved during the application of the electric field. In the case of yeast, the viable cells were distinguished from the dead ones using the fluorescent FUN-1 cell stain (Figure 3.3a-inset). The cytoplasm of the metabolically active yeast cells appeared dull green in color and displayed evidence of orange-red fluorescent intravacuolar structures whereas, the cytoplasm of dead cells appeared very bright fluorescent green-yellow. The proportion of metabolically active cells (~ 90 %) remained approximately constant before and after applying the AC field for approx. 2 h. This
confirmed that the electric field does not affect the viability of yeast cells within our experimental conditions.

The viability experiments for mouse fibroblast cells were performed using the Trypan Blue dye that only penetrates to the cytoplasm of cells whose membranes are damaged (Figure 3.3b-inset). The cells which absorb the dye turn different shades of blue and this allows us to monitor the health of the cell in real-time. We observed that in the absence of electric field, approx. 90% of the fibroblast cells stayed alive for up to 1 – 1.5 h at room temperature. These observations were made from the time the cells were trypsinized and resuspended in the new buffer media. When an external electric field was applied, no detectable change in the viability of fibroblasts was noted up to 15 V mm\(^{-1}\) field intensities.

Our collective data from these experiments suggest that both yeast and fibroblast cells retain their metabolic activity after treatment on the DEP chips. In general, the fibroblast cells appeared to be more susceptible to small changes in their environment (such as, temperature, pH, CO\(_2\) content, electrolyte concentration and so on) than to the applied electric field. This is likely due to the fact that unlike yeast, fibroblast cells do not possess a robust cell wall that protects their cell membrane. In order to prolong the viability of fibroblast cells in the future, these experiments can be performed inside a closed plastic chamber which is maintained at 37 °C temperature and at a constant 5% CO\(_2\) supply.

### 3.4.2 Effect of Operating Parameters on the Assembly of Cell Chains

After establishing that the cells stay alive at the given experimental conditions, next we identified and characterized the experimental parameters that might affect the mechanism
and the rate of cell chain assembly. The parameters recognized were voltage and frequency of the applied electric field, pH of the suspension, electrolyte concentration in the buffer media, and the concentration of cells in the suspension. The experimental details and the conclusions drawn for the studied parameters are given below.

(i) Effect of Applied Field Strength and Frequency. The dielectrophoretic response of yeast cells was explored as a function of both field strength and frequency (Figure 3.4). First, an AC field of 20 V was applied to a yeast cell suspension of concentration 0.1 % w/v and the frequency was systematically varied from 10 Hz to 5 kHz in increments of 200 Hz or more. All other parameters were held constant during this time. The cells that were captured in chains of different lengths were counted using the Image-Pro Plus analyzing software (Media Cybernetics, MD) and the average length of the cell chains was determined at each condition. This entire procedure was repeated for voltages 30, 40, 50 and 55 V.

The effect of voltage on the cell assembly was straightforward. When the voltage was increased, the average cell chain length increased at all frequencies because the strength of the electric field is the main driving force for dielectrophoretic assembly. In principle, longer chains could be formed at even higher voltages but this was not practical since increasing the voltage above 55 V generated undesirable water electrolysis and electro-osmotic flows. Frequency had an opposite effect on cell assembly such that the lengths of the chains increased as frequency was decreased. No readings could be taken below 10 Hz because the cells vibrated due to electrophoretic mobility. The electrohydrodynamics of the bulk fluid also became significant at such low frequencies.
The above frequency data correspond well with the concept that cells (and colloidal particles) show a large dispersion in the low-frequency range referred to as the $\alpha$-dispersion in biophysics or the Low Frequency Dielectric Dispersion (LFDD) in colloidal chemistry.\textsuperscript{48} This dispersion is related to the macroscopic polarization of the induced double layer around the cells and has a characteristic relaxation frequency of

$$\omega_{\text{char}} = \frac{2DM}{R^2}$$

where, $D$ is the diffusion coefficient of the ions in the double layer and $M$ is a dimensionless factor that accounts for the electro-osmotic contribution of the ion flux of the double layer.

At pH 6.3, yeast cells are negatively charged (isoelectric point of yeast cells is pH 4)\textsuperscript{49, 50} and so they are surrounded by positive counter-ions (mostly Na$^+$ ions in our case) in the suspension. When an external field is applied, the counter-ionic cloud moves in a direction opposite to the one of the charged cell and effectively forms a larger dipole in addition to the smaller dipole induced inside the cell. The re-orientation of the dipoles to follow the phase of the oscillating AC field is limited by the mobility of the counter-ions in the double layer and so, as the frequency increases and approaches the characteristic relaxation frequency, the ions can no longer polarize and there is a net decrease in the induced dipole moment of the cells. This is likely the reason why the rate and as a result, the length of the cell chains decreases at higher frequencies.
For the yeast cells in our system, the characteristic relaxation frequency obtained using equation 4 is \( \omega_{\text{char}} \approx 800 \text{ Hz} \) which rightfully suggest that while the cell interior may or may not be easily polarizable, the contributions of the double layer polarization to the dielectrophoretic attraction cannot be ignored at low frequencies. The values of the physical parameters used in this calculation are reported in Table 3.1. The zeta potential of the cells was estimated from the electrophoretic mobility data reported in the literature.\(^{49, 51}\) This value of zeta potential was then used to calculate the cell’s surface charge density using the simplified Grahame equation.\(^{48}\) The values of all other parameters were either calculated at the given experimental conditions or borrowed directly from the literature.\(^{48}\)

(ii) Effect of pH. The effect of frequency on the rate of cell chaining highlighted the importance of counter-ionic mobility for forming field induced dipoles. The counter-ionic polarization can also be suppressed by changing the pH of the suspension and therefore, pH was expected to be another key parameter in controlling the cell assembly process. We varied the pH of a 0.1 % w/v yeast cell suspension from 2.8 to 11.5 in approximate increments of 0.7 using 1 N HCl or 1 N NaOH and studied the process of cell chain formation as a function of frequency (Figure 3.5). The applied electric field strength was 10 V mm\(^{-1}\). No dielectrophoretic movement of the cells was observed below c.a. pH 6, however, electrohydrodynamic flows did cause some cells to align near the electrodes at low frequencies between pH 3 and 4. The cells readily organized into chains above pH 6.2 but the rate of cell assembly decreased as the frequency was increased, which is consistent with our previous frequency results. Cell chaining was accompanied by free convection of the bulk fluid above pH 11.1 although the degree of convection was less at higher frequencies.
Our observations can be explained by the effect that the pH of the suspension has on the counter-ionic atmosphere surrounding the yeast cells. Altering the pH of the suspensions affects the net charge of the cell surface which in turn has an influence on the concentration of counter-ions in the double layer. The yeast cells carry no net surface charge at the isoelectric point (IEP) pH 4.49 Above the IEP, cells are negatively charged due to the presence of weakly dissociating anionic groups such as carboxyl and phosphate. At pH values smaller than the IEP, cells carry positively charged NH₃⁺ groups. When the pH of the suspension is maintained close to the IEP, the presence of only a small number of surface charges leads to a thin double layer that cannot induce a large enough dipole moment in the applied field similarly to the case of high frequency. In the cases of very high (> 11) or low (< 3) pH values, we see not only chaining but also free convection of the bulk fluid which is due to electro-osmotic flow of the large number of negative and positive ions in the system. At all other pH values, the cells chain mainly due to the macroscopic polarization of the double layer.

(iii) Effect of Electrolyte Concentration. Another important parameter that effects the polarization of counter-ionic double layer is the amount of electrolyte in the media. To determine its effect on the cell assembly process, we performed experiments in PBS media of several different dilutions. In physiological buffers containing ~ 0.15 M electrolyte concentration, undesirable macroscopic fluid currents were generated due to electro-osmosis and water electrolysis. These effects almost completely disappeared below 1.5 mM electrolyte, however, this low salt concentration caused osmotic pressure induced swelling of the mouse fibroblast cells which eventually lead to cell bursting. The reduction in osmotic
pressure outside the fibroblast cell membranes was compensated by adding dextrose to the suspensions and we noted that the cells indeed stayed viable for a longer period of time. An adverse consequence of adding dextrose to the suspension is that it increases the viscosity of the media and this in turn decreases the rate of cell assembly.

In the experiments involving yeast cells, there was slight but detectable increase in the assembly rate as the electrolyte concentration was decreased from 1.5 mM all the way up to deionized water. The yeast cells also maintained viability for the entire range of electrolyte concentrations studied which can be attributed to the rigid cell wall that protects their cell membrane. The decrease in the rate of cell chaining at high electrolyte concentrations is again dependent on the formation of a thin double layer that cannot polarize similarly to the case of IEP. These data reveal that the dielectrophoretic assembly of the fibroblast cells can be effectively performed but only within a very narrow range of electrolyte concentrations whereas, the yeast cells can be assembled in a wide range of electrolyte concentrations similar to the case of synthetic colloidal particles.

(iv) Effect of Cell Concentration. The rate of cell assembly directly increased with the increase in the concentration of cells in the suspension. We also observed that at higher concentrations of cells, the lateral attraction between adjacent cell chains lead to the formation of partially close-packed 2D arrays in localized areas of the sample. When we viewed the sample at low-magnification, the chains appeared to organize in stripes extending perpendicular to the direction of the electric field. An overall 2D foam-like structure was obtained in the cases where the concentration of cells was sufficient to form a monolayer (see
Figure 3.12a). The role of electric fields in forming uniform 2D arrays will be discussed in greater detail further in this chapter.

3.4.3 Numerical Simulation of Dielectrophoretic Cell Assembly

To understand how the induced dipolar interactions align cells into chains, it was necessary to develop a model that would enable us to calculate the magnitudes and direction of the DEP forces that are involved in the various stages of assembly. Since the magnitude and direction of the DEP force depends on the applied electric field, the electric field distribution in the experimental setup was determined by performing 2D electrostatic calculations in FEMLAB multiphysics modeling package (COMSOL; Burlington, MA). The spherical cells were modeled in the 2D electrostatic calculations as thin cylinders (or disks) of height, $h$. To represent the system most accurately, the height of the cylinder was chosen to be comparable to the cell diameter $(h = 2R)$ in all our calculations. It was also sufficient to simulate the field distribution using two cells in order to capture the physics of assembly in dilute solutions. The AC electric field distribution was calculated for one positive half cycle of the applied voltage.

The numerical procedure for capturing the experimentally observed cell dynamics using FEMLAB consisted of several steps. The geometry of the system viewed top-down was specified to scale and the cells were assigned initial coordinates (Figure 3.6a). The boundaries were the top gold electrode (applied voltage = 40 V, positive half cycle of the AC field) and the bottom gold electrode (ground). The two boundaries to the left and right were taken to be electrically symmetrical. The subdomains consisted of water containing 1.37 mM
electrolyte \((\varepsilon_m = 79\varepsilon_0, \ \sigma_m = 0.017 \text{ S})\) and yeast cells \((\varepsilon_c = 1675.5\varepsilon_0, \ \sigma_c = 0.039 \text{ S})\). Here, \(\varepsilon_0 = 8.85 \times 10^{-12} \text{ C}^2 \text{ J}^{-1} \text{ m}^{-1}\) is the dielectric permittivity of free space. The complex dielectric permittivity and conductivity of yeast cells were calculated by using multi-shell models (model details and numerical parameters are provided in Appendix B). The complex polarizabilities of the media and cells were calculated as a function of frequency \((\omega)\) by using equation \(\varepsilon_{m,c} = \varepsilon_{m,c} - \frac{j}{\omega} \sigma_{m,c}\).

Next, the solution space was divided into a number of small triangular elements. There were no mesh elements across subdomains or boundaries as the physical properties inside the elements remain constant. The solver was then initialized to solve the Poisson equation \((\nabla^2 \phi = -\frac{\rho}{\varepsilon_0})\) for all elements in the mesh and the electric field distribution inside the system was obtained. The electrostatic force vector was generated by the boundary integration of the Maxwell stress tensor on the exterior surface of each cell \((\vec{F}_{EF} = -\oint_S \left( -\frac{1}{2} \vec{E} \cdot \vec{D} + (\vec{n} \cdot \vec{E}) \vec{D}^T \right) dS)\). This force was used to calculate the velocity of the cells from the hydrodynamic resistance, \(\vec{v} = \frac{\vec{F}_{EF}}{6\pi \eta r}\). The cells were displaced in the direction of the velocity and their new coordinates were obtained for the next stage of the simulation. The electrostatic field distribution and force vectors were recalculated for the new geometry and the process was repeated iteratively until the cell boundaries overlapped.

Three time-lapsed images taken from the simulation performed at 100 Hz are shown in Figure 3.6b – d. The results from the simulation illustrate that the electric field intensity is
highest above and below the cells in the direction of the electric field, which indicates that the cells are more polarizable than the media at this frequency. The field lines near the cells are distorted and this generates a local field gradient that attracts the cells towards each other by positive DEP. As a result, the cells align collinear to the field. These results of the iteration procedure compared with the actual experimental observations in Figure 3.6 show that the two are in remarkable agreement.

The accuracy of the numerical procedure was also verified by comparing its results to the analytically calculated magnitude of the dipolar attraction force between two cells. Since the cells were modeled as thin cylinders with height, \( h = 2R \), the analytical formula for the dipole-dipole interaction between spherical particles (equation 3) was modified to

\[
F_{\text{chain}} = -C \pi \varepsilon_m R h \operatorname{Re} |K(\omega)|^2 E^2.
\]

Here, the coefficient \( C \) depends on the relative positions of the two cylinders. The numerical and analytical values obtained for the dipolar attraction between two cells at different positions are compared in Table 3.2. The numerical and analytical results correlated very well for the cases when the cells are separated by distances greater than \( \sim 0.5R \); however, a deviation of 1 - 2 orders of magnitude occurred when the cells were very close to each other. This deviation is expected because the analytical calculations do not account for the higher order mutual polarization effects that become significant at small intermolecular distances and strongly increase the attraction.\(^{56}\) The velocities of the cells after accounting for the hydrodynamic resistance were obtained to be of the order of \( \sim 1 \mu m \ s^{-1} \), which corresponds well to the experimental data. These results suggest that the model correctly captures the dynamics and magnitudes of forces involved in the cells assembly process.
3.4.4 Dielectrophoretic Co-Assembly of Cell-Particle Binary Systems

One important application of the DEP-driven assembly can be the co-organization of cells and synthetic particles to form biocomposites which can be utilized for drug delivery, sensors, components of microsurgery, smart hybrid materials and so on. Since both the chaining and dielectrophoretic forces depend on the size and material property of the cells and synthetic particles, a variety of effects can be expected to play a significant role in the co-assembly process as well as in the types of structures that result. Therefore, in order to evaluate the effect of field on cell-particle mixtures, two kinds of suspensions – (i) cells with similar sized latex particles and, (ii) cells with smaller sized latex particles, were examined. The detailed results quite expectedly depended strongly on the frequency of the applied field.

(i) Binary Suspensions of Cells and Similar Sized Latex Particles. Latex particles of 5.0 µm diameter were homogeneously mixed with yeast cells in 1:1 ratio. This particular size of the latex was chosen to be comparable to that of the cells in order to study only the effect of dielectric permittivity on the co-assembly process. When AC fields of strengths between 4 - 20 V mm\(^{-1}\) and frequencies between 10 - 1200 Hz were applied to the mixed suspension, we observed three distinct patterns of chain formation (Figure 3.7). This process appeared to be equivalent to an interaction-induced phase separation. In the indistinguishable mixing region, both the cells and latex spheres exhibited a similar strength of dipolar interaction and they were both uniformly distributed throughout the entire length of the chains. Above 400 Hz and at intermediate field strengths (\(\sim 12\) V mm\(^{-1}\)), the proportion of latex spheres in the chains increased dramatically whereas, the cells interacted rather weakly with the other neighboring cells and latex particles. This type of cell-particle interaction
behavior was termed the transition region. At the highest voltages and frequencies applied above 800 Hz and 16 V mm$^{-1}$, the cells eventually phase separated from the latex completely and only latex particle 1D chains were left behind.

We believe that the observed phenomenon of cell segregation is related to the relative polarizability difference between the cells and latex particles being a strong function of the frequency. At the given experimental conditions, the values of the surface charge densities of the cells and latex are $\sim 0.18 \, \mu\text{C cm}^{-2}$ and $3.1 \, \mu\text{C cm}^{-2}$, respectively. These surface charges give rise to a counter-ionic diffuse double layer that is thinner around the cells in comparison to the latex particles. At low frequencies of the applied electric field, in spite of the difference in the thickness of double layer, the induced dipolar polarization is sufficiently strong enough in both cells and latex to form uniformly mixed cell-particle chains. As the frequency of the applied field increases, the polarizability of cells decreases more rapidly in comparison to the latex particles because of the limited mobility of the counter ions. The cells phase separate once the frequency of the field exceeds their characteristic relaxation frequency ($\omega_c \cong 800$ Hz) but the latex particles continue to form chains. The reason why the phase transition is not observed at low voltages is simply a kinetic effect. When the field strength is very low, the dielectrophoretic and particle chaining forces are too weak to align particles that are more than a few particle-diameters away. Therefore, cells and latex that are close to each other are non-preferentially pulled into forming small chains of 2 - 3 particles or cells.

The characteristic relaxation frequency of the latex microspheres, $\omega_p \sim 3$ kHz, was calculated to be not only higher than the characteristic relaxation frequency of the cells but also to lie outside the range of frequencies studied experimentally. This good correlation
between our calculations and experimental data confirms that the co-assembly dynamics is indeed dependent on the complex dielectric permittivity of the cells and latex particles. This method can provide a potential tool to predict the types of structures that can be dielectrophoretically assembled from almost any kind of cell-particle mixture containing particles of comparable sizes.

(ii) Binary Suspensions of Cells and Smaller Sized Latex Particles. Next we studied the combined effect of complex dielectric permittivity and particle size on the co-assembly process. Mixed suspensions comprising of 0.2 % w/v 5 µm yeast cells and 0.025 % w/v 1.0 µm fluorescent latex particles were prepared and the same procedure of applying the electric field was repeated as described above. Application of the AC field in the low-frequency range of 10 – 200 Hz always lead to the entrapment of the small latex particles in-between the cell junctions. A typical high-magnification optical micrograph of the cell-particle linear clusters is shown in Figure 3.8a. A complementary image of this micrograph taken using fluorescence microscopy indicates how the cells and latex particles are organized into chains of alternate confirmation (Figure 3.8b). Increasing the frequency above 10 kHz resulted in the phase separation of cells and latex. The phenomenon of latex particle entrapment between cells was mostly observed at low electrolyte concentrations; increasing the electrolyte concentration leads to the suppression of the co-assembly process.

To understand the role of electric field in the cell-particle chain formation, the co-assembly dynamics was simulated in FEMLAB using the model developed above for the field induced interaction between two cells (refer Figure 3.6). Again for the simplicity of calculations, the actual experimental setup was represented by a 2D system consisting of only
two cells and two latex particles placed between parallel coplanar electrodes (Figure 3.9a). The dielectric permittivity and conductivity of 1.0 \( \mu \text{m} \) latex particles (\( \varepsilon_p = 2.55\varepsilon_0, \sigma_p = 0.02 \text{ S} \)) was calculated by using multi-shell models (see Appendix B for detailed calculations and numerical parameters).\textsuperscript{51, 58, 59} The complex permittivity values for yeast cells and PBS media were the same as in the previous cell model. All calculations were performed for a constant applied field of one positive half cycle.

The sequential time-evolved images of the electric field distributions and particle rearrangement at various stages of the assembly are presented in Figure 3.9b - d. The field strength and frequency were 13.3 V mm\(^{-1}\) and 100 Hz, respectively. The simulation demonstrates that both 1.0 \( \mu \text{m} \) particles and cells are more polarizable than the media at the given conditions. The electric field draws the small synthetic particles by positive dielectrophoresis towards the regions of highest localized field intensities that exist directly above or below the cells (along the direction of the field). This in turn attracts the next cell and as a result, the latex particle gets trapped between the cells. Thus, the capture of smaller sized latex particles between cells is a field driven process. In some simulations, the cells were also first drawn towards each other and then followed by particle capture between them (results not shown here). This process is conceptually similar to the one described and gives the same end result as in Figure 3.9d. The route to assembly depends only on the initial coordinates of the cells and latex particles. Our experimentally observed co-assembly dynamics (Figure 3.9e – g) correspond very well with the simulation. The observations that there is no co-assembly at high frequencies or high electrolyte concentration is consistent with our results obtained for cell-only chaining.
3.4.5 Cell Chains Made Permanent by Functionalized Microparticles and Ligands

We have successfully shown by now how the electric field plays a key role in forming various architectures of cell-cell and cell-particle chains, the morphology of which can easily be tuned by controlling a few simple parameters. These assemblies are, however, not permanent and therefore cannot be employed in forming any useful functional material. Since an important consequence of our field-driven co-assembly process is that smaller sized dielectric particles of almost any composition can be dielectrophoretically incorporated between the cells, we can expect the small particles to serve as “biocolloidal glue” if their surface is functionalized with a ligand which has an irreversible affinity for the cell surface. This could not only allow assembly of permanently stable biocomposites but also to further engineer the specific functionality of the hybrid biomaterial by synergistically combining the property of the binder particles to the ones of the cells.

In order to realize this goal, we performed experiments in which different types of ligand-functionalized organic and inorganic microparticles were used as permanent binding units. The binding of the cells and functionalized particles was accomplished by mainly two types of interactions – electrostatic and bio-specific (Figure 3.10a and b). For electrostatic binding of negatively charged cells, we used positively charged 1.8 µm iron oxide particles with amine end groups. When an AC field was applied to the cell-particle mixtures, the binder particles were invariably captured between the cells and irreversibly attached to them in less than 10 min. Though this process yielded permanent chains, it was difficult to prevent their adhesion to the negatively charged glass surface. The problem of adhesion was overcome by using 1.0 µm FITC-labeled fluorescent microparticles which had chemically
attached lectins on their surfaces. Lectin is a type of protein that binds bio-specifically to the saccharide functional groups on the cell’s surface (in the presence of small amounts of Ca\(^{2+}\) ions).\(^4\) Permanent binding of cells was achieved in 30 - 45 min and the results were highly reproducible. In all these experiments, the morphology of the cells chains could also be controllably modified from one system to another. For example, chains prepared with higher ratio of particles to cells were more rigid. Also, chains formed at smaller frequencies were longer (consistent with our previous results).

We also demonstrated that one can make responsive cell “wires” by using binder particles which have an intrinsic magnetic functionality. For example, permanent chains of yeast and mouse fibroblast cells were co-assembled with 0.95 µm lectin-coated paramagnetic particles. These chains could then be transported and rotated rapidly (milliseconds) under the field of a small permanent magnet (Figure 3.10c and d). The chains did not fracture in spite of shear flow and remained stable over a period of few days following the experiment. One potential application of these magnetic composite materials could be a real-time biosensor where the manipulation in external magnetic fields could allow for the rapid alignment of the cell wires between micropatterned electrodes. This technique could also easily be extended to other systems in which the electrical, optical or any other functionality of the binder particles could be imparted to the entire cell structure to create smart materials.\(^4\)

The permanent binding of cells could alternatively be performed by using only ligands. We demonstrated this by using three different kinds of ligands that attached to the cells through either electrostatic or biospecific interactions (Figure 3.11). The positively charged polymer PDMAEMA electrostatically attached to the negatively charged yeast cells
but it was again difficult to prevent the chains from adhering to the glass substrate. D-mannose and lectin, on the other hand, facilitated irreversible bio-specific attachment by binding to the constituent carbohydrate receptors (such as, surface lectins etc.) or polysaccharides present on the cell wall, respectively.\textsuperscript{60, 61} Details of the results from these and from a few other additional binding and control experiments are summarized in Table 3.3.

3.4.6 Cell 2D Arrays

We have effectively demonstrated by now how the combined principles of dielectrophoresis and particle chaining forces can be utilized for directed assembly of cells and synthetic particles into 1D chains. Our next major challenge was to assemble the cells into 2D arrays. Our previous results for 2D crystal assembly of latex and silica particles using a two-electrode chip have shown that the crystallization process proceeds in two stages.\textsuperscript{40, 41} In the first stage, particle chains are formed because of attractive dipolar interparticle interactions. In the second stage, dipoles in the adjacent particle chains interact laterally to form hexagonally close-packed 2D crystals. When we used the two-electrode chip in our initial attempt to form cell 2D arrays, we observed that the cells formed 1D chains (similar to the ones observed with latex and silica particles) as an intermediate structure, but the lateral dipolar interactions between the cells were too weak to assemble the chains into long-range 2D arrays (Figure 3.12a). The reasons for the lack of attraction between cell chains we believe are that the cells are polydisperse and they polarize weakly in comparison to the synthetic microparticles.
To overcome the problem of inhomogeneous packing, we used a four-electrode chip configuration (see Figure 3.2b) in which the four orthogonally arranged point electrodes allowed us to apply the electric field in two perpendicular directions. The voltage source was connected to any one pair of adjacent electrodes and the opposite pair was grounded. The AC field was first applied to the cell suspension in one direction for ~15 min. By the end of this time, the cells were settled at the bottom of the chip and they were aligned in the direction of the electric field via axial dipolar interactions. The field was then switched to the other electrode pair to let the cells re-align along the perpendicular direction. The switching process was repeated approx. every 5 min for a total duration of 30 – 45 min until we managed to draw the cells into uniformly close-packed arrays.

Figure 3.12b shows a high-magnification optical micrograph of a typical single-layer 2D array of yeast cells assembled in 30 min using the four-electrode chip. The volume fraction of the cell suspension was 0.08 % w/v (adequate to form a monolayer). To confirm that the formation of close-packed 2D arrays is an intrinsic characteristic of the on-chip field driven process, we performed a control test in which the same concentration of yeast cells were allowed to sediment freely under gravity (no electric field) for 30 min in the same experimental setup (Figure 3.12c). No close-packed 2D cell structure was obtained in the absence of the electric field which proves that the two-directional alignment and annealing in AC fields is the main driving force for 2D “crystallization” of cells.

(i) Magneto-Responsive Permanent Cell Membranes. We know from our cell-particle chaining experiments that the capture of functionalized binder particles between cells during the process of field-induced organization is critical for the irreversible biocomposite
assembly. The same approach could be used in the 4-electrode chip for the assembly of large (~ cm²) permanent magnetic biomembranes from live cells. Single layer membranes made of organized yeast and fibroblast cells bound by lectin-coated magnetic particles are shown in Figure 13.13a and c. The intimate structure of the membranes consisting of cells and particles could be observed by Field Emission SEM after fixation with glutaraldehyde and sputtering with gold (Figure 13.13b). Due to their intrinsic magnetic functionality, the biomembranes assembled on the chip could be stretched, translated, folded and rotated in 2D and 3D by external magnets (Figure 13.14a). The magnetic response of the biomembranes could also be used to extract them from the chamber where they are assembled (Figure 13.14b). The ability to assemble, transport, and manipulate live cell biomembranes has obvious significance for the creation of artificial tissues and cell assemblies for possible surgical applications.

A field emission scanning electron microscopy (FESEM) image of a yeast cell membrane fixed with glutaraldehyde showed that the particles were embedded between the closely-packed cells (Figure 3.13a). This also implies that our simulation of alternating configuration of cells and binder particles can effectively be extended to a 2D system. The biomembranes assembled on the chip were rotated, folded and translated in 2D and 3D using external magnetic fields (Figure 3.13b and c). We were also able to demonstrate that the membranes could be removed from the chips where they were assembled and transported to the site of further implementation. For this purpose, a thin and transparent Teflon tubing was attached to the four-electrode microchamber and the membranes were flown out of it using the pull of a small magnet (Figure 3.13d). The ability to manipulate these live cellular membranes using external magnetic fields could now be harnessed for biomedical
applications which require rapid orientation and replacement of cell patches that are used as wound dressings. The cell membranes may also be used as artificial tissues for microsurgery and prosthetics, advanced drugs and vaccines, and bio-filters.

3.5 Conclusions

We have reported the use of alternating electric fields for the rapid and directed co-assembly of live cells and synthetic colloidal particles into freely suspended biomaterials. Operating conditions such as the electrode geometry, voltage and frequency of the applied field, pH, electrolyte and cell concentration in the buffer media, and particle size are shown to be the essential tools in controlling the rate of assembly and the morphology of the resulting structures. Experimental observations and electrostatic simulations of the cell-particle co-assembly dynamics illustrate that at low frequencies, smaller sized synthetic particles get captured in-between the cell junctions by positive dielectrophoresis. By applying this phenomenon and using various types of surface-functionalized synthetic colloidal particles as binding units, we demonstrate how permanent cell structures can be assembled rather easily on a chip. The subsequent utilization of lectin-coated paramagnetic particles, allowed the fabrication of stable responsive cell “wires” and cell membranes which could be freely manipulated inside the chip and extracted from it using external magnetic fields.

Biocomposite structures similar to the ones shown here could be used in applications where the functionality of the synthetic particles complements the one of the cells. The particles can impart magnetic, electronic or optical properties to the assemblies.1, 4, 63, 64 One of the potential applications of cell-particle assemblies on a chip is in biosensors with
electrical detection of changes in cell impedance imparted by toxins or changes in the environment. Such devices have been previously fabricated by sedimenting single cells onto micropatterned electrodes. The serial connection of cells in “wires” can increase the sensor sensitivity to single events such as cell death, poisoning, membrane potential shift and ion channel activity. The parallel connection of multiple chains in membranes could increase the precision of detection of low dose toxins that change the impedance of each cell only slightly. The ability to manipulate the cell arrays with magnetic fields might be used for rapid alignment and replacement of the cell patches. Co-assemblies with metallic nanoparticles could allow easier and more reliable electrical interfacing. The cell-particle arrays can also find application as artificial tissues for microsurgery, advanced drugs and vaccines, or “smart” biomaterials or reporters that respond to changes in the chemical composition of the environment.

3.6 Acknowledgements
The authors would like to thank Michael Weiger and Dr. Jason Haugh at NCSU for generously providing the NIH/3T3 mouse fibroblast cells, Dr. Genzer’s lab for providing PDMAEMA, Dr. Anka Veleva for access to ImagePro software, and Valerie Knowlton and Dale Batchelor for assistance with the SEM imaging. This study was supported by NER, CAREER and NIRT grants from the National Science Foundation (NSF).
Figure 3.1 Schematic of the types of cell-cell and cell-particle assemblies formed using dielectrophoresis in AC electric fields.
Figure 3.2 Schematic of the chips used for the dielectrophoretic assembly of live cells: (a) Parallel coplanar gold electrodes for making 1D chains and, (b) Orthogonally arranged four-point brass electrodes for fabricating uniform 2D arrays.
Figure 3.3 Chains assembled from live cells using AC electric fields. (a) Yeast (*S. cerevisiae*) cell chains under 15 V mm⁻¹ and 50 Hz electric field. Inset: The viability of the cells remains preserved in the electric field after 2 h as indicated by the gutted appearance of the cell interior; (b) NIH/3T3 mouse fibroblast cells under 10 V mm⁻¹ and 10 kHz electric field. Inset: Magnified view of the black rectangle shown in (b). The blue color indicates a dead cell. Most cells stay alive during the assembly process for up to 1 h. [Please note that there is no detectable difference in the responses of the dead and the live cells at the low frequencies used].
Figure 3.4 Effect of the applied AC electric field on the cell chain length; longer chains are formed at higher voltages and lower frequencies. Each data point is an average of count from 10 different images.
**Table 3.2** A list of physical parameters used for the calculation of the characteristic relaxation frequency of yeast cells.

<table>
<thead>
<tr>
<th>Physical Parameters</th>
<th>Values</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R$</td>
<td>Cell radius</td>
<td>$2.5 \times 10^{-6}$ m</td>
</tr>
<tr>
<td>$D$</td>
<td>Diffusion coefficient of Na$^+$ ions</td>
<td>$1.33 \times 10^{-9}$ m$^2$ s$^{-1}$</td>
</tr>
<tr>
<td>$q$</td>
<td>Elementary electron charge</td>
<td>$1.6 \times 10^{-19}$ C</td>
</tr>
<tr>
<td>$\sigma_q$</td>
<td>Surface charge density</td>
<td>$1.77 \times 10^{-3}$ C m$^{-2}$</td>
</tr>
<tr>
<td>$k_B$</td>
<td>Boltzman constant</td>
<td>$1.38 \times 10^{23}$ J K$^{-1}$</td>
</tr>
<tr>
<td>$T$</td>
<td>Temperature</td>
<td>298 K</td>
</tr>
<tr>
<td>$\varepsilon_m$</td>
<td>Permittivity of the medium</td>
<td>$7.08 \times 10^{-10}$ F m$^{-1}$</td>
</tr>
<tr>
<td>$\kappa$</td>
<td>1/Debye length</td>
<td>$1.04 \times 10^{8}$ m$^{-1}$</td>
</tr>
<tr>
<td>$\zeta$</td>
<td>Zeta potential</td>
<td>-0.017 V</td>
</tr>
</tbody>
</table>

$$C'_d = \varepsilon_m \kappa \cosh \left( \frac{q\zeta}{2k_BT} \right)$$
Effective capacitance of the bound layer

$$M = 1 + \frac{q\sigma_q}{k_BT C'_d}$$
Dimensionless factor

$$7.78 \times 10^{-2} \text{ F m}^{-2}$$

$$1.89$$
**Figure 3.5** Effect of pH on the dielectrophoretic assembly of cell chains determined as a function of frequency. The electric field intensity is 10 V mm$^{-1}$. 
Figure 3.6 Snapshots of the electrostatic simulation at various stages of cell assembly compared with the actual experimental images. The AC electric field is 13 V mm\(^{-1}\) and 100 Hz in both cases. (a) Boundary conditions and the geometry specified in the model. (b - d) Time-lapsed simulation of the field intensity distributions and cell re-arrangement using FEMLAB are color coded. The higher polarizability of the cells leads to attraction by positive dielectrophoresis. (e - g) Actual experimental images capturing the dynamics of cell alignment in AC field.
Table 3.3 Force calculations for interactions between cells. Comparison between numerical and analytical values obtained for dipolar forces between yeast cells at two different positions.

<table>
<thead>
<tr>
<th>Dipole Interaction</th>
<th>Numerical Calculations</th>
<th>Analytical Calculations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{Num} = - \frac{1}{S} \int \left( \frac{1}{2} E \cdot D + (n_1 \cdot E) \cdot D^T \right) dS$</td>
<td>$F_{Anal} = C \pi \varepsilon \rho \cdot R \Re \left( \frac{\varepsilon_c - \varepsilon_m}{\varepsilon_c + \varepsilon_m} \right)^2 E^2$</td>
</tr>
<tr>
<td></td>
<td>$3.22 \times 10^{-14} \text{ N}$</td>
<td>$3.44 \times 10^{-14} \text{ N}$</td>
</tr>
<tr>
<td></td>
<td>$1.4 \times 10^{-11} \text{ N}$</td>
<td>$6.25 \times 10^{-13} \text{ N}$</td>
</tr>
</tbody>
</table>
Figure 3.7 Morphology diagram showing the interaction-induced phase separation of yeast cells and 5 µm sulfate-stabilized latex spheres as a function of field strength and frequency. The different regions in the phase diagram indicate: (a) Homogeneous distribution of cells and latex particles through the entire length of the chains, (b) Weaker interaction of cells with neighboring cells and latex particles leading to increased proportion of latex particles in the chains, and (c) Complete phase separation of the cells resulting in latex particle-only chains.
Figure 3.8 Alternating linear composite clusters of yeast cells (0.2 % w/v) and 1 µm green fluorescent latex particles (0.025 % w/v) assembled at 15 V mm\(^{-1}\) and 200 Hz AC electric field. (a) Optical micrograph taken after the field was applied for 45 mins. (b) Complementary image of (a) acquired using fluorescence microscopy. The green fluorescent stripes indicate the relative position of the latex microparticles between the cells.
Figure 3.9 Time-lapsed images of the co-assembly dynamics of a cell-particle system at 100 Hz. (a) Specifications of the system geometry and boundary conditions used in the model. (b - d) Three snapshots of the dynamic simulation, where the field intensity distribution is color coded. The higher polarizability of the cells leads to attraction and chaining. The simulation illustrates how one of the particles is captured in the higher intensity area between the cells in the “chain”. This process is observed experimentally (e - g) and is used to bind the structure.
Figure 3.10 Permanent cell chains using functionalized binder particles. (a) Yeast cells with 1.8 µm amine-terminated iron-oxide particles. (b) Yeast cells with 1.0 µm lectin-coated fluorescent latex particles. (c) Yeast cells with 0.95 µm lectin-coated magnetic particles. (d) Mouse fibroblast cells with 0.95 µm lectin-coated magnetic particles. Insets show chain rotation using an external magnet.
Figure 3.11 Permanent binding of yeast cell chains using (a) poly(dimethyl aminoethyl methacrylate) (PDMAEMA), (b) Concanavalin-A lectin, (c) D-Mannose, and (d) Both Concanavalin-A lectin and D-Mannose.
Table 3.3 A list summarizing the binding and control experiments performed on yeast and NIH/3T3 mouse fibroblast cells. The applied AC fields are of strengths 5 - 17 V mm\(^{-1}\) and frequencies 50 Hz – 10 kHz. The concentrations of cells are between 0.01 - 0.2 % w/v.

<table>
<thead>
<tr>
<th>Types of binding agents</th>
<th>Concentration of binding agents</th>
<th>Exposure to electric field</th>
<th>Permanent binding of yeast (Y) and fibroblast (F) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Functionalized Particles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 (\mu)m amine-terminated magnetic particles</td>
<td>0.013 % w/v, 0.036 % w/v</td>
<td>10 min, 10 min</td>
<td>Yes (Y), Yes (F)</td>
</tr>
<tr>
<td>1.0 (\mu)m fluorescent aldehyde/sulfate latex particles</td>
<td>0.02 % w/v</td>
<td>45 min</td>
<td>Yes (Y)</td>
</tr>
<tr>
<td>1.0 (\mu)m lectin-coated fluorescent latex particles</td>
<td>0.025 % w/v</td>
<td>45 min</td>
<td>Yes (Y)</td>
</tr>
<tr>
<td>1.0 (\mu)m lectin-coated fluorescent latex particles + D-mannose</td>
<td>0.025 % w/v + 60 mM</td>
<td>45 min</td>
<td>Yes (Y)</td>
</tr>
<tr>
<td>1.8 (\mu)m and 0.95 (\mu)m lectin-coated paramagnetic particles</td>
<td>0.018 % w/v, 0.017 % w/v</td>
<td>1 h, 30 min</td>
<td>Yes (Y), Yes (F)</td>
</tr>
<tr>
<td>1.8 (\mu)m and 0.95 lectin-coated paramagnetic particles + D-mannose</td>
<td>0.013 % w/v + 38 mM</td>
<td>30 min</td>
<td>Yes (Y)</td>
</tr>
<tr>
<td>1.0 (\mu)m fibronectin-coated latex particles</td>
<td>0.016 % w/v</td>
<td>45 min</td>
<td>Yes (F)</td>
</tr>
<tr>
<td><strong>Ligands</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDMAEMA</td>
<td>(7.5 \times 10^{-4}) % w/v</td>
<td>30 min</td>
<td>Yes (Y)</td>
</tr>
<tr>
<td>Lectin</td>
<td>(5 \times 10^{-4}) % w/v</td>
<td>1 h</td>
<td>Yes (Y)</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>43.5 mM</td>
<td>30 min</td>
<td>Yes (Y)</td>
</tr>
<tr>
<td>Lectin + D-Mannose</td>
<td>(5 \times 10^{-4}) % w/v + 60 mM</td>
<td>45 min</td>
<td>Yes (Y)</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 (\mu)m sulfate-stabilized polystyrene latex microspheres</td>
<td>0.013 % w/v</td>
<td>1 h</td>
<td>No (Y)</td>
</tr>
<tr>
<td>1.8 (\mu)m amine-terminated magnetic particles + excess BSA</td>
<td>0.013 % w/v</td>
<td>1 h</td>
<td>No (Y)</td>
</tr>
</tbody>
</table>
Figure 3.12 Two-directional alignment and annealing in AC fields for assembling uniform 2D cell arrays. (a) No close-packed 2D yeast cell array was obtained using two parallel coplanar electrodes; (b) Organization of yeast cells into close-packed 2D arrays using the 4-point electrode setup. The field was applied for 30 min; (c) Random deposition of yeast cells that were freely sedimented under gravity-only force (no electric field) for 30 min.
Figure 3.13 Micrographs of permanent cell membranes bound together by Con-A functionalized microparticles. (a) Large magnetic yeast cell membrane at low microscope magnification; note that the membrane is only one cell layer thick. (b) SEM image of a closely-packed fixed yeast membrane. (c) NIH/3T3 mouse fibroblast membrane.
Figure 3.14 Magnetic manipulation of responsive cell membranes. (a) Folding of a large magnetic yeast cell membrane by an external magnetic field. (b) Extraction and transport of a yeast cell membrane through thin Teflon tube using an external magnet. Inset: Arrow points to the actual position of the membrane in the tubing attached to the chip used in the experiments.
3.7 References


(47) The random displacement of the yeast cells due to Brownian motion was compared to their sedimentation due to gravity by calculating the Peclet number which is given by,

\[
Pe = \frac{hU}{D} = \frac{\left( \frac{2\Delta \rho gh r^2}{9 \mu} \right)}{\left( \frac{k_B T}{6 \pi \mu r} \right)}.
\]

Here, \( h \) is the characteristic displacement (assumed to be the order of the cell diameter), \( U \) is the sedimentation velocity, \( D \) is the cell diffusion coefficient, \( \Delta \rho \) is the different in the densities, \( g \) is the acceleration due to gravity, \( r \) is the cell radius, \( \mu \) is the medium viscosity, \( k_B \) is the Boltzmann constant, and \( T \) is the temperature.


(57) The value of the surface charge density of 5 micron latex particles was used from the information data sheet provided by the vendor, Interfacial Dynamics Corp. (OR).


(62) The permanent membrane comprising of yeast cells and 0.95 micron lectin-functionalized paramagnetic particles was transferred on to a Nochromix cleaned glass cover slip using a transfer pipette. The membrane was washed twice using DI water in order to remove any excess BSA, surfactant, unbound cells and unbound lectin-functionalized particles from the media. The cells were fixed in 2 % w/v glutaraldehyde for 2 - 3 h and then dehydrated by washing with a series of progressively concentrated ethanol solutions (20, 40, 60, 70 % v/v) for 10 min each. They were finally left inside 95% ethanol solution for ~ 1 h. The cells were dried using a carbon dioxide critical point dryer. The dried samples were immediately mounted on top of a SEM stub and stored in a desiccator overnight. SEM: The samples were sputtered with a thin layer of gold (~10 Angstroms) and then viewed with an FESEM (JEOL 6400F) using a 5kV accelerating voltage.


Chapter 4

Latex Agglutination Detection by Electrochemical Impedance
4.1 Abstract

We describe a new class of impedance based immunosensors in which the agglutination of latices is monitored by electrochemical impedance spectroscopy (EIS). Antibody-conjugated latex microspheres agglutinate in the presence of target antigens in suspension. As the particles sediment into microscale channels between interdigitated electrodes, an impedance spectrum is generated that depends strongly on the agglutinated morphology and the sedimentation rate of the latex particles. Reproducible results are obtained in the 0.1 – 1 MHz frequency range. An equivalent circuit model of the system shows that the impedance is governed primarily by electrical double layer interactions close to the electrode surface. The effects of sedimentation time, particle type, size, and concentration are also characterized in detail. The method holds promise for developing sensitive and miniaturized sensors for rapid immuno-testing.
4.2 Introduction

Key advances have been made in the development of rapid and miniaturized biosensors in the last decade. This progress has been stimulated, in part, by exploiting highly specific recognition of biological systems (DNA, antigen/antibody, streptavadin/avidin, sugar/lectin etc.) in important applications such as pregnancy tests, clinical diagnostics, food and water quality control and environmental analysis. To exploit the affinity nature of biomolecular interactions, it is often necessary that they be conjugated to a probe that transduces signal upon recognition. Previous biosensors have been based on surface plasmon resonance, fluorescence, calorimetry, waveguide and piezoelectric properties of the transducer. Of particular interest are systems in which the specific binding event between biomolecules is measured using electrical signals. The use of electric fields in such devices makes them adaptable for automation, allows integration of many sensors into parallel arrays on a single chip and reduces the time and cost of analysis. A few examples of electronic biosensors reported in the literature include in-situ assembly of colloidal particles into sensor patches, selective bacterial detection using dielectrophoretic impedance measurement and transduction of cell-surface enzymatic activity into electrical signals.

In practice, many immunological assays are performed by means of latex agglutination tests (LATs) which provide a simple and versatile tool for immuno-detection. The principle of LATs is based upon the very specific interaction of antigens (Ag) with the antibodies (Ab) that are covalently bound to latex particles of micron or sub-micrometer size. Small concentrations of antigens are sufficient to agglutinate and precipitate antibody-conjugated latex particles from dilute suspensions. The results are typically read-out by using
light scattering techniques such as spectrophotometry, nephelometry, scanning laser microscopy or just by visual inspection (Figure 4.1). These detection methods are widely used to provide good results in both clinical diagnostic tests (yes/no type qualitative results) and in laboratory-based immunoassays (quantitative results).

This chapter describes a new approach in which LATs are carried out on top of chips with micron-gap interdigitated electrodes and the precipitation of particles by antigens is measured by electrochemical impedance spectroscopy (EIS) (see Figure 4.1). Such type of sedimentation based EIS detection of agglutination assays in confined geometries has no analogue in the literature to the best our knowledge. The studies related closest to this research are reported by Suehiro et al. and Smith and coworkers who demonstrate that the quantitative estimation of biological cell concentration in aqueous media and the aggregation of nanoparticles by a target molecule can be made by using dielectrophoretic impedance measurements.\textsuperscript{12, 13} In the previous immunoassay studies based on EIS, the antibody is typically immobilized on a conductive support (i.e., gold) and the electrical properties of the double layer at the interface are modified when the antibody reacts with the antigen of interest.\textsuperscript{14-17} This process is time consuming due to the slow diffusion of antigens to the surface and also requires additional washing steps. In our technique, we eliminate the immobilization step by allowing antibody-conjugated latex particles to bind with the antigens in suspension. Since the formation of an antigen-antibody complex significantly changes the morphology, concentration and sedimentation rate of the agglutinated particles, there is a dramatic shift in the impedance spectra if there are antigens present. The sedimentation path length is purposely kept small (order 100 \(\mu\)ms) in order to obtain results within a few minutes.
Micron-gap interdigitated electrodes were used since they are known to have high sensitivity\textsuperscript{18,19} and they can be fabricated at a relatively cheaper cost.

In the following sections, we report a systematic study of the effect of latex particles sedimenting on or in the near vicinity of interdigitated microelectrodes. The qualitative shift in the impedance signal due to agglutination by antigen is used as a versatile tool for designing a new type of EIS based particle-agglutination immunosensor. A simple circuit model is proposed to interpret the response of the sensor. The effects of various operating parameters on the sensor performance are also investigated in detail.

4.3 Experimental Procedure

4.3.1 Materials

White, surfactant-free, sulfate-stabilized polystyrene latex microspheres of 1.0, 2.0, 4.0 and 8.7 µm diameters were purchased from Interfacial Dynamics Corp., OR. Goat anti-rabbit immunoglobulin (IgG) (H&L)-Fluoresbrite\textsuperscript{TM} Carboxylate YG beads of 1.03 µm diameter were obtained from Polysciences Inc., PA. Rabbit IgG acting as their antigens were purchased from Calbiochem, CA. The following reagents were also used in the experiments: phosphate buffered saline tablets and bovine serum albumin (Sigma-Aldrich, MO); Tween 20 (Ted Pella, Inc., CA); deionized (DI) water of resistivity ~ 18.2 MΩ cm (Millipore RiOs purification system, MA).
4.3.2 Sample Preparation

**Latex Suspension:** A phosphate buffered saline (PBS) solution containing 0.137 M NaCl, 0.0027 M KCl, and 0.01 M phosphate buffer was prepared by dissolving one PBS tablet in 200 mL of DI water. The latex microspheres were centrifuged at 3000 g for 20 min and washed twice with the PBS solution containing 0.19 % w/v of non-ionic surfactant Tween 20. The washing steps were necessary to remove any preservatives present in the media. The surfactant was added to prevent any non-specific aggregation or adhesion of the latex particles. The final latex particle concentrations in the samples were adjusted to 0.06 – 3.25 % w/v.

**Latex-IgG Suspension:** A 100 µL volume of 1.3 % w/v goat anti-rabbit IgG-conjugated latex particles (henceforth referred to as latex-IgGs) was mixed with 1000 µL of PBS solution containing 0.19 % w/v of Tween 20. The latex-IgG suspension was centrifuged at 3000 g for 20 min and the supernatant was removed. The latex-IgG particles were resuspended in 500 µL PBS solution containing 2 mg mL⁻¹ bovine serum albumin (BSA). BSA was routinely added to prevent non-specific binding of the antigens to the surface of latex-IgG particles during the immunoassays. The suspension was sonicated for 5 min to ensure that all latex-IgG particles were well dispersed and set aside for 30 min. The suspension was centrifuged at 3000 g for 20 min and washed twice with PBS to remove any excess BSA. The latex-IgG particles were finally resuspended in PBS containing 0.19 % w/v Tween 20 to obtain samples with final latex-IgG concentrations between 0.06 – 3.25 % w/v.

**Antigen Suspension:** The irreversible agglutination of latex-IgG particles was carried out using complementary rabbit IgGs. Since both under- and oversaturation of antigens can
prevent cross-linking of latex-IgG particles, it was first necessary to determine the optimal amount of rabbit IgG antigens that would be required to agglutinate any given concentration of latex-IgGs in our system. To this end, a preliminary set of trial and error experiments were performed with 10 µL aliquots of 3.25 % w/v latex-IgG particles, each mixed and incubated with different concentrations of rabbit IgGs. The mixed suspensions were gently shaken by hand every 30 min to keep the particles in a dispersed state. The extent of agglutination in each aliquot was observed every 30 - 45 min by dispensing 1 µL sample on a glass slide (Fisher Scientific, PA) and viewing it by an Olympus BX61 optical microscope. The results of the systematic study revealed that the highest degree of agglutination in 3.25 % w/v latex-IgG suspensions occurs with 1 µg mL⁻¹ antigen in ~ 4.5 h. This empirically derived value formed the basis of calculating optimal antigen concentrations required for all other latex-IgG suspensions used in our experiments (Table 4.1).

**4.3.3 Experimental Apparatus**

Interdigitated electrode structures with gap sizes of 10 µm were microfabricated on a glass surface via photolithography. These electrodes were formed by depositing a 100 nm thick layer of gold onto a 10 nm thick layer of chromium. A photomask defining the shape and size of the electrodes was used to apply an etch-resist layer, and then the excess metal was removed. A schematic of a typical microelectrode chip used is shown in Figure 4.2a. When the experiments involved only latex particles, the electrode chips were reused after extensive washing with DI water. In all other cases, a fresh electrode set was used for each experiment.
in order to circumvent the problem of immunoglobulins sticking irreversibly to the glass and gold substrate.

A transparent microfluidic flow-chamber (5 × 2 × 0.15 mm, Grace BioLabs, OR), in which the latex particles were suspended, was sealed onto the glass surface surrounding the electrode pattern (Figure 4.2b). The chamber consisted of two ports, one for injecting suspensions manually using a micropipette and the other for withdrawing them controllably by means of a variable flow peristaltic pump with Tygon tubing (Cole-Palmer, IL). An AC oscillation of 10 mV was applied to the electrodes over a frequency range of 1 Hz – 1 MHz and the electrochemical impedance was measured in a potentiostatic two-electrode configuration mode using an IM6E Zahner electrochemical impedance spectroscope (Kronach, Germany). The EIS data were fitted by the ZSimpWin software (Version 2.00) supplied by EChem Software, MI.

4.3.4 Methods

Prior to loading the particle suspension, the chamber was flushed with ~ 500 µL of DI water. The first impedance reading was obtained with only DI water. This blank reading was necessary to ensure that 1) the chip was clean (an especially important step in cases where the chip was reused) and 2) the readings were stable (no sample leakage through the chamber or oxidation of the gold electrodes at the given conditions). The water in the chamber was then replaced and a second reference reading was taken with 0.01 M PBS solution containing 0.19 % w/v Tween 20. Finally, the chamber was loaded with latex (or, latex-IgG) suspension and the ports were sealed to minimize evaporation. For experiments involving LATs, latex-
IgGs and antigens were mixed 1 min prior to the loading step, sonicated for ~ 30 s and then inserted into the chamber. Impedance readings were taken every 10 min starting from this instant. The end results were reported as % impedance change from the reference PBS solution, with the aim to 1) minimize background signal in cases where the chips were washed and reused, and 2) account for the slight variation in signal that may occur from one experiment to another while sealing the flow-chamber on the chip.

The appropriate duration of each impedance experiment was estimated by calculating the minimum time required for complete sedimentation of particles. First the terminal velocity of the particles was calculated using the equation,

$$v_t = \frac{V_p (\rho_p - \rho_w) g}{3\pi \eta D_p}$$

Here, $V_p$ is the volume of the particle, $\rho_p$ is the density of the particle, $\rho_w$ is the density of water, $g$ is the acceleration due to gravity, $\eta$ is the viscosity of the medium, and $D_p$ is the diameter of the particle. For 1.0 µm latex particles, the terminal velocity is $3.06 \times 10^{-8}$ m s$^{-1}$. Next, the settling time of approximately 82 min was computed for sedimentation taking place inside a 0.15 mm thick chamber. The experiments were conducted for 90 min to ensure that the signal reached saturation. Experiments with 2, 4 and 8.7 µm size latex particles were performed for shorter times, viz. 30, 10 and 5 min, respectively.
4.3.5 LATs by TIRF Microscopy

The changes in the sedimentation rates of latex-IgG particles due to the agglutination by antigens were observed with a prism-based Total Internal Reflection Fluorescence (TIRF) microscope (Axioskop 2FS; Carl Zeiss MicroImaging, Inc.) using a 20x water immersion objective. The TIRF excitation source was a tunable wavelength laser head emitting lines of 488 nm wavelength (Melles Griot, CA). The light was used at maximum power (60mW). Digital images were acquired with 2 × 2 binning every 10 – 15 s over 50 – 70 min using an ORCA ER-cooled CCD camera (Hamamatsu, NJ) and later analyzed with Metamorph software (Universal Imaging, PA).

4.4 Results and Discussion

The impedance spectrum was first measured in DI water (Figure 4.3a). The impedance behavior is largely capacitive at low frequencies as seen by the high phase angle. This is due to the formation of an electrical double layer at the electrode surface. At intermediate frequencies, the phase angle decreases and the sensor behaves more like a resistor. This can also be noticed by the appearance of a plateau in the impedance data which exists due to the bulk resistance of the DI water. At even higher frequencies, the plateau disappears and again a capacitance component can be noticed which corresponds to the dielectric behavior of water ($\varepsilon_r = 78$).

For interpretation of these results, the experimental data were fit to an equivalent circuit model. The system was most accurately represented by a constant phase element $Q_{dl}$ for the electric double layer at the electrode surface, $Q_{d}$ for the dielectric behavior of water,
$R_{sol}$ representing the resistance of the bulk solution, and $R_{surf}$ as the resistance of the double layer at the glass surface. The impedance of a constant phase element is defined as $Z_{CPE} = \frac{1}{Q(j\omega)^n}$, where $n = 1$ for a pure capacitor and $n = 0$ for a perfect resistor. From the fitting, $n$ for the double layer is obtained as 0.77. The phase angle is then not $\Theta = -90^\circ$ but $\Theta = -90^\circ n \cong -70^\circ$ which is most probably related to the electrode surface having mild roughness or defects. The fitted values of the capacitive parts of $Q_{DL}$ and $Q_{DI}$ are 56 nF and 1.64 nF, respectively. A higher capacitance here means that more charge can be stored in the double layer. The values of the resistances obtained from the model are $R_{sol} = 4.6$ MOhm and $R_{surf} = 84$ kOhm. A similar magnitude of the solution resistance was obtained using the numerical formula derived by Gerwen et al.\textsuperscript{18}

In physiological buffer media, the resistance of the solution can no longer be resolved from the spectrum as the behavior of the sensor is capacitive up to very high frequencies (Figure 4.3b). This capacitance is then the parallel capacitance of the double layer and the dielectric. Since the two capacitances cannot be distinguished from the model fit anymore, we used a slightly modified circuit model for the fitting. The new model includes a constant phase element $Q_{DI}$ representing the combined effect of double layer and dielectric behavior for the bulk and $Q_{DL}$ accounting for only the electric double layer formation at the electrodes. The values of the capacitive parts of $Q_{DL}$ and $Q_{DI}$ obtained from the model are 344 nF and 152 nF, respectively. The fitted values of resistances are $R_{sol} = 21$ Ohm and $R_{surf} = 2.4$ kOhm, which is consistent with what one would expect from ionic theory. The PBS solution is more conductive than DI water because there are large quantities of mobile ions present in the
system (concentration of electrolyte ~ 0.15 M). These ions can also freely adsorb at the solid-liquid interface which is why the values of the surface conductance and capacitances are also greater.

### 4.4.1 Effect of Operating Parameters

After designing an operating procedure and developing a fair understanding of the electrical cell, our next goal was to fully characterize the sensor by investigating in detail the different parameters that can affect its performance. The experimental parameters which were recognized to influence the impedance spectra were frequency of the applied electric field, sedimentation time, particle concentration and particle size. The effects of each of these operating parameters are described in detail below. Since impedance responses of many different experiments are compared, all results are reported as % impedance change from the reference PBS solution.

**(i) Effect of Frequency.** The stability of the immunosensor was first tested over the entire 1 Hz – 1 MHz frequency range by monitoring its impedance response to DI water and PBS solution over 90 min. Both measurements showed significant drift over time below 0.1 MHz (Figure 4.4). We believe this is due to the swelling of the epoxy-based adhesive polymer or its slow diffusion into the flow-chamber. The drift was negligible above 0.1 MHz. Therefore, 0.1 – 1 MHz was chosen to be a suitable frequency range for operating a stable sensor and all future investigations were performed at these conditions. Previous studies involving zeta potential measurements of latex particle also pointed out that $0.1 \text{ MHz} < f < $
10 MHz frequency range is most suitable for impedance spectroscopy because it is least susceptible to electrode polarization effects.\(^{20}\)

(ii) **Effect of Sedimentation Time and Particle Concentration.** Once the appropriate frequency range for operating the immunosensor was identified, different latex-IgG suspensions of particle concentrations ranging from 0.06 - 3.25 % w/v were prepared. The particles in each suspension were allowed to freely sediment on the microelectrode surface for 90 min and the change in impedance at 1 MHz was plotted as a function of time for each concentration (Figure 4.5a). In the beginning, when the particles were fully dispersed, the impedance of all latex-IgG suspensions was lower than the impedance of the physiological buffer media alone (i.e., negative on the y-axis at t = 0 min). This observation is consistent with the one reported previously in other impedance studies.\(^{21}\) As the particles accumulated on the surface with time, the impedance of the more concentrated suspensions (> 0.44 % w/v) increased monotonically at first and then eventually leveled off. On the other hand, impedance decreased for the more dilute samples (< 0.44 % w/v). At 0.44 % w/v, which also happens to be the approximate concentration at which 1.0 \(\mu\)m particles form a monolayer at the surface upon complete sedimentation, the impedance remained more or less constant.

Analogous results were obtained when similar experiments were repeated with 1.0 and 2.0 \(\mu\)m non-functionalized sulfate-stabilized latex particles (Figure 4.6). The outcomes indicate that the impedance experiments are reproducible at high frequencies. The presence of IgGs on the particle surface has little effect on the impedance response. Therefore, non-
conjugated latex microspheres were used as model particles for all the subsequent characterization experiments instead of latex-IgG particles.

To understand why the impedance changes with the particle accumulation at the surface, we fitted the impedance data obtained for 3.25 % w/v latex-IgG suspensions to our PBS model (Figure 4.7). The fitting shows the evolution of the constant phase elements, $Q_{DI}$ and $Q_{DL}$, during the sedimentation of particles. Both phase angles are obtained to be around $-80^\circ \ (n \approx 0.9)$ and remain constant during the process. The capacitance value of $Q_{DL}$ drops by almost 60 %, which implies that the largest effect of the sedimenting latex particles is their interference with the electric double layer. Similarly, the time-dependent results for $R_{sol}$ and $R_{surf}$ indicate that there is more resistance to current flow due to collection of particles between the electrode surfaces. Therefore, it is not the bulk of the solution but the events taking place near the surface which control the impedance change of the entire system.

Next, we aimed to resolve the reason for the sudden change in slope above and below the monolayer concentrations. For this purpose, the impedance data obtained with 1 and 2 µm latex suspensions was plotted as a function of particle concentration as shown in Figure 4.5b. In both systems the overall impedance increased with increasing concentration but the curves displayed characteristic localized peaks near approx. monolayer concentrations 0.44 and 0.85 % w/v, respectively. The unique footprint of monolayer concentrations was also accompanied by an unexpected change in the relative impedance values of the two curves; the impedance of 1 µm suspension was greater below and smaller above these monolayer concentrations as compared to the 2 µm sample. Based on our current understanding of the
system we speculate that the efficient homogeneous packing of the latex particles causes maximum hindrance to current flow but further investigation is required in this area. In general, the results appear promising for analyzing various physical properties of colloidal suspensions, such as the composition of heterogeneous samples.

(iii) Effect of Particle Size. The effects of sedimentation time and particle concentration highlight the importance of changes in the double layer interactions due to the accumulation of particles between the electrodes. The stability and homogeneity of the electric double layer, influenced by the proximity of the particles to the surface, can also depend on the size of the latex particles. We studied the effect of particle size by measuring the impedance of 1, 2, 4 and 8.7 µm sized latex suspensions at 1 MHz and plotting them as a function of particle surface area (Figure 4.8). The latex concentrations were fixed at 0.44 % w/v and therefore, all colloidal suspensions had only particles enough to form either a complete or sub monolayer. We observed that the impedance decreased monotonically with increasing particle size, which is in good agreement with our results (Figure 4.5b).

4.4.2 Electrochemical Impedance Measurements of Latex Agglutination Tests

After establishing the role of operating parameters in the sensor performance, we now focus our attention towards utilization of EIS technique for characterizing latex agglutination tests. The aggregation of IgG-conjugated particles using complementary antigens has been well studied.9-11 A particle agglutination test should possess the following: (i) the latex suspension should not be under or oversaturated with antigens; (ii) the particle size and sample volume should be commensurate with the light scattering technique used for detection; and (iii)
particles should be adequately small to allow fast diffusion in suspension and sufficiently large to have as many binding sites per particle as possible. Agglutination tests are typically carried out with 0.1 – 1 µm sized particles and often require that the concentration of antigens be optimized experimentally.

We carried out an empirical study to determine the optimal antigen concentration needed to agglutinate 3.25 % w/v latex-IgG particles. The results showed this concentration to be ~ 1 µg mL\(^{-1}\) (see Table 4.1) and the entire agglutination process took place in approx. 4.5 h. Since, the kinetics of agglutination in suspension is opposed by the fast sedimentation of the latex-IgG particles inside the thin flow chamber (in less than 90 min), optimizing the agglutination process becomes a key step towards enhancing the immunosensor performance. The height of the chamber in our experiment setup is thin to obtain more rapid results. Alternatively, a signal of higher strength could possibly be attained by either increasing the chamber thickness or decreasing the particle size, but in both cases the response times are expected to be longer.

The kinetics of aggregate growth rate in the first 90 min visualized by optical microscopy are shown in Figure 4.9. From these images it is apparent that the latex-IgGs are not fully agglutinated during this time and an assortment of aggregated structures exists (dimers, trimers and so on). Even though the size of agglutinated clusters is small, it causes a detectable change in impedance when the agglutination assay is carried out on top of micropatterned electrodes. Figure 4.10 shows the time dependent impedance response of the sensor at 1 MHz. The impedance of the latex-IgG particles decreases by almost 5 % in the presence of antigens consistent with our particle concentration and particle size results.
The main reason for the quantitative impedance shift in Figure 4.10 was speculated to be related to the small differences in the morphology of the agglutinated and non-agglutinated latex-IgG particles that can significantly modify the rate at which the electrode surface area gets occupied by the particles. For further independent evidence to confirm this hypothesis we carried out supporting experiments in which latex-IgG suspensions containing as low as 0.06 % w/v particles were agglutinated and the process was observed using a Total Internal Reflection Fluorescence (TIRF) microscope. TIRF microscopy technique was used instead of regular optical microscopy because it allows the investigation of the changes taking place within a very thin region of the sample, usually 100 nm above the glass surface, by selectively illuminating and exciting the fluorophores in that area. The excitation is produced by using evanescent waves that are generated only when the incident light is totally reflected at the glass-water interface (Figure 4.11a).

The results of the TIRF experiments shown in Figures 4.11b and c illustrate that the fluorescence intensity of the sedimenting latex-IgG particles gets saturated faster when there are no antigens present. This result is somewhat counterintuitive as it wrongly suggests that the surface concentration and sedimentation rate of non-agglutinated latex-IgG particles are higher than agglutinated ones, which is practically not possible. To interpret these results correctly, one must consider the total surface area covered by the particles. Due to the presence of antigens in the system, latex-IgG particles get agglutinated into bigger agglomerates that sediment rather quickly. However, only a fraction of the particles in the precipitated aggregates contribute towards the fluorescence intensity signal because they are in near vicinity of the surface (see Figure 4.1). Non-agglutinated particles on the other hand
are more homogeneously distributed on the surface and therefore, generate a stronger fluorescent intensity signal. This is the same rationale why the impedance of smaller sized latex suspensions is stronger than the larger ones. The TIRF results are also consistent with the impedance data in Figure 4.10.

Based on our observations it is evident that immunodetection via latex agglutination and TIRF microscopy has potential for use in biosensors. However, among the many requirements for designing an efficient biosensor are not only to assemble a miniaturized device that is sensitive and produces rapid results but also one that has the capacity to be fully automated, parallelized and integrated into micro-Total Analysis Systems (\(\mu\)-TAS). Electric fields are better suited for use in such devices. In the following section, we discuss how the interfacing of EIS technique with LATs can be used for designing simple and semi-automated immunosensors.

### 4.4.3 EIS-Agglutination based Programmable Immunosensors

In order to utilize LATs as functional and electronic immunosensors, it is necessary to formulate a general procedure that can accept or reject a sample based on a simple criterion. One of the many conditions that satisfied this requirement is the change in slope of the impedance curve at high frequencies. For example, when antigens were added to the 3.25 % w/v latex-IgG suspension, the second derivative of the slope of the impedance curve lying between 0.1 MHz and 1 MHz changed from negative to positive within the first 10 min (Figure 4.12). The slope did not change if there were no antigens present. This property of the sensor, wherein, the presence of antigen is displayed as a simple yes or no type digital
response could be used in components of μ-TAS for applications like separations, clinical and forensic analysis and point-of-care clinical diagnosis.23

To determine the threshold particle concentration beyond which there will be no discernable signal in 10 min, we performed experiments with several different concentrations of latex-IgG suspensions (0.06 – 3.25 % w/v). The results from a few of these experiments are presented in Figure 4.13. No detectable change was recorded below 2.05 % w/v latex-IgG concentration. Thus, the optimal antigen concentration at this condition, i.e., 0.63 μg mL⁻¹ was considered the lowest limit of detection (LOD) for the immunosensor. This LOD is not as sensitive compared to some of the other immunoassay techniques reported in the literature.24-27 Nevertheless, the sensor appears applicable as a substitute to most of the other qualitative latex agglutination tests available today for detection of specific diseases and pathogens.28, 29 A number of possibilities for the future improvement and optimization of the method exist, such as exploring other types of biomolecule-ligand pairs which have faster binding kinetics, performing multiple experiments simultaneously on a single chip, and possibly increasing the sensitivity by going to higher frequencies.

4.5 Conclusions

We have demonstrated a novel approach for detecting results of latex agglutination tests by using the EIS technique. The exploratory results show that the addition of minute amounts of antigen to the antibody-conjugated latex suspensions brings about significant qualitative changes in the impedance signal within 10 min. This result could allow using the digital type yes or no response of the immunosensor in components of μTAS. As the sensors are
assembled from commercially available particles, the principle of detection can be universally extended to most other assays involving highly specific biomolecular interactions (e.g. DNA, streptavadin/biotin, sugar/lectin etc.).

EIS based detection of sedimenting particles also provides a useful platform for characterizing different physical properties of colloidal suspensions, such as concentration, size and composition of the constituent particles. The detection mechanism is, in principle, analogous to the one of currently available high speed single cell/particle counting devices,\textsuperscript{30} e.g. the coulter counter, but there are additional advantages for commercial applications. For instance, the technique is scalable as focusing of particles to a small detection volume is not required. This unique feature of the device has major potential to be utilized as a simple alternative to the current light scattering techniques used for analyzing voluminous colloidal suspensions. While quite a few studies have appeared that reported on using impedance for measuring properties (electro-kinetic mobility, zeta potential, particle behavior \textit{etc.}) of dispersed latex particles,\textsuperscript{20, 21, 31} the research on impedance change due to sedimented particles is still in its infancy.

\textbf{4.6 Acknowledgements}

We are grateful to Dr. Sonia Grego from RTI International, NC for providing the micro-patterned electrode chips. We thank Dr. Peter Fedkiw and Rameshwar Yadav for access to the EIS equipment. We also appreciate the help of Dr. Jason Haugh and Michael Weiger in the usage of TIRF microscope. This study was supported by the grants from the National Science Foundation (NSF).
Figure 4.1 Schematics of the principle of latex agglutination tests (LATs) and the various methods used for detecting their results.
Table 4.1 A list of latex-IgG concentrations used in the experiments. The values on the right show the optimal antigen concentrations needed for their agglutination.

<table>
<thead>
<tr>
<th>Latex-IgG Concentration (% w/v)</th>
<th>Optimal Antigen Concentration (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.25</td>
<td>1</td>
</tr>
<tr>
<td>2.05</td>
<td>0.631</td>
</tr>
<tr>
<td>1.25</td>
<td>0.385</td>
</tr>
<tr>
<td>0.58</td>
<td>0.179</td>
</tr>
<tr>
<td>0.44</td>
<td>0.135</td>
</tr>
<tr>
<td>0.06</td>
<td>0.018</td>
</tr>
</tbody>
</table>
Figure 4.2 Experimental setup (not drawn to scale). **Top view:** Design of glass chip with the micropatterned interdigitated electrodes. Electrodes 1 and 3 are energized against electrodes 2 and 4. Dotted line marks the inner edge of the microfluidic chamber. **Side view:** Sample is loaded into the microfluidic chamber encapsulating the interdigitated electrodes. The inlet and outlet ports are sealed to avoid evaporation. Sedimentation of particles is measured using electrochemical impedance spectroscopy (EIS).
Figure 4.3 Fitting of the impedance data to an equivalent circuit model: (a) DI water and (b) physiological media. Constant phase elements \(Q_{DL}\) and \(Q_{DI}\) represent the non-ideal capacitive behavior of the electric double layer and dielectric, respectively. \(R_{sol}\) and \(R_{surf}\) represent the resistances of the solution and surface, in that order.
Figure 4.4 Impedance spectra obtained with DI water and physiological buffer medium. The significant drift in impedance below 0.1 MHz frequency results possibly due to the swelling of the adhesive polymer or its slow diffusion into the flow chamber.
Figure 4.5 Effect of (a) sedimentation time and (b) particle concentration on the impedance spectra at 1 MHz. The results in (a) are reported for 1 µm sized latex-IgG particles. The results in (b) are shown for 1 and 2 µm latex after the particles have sedimented completely in 90 and 30 min, respectively. Characteristic local peaks in impedance are registered near the monolayer concentrations of the two particles.
Figure 4.6 Effect of sedimentation time on the impedance spectra at 1 MHz for (a) 1 µm and (b) 2 µm sized latex particles. The pattern of impedance change is similar to that of latex-IgG particles.
Figure 4.7 Time dependent impedance response fitted to the PBS model as 3.25 % w/v latex-IgG particles sediment and accumulate on the electrode surface. (a) Equivalent circuit model. (b) Evolution of the angles and capacitive parts of the constant phase elements for the electric double layer and dielectric. (c) Evolution of the solution and surface resistances. The fitting is performed for impedance data between 1 Hz – 1 MHz.
Figure 4.8 Effect of particle size on the impedance spectra at 1 MHz. The readings are taken for 1, 2, 4 and 8.7 µm latex after the particles have sedimented completely in 90, 30, 10 and 5 min, in that order. The concentration of latex particles is 0.44 % w/v in all cases. Note that 0.44 % w/v concentration corresponds to the monolayer coverage of 1 µm latex particles.
Figure 4.9 Time-lapsed optical microscopy images showing how the agglutination of 3.25 % w/v latex-IgG particles with 1 μg mL⁻¹ antigen proceeds in (a) 1 min, (b) 30 min, (c) 60 min, and (d) 90 min. These images are acquired after diluting the sample 100x and dispensing 10 μL of it on a glass slide under a glass cover slip.
**Figure 4.10** The dynamic change in impedance at 1 MHz before and after antigen (1 \( \mu \text{g mL}^{-1} \)) is added to the latex-IgG suspension (3.25 % w/v). Addition of antigen alone has little effect on the impedance at this frequency.
Figure 4.11 Detection of latex agglutination results using Total Internal Reflection Fluorescence (TIRF) microscopy. (a) Schematics of the principle of TIRF microscopy. (b) A timed comparison of the fluorescence intensity as latex-IgG particles (0.06 % w/v) sediment in the absence and presence of antigen (0.018 µg mL⁻¹). The intensity gets saturated much faster without antigens. (c) Average fluorescence intensity of the sedimenting particles plotted as a function of time.
Figure 4.12 Qualitative detection of antigen by the second derivative method. (a) Impedance spectra measured between 0.1 – 1 MHz as the latex-IgGs (3.25 % w/v) sediment in the presence and absence of antigens during 90 min. (b) Second derivative of the impedance signal changes from negative to positive in the presence of antigens - ideal behavior for a biosensor. The qualitative response of the sensor is registered equally well at 10 and 90 min. [Note: scales different on Y-axes].
Figure 4.13 Performance of the impedance based immunosensor at 10 min for three different concentrations of latex-IgG suspensions: (a) 2.05 % w/v, (b) 1.25 % w/v, and (c) 0.06 % w/v. The corresponding antigen concentrations are 0.631, 0.385, and 0.018 µg mL⁻¹, respectively. Immunosensor works effectively only for high particle concentrations.
4.7 References


Chapter 5

Summary and Outlook
5.1 Summary

The theme of this Dissertation is the use of colloidal particle assemblies to develop new types of electrically functional biosensors on a chip. Three specific topics were considered: 1) self-organization of surface-modified nanoparticles on an organic matrix composed of surface-tethered antibody layer, 2) directed co-assembly of live cells and surface-functionalized particles in suspension using AC electric fields, and 3) agglutination and detection of freely suspended surface-active microspheres on horizontal microelectrodes. The resultant structures were made permanent by irreversible biological binding. The common emphasis in all three projects was to understand the fundamentals of particle-particle interaction or particle interaction with the externally applied field. The routes of assembly, types of synthetic and biological particle suspensions, and geometries of the on-chip electrodes were investigated. Detailed modeling of the systems was carried out at each stage to identify the various controlling parameters. This knowledge proved enabling for fabricating optimized particle-based immunoassays, for controlled assembly of responsive composite biomaterials and for designing electronically programmable latex agglutination tests.

In the first project, we demonstrated the use of metallic nanoparticles as secondary labels in sandwich immunoassays. A receptor layer formed by the chemical immobilization of primary antibodies to the substrate was employed to bind complementary antigens from dilute samples. Antibody-conjugated gold nanoparticles were then self-assembled on this irreversibly bound two-dimensional layer of antigens on the surface. The gold particles assemblies provided a convenient tool for antigen detection. Using the well known silver enhancement technique they could be enlarged by almost 5 orders of magnitude and
visualized by naked eye. Quantification of results in terms of optical darkness ratio (ODR) was performed by analyzing the silver enhanced spots by transmitted-light optical microscopy. By silver nucleating a concentration gradient sample of gold nanoparticles affixed via surface-grafted APTES molecules, a direct correlation between the surface coverage by particles and ODR was established.

The silver enhanced spots were further used to survey the effect of various parameters governing the formation of a sandwich configuration assembly. The concentration and incubation times for each reagent were varied individually and the adsorption kinetics at each binding step was matched against a mass-transfer model. This systematic characterization allowed us to optimize the overall performance of the assay and to understand the effect of factors controlling the binding process, such as steric hindrance, orientation and density of the surface receptors. On the basis of this analysis, a general semiquantitative algorithm was formulated that could be implemented to determine (a priori) the minimum operation time of any assay. In the future, the conductive silver patch could be interfaced with on-chip microelectrodes for direct electrical readout of the results. The electronic measurements could further allow parallel processing of a series of immunoassays on a single chip, each of which can be miniaturized below the size restrictions of conventional optical detection.

In the second project, we illustrated how the on-chip dielectrophoretic co-assembly of live cells and synthetic microparticles could be utilized to create a novel class of composite biomaterials combining the functionality of the cells and the particle. The key to the assembly of the cell-particle composites was the electric field-driven sequestration of the particles into the interstitial space between the cells. This process made possible the binding
of the cells into a permanent material. These new structures included freely suspended, permanently bound chains and one-layer thick membranes from live cells and particles. The cells in the biocomposites remained alive, while the composite materials possessed the functionality of the particles - e.g., they could be manipulated magnetically and eventually interfaced electrically. The physical origins of the sequestration effect were identified by performing a numerical simulation of the field distribution in the complex cell-particle system. The simulation compared favorably to the experimentally observed particle dynamics and pointed the way to a quantitative interpretation of such processes.

This work was predominantly exploratory and fundamental, but the types of functional biomaterials formed could eventually find application in sensors, microassays, microsurgery, or as responsive biomaterials. The electrically controlled assembly is rapid and precise and can be used for automated on-chip fabrication. The broader impact of this research related to how these methods of colloidal assembly could be transcribed into the biological domain. The field of colloidal assembly has been investigated intensively, but mostly using synthetic micro- and nanoparticles. The areas of applications of materials assembled from synthetic particles are relatively limited compared to the potential and functionality of cell-based biomaterials. Our findings show how live cells can be treated as building blocks and assembled into materials with a potentially rich area of applications.

The third project that we carried out explored a new and facile route to measure the results of latex agglutination tests (LATs) electronically. The agglutination of antibody-conjugated latex microspheres in the presence of complementary antigens took place inside a custom-designed thin chamber above horizontal interdigitated microelectrodes. As the
agglutinated particles sedimented, a unique impedance spectrum was detected using an electrochemical impedance spectroscopy (EIS). This impedance signal depended mainly on the agglutinated morphology and sedimentation rate of the latex particles. An equivalent circuit model was proposed for the system. It revealed that the impedance is governed mainly by the double layer interactions close to the electrode surface. Supporting optical and TIRF microscopy experiments helped us to gain insight into the kinetics of aggregate growth and sedimentation.

We observed that the slopes of impedance spectra at high frequencies changed from negative to positive after agglutination. This qualitative shift in the signal behavior proved to be a useful criterion to design digitally programmable immunosensors wherein, the presence of antigen could be detected as a simple yes or no type response. The results were obtained reproducibly and rapidly within 10 min. The lowest limit of antigen detection was 0.63 µg mL\(^{-1}\) which shows that there is still room for improvement and optimization. An in-depth characterization performed by studying the effect of different experimental parameters on the sensor performance, highlighted the potential of this technique to substitute the current light scattering methods used for analyzing physical properties of colloidal suspensions. The electrical detection mechanism makes possible for this to be used in a micro total analysis system (µTAS).

5.2 Outlook: Rocky Road from Science to Engineering

In this Dissertation, we investigated the directed assembly of colloidal particles and synthesized microscopic structures with electrical and biological functionality. These
assemblies, however, represent only a small fraction of the useful structures that can be fabricated using colloidal assembly, an area which is now thriving and attracting significant public attention and research funding.¹ Inasmuch many of the structures assembled either include nanoparticles, nanowires and nanotubes, or possess interesting properties derived from their nanoscale structural features, colloidal assembly is considered to be one of the major fabrication tools in nanoscience and nanotechnology.²⁻⁷ Yet, the immense array of exciting achievements in research has not yet been matched by a commensurate progress in applications and manufacturing. To a large extent, this is because the area is still in its infancy, and the academic rewards in creatively developing new materials and methods outweigh the urge to consider more mundane tasks of reducing the discoveries to practice. However, in addition to this, there appear to be multiple and serious challenges that the materials and nanoscience researchers today need to face and solve on the road to manufacturing and commercialization of technologies based on particle assembly. We define and discuss some of the challenges here as viewed from our perspective.

The first major challenge facing the investigators today is the transition from synthesis and demonstration to engineering and process design. Many of the materials reported to date have been obtained in small domains or small volumes. While it may be relatively easy to assemble a structure of certain type on the scale of millimeters to centimeters, controlling and repeating the process over and on large scales might be much more problematic. The small sizes and large specific area of the particles means that the assembly processes in liquids are governed by colloid and surface interactions, including van der Waals, electrostatic, steric and hydrophobic.⁸ These interactions are usually difficult to
adjust precisely and thus the control of the assembly process can be difficult and unreliable. Another common problem with such processes is the relatively large times (minutes to hours) required for the assembly, which most often are needed because the particles have to come together slowly, re-arrange and form an organized structure. Such processes typically can't be speeded up easily as they are kinetically restricted by the relatively small diffusion rates of the particles.

A few other major problems with the synthesis methods arise when considering the related issues of time, scalability and cost. As most of the materials synthesis processes yield products of microscopic size, scaling up to the usual volumes of materials fabrication might not be straightforward, and in some cases can be a daunting challenge. The increase of the size of the fabrication device might also be problematic due to the emergence of thermal and chemical gradients in the system and the related tasks of control and maintenance of uniform process conditions. The scalability can also be impeded by the slow speed of assembly. Processes that take a long period to complete are not suitable for industrial fabrication. In general, the scalability and cost of fabrication appear to severely limit the number of applications of particulate materials. In that regards, it should be pointed out that not all of the proposed or perceived applications of the nanostructures and materials formed by colloid assembly are well defined and viable.

The evaluation of the future potential of any structure and technology is difficult and somewhat arbitrary process. Still it makes sense to try and assess the technology potential of some of the structures made by colloidal assembly and to seek and identify some general trends that may help in bringing these materials closer to practice. We attempt to classify and
compare some of the most intensively studied materials in terms of cost of fabrication, which includes synthesis of particles and process of assembly (Figure 5.1). On the other axis of the graph we plot the expected value added of the materials after the assembly, based on the projected complexity and cost of the technology where they will be applied. In order to "calibrate" the graph, we have also included the expected domain positions of three technologies widely used in practice, the synthesis of simple inexpensive particles, such as polymeric spheres, the synthesis of more complex synthetic particles, such as ones with physical or chemical anisotropy,\(^9\)\(^{-11}\) and the common photolithography based fabrication of electronic microcircuits on a chip.

The position of the domains reflects a few trends in materials manufacture costs. As a rule, the cost of fabrication of well-ordered structures is higher because of the higher cost of monodisperse suspensions, the longer time required for the assembly and the need to reduce or remove defective structures. Amongst the well-defined colloidal architectures, structures with lower dimensionality have higher fabrication costs since they require larger effort and longer time for assembly. These materials are, however, of greater commercial value due to the growing demand of 1D wires in hi-tech applications like electrical circuits and sensors. To bridge the existing gap between science and engineering, one needs to focus on the creation of high value added materials with low cost of fabrication. One way to achieve this goal, in our perspective, is to use naturally occurring “smart” colloidal particles, such as live cells, since they are ubiquitous, they possess advanced functionalities to perform bio related operations and, only a few cells can effectively carry out the same task that would otherwise require many synthetic particles. We demonstrated how live cells can be treated as building
blocks and assembled into functional biomaterials with potential applications in sensors, prosthetics and drug delivery systems. This approach is by no means absolute or complete. The rapidly advancing discipline of nanoscience is forever cultivating new ideas that can change the course of research altogether. We can only hope that the work presented in this Dissertation will contribute at least an inch towards moving closer to this vision.
Figure 5.1 A hypothetical evaluation of the value added for different types of colloidal assemblies vs. the cost of their fabrication. Materials positioned to the left and the top side of the graph are more likely to find practical applications. The likely positions of simple and complex synthetic particles and microelectronic chips are shown for comparison. We demonstrate how high value added bioelectronic materials can be assembled on a chip using directed assembly of biological and colloidal particles.
5.3 References

(1) Latest funding trends indicating growing interest in active nanostructures can be found at the NSF National Nanotechnology Initiative (NNI) website: http://www.nsf.gov/crssprgm/nano/


Appendix A

Protocols Used for Conjugating Proteins to Colloidal Particles
A.1 Chemical Conjugation of Concanavalin A Lectin to 1.0 µm Fluorescent Aldehyde/Sulfate Latex Microspheres

A.1.1 Reagents

All of the following reagents were purchased from Interfacial Dynamics Corp., OR: 0.025 M phosphate buffered saline at pH 6.2 (henceforth referred to as PBS (I)); 0.1 M phosphate buffered saline at pH 7.2 (henceforth referred to as PBS (II)); storage buffer containing 0.1 M phosphate buffered saline at pH 7.2, 0.1 % glycine and 0.1 % NaN₃.

Note: Glycine is used in the storage buffer to fill any reactive sites on the surface of the microspheres which have not been covered by the protein. This is to reduce non-specific binding. Bovine Serum Albumin (BSA) may be used for the same purpose if desired. The NaN₃ is present as a biocide. If the latex is kept sterile, NaN₃, which is not compatible with cell or tissue culture, can be omitted.

A.1.2 Preparation of Latex Suspension

(1) 0.5 mL of 2 % w/v 1.0 µm aldehyde/sulfate latex microspheres in DI water were homogeneously mixed with 1 mL of PBS (I).

(2) The suspension was centrifuged at 3000 g for 20 min using Marathon micro-A centrifuge (Fisher Scientific, PA).

(3) The supernatant was removed and the pellet was resuspended in 1 mL of PBS (I).

(4) The suspension was centrifuged again at 2700 g for 20 min.

(5) The supernatant was removed and the pellet was resuspended in 0.5 mL PBS (I). The concentration of the latex suspension was now ~ 2 % w/v solids.
A.1.3 Functionalization of Latex Microspheres with Concanavalin A Lectin

(1) A 0.1 % w/v solution of lectin was prepared in 0.5 mL of 1:1 v/v mixture of PBS (I) and PBS (II) (from here on, 1:1 v/v mixture of PBS (I) and PBS (II) mixture will be referred to as PBSS).

(2) 0.5 mL of the 0.1 % w/v lectin solution in PBSS was mixed with an equal volume of 2 % w/v latex suspension in PBS (I). [Note: This lectin concentration is ca. 200 % of that required for complete monolayer coverage of the particle surface. This order of addition will ensure the best coating of the particles with the least possibility of aggregation].

(3) The latex-lectin mixture was incubated overnight with constant gentle mixing at room temperature.

(4) The mixture was centrifuged at 3000 g for 20 min to remove any unbound lectins from the lectin-conjugated latex microspheres.

(5) The supernatant was removed and retained for concentration determination.

(6) The pellet was resuspended in 10 mL of PBS (II) and the suspension was centrifuged again to sediment the lectin-functionalized latex microspheres.

(7) Step 6 was repeated twice to obtain a total of 3 washes.

(8) Finally the lectin-functionalized latex microspheres were resuspended in 1 mL of storage buffer to obtain a final concentration of 1 % w/v.

(9) The suspension was stored at 4°C until used. [Note: Do not freeze].
A.2 Chemical Conjugation of Concanavalin A Lectin to 1.8 µm Amine-Terminated Magnetic Iron Oxide Particles

A.2.1 Reagents

All reagents were purchased from Bangs Laboratories, Inc., IN. They are listed in Table A.1.

A.2.2 Activation of Magnetic Particles

(1) 5 mL of amine-terminated 1.8 µm magnetic particles (as supplied) were transferred to a reaction flask that could easily contain the maximum volume of 50 mL to be used in the next coupling procedure. (A 50 mL tissue culture flask or conical tube is typically used).

(2) Coupling Buffer was added to the above suspension to obtain a final mixture volume of 25 mL and the mixture was shaken vigorously. The mixture was centrifuged at ~ 8000 g for 10 min. The supernatant was removed leaving the magnetic particles as a wet cake on the container wall. The magnetic particles concentration should now be 1 % w/v.

(3) Step 2 was repeated three times.

(4) The total amount of the suspension was separated into 2 tubes of equal volumes. One tube was refrigerated in the Coupling Buffer for future use. The activation protocol was followed with the second tube (~ 10 mL with concentration of 1 % w/v).

(5) 5 mL of 5 % w/v glutaraldehyde was added to the wet cake and the mixture was shaken vigorously. The mixture was then rotated at room temperature for 3 h.

(6) The magnetic particles were pulled to one side perpendicular to the gravity using an external magnet and the unreacted glutaraldehyde was separated.

(7) Step 2 was repeated three times.
A.2.3 Functionalization of Magnetic Microspheres with Concanavalin A Lectin

(1) A 0.1 % w/v solution of lectin was prepared in 0.5 mL of PBSS.
(2) 4 mg of lectin was added to 1 mL of Coupling Buffer to obtain a lectin concentration of 0.4 % w/v.
(3) 75 µL of the lectin solution was removed and added to 1 mL of Coupling Buffer. It was labeled as Pre-Coupling Solution and set aside for coupling efficiency determination.
(4) 1 mL of 0.4 % w/v lectin solution was added to 5 mL of 1 % w/v glutaraldehyde-activated magnetic particles from step 7 in A.2.2.
(5) The mixture was shaken vigorously and rotated for 16 - 24 h at room temperature.
(6) The magnetic particles were pulled to one side perpendicular to the gravity using an external magnet and the supernatant was separated. The supernatant was labeled and set aside for coupling efficiency determination.
(7) 5 mL of Glycine Quenching Solution was added to the above suspension. The mixture was shaken vigorously and then rotated for 30 min at room temperature.

A.2.4 Washing and Dilution of the Conjugated Magnetic Particles

(1) The magnetic particles were pulled to one side perpendicular to the gravity using an external magnet and the supernatant was separated.
(2) 50 mL of Wash buffer was added and the mixture was shaken vigorously.
(3) The above two steps were repeated three times. (The supernatant was saved each time).
(4) The 1 % w/v conjugated magnetic particles were stored in the refrigerator at 2 - 8°C as a suspension in the Wash Buffer. Do not freeze or dry. [Please note: The conjugated
magnetic particles will settle with prolonged storage and should be shaken vigorously each time before use.]

**A.2.5 Coupling Efficiency**

(1) The spectrophotometer (Jasco V550) wavelength was set to 280 nm. A blank reading was taken with the Coupling Buffer.

(2) The absorbance of the Pre-Coupling Solution from step 3 in A.2.3 was measured. A further dilution may be necessary to read an absorbance depending upon the amount of lectin added.

(3) The absorbance of the Post-Coupling Supernatant from step 4 A.2.3 was measured. A further dilution may be necessary to read the absorbance.

(4) The coupling efficiency was calculated as the % lectin uptake using the formula shown below:

\[
\text{OD}_{280\text{Pre-Coupling Solution} \times (D)} - \text{OD}_{280\text{Post-Coupling Supernatant} \times (D)} \times 100
\]

\[
\text{OD}_{280\text{Pre-Coupling Solution} \times (D)}
\]

Here, \(D\) = Dilution Factor. The typical % lectin uptake is > 60.

**A.3 Physical Adsorption of Fibronectin to 1.0 \(\mu\text{m}\) Sulfate-Stabilized Latex Microspheres**

(1) A suspension containing 1 % v/w concentration of 1.0 \(\mu\text{m}\) sulfate-stabilized latex microspheres was prepared in DI water.
(2) The latex suspension was washed twice by centrifuging at 3000 g for 20 min using Marathon micro-A centrifuge (Fisher Scientific) and replacing the supernatant with DI water.

(3) 10 µL of 0.1 % w/v fibronectin solution were homogeneously mixed with 0.5 mL of the latex suspension.

(4) The mixture was vortexed for 1.5 h and then refrigerated overnight.

(5) The unbound fibronectin protein was removed by centrifuging the suspension at 3000 g for 20 min and replacing the supernatant with 0.1 mM PBS solution.

(6) 5 µL of 10 % w/v bovine serum albumin was added to the final suspension to prevent non-specific aggregation of the fibronectin-conjugated latex particles.
Table A.1. A list of reagents used for conjugating Concanavalin A lectin to amine-terminated magnetic iron oxide particles.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
<th>Materials</th>
<th>Preparation Instructions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coupling Buffer</strong></td>
<td>0.01 M pyridine</td>
<td>0.8 mL pyridine</td>
<td>Add 0.8 mL pyridine to 900 mL distilled water. Adjust to pH 6.0 with 6 N HCl. Fill to 1 L with water.</td>
</tr>
<tr>
<td><strong>Glycine Quenching Solution</strong></td>
<td>1 M glycine</td>
<td>7.5 g glycine</td>
<td>Dissolve 7.5 g glycine in 90 mL distilled water and adjust to pH 8.0 with 10 N NaOH. Fill to 100 mL with water.</td>
</tr>
<tr>
<td><strong>Wash Buffer</strong></td>
<td>0.01 M Tris 0.1 % NaN₃ 0.1 % w/v BSA 0.15 M NaCl 0.001 M EDTA</td>
<td>1.21 g Tris 1.0 g NaN₃ 1 g BSA 8.7 g NaCl 0.37 g EDTA</td>
<td>Dissolve solids in 900 mL distilled water. Adjust to pH 7.4 with 10 N NaOH or 6 N HCl as required. Fill to 1 L with water.</td>
</tr>
</tbody>
</table>
A.4 References

(1) Protocol adapted from Interfacial Dynamics Corp. (OR) - “Coupling of Proteins to IDC UltraClean Aldehyde/Sulfate Latex”.
(2) Protocol adapted from data Sheet #546 provided by Bangs Laboratories, Inc. (IN).
(3) Protocol provided by Molecular Probes (OR) - “Working with FluoSpheres Fluorescent Microspheres”.
Appendix B

Polarizability Calculations using Multi-Shell Models
B.1 Multi-Shell Model for Yeast Cells

We developed a numerical procedure for evaluating the effective polarizability of yeast cells as a function of frequency. In this model, the multi-shelled structure of the yeast cell was substituted by a homogeneous sphere of complex permittivity $\varepsilon'' = \varepsilon_1'' - \frac{j}{\omega} \sigma_1''$ (Figure B.1b), the effective polarizability of which was equivalent to that of the original yeast cell (Figure B.1a). The procedure includes the following steps:

1. The complex permittivities of the various shells comprising the yeast cell were expressed as follows:
   
   **Cytoplasm:**  
   \[ \varepsilon_2 = \varepsilon_2 - \frac{j}{\omega} \sigma_2 \]  
   \[ (1) \]
   
   **Membrane:**  
   \[ c_m = c_m - \frac{j}{\omega} g_m \]  
   \[ (2) \]
   
   **Cell wall:**  
   \[ \varepsilon_1 = \varepsilon_1 - \frac{j}{\omega} \sigma_1 \]  
   \[ (3) \]
   
   **Counter-ionic layer:**  
   \[ \varepsilon_{EDL} = \varepsilon_{EDL} - \frac{j}{\omega} \sigma_s \]  
   \[ (4) \]

2. The complex permittivities of the cytoplasm and the membrane were replaced with a homogeneous sphere of equivalent complex permittivity.

3. The complex permittivities of the equivalent homogeneous sphere in step 2 and the cell wall were replaced with a homogeneous sphere of equivalent complex permittivity.
4. The complex permittivities of the equivalent homogeneous sphere in step 3 and the thin counter-ionic layer were replaced with a homogeneous sphere of equivalent complex permittivity.

The final expression for the complex permittivity of the yeast cell in terms of the shell parameters is given by equation (5). The values of each parameter are given in Table B.1.

\[
\varepsilon_1'' = \varepsilon_1 \left[ \left( \frac{R_o}{R} \right)^3 + 2 \left( \frac{\varepsilon_2' - \varepsilon_1}{\varepsilon_2' + 2\varepsilon_1} \right) \right] + \varepsilon_{\text{EDL}} \quad (5)
\]

where, \( \varepsilon_2' = \frac{c_m R \varepsilon_2}{c_m R + \varepsilon_2} \), \( \varepsilon_{\text{EDL}} = \frac{2\Delta\varepsilon_m}{R} \) and \( \sigma_s = 2\sigma_m R_o^{-1} \kappa^{-1} \exp\left( \frac{ze\xi}{2kT} \right) - 1 \)
B.2 Multi-Shell Model for Latex Particles

A model similar to the one described above for yeast cells was used to calculate the effective polarizability of latex particles in Figure B.2a. The equivalent conductivity of the simplified structure in Figure B.2b was given by equation (6). The values of each parameter are given in Table B.2.

\[ \sigma_p' = \sigma_p + \sigma_s \]

where \[ \sigma_s = \frac{\gamma \mu_{Na}}{R}. \]  

(6)
Figure B.1. (a) Multi-shell model for the polarizability of yeast cell. (b) Simplified model of yeast cell with effective polarizability equivalent to the multi-shell type structure in (a).
**Table B.1.** Numerical parameters for calculating effective polarizability of yeast (*S. cerevisiae*) cells.\(^1\,^2\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm dielectric constant</td>
<td>(\varepsilon_2)</td>
<td>(5.31 \times 10^{-10})</td>
<td>(\text{C}^2,\text{J}^{-1},\text{m}^{-1})</td>
</tr>
<tr>
<td>Cytoplasm conductivity</td>
<td>(\sigma_2)</td>
<td>0.5</td>
<td>(\text{S},\text{m}^{-1})</td>
</tr>
<tr>
<td>Membrane capacitance</td>
<td>(c_m)</td>
<td>0.01</td>
<td>(\text{F},\text{m}^{-2})</td>
</tr>
<tr>
<td>Membrane transconductance</td>
<td>(g_m)</td>
<td>0</td>
<td>(\text{S},\text{m}^{-2})</td>
</tr>
<tr>
<td>Cell wall dielectric constant</td>
<td>(\varepsilon_1)</td>
<td>(5.75 \times 10^{-10})</td>
<td>(\text{C}^2,\text{J}^{-1},\text{m}^{-1})</td>
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<tr>
<td>Cell wall conductivity</td>
<td>(\sigma_1)</td>
<td>0.1</td>
<td>(\text{S},\text{m}^{-1})</td>
</tr>
<tr>
<td>Inner radius</td>
<td>(R)</td>
<td>(2.0 \times 10^{-6})</td>
<td>(\text{m})</td>
</tr>
<tr>
<td>Outer radius</td>
<td>(R_o)</td>
<td>(2.5 \times 10^{-6})</td>
<td>(\text{m})</td>
</tr>
<tr>
<td>Double layer thickness</td>
<td>(\Delta)</td>
<td>(3.0 \times 10^{-9})</td>
<td>(\text{m})</td>
</tr>
<tr>
<td>Debye length</td>
<td>(\kappa)</td>
<td>(9.60 \times 10^{-9})</td>
<td>(\text{m})</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>(\zeta)</td>
<td>-30</td>
<td>(\text{mV})</td>
</tr>
<tr>
<td>Counter-ionic layer dielectric constant</td>
<td>(\varepsilon_{\text{EDL}})</td>
<td>(1.66 \times 10^{-12})</td>
<td>(\text{C}^2,\text{J}^{-1},\text{m}^{-1})</td>
</tr>
<tr>
<td>Counter-ionic layer conductivity</td>
<td>(\sigma_s)</td>
<td>(8.61 \times 10^{-5})</td>
<td>(\text{S},\text{m}^{-1})</td>
</tr>
<tr>
<td>Medium dielectric constant</td>
<td>(\varepsilon_m)</td>
<td>(6.9 \times 10^{-10})</td>
<td>(\text{C}^2,\text{J}^{-1},\text{m}^{-1})</td>
</tr>
<tr>
<td>Medium conductivity</td>
<td>(\sigma_m)</td>
<td>0.017</td>
<td>(\text{S},\text{m}^{-1})</td>
</tr>
<tr>
<td>Angular frequency</td>
<td>(\omega)</td>
<td>628</td>
<td>(\text{Rad},\text{s}^{-1})</td>
</tr>
<tr>
<td>Effective cell dielectric constant</td>
<td>(\varepsilon_i^{''})</td>
<td>(1.48 \times 10^{-8})</td>
<td>(\text{C}^2,\text{J}^{-1},\text{m}^{-1})</td>
</tr>
<tr>
<td>Effective cell conductivity</td>
<td>(\sigma_i^{''})</td>
<td>0.039</td>
<td>(\text{S},\text{m}^{-1})</td>
</tr>
</tbody>
</table>
Figure B.2. Models of latex microspheres in aqueous media: (a) Thin conductive counter-ionic layer surrounding the latex microsphere. (b) Simplified structure with effective polarizability equivalent to the shell type structure in (a).
Table B.2. Numerical parameters for calculating the effective polarizability of 1 µm latex particles. The values of these parameters were taken from Ref. [2] and from the information data sheet provided by the vendor, Interfacial Dynamics Corp. (OR).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle dielectric constant</td>
<td>(\varepsilon_p)</td>
<td>(2.26 \times 10^{-11})</td>
<td>(C^2 \cdot J^{-1} \cdot m^{-1})</td>
</tr>
<tr>
<td>Particle conductivity</td>
<td>(\sigma_p)</td>
<td>(1.0 \times 10^{-15})</td>
<td>(S \cdot m^{-1})</td>
</tr>
<tr>
<td>Particle radius</td>
<td>(R_p)</td>
<td>(5.0 \times 10^{-7})</td>
<td>(m)</td>
</tr>
<tr>
<td>Medium dielectric constant</td>
<td>(\varepsilon_m)</td>
<td>(6.95 \times 10^{-10})</td>
<td>(C^2 \cdot J^{-1} \cdot m^{-1})</td>
</tr>
<tr>
<td>Medium conductivity</td>
<td>(\sigma_m)</td>
<td>0.017</td>
<td>(S \cdot m^{-1})</td>
</tr>
<tr>
<td>Surface charge density</td>
<td>(\gamma)</td>
<td>(9.5 \times 10^{-2})</td>
<td>(C \cdot m^{-2})</td>
</tr>
<tr>
<td>Sodium ion mobility</td>
<td>(\mu_{Na})</td>
<td>(5.24 \times 10^{-8})</td>
<td>(m^2 \cdot S \cdot C^{-1})</td>
</tr>
<tr>
<td>Particle surface conductivity</td>
<td>(\sigma_s)</td>
<td>0.02</td>
<td>(S \cdot m^{-1})</td>
</tr>
<tr>
<td>Effective particle conductivity</td>
<td>(\sigma_p')</td>
<td>0.02</td>
<td>(S \cdot m^{-1})</td>
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</tbody>
</table>
B.3 References

