Spring 2004

Particle size dependence on immobilized molecular sensors : assay of nucleic acid probes attached to paramagnetic particles

Celin Sunny
New Jersey Institute of Technology

ABSTRACT

PARTICLE SIZE DEPENDENCE ON IMMOBILIZED MOLECULAR SENSORS: ASSAY OF NUCLEIC ACID PROBES ATTACHED TO PARAMAGNETIC PARTICLES

by

Celin Sunny

Nanotechnology is concerned with materials and systems whose structure and components exhibit novel and significantly improved physical, chemical and biological properties, phenomena and processes due to their nano scale size.

The transition from micro scale to nano scale leads to a number of changes in physical properties, possibly including new physical principles, some of which may be yet to be discovered. One of the major factors in this is the increase in ratio of surface area to volume.

This thesis research uses the increase in ratio of surface area to volume property of going from macro to micro to nano scale to pose the hypothesis that the increase in surface area to volume ratio as particle size gets smaller improves the functionality of immobilized molecular sensors.

In this thesis work we have successfully immobilized pseudo molecular beacons to paramagnetic particles of different sizes and shapes. The research has surprisingly concluded that the relationship between specific binding capacity and performance as measured by fluorescence binding capacity or performance labeling did not correlate inversely with particle sizes at fixed particle mass as originally postulated.

PARTICLE SIZE DEPENDENCE ON IMMOBILIZED MOLECULAR SENSORS: ASSAY OF NUCLEIC ACID PROBES ATTACHED TO PARAMAGNETIC PARTICLES

by

Celin Sunny

A Thesis
Submitted to the Faculty of
New Jersey Institute of Science and Technology
in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biomedical Engineering

Department of Biomedical Engineering

May 2004

APPROVAL PAGE

PARTICLE SIZE DEPENDENCE ON IMMOBILIZED MOLECULAR SENSORS: ASSAY OF NUCLEIC ACID PROBES ATTACHED TO PARAMAGNETIC PARTICLES

by

Celin Sunny

Dr. Michael Jaffe, Thesis Advisor — Date
Research Professor of Biomedical Engineering, NJIT

Dr. Roger Cubicciotti, Thesis Advisor — Date
President, Nanomedica Inc.

Dr. David Kristol, Committee Member — Date
Associate Chair and Professor, Biomedical Engineering, NJIT

Dr. Patricia Soteropoulos, Committee Member — Date
Director, Center for Applied Genomics

BIOGRAPHICAL SKETCH

Author: Celin Sunny

Degree: Master of Science

Date: May 2004

Undergraduate and Graduate Education:

- Master of Science in Biomedical Engineering,
  New Jersey Institute of Technology, Newark, NJ. 2004.

- Bachelor of Science in Biomedical Engineering
  Cochin University of Science and Technology

Major: Biomedical Engineering

Dedicated to my loving husband and dearest daughter

With Love.....

For all the countless ways you find to show how much you care!

ACKNOWLEDGMENT

I would like to thank to Dr. Michael Jaffe, my graduate advisor for providing valuable resources, insight, and intuition for my research. His constant support, encouragement and reassurance were instrumental in the completion of my thesis. His valuable advice and comments helped me in improving the quality of my work. I am especially grateful that he let me pursue collaborations and seek advice outside the laboratory.

I would like to express my deepest appreciation and gratitude to Dr. Roger Cubicciotti, President, Nanomedica Inc. who gladly agreed to also be my advisor and use the facilities at nanomedica for my research. He taught me the skills on how to conduct systematic research. He was always willing to find time to help me out amidst his busy schedule. I enjoyed working with him, and I especially valued his inspiration, insight and immeasurable support and encouragement.

I am indebted to Dr. David Kristol, for kindly agreeing to serve as a committee member and his support. Special thanks are given to Dr. Patricia Soteropolous for agreeing to participate as a committee member and for her advice and insights. I would like to also thank Dr. David Schwartz, Solulink Inc., for finding time to answer our queries.

Furthermore, my friends Shobana and Kimberely from the Biomedical Engineering department and especially Ms. Daya Sooryadas are deserving of recognition for their support.

Most of all, I would like to thank my dearest daughter, Vanessa Zacharia and my beloved husband, Mr. Ajumon Zacharia for their love, understanding, patience and immeasurable support.

TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

Chapter **Page**

LIST OF TABLES

LIST OF TABLES
(Continued)

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The development of sensitive specific devices (e.g., molecular sensors) for the detection, diagnosis and monitoring of diseases, drug discovery, environmental detection of pollutants and/or biological agent has been an active area of research for the past four decades. One of the most significant problems in developing reliable sensors is controlling and reproducing attachment of macromolecules, particularly biomolecules to inorganic substrates/materials.

This thesis research is designed to demonstrate the ability of nanotechnology to develop tools that allow characterization and detection of single cells, single molecules and single molecular events, e.g. DNA hybridization and protein-protein interactions etc.

This research work has successfully designed nucleic acid sequences as pseudomolecular beacons and immobilized them to paramagnetic particles of different sizes i.e. from 1 micron to ~ 9 micron. To different types of paramagnetic particles spherical particles and comet shaped particles were evaluated for use as attachment substrates. The oligos were immobilized using novel bioconjugation chemistry from Solulink Inc.

The long term goal of this work is to exploit the improved physical, chemical and/or biological properties of materials in nano scale by gaining control of the structures at atomic and molecular levels and learn to efficiently engineer smart devices.

This field of research requires attention to several emerging disciplines which are discussed in the next chapter.

CHAPTER 2

BACKGROUND

2.1 Nanobiotechnology

Nanobiotechnology is an emerging area of scientific and technological opportunity. It is an area that brings together the field of nanotechnology and biotechnology. Nanobiotechnology applies the tools and processes of nano/microfabrication to build devices for studying biosystems. This field illustrates the interdisciplinary nature of the thesis work, which incorporates elements of both nanotechnology and biotechnology.

2.2 Nanotechnology

Nano is a Greek prefix that defines the smallest (1000 times smaller than micrometer) natural structures or materials and nanotechnology, a most recent and highly energized discipline of science and technology that deals with manipulation and use of these structures/materials typically having the dimensions up to 100 nm with superior electrical, chemical, mechanical or optical properties [1].

One of the first to articulate futuristic nanotechnology concepts was nobel laureate Richard Feynman. In late 1959 he presented a lecture at the California Institute of technology titled "There is plenty of room at the bottom". He talked about the principles of nanotechnology even before the word existed. In his talk Feynman exposed his audience with a technological vision of extreme miniaturization.

The essence of nanotechnology is the ability to work at the molecular level, atom by atom, to create relatively larger and more complex structures with fundamentally new molecular organization. For development of commercial applications (as distinct from

basic research), such new molecular organization ideally enables or provides a useful function in which case the structure can be legitimately referred to as a "device".

The ability to engineer a nanoparticle, nanomaterial or nanodevice to utilize its property of small size and increased surface area to volume ratio brings the possibility of detecting a very small amount or low concentration of analyte molecules compared to materials in the macro or micro scale.

Nanometer sized objects possess remarkable self-ordering and assembly behaviors under the control of forces quite different from macro objects. These unique behaviors are what make nanotechnology possible, and by increasing our understanding of these processes, new approaches to enhancing the quality of human life will surely be developed.

Nanotechnology is expected to transform our lives in this decade. As scientists and engineers exploit the tools of nanotechnology to design closer to the molecular level, nanoscale materials engineering will have an increasingly important impact on a number of sectors, including biotechnology, electronics, energy, and industrial products. Nanotechnology should let us make almost every manufactured product faster, lighter, stronger, smarter, safer and cleaner [2].

Examples of advances in the application of nanotechnology are Advanced Computing – Nanoelectronic devices based on quantum dots, nano-wires and molecular switches will enable next-generation computer chips, Cancer Treatment – Nanoparticles are being developed for the targeting and destruction of breast cancer cells, Energy Storage – Cathodes fabricated from nanomaterials promise rechargeable batteries with longer lifetimes, Engineered Textiles – Nanofibers improve the properties of lightweight

protective gear for public safety and defense professionals, Environment – Nanomaterial-based photocatalysts clean the environment and yield surfaces with "self-cleaning" properties, Packaging – Nanocomposite barrier plastics can increase the shelf life of various foods and beverages, Pharmaceuticals – Antimicrobial nanocoatings on wound dressings kill bacteria, reduce inflammation and promote healing.

The nanoscale offers the potential for orders of magnitude improvements in sensitivity, selectivity, response time, and affordability. As surfaces get smaller there is increased surface area to volume ratio. This thesis research is interested in the ability of nanotechnology to develop tools that allow characterization and detection of single cells, single molecules and single molecular events, e.g. DNA hybridization and protein-protein interactions etc.

2.2.1 Nanomaterials

Nanomaterials have numerous commercial and technological applications, including analytical chemistry; drug delivery; bioencapsulation; and electronic, optical, and mechanical devices. In addition, this field poses an important fundamental question - how do the electronic, optical, and magnetic properties of a nanoscopic particle differ from those of a bulk sample of the same material? This issue is of particular importance because all properties of a material change as the particle size approaches molecular dimensions and because it is often the unique properties of the nanomaterial that make it useful for a particular application [3].

The use of miniaturized solid-phase surfaces for hybridization analysis has become increasingly more attractive for nucleic acid detection and analysis. Many platforms utilize solid-support bound deoxyoligonucleotide probes to hybridize, and

thereby capture, single-strand targets. Therefore, surfaces suitable for the immobilization of DNA have become an increasingly important biological tool in recent years. Hybridization of nucleic acid targets with tethered deoxyoligonucleotide probes is the central event in the detection of nucleic acids on microarrays or other high-throughput solid-phase-based assays. Choosing the correct surface as a solid phase is a critical step in assay development. Molecules may be immobilized either passively through hydrophobic or ionic interactions or covalently by attachment to activated surface groups. Noncovalent surfaces are effective for many applications; however, passive adsorption fails in many cases. Covalent immobilization is often necessary for binding molecules that do not adsorb, adsorb very weakly, or adsorb with improper orientation and conformation to noncovalent surfaces. Covalent immobilization may result in better biomolecule activity, reduced nonspecific adsorption, and greater stability [4].

Arrays of DNA molecules, either as double-stranded segments or as short single-stranded oligo-deoxyribonucleotides, have been utilized for drug development, DNA sequencing, medical diagnostics, nucleic acid–ligand binding studies and DNA computing. The principal advantages of using surface-bound oligonucleotide over those in solution include ease of purification, conservation of material and reagents, reduction of interference between oligonucleotides and facilitated sample handling. Previously explored surfaces for immobilization of DNA include latex beads, polystyrene, carbon electrodes, gold and oxidized silicon or glass. Each has unique surface characteristics that can affect the performance of an assay [5].

The goal of surface modification is maximization of performance. The critical assay parameters that determine the success of the surface modification and, ultimately,

the commercial success of the assay are as follows [6]: (1) The ability to achieve targeted sensitivity, (2) Maximum specificity with minimal nonspecific adsorption. (3) Adequate reactions and kinetics of the ligand with its target molecule. (4) Stability of the ligand, which increases reproducibility and extends shelf life. (5) Ease of use.

Selection of the solid phase is also often influenced by the availability of compatible instrumentation and robotic systems in research. Multiple-well polystyrene plates have gained widespread acceptance in part because pipetting, washing, and its signal detection are easily automated. Other advantages include the ability to analyze multiple samples simultaneously and compatibility with a number of different detection systems (e.g., colorimetric, fluorescent, and chemiluminescent).

2.2.2 Particles as Solid Supports

The application of small particles for *in vitro* diagnostics has been practiced for nearly 40 years. This is due to a number of beneficial factors including a large surface area to volume ratio, and the possibility of ubiquitous tissue accessibility. In the last decade increased investigations and developments were observed in the field of nanosized magnetic particles (the term nanoparticle being used to cover particulate systems that are less than 1μm in size, and normally below 500 nm).

The use of synthetic microparticles in bioanalysis originated in the mid-1950s, with the invention of latex agglutination tests by Singer and Plotz. These simple, ingenious tests use suspended latex microparticles (diameter ~1m) that are chemically derivatized with a desired antibody. In early versions of this test, a drop of homogeneous milky-white antibody-labeled suspension was applied to a glass slide and mixed with a drop of the analyte solution. The analyte chemically links adjacent latex particles by

binding to antibody sites on these particles, resulting in agglutination (or clumping together) of the particles into what looks like curdled milk [3].

Magnetic particles offer some attractive possibilities in biomedical engineering. They have controllable sizes ranging from a few nanometres up to tens of nanometres, which places them at dimensions that are smaller than or comparable to those of a cell (10–100μm), a virus (20–450nm), a protein (5–50nm) or a gene (2nm wide and 10–100 nm long). This means that they can 'get close' to a biological entity of interest. Indeed, they can be coated with biological molecules to make them interact with or bind to a biological entity, thereby providing a controllable means of 'tagging' or addressing it. The nanoparticles are magnetic, which means that they obey Coulomb's law, and can be manipulated by an external magnetic field gradient. This 'action at a distance', combined with the intrinsic penetrability of magnetic fields into human tissue, opens up many applications involving the transport and/or immobilization of magnetic nanoparticles, or of magnetically tagged biological entities. In this way they can be made to deliver a package, such as an anticancer drug or a cohort of radionuclide atoms, to a targeted region of the body, such as a tumor. Third, the magnetic nanoparticles can be made to resonantly respond to a time-varying magnetic field, with advantageous results related to the transfer of energy from the exciting field to the nanoparticle. For example, the particle can be made to heat up, which leads to their use as hyperthermia agents, delivering toxic amounts of thermal energy to targeted bodies such as tumors; or as chemotherapy and radiotherapy enhancement agents, where a moderate degree of tissue warming results in more effective malignant cell destruction. These, and many other potential applications,

are made available in biomedicine as a result of the special physical properties of magnetic nanoparticles [7].

Advances in nanofabrication techniques are opening up a wide array of highly sophisticated biomedical applications for smaller and smarter magnetic particles for use in targeted drug delivery, ultra-sensitive disease detection, gene therapy, high throughput genetic screening, biochemical sensing, and rapid toxicity cleansing. Each of these disparate applications hinges on the apparently benign relationship between magnetic fields and biological systems; field strengths required to manipulate nanoparticles have no deleterious impact on biological tissue and the biotic environment does not shield efforts to detect internal magnetism. This makes magnetic nanoparticles attractive as in vivo probes or in-vitro tools to extract highly desired information on biochemical system[8].

Over the past decade, a number of biomedical applications have begun to emerge for magnetic micro- and nanoparticles of differing sizes, shapes, and compositions. Many applications use iron oxide particles (usually Fe2O3 or Fe3O4. These particles are available with diameters ranging from ~300 nm to less than 10 nm for nanoscale applications. They exhibit superparamagnetic behavior, magnetizing strongly under an applied field, but retaining no permanent magnetism once the field is removed.

This on/off magnetic switching behavior is a particular advantage in magnetic separation, one of the simplest applications. Magnetic separation is now well established as a viable alternative to centrifugal separation of complex chemical or biological solutions.

To have practical utility in sensing devices, each paramagnetic particle (PMP) should also have surface properties that allow antibodies or recognition units to be linked to the particles. For example: Iron oxide particles are first encased in a biocompatible coating to form tiny beads. The beads are then 'functionalized', that is, their surfaces are treated with a biological or chemical agent known to bind to a specific target. Upon placing the beads in solution, any target cells or molecules will latch onto the functionalized surfaces. Figure 2.1 shows an iron oxide particle coated with a thin layer of gold. The particle is functionalized using a sulphur group and a covalent bond is used to attach this group to the gold surface.

A permanent magnet placed at the side of the solution beaker induces a magnetic moment in each of the freely floating beads and sets up a field gradient across the solution. The now-magnetized beads will move along the field lines and clump together by the magnet, separating their bound targets from the bulk solution.

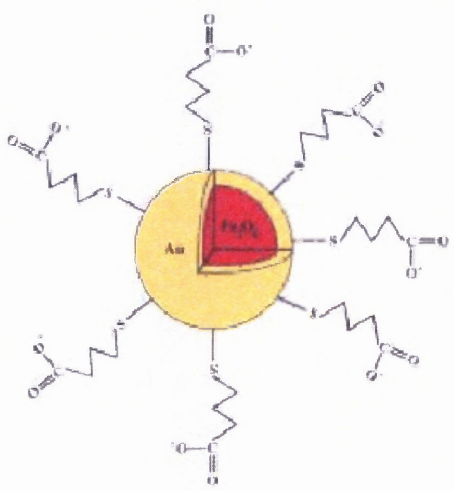


Figure 2.1 Iron Oxide Particle Coated with a Thin Layer of Gold.

A great deal of research is being done focusing on the use of magnetic nanoparticles to design smart sensors. Paulo Freitas, professor of physics at the Instituto

Superior Técnico in Lisbon, has been conducting research on different superparamagnetic iron oxide particles as markers for a table-top genetic screening device. The particles are first functionalized with streptavidin, to enable binding of targets containing biotinyl groups. A surface is prepared with a coating of biomolecular probes that will also bind with complementary target species, creating a 'gene chip'. The intended end result is similar to a sandwich: the target hybridizes with the fixed molecular probe and links to the functionalized magnetic label. Its capture is recorded by magnetoresistive sensors beneath the gene chip (Figure 2.2) [9].

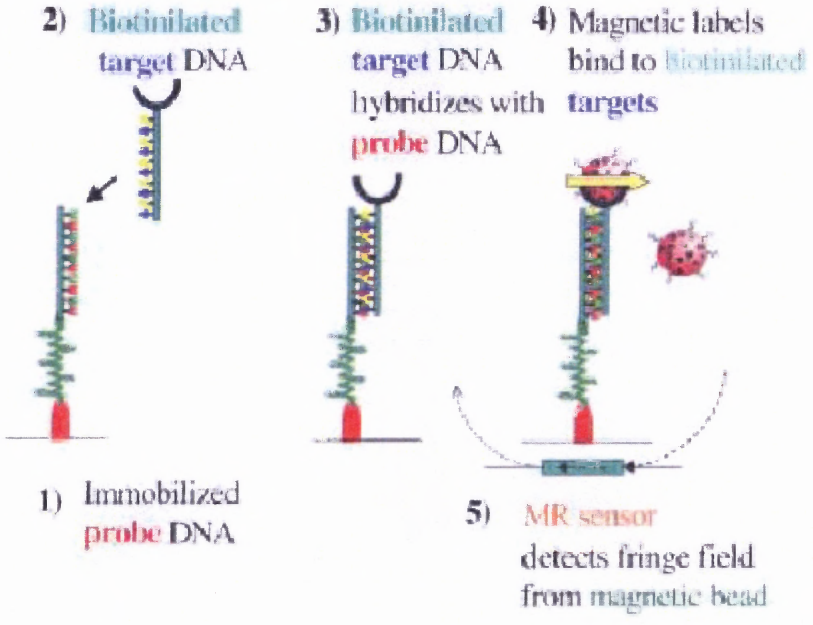


Figure 2.2 Attachment of DNA Target to Immobilized DNA Probe.

Sensor development using magnetic, paramagnetic and superparamagentic particles are still at a very early stage and many challenges remain. The tendency of Fe or Co particles to aggregate would prevent a sensor from working effectively. Polymer-coated iron oxide particles have the advantage of moving freely, but they have a far smaller magnetic moment for their size. Micron-sized particles may impede biomolecular

recognition and interaction, hampering the sensor's efficacy, but particles smaller than 100 nm may prove difficult to detect individually [8].

2.3 Biotechnology

2.3.1 Biosensors

A Sensor is a device, which responds to an input quantity by generating a functionally related output usually in the form of an electrical or optical signal. Sensor technology is seen as an integral element in the overall development of products and services. In fact it has emerged as a key technology to support a wide variety of research and industrial applications. Sensor technology is an interdisciplinary technology. The development of sensors combines the areas of physics, chemistry, advanced materials and molecular biology with electronics and signal processing.

Biosensors are small devices which utilize biological reactions for detecting target analytes. Such devices intimately couple biological recognition elements (which interact with the corresponding target analyte) with a physical transducer that translates the biorecognition event into a useful electrical signal. Common transducing elements such as optical, electrochemical or mass-sensitive devices generate light, current or frequency signals, respectively. There are two types of biosensors, depending on the nature of the recognition event. Bioaffinity devices rely on the selective binding of the target analyte to a surface-confined ligand partner (e.g. antibody, oligonucleotide). In contrast, in biocatalytic devices, an immobilized enzyme is used for recognizing the target substrate (e.g. Sensor strips for personal monitoring of diabetes) [12].

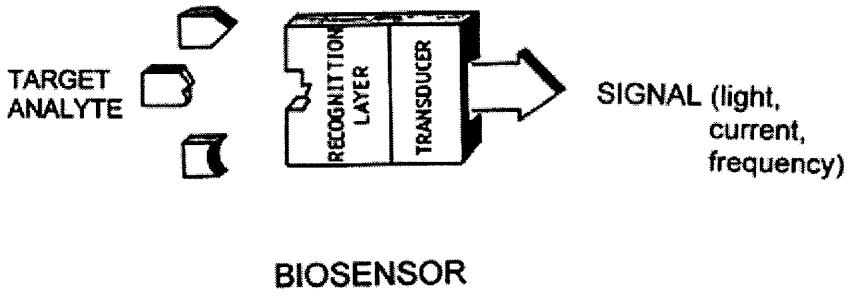


Figure 2.3 Biosensors: The Intimate Coupling of Biorecognition and Signal Transduction.

A biosensor can be said to comprise of the following components: a biological sensing element, a transducer and a data processor. The biosensor's biological component provides specificity: the ability to selectively recognize one type of chemical or event. Its transducer confers sensitivity: the ability to transform the low energy of the biological event into a measurable signal [13].

DNA biosensors, based on nucleic acid recognition processes, are rapidly being developed with the goal of rapid, simple and inexpensive testing of genetic and infectious diseases, and for the detection of DNA damage and interactions. Sequence-specific hybridization biosensors rely on the immobilization of a single stranded DNA probe onto the transducer surface. The duplex formation can be detected following the association of an appropriate hybridization indicator or through other changes accrued from the binding event.

There are many ways to detect DNA hybridization. Optical detection of DNA hybridization is frequently used. The immobilized probe is usually tagged with a fluorescent label. DNA optical biosensors commonly rely on a fiber optic to transduce the emission signal of a fluorescent label. Walts group developed a fiber-optic DNA sensor array for simultaneous detection of multiple DNA sequences. Hybridization of

fluorescently-labeled complementary oligonucleotides was monitored by observing the increase in fluorescence that accompanied binding [18].

Yet another approach to DNA analysis taken by Vo-Dinh, is to design an array of charge-coupled device (CCD) detectors and attach to their surface specific sequences of DNA. Free DNA strands are labeled with fluorescent molecules and hybridized to the bound DNA. When illuminated with laser light of the correct wavelength, the fluorescent tags of the hybridized DNA emit light signals that are detected by the individual CCD detectors behind each DNA site.

Each of the CCD pixels has a different but known short DNA sequence bound to it, therefore the sequence of the piece of the longer DNA strand that hybridizes with the short strand can be identified. By assembling all the information from the pixels, a larger portion of the DNA sequence is obtained. Since bacteria have unique DNA sequences, these hybridization methods hold great promise as the basis for new techniques to rapidly analyze DNA, characterize its source, and identify bacteria [19].

Fluorescence sensing remains the most widely used methodology in biotechnology. Separation technologies, such as capillary array electrophoresis and micro-array technology use fluorescent labeling for the detection of DNA and proteins. Fluorescence detection offers exquisite sensitivity, specificity and compatibility with standard biochemical reactions, such as polymerase chain reaction (PCR).

Traditional bio-fluorescence readers use bulky and discrete elements, which are expensive and require a large footprint and precise alignment. The advantages of integrated biological analysis systems are reduced when these systems rely upon large and fragile optical sensing equipment. Integrated on-chip sensing architectures make

portable and robust medical care equipment practical. As a result of the recent explosion in optoelectronics for telecommunications, a variety of interesting and useful integrated optical sensing architectures can now be realized [5].

A different optical transduction, based on evanescent wave devices, can offer real-time label free detection of DNA hybridization. These biosensors rely on monitoring changes in surface optical properties (shift in resonance angle due to change in interfacial refractive). Calorimetric DNA detection is another way to detect the DNA hybridization. The plasmon resonance absorption of colloidal gold particles has recently been exploited as a DNA-detection method.

Mirkin et al. attached 13-nm-diameter gold nanoparticles to single-stranded oligonucleotides [17]. The plasmon resonance absorbance for these particles has a maximum at 520 nm, and the particles appear red. When a linking oligonucleotide was added, the gold nanoparticles agglutinated, and the color changed from red to purple. To attach the single-stranded oligonucleotides to the gold nanoparticles, a gold sol was stirred with a solution of terminally thiolated, 28 base-pair DNA. The first 13 nucleotides served as a spacer from the nanoparticle surface; the last 15 were the recognition element for the target. Two different recognition-element sequences were used The target DNA was a 30 base-pair strand, the first 15 bases of which are complementary to the first recognition element, and the remaining 15 complementary to the second recognition element. When the target DNA was added to the modified colloidal suspension, the target linked the individual colloid particles into a polymeric network. Because the nanoparticles were now much closer together, the plasmon resonance band shifted, and the color changed. Although this detection method had not yet been optimized, the

detection limit was 10fmol for the target oligonucleotide. An additional benefit of this particular method is that it allows visual detection, especially if the sample is developed on a solid support.

Bioelectronic devices for the electronic transduction of the formation of antigen–antibody complexes on surfaces include the application of piezoelectric crystals, and particularly quartz crystals, as a microgravimetric sensing device. The frequency change of a quartz crystal (Δf), results from a mass change (Δm) occurring on the crystal. Thus, mass changes that occur on a quartz crystal as a result of the association of an antibody or antigen, or the dissociation of an antigen–antibody complex by an analyte antigen, can be microgravimetrically probed by a quartz crystal microbalance. For example, a maleimide-monolayer-functionalized-gold-electrode was reacted with the anti-immunoglobulin G(IgG) $F(ab')_2$ antibody that couples to the anti-*Chlamydia trachomatis* lipopolysaccharide antibody. The latter interface specifically binds to the *Chlamydia trachomatis* cells and the process is transduced by the microgravimetric assay of the formation of the antibody–cell complex on the piezoelectric crystal [10].

Today's bioelectronic sensors generally consist of an electronic transducer whose surface is engineered to support a biomolecule-bearing film, monolayer, or multilayer of controlled thickness. Often, these substrates are functionalized with nucleic acids, proteins, or ligands that recruit their binding partners through affinity interactions. The electronic communication between the biomaterials and the respective transducers is the essence of tailored bioelectronic devices.

The electronic communication can proceed in two directions:

(a) the biological events, such as recognition or catalysis, which occur on the transducer, might be reflected by an electronic signal leading to the formation of electronic biosensors; or

(b) electronic signals, such as potential, might activate biological functions, such as biocatalysis, thus enabling the development of electrically-driven biotransformations [10].

Bioelectronics is a progressing interdisciplinary research field that involves the integration of biomaterials with electronic transducers, such as electrodes, field-effect-transistors or piezoelectric crystals. Surface engineering of biomaterials, such as enzymes, antigen–antibodies or DNA on the electronic supports, controls the electrical properties of the biomaterial–transducer interface and enables the electronic transduction of biorecognition events, or biocatalyzed transformation, on the transducers [10].

Biosensors of tailored sensitivities and specificities can be developed at the nano scale using nano structures. The nanoscale should offer the potential for orders of magnitude improvements in sensitivity, selectivity, response time, and affordability of nansensors. Nanostructures, with their light-weight, small size and high surface-to-volume ratio, will improve by orders of magnitude the capability to detect chemical, biological, radiological, explosive (CBRE) agents with high sensitivity and selectivity. Protective devices can be made to protect through filtration, adsorption, or neutralization of agent leveraging on nanoporosity and high surface-to-volume of nanomaterials. With nanotechnology the detection of a single CBRE entity comes within the realm of possibility.

To build a nanosensor, nanotechnology can be utilized to fabricate a sensing area that allows detection of binding events for only a few or single molecules. The procedure

will be to selectively immobilize a binding agent on the sensor area, and to monitor the change of the electrical, mechanical or optical properties of the "smart sensor" as a function of the binding reaction with the target. Improved sensitivity will be a key parameter in nanoscale assays, where minimum of sample and reagents are used. By studying molecules and cells one by one, information is made accessible that would otherwise be averaged out in standard population based assays.

2.3.2 DNA Probes

One of most specific molecular recognition or sensing event takes place when a strand of nucleic acid anneals to its complement. A single-strand of oligonucleotide probe can find a complementary strand in the presence of large excess of other nucleic acid. In order to detect the hybridization reaction, either the probe has to be labeled or the target has to be labeled.

DNA probes are single-stranded DNA or RNA. They hybridize to a target DNA or RNA sequence. Probe and target base sequences must be similar to each other but, depending on conditions, do not necessarily have to be exactly identical, *i.e.* their complementarities is not always 100%. The probes must be labeled otherwise the hybridization cannot be detected. DNA probes are used in various blotting and *in situ* techniques for the detection of nucleic acid sequences. In medicine they can help in the identification of microorganisms and the diagnosis of infectious, inherited and other diseases.

Radiolabels and non-radioactive labels are the two kinds of probe labels. ^{32}P, ^{35}S, ^{125}I, ^{3}H are used as radio isotope labels for nucleic acid probes and the labels detected by autoradiography or Geiger-Muller counters. Radiolabeled probes are the most sensitive,

e.g. ^{32}P labeled probes can detect single-copy of a gene in only 0.5 μg of DNA. High sensitivity means that low concentrations of probe-target hybrid can be detected. However, radiolabeled probes less popular today because of safety considerations. Non-radioactive labels are generally not as sensitive as radioactive labels. They are safer than radiolabels and do not require dedicated rooms, glassware, equipment and staff monitoring.

Some examples of non-radioactive labels are (i) Enzymes: The enzyme is attached to the probe and its presence usually detected by reaction with a substrate that changes color e.g. alkaline phosphatase and horseradish peroxidase, (ii) Chemiluminescence: In this method chemiluminescent chemicals attached to the probe are detected by their light emission using a luminometer, (iii) Fluorescence: Chemicals attached to probe fluoresce under UV light. (iv) Antibodies: An antigenic group is coupled to the probe and its presence detected using specific antibodies. Also, monoclonal antibodies have been developed that will recognize DNA-RNA hybrids. The antibodies themselves have to be labeled, *e.g.* using an enzyme.

2.3.3 Molecular Sensors

Molecular sensor is a molecule, typically a biomolecule or biomemetic molecule that performs two distinct functions in detecting target molecules. The first function is specific binding, wherein the sensor molecule specifically recognizes a target molecule. The second function is the generation of a target-dependent signal. The molecular sensor used in this research is a pseudo molecular switch similar to the molecular beacons invented by Kramer and Tyagi [16].

2.3.4 Molecular Beacons

Molecular beacons are oligonucleotide probes that can report the presence of specific nucleic acids in homogeneous solutions. As originally designed, a molecular beacon is a single-stranded oligonucleotide probe containing a sequence complementary to the target that is flanked by self-complementary termini, and carries a fluorophore and a quencher at the 3'- and 5'-ends. In the absence of the target, these molecules form closed stem-loop structures in which the fluorophore and quencher are in close proximity, which quenches the fluorescence. In the presence of the target, the molecular beacon forms a complex with its target, which dissociates the fluorophore from the quencher. Once the fluorophore and quencher are spatially separated, the fluorescence increases and quantitatively signals the presence of the target [16].

In order to design molecular beacons that function optimally under a given set of assay conditions, it is important to understand how their fluorescence changes with temperature in the presence and absence of their targets. At low temperatures molecular beacons exist in a closed state, the flurophore and quencher are held in close proximity to each other by hairpin system, and there is no fluorescence. At high temperature the helical order of the stem gives way to a random-coil configuration, separating the flurophore from the quencher and restoring fluorescence. The temperature at which the stem melts depends upon the GC content and the length of the stem sequence. If a target is added to a solution containing a molecular beacon at temperatures below the melting temperature of its stem, the molecular beacon spontaneously binds to its target, dissociating the stem, and become fluorescent. At low temperatures, the probe-target hybrid remains fluorescent but as temperature is raised the probe dissociates from target

and tends to return to its hairpin state, diminishing the fluorescence significantly. The temperature at which the probe-target hybrid melts apart depends upon the GC content and the length of the probe sequence.

Owing to their stem, the recognition of targets by molecular beacons is so specific that single-nucleotide differences can be readily detected. Because of this property, molecular beacons have been used for the detection of RNAs within living cells, for monitoring the synthesis of specific nucleic acids in sealed reaction vessels, for homogenous one-tube assays for genotyping single-nucleotide variations in DNA and for multiplex PCR's for the detection of four different pathogenic retroviruses.

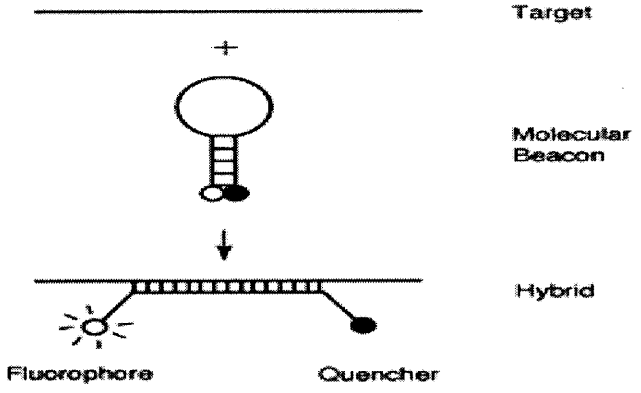


Figure 2.4 Schematic drawing of mechanism of signaling by molecular beacons.

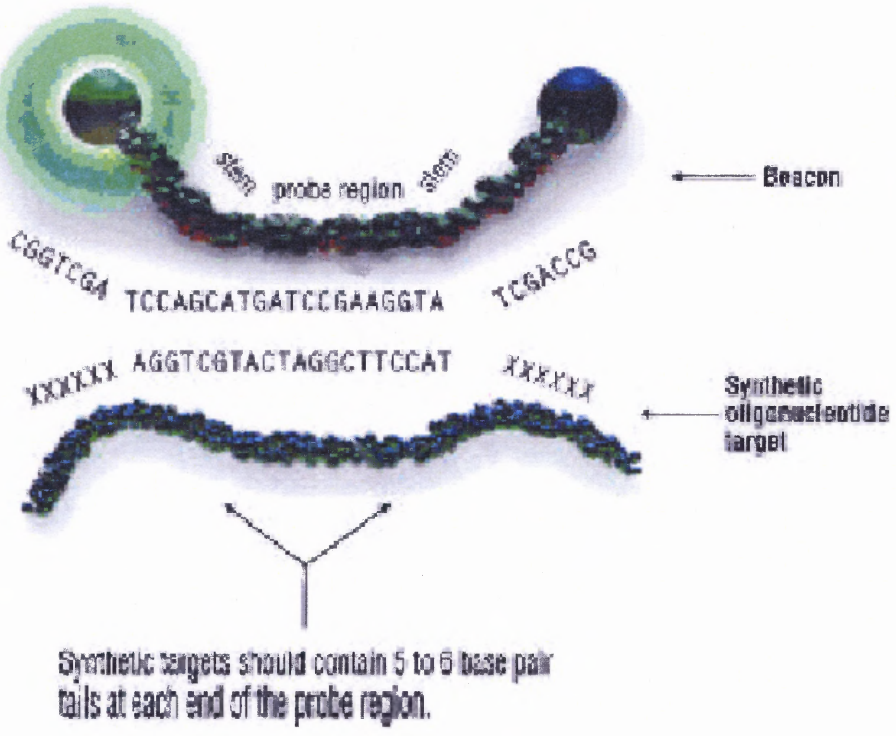


Figure 2.5 Design of a synthetic oligonucleotide for use as a molecular beacon target.

Capture via hybridization of nucleic acid targets by deoxyoligonucleotide probes attached to a solid support surface is crucial for target detection on DNA microarrays and other high-throughput solid-phase-based assays. Riccelli et al. studied the capture of linear target DNA by hairpin and linear probes coupled to a solid support surface [14]. There were two inter-related components of the study. The first was to define standard experimental parameters for performing hybridization assays on microtiter plates. This required precise determination of the coating capacity of microtiter plate surfaces with DNA probes and evaluation of the saturation (binding) capacity of the coated plates. The second component of the study was to employ the developed and characterized assay system to measure hybridization rates and stabilities of hairpin and linear capture probes. The results show that target capture by hairpin probes is faster than with linear probes and that, once formed; hairpin complexes are thermodynamically more stable. This is despite

the fact that the coupling density on the surface for linear probes was nearly twice that for hairpin probes. At 25 and 45°C and all target concentration examined, rates of hybridization for hairpins are at least twice those of their linear probe counterparts. Length of the single-stranded capture region was also found to affect target capture and in general longer capture regions were favored.

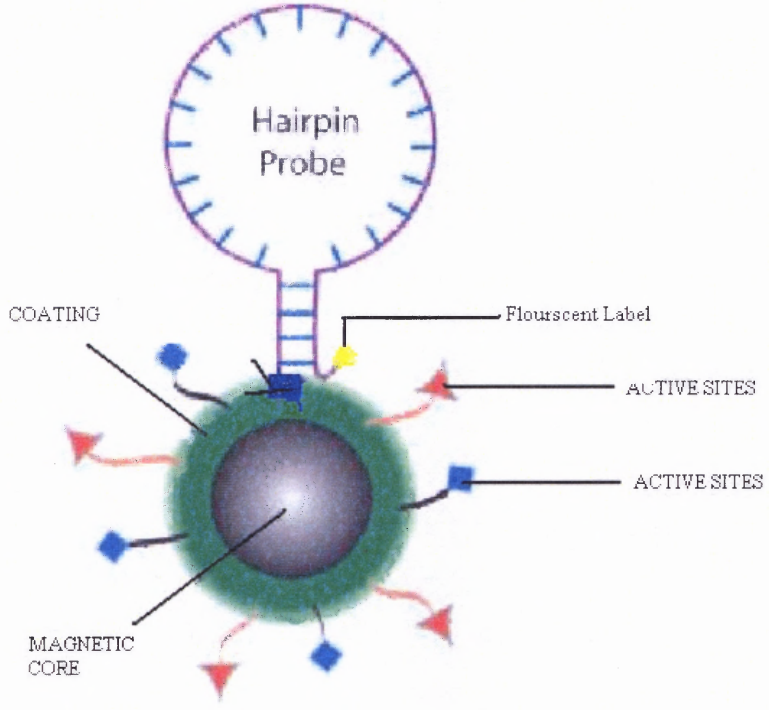


Figure 2.6 Attachment of Hairpin Probe to Paramagnetic Particles.

2.4 Bioconjugation

Bioconjugation is defined as the linking of biomolecules to other biomolecules, polymers, small molecules, surfaces or metals (Figure 2.7). Bioconjugation is a critical enabling component of in vitro and in vivo diagnostics and in-vivo therapies.

Bioconjugation requires identifying a conjugation scheme that forms a stable linkage between the molecules, yet does not affect the inherent function of either coupling partner. Examples of biomolecules used in conjugates include enzymes, antibodies, peptides (synthetic and natural), oligonucleotides (synthetic and natural) and carbohydrates. Other types of molecules widely conjugated to biomolecules include fluorescent reagents, metal chelates and drugs. Yet another type of conjugation is the attachment (immobilization) of biomolecules to solid surfaces such as plastics, glass, metal aggregates and beads, which has broad applications in genomics, proteomics and the diagnostic industry in general. For ideal bioconjugation to occur, first, the reactive moieties (X) must react efficiently, yet must not crosslink with the biomolecule itself. Second, the linkers (A and B) need to be reactive enough to couple with high efficiency, yet stable enough so as to be stored in solution for long periods. Finally, the linkage should be stable over broad pH and temperature ranges, while still allowing cleavability to be introduced if desired. It is also extremely important that no non-specific (i.e.non-covalent) binding/sticking of one biomolecule to the other biomolecule occurs which would lead to high background interference in any assay. It would be further advantageous if the functional reactive moieties (A and B) could be modified for incorporation onto a biomolecule synthesized using standard solid phase methods.

There are three major bioconjugation/immobilization methods in current use. One is biochemical (biotin/avidin), and two are chemical (amine/active esters and maleimide/thiol).

Avidin/Biotin: Biotin is a natural small-molecule ligand that binds to its protein receptor, avidin (molecular weight 66 kD), with an extremely high binding affinity (1015 M).

Biochemists have taken advantage of this high affinity by incorporating biotin onto one biomolecule and conjugating avidin to another biomolecule, then coupling the two conjugates together to form the final product.

Amine/Active Ester: Due to the limited stability of active esters in aqueous milieu the amine/active ester conjugation couple has extremely limited utility. It is mainly used to conjugate small molecules such as fluorophores to biomolecules such as proteins or synthetic oligonucleotides.

Maleimide/Thiol: The maleimide/thiol couple has been used extensively in conjugating biomolecules. Current ELISA (enzyme linked immunosorbant assay) antibody-enzyme conjugates are prepared using this method. A wide variety of conjugates used for in vivo and in vitro applications have also been prepared using this chemical conjugation couple.

All three methods have serious inherent flaws that reduce their utility in many bioconjugation applications. They do not fit the description for the ideal bioconjugation couple mentioned above [15].

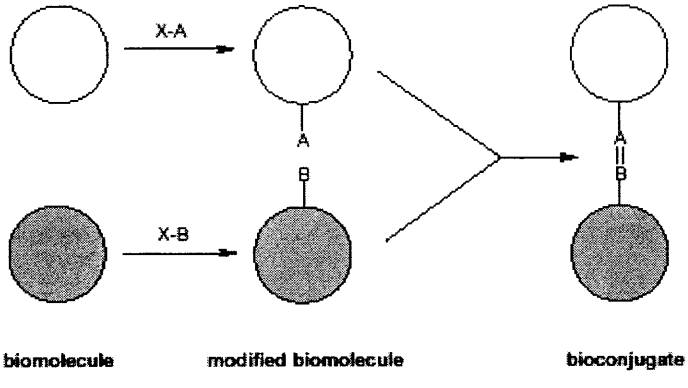


Figure 2.7 Biomolecule Modification.

2.5 Immobilization using Solulink Chemistry

HydralinK is a novel bioconjugation system for the conjugation and immobilization of peptides, proteins, carbohydrates, DNA and RNA. The technology is based on the reaction of a 2-hydrazinopyridyl moiety with benzaldehdye moiety to yield a stable bis-aromatic hydrazone. The chemistry is highly selective, stable in solution and not susceptible to non-specific binding, making it superior to conventional methods of bioconjugation such as malemide/thiol and avid biotin.

The technique can be readily engineered to link small molecules (peptides, flurophores), biomolecules proteins, DNA, RNA) or other molecules to solid surfaces (glass, plastic, latex, silica beads), for application in proteonomics, genomics, drug discovery, therapeutics and diagnostics. The implementation of the technology is extremely simple. Benzaldehyde moiety on surfaces is effected by treatment with succinimidyl 4-formylbenzoate. To introduce 2-hydrazinopyridine moiety is introduced by using SANH.

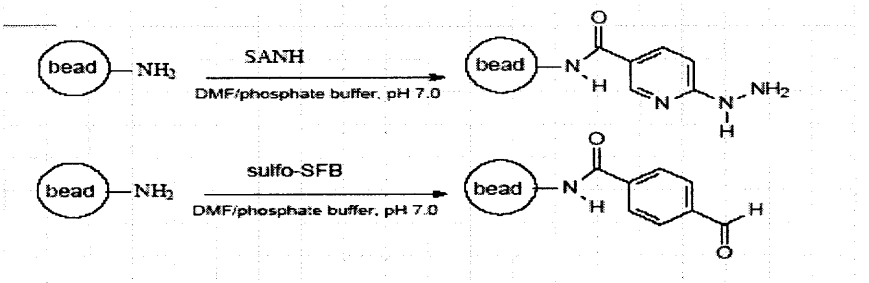


Figure2.8 Modification of beads using SANH and SFB.

Biomolecule modified with hydrazine and aldehyde modification reagents are then mixed to give the hydrazone-mediated conjugate. The reaction is optimally carried out at pH 4.7, but reaction also can occur up to 7.3, but the reaction will be very slow.

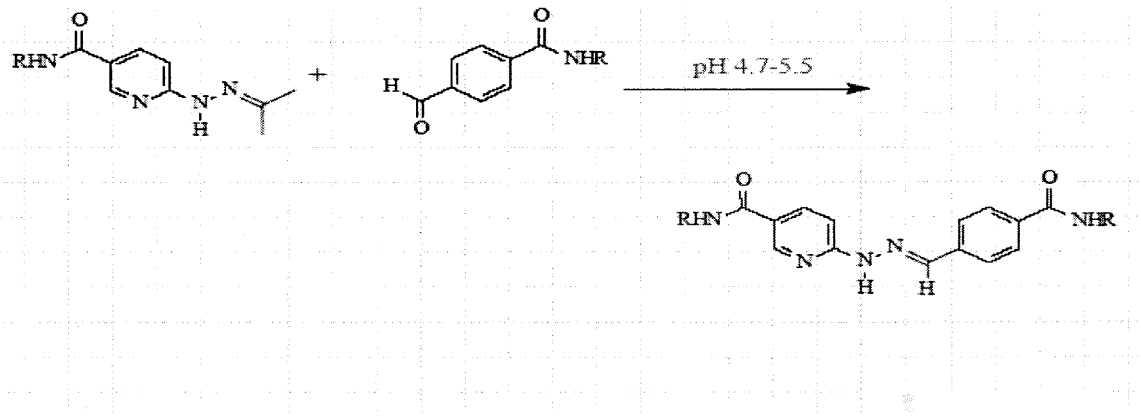


Figure 2.9 Bioconjugation of hydrazine and aldehyde modified moiety.

Advantages of the HydralinK bioconjugation system are: (1) Enhanced stability of biomolecules modified with hydrazines and aldehydes leads to increased ease of use, reproducibility and efficiency. The extended stability allows modified proteins to be prepared days or weeks in advance of conjugation or immobilization and reaction conditions to be optimized prior to scale up (2) The level of incorporation of both 2-hydrazinopyridyl and benzaldehyde can be easily quantified spectrophotometrically (3) The conjugation reaction is highly selective, eliminating non-specific interactions (4) Fast reaction kinetics (5) Amenable to solid phase synthesis.

CHAPTER 3

RESEARCH OBJECTIVE

The purpose of the research is to combine emerging capabilities in nanotechnology and biotechnology with the final aim of designing sensitive devices for detecting and quantifying nucleic acid targets. The work done in this thesis is:

1. Design oligonucleotide sequences as pseudomolecular beacons.
2. Immobilize fluorescent labeled probe oligonucleotide nucleotide sequences to paramagnetic particles using efficient immobilization chemistry.
3. Optimize the chemistry to get maximum probe attachment.
4. Analyze functionality of the probe by hybridizing target oligonucleotide sequence to the immobilized particles.
5. Perform 1 and 2 on particles with different sizes and 2 different shapes (spherical, irregular).
6. Study the effect of particle size on immobilization of molecular sensors.

The long term goal of this research work is to develop in-vitro sensors for detection of biological targets.

CHAPTER 4

EXPERIMENTS

4.1 Immobilization of FTBProbes to Microtiter Plate

Experimental Objective: To covalently immobilize fluorescently labeled amino linked oligonucleotide nucleotide to glutaraldehyde modified BSA coated on black polystyrene microtiter plate wells.

Reagents:

1. Coating Buffer = $NaPO_4$ pH 7.0
2. Wash buffer = PBS Azide - 10mM $NaPO_4$, 150mM NaCl, 0.25% NaN_3 pH 7.1 (No Tween, No Salt).
3. Bovine Serum Albumin (BSA)
4. FTBProbe-080803 :
   5'- CGA TCG CGA CAT TCC ATC GTG CCC GAT CGA AAA A-3'
   The oligonucleotide sequences were synthesized by MWG Biotech Inc.
5. Glutaraldehyde (GA)
6. Glycine

Steps:

1. The microtiter plate was coated with 200µl of varying concentration of BSA/well
2. A Dilution Series for [BSA] was made according to table 4.1
3. The prepared plate was covered with Saran wrap and incubated overnight at 2°C – 8°C. (4°C).
4. The BSA coated plate was washed after incubation overnight three times in wash buffer.
   Stock = 16.47mg/ml.

A Dilution was prepared for GA according to table 4.2

Table 4.1 BSA Dilution Series for Microtiter Plate Adsorption

Tube #	[BSA] µg/ml	Stock BSA	Coating Buffer ml	Total Volume ml
A	20.0	12.1µl	10.0	10.012
B	10.0	2.0ml	2.0	4.0
C	5.0	1.0ml	3.0	4.0
D	2.0	0.4ml	3.6	4.0
E	1.0	0.4ml	3.6	4.0
F	0	0	4.0	4.0

Table 4.2 Glutaraldehyde Dilution Series for Aldehyde Modification of Amine Groups

Tube #	[GA] %	Stock GA µl	Buffer ml	Total Volume ml
A	5	660	2.54	2.60
B	1	550	2.0	2.55
C	0.2	100	2.4	2.5
D	0	0	2.5	2.5

5. GA dilutions were added at 100µl/well to the microtiter plate after the wash procedure.

6. The plates were incubated overnight at 2°C – 8°C. (4°C)

7. After overnight incubation, GA from plates was washed using PBS Azide wash buffer.

8. Dilution series for FTBProbe was made.
   Stock FTBProbe = 100 µM

Table 4.3 Dilution series for FTBProbe

Tube #	[FTBProbe] µM	F-TB stock µl	Coating Buffer ml	Total Volume ml
A	10	200	1.8	2.00
B	3	450µl A	1.05	1.5
C	1	140µl A	1.26	1.4
D	0.3	140 µl B	1.26	1.4

9. 50 µl of the prepared FTB dilutions was added according to designed protocol.
10. The plate was incubated overnight at 2°C – 8 °C (4°C).
11. After overnight incubation, the plates were washed three times in PBS Azide wash buffer.
12. 1 M glycine in $NaPO_4$ 10mM (pH 8.0) buffer was prepared. Glycine was added to quench unreacted aldehyde with the amine group of the acid. 200µl of Glycine/well was added and incubated at room temperature for 1 hour. After incubation, the plates were washed three times with PBS Azide (pH 7.1).
13. The microtiter plate was read at 5.0 volts in fluorolite 1000 microtiter plate reader

Experiment result:

The reading from the microtiter plate reader suggests that there is no significant immobilization of FTB oligonucleotide probe to the microtiter plate. This could be because of the low surface area offered for coating and binding by the microtiter plate well. To get higher surface area, the substrate for immobilization was changed to paramagnetic particles. The advantages of using the amine terminated biomag plus particles or any paramagnetic particle over macro surfaces include:

1. Large surface area compared to microtiter plate.
2. Particles are already coated with silane amine group.

4.2 Experiment 2

The objective of this experiment was to:

1. Immobilize the FTBProbe and unlabelled Target-NH2 onto the BioMag particles using Glutaraldehyde Chemistry.

2. Hybridize the immobilized FTBProbe with a quencher and the immobilized unlabelled Target-NH2 with a FTBProbe respectively.

4.2.1 Experimental Objective A: Immobilization of FTBProbe and unlabeled Target-NH_2 to 1 micron biomag plus particles.

Buffers:

1. 100ml of 1M Dibasic Na_2HPO_4

2. 200ml of 1M Monobasic NaH_2PO_4

3. 500ml of 0.1M $NaPO_4$ pH 8.0– made using the Dibasic and Monobasic

Dilution Series:

1. FTBProbe – 10nmoles, 3.3nmoles, and 1nmoles

2. TBTarget-NH_2 – 5nmoles, 1.67nmoles and 0.5nmoles

3. BioMag - 1000µg/µl, 200µg/µl, and 40µg/µl

Materials:

1. FTBprobe-080803

2. Q-TBtarget-080803

3. 1 Micron paramagnetic particles from Polysciences

Experimental Procedure:

Immobilization was done using three concentrations each of oligonucleotide and target and 2mg PMP particle for each condition at 12.5mg PMP/ml. i.e. 2* 6 = 12mg PMP was needed. But double the amount of PMP was taken i.e. 2mg * (3 + 3) * 2 = 24mg PMP

(approx 25mg PMP). Therefore 0.5ml was taken from the 50mg/ml stock PMP = 25mg/ml.

Steps:

1. 0.5ml of Biomagplus PMP particles was taken and pipetted into a glass test tube containing 5 ml of PH 8.0 buffer.

2. The test tube was then placed in a magnetic rack to separate the particles and the residual buffer volume was decanted.

3. The paramagnetic particles were washed three times in 100mM $NaPO_4$ pH 8.0 buffer.

4. While keeping the washed particles in the magnetic rack, 1.6ml of 100mM $NaPO_4$ buffer was added. 400ml of Glutaraldehyde (25%) was added to the test tube. The tube was vortexed, capped and placed on a shaker. The tube was incubated in the shaker for 5 hours.

5. After incubating for 5 hours, the particles were washed seven times in 100mM $NaPO_4$ pH 8.0 buffer.

6. After washing, the particles were resuspended in 250µl of pH 8.0 buffer.

7. A dilution series of probe/ target was prepared as shown in table 2.1 and 2.2.

8. Concentration of stock FTBProbe = 100µM.

Table 4.4 Preparation of Dilution Series for FTBProbe

Concentration of stock FTBProbe = 100µM

Tube #	Oligonucleotide nmoles	Volume of oligonucleotide stock (µl)	Buffer µl
P10	10	100	0
P3.3	3.3	33.3	66.7
P1.0	1	10	90.0

Table 4.5 Preparation of Dilution Series for TBTarget

Concentration of stock TB Target – NH_2-080803 = 50μM

Tube #	Oligonucleotide nmoles	Volume of oligonucleotide stock (μl)	Buffer μl
T5	5	100	0
T 1.67	1.67	33.3	66.7
T 0.5	0.5	10	90

1. Six test tubes were taken and labeled P10, P3.3, P1.0, T5, T1.67, T0.5. The dilutions were prepared according to the table 2.1 and 2.2.

2. 20μl of 10mg/ml BioMagplus PMP was added to each of the six test tube containing probe/target and the particles were allowed to separate in a magnetic rack immediately.

3. While the tubes were in the magnetic rack 10μl of supernatant was withdrawn from each tube. The supernatants were taken to give the input supernatant counts.

4. The 10μl supernatant withdrawn was resuspended in 90μl of 100mm $NaPO_4$ pH 8.0 buffer (1/10 dilution of supernatant). This dilution allowed us to determine the percentage of oligonucleotide/target offered to the PMP for immobilization.

5. The six tubes with probe/target with the BioMag were vortexed thoroughly and were kept in the shaker at an angle for overnight incubation.

Post Immobilization Steps:

1) After Overnight incubation the tubes were taken off the shaker and put on the magnetic separator rack. To get output counts, after the particles were fully separated, 10μl of the supernatant was taken and diluted in 90μl of 100mm $NaPO_4$ pH 8.0 buffer (1/10 dilution of output supernatant).

2) The immobilized particles were washed three times in 1ml 100mM $NaPO_4$ pH 8.0 buffer.

3) After washing the particles were reconstituted in 200μl of blocking buffer (PBS Azide + BSA).

4) 1ml of 1M Glycine (pH 8.0) was added to the tubes, vortexed and incubated in the shaker for 1 hour.

5) After incubation, the particles were washed three times with 1ml 100mM $NaPO_4$ (pH 8.0) buffer.

6) The particles with oligonucleotides immobilized were reconstituted in 200µl of blocking buffer (PBS Azide + BSA).

7) A dilution series for PMP particles was made with concentrations (100µg/ml, 200 µg/ml and 40µg/ml).

Table 4.6 Dilutions for PMP

Tube #	PMP (µg/ml)	Stock µl	Buffer µl
A	1000	120	1080
B	200	200	800
C	40	40	960

8) A protocol was designed to read the input and output dilutions of the FTB-probe and the different concentrations of oligonucleotides immobilized to particles.

Experiment Results:

Successful immobilization of FTBProbe to BioMag particle was observed. Only 5 – 10% of oligonucleotide immobilization was observed from input and output counts.

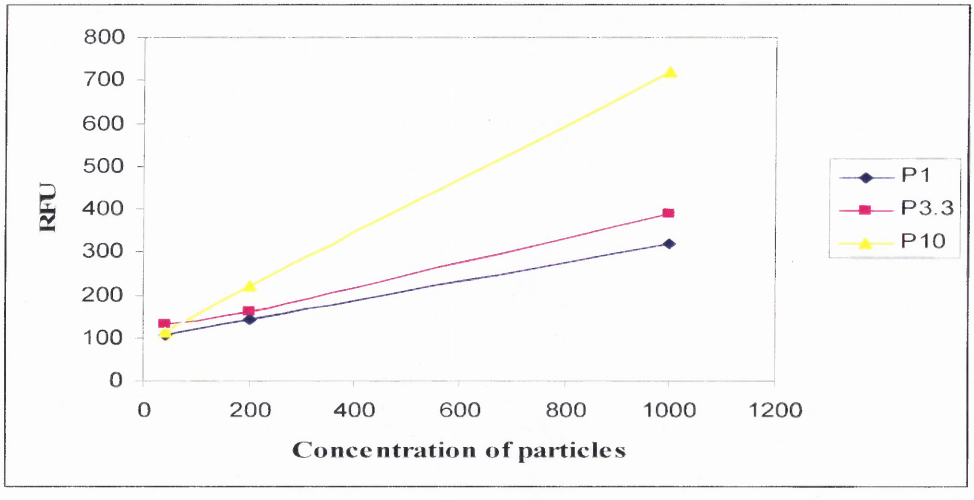


Figure 4.1 Titration Curve for GA Chemistry Based Probe immobilization on Particle.

Table 4.7 Input / Output data and dilution of glutaraldehyde activated FTB probe immobilized particles

	1	2	3	4	5	6	7	8	9	10	11	12	**Protocol** Dilution
A	-	1297	341	293	133	252	109	162	347	149	213	669	Particle Dilution
B I/P	-	1263	340	89	131	274	84	163	374	103	208	615	40µg/ml 200µg/ml 40µg/ml
C	-	1385	418	321	139	298	116	170	416	719	218	633	
D	-	1350	253	99	209	302	99	174	413	98	225	607	Probe Concentrations
E	150	1425	376	450	158	327	103	184	496	101	243	682	P1= 1nmole P3.3=3.3nmole P10=10nmole
F O/P	155	1382	357	127	138	336	122	179	476	119	252	766	
G	163	1375	382	443	178	365	116	191	PH8.8 92	130	239	801	
H	162	1277	229	115	154	335	94	177	PH8.8 93	107	256	785	
Reading	P1	P3.3	P10	C40	B200	A1000	C40	B200	A1000	C40	B200	A1000	50 µl / Well
	2.0Volts			P1			P3.3			P10			
	VOLTAGE = 7 ; NO FILTER APPLIED {IND;1.3ND}												

4.3 Optimization of Solulink Chemistry for Immobilization of Oligonucleotide to Paramagnetic Particles of size 1 micron.

Objective: To optimize the amount of SANH to activate the paramagnetic particles and the amount of SFB to activate the oligonucleotides and to test the effectiveness of the probe based assay using quencher labeled targets.

4.3.1 Experiment (i):

Objective: Immobilization of fluorescently labeled oligonucleotide probes to PMP particles using solulink chemistry and determining % of uptake of oligonucleotide by measuring input and output supernatant counts.

Materials:

1. Paramagnetic particles from Polyscience of size 1 micron.
2. FTB- probe :

   5'- CGA TCG CGA CAT TCC ATC GTG CCC GAT CGA AAA A-3'
3. Sodium phosphate buffer pH6.0
4. Sodium Phosphate/ Sodium Chloride buffer pH 7.8.
5. Solulink S-9002 HydralinK kit.

Experimental Procedure:

Particle preparation: Three batches of 10mg particle at 20mg/ml were taken in three test tubes to be activated with three varying concentrations of SANH per particle. The number of amines per PMP for the 1 micron biomagplus particles is about 240μmoles/gram.

Test Tube A: The condition in this test tube was decided to be 3eq SANH/ eq PMP – NH_2.

Test Tube B: The condition in this test tube was decided to be 1eq SANH/ eq PMP – NH_2

Test Tube C: The condition in this test tube was decided to be 3.0eq SANH/ eq PMP – NH_2 .

Table 4.11 Molar Ratio of SANH Activation for Modifying particles

Condition	Molar Ratio	PMP Volume (μL)
A	0.3eq /1eq	200
B	1eq/ 1eq	200
C	3.0eq/1 eq	200

All the particles were washed three times in pH 7.8 buffer by vortexing, magnetically separating and decanting. After the final wash, test tubes 1, 2, and 3 were resuspended in 100μL of pH 7.8 phosphate buffer. Final concentration for all particles before activation is 20mg/ml per condition.

Activation of particles: The particles were modified with a hydrazine linker using SANH. According to solulink SANH was to be prepared at 5.5mg of SANH/ 100μL of DMSO. To make 70μL of the SANH solution, 3.85mg of SANH was dissolved in 70μL of DMF. Care was taken to ensure that most of the SANH goes into solution. SANH was added to the three test tubes with 200μL of PMP solution in each according to the molar ratio as shown in the table. The test tubes were then incubated on a shaker at room temperature (20° – 25° C) for 2.5 hours

Oligonucleotide activation: FTBprobe-080803 oligonucleotide probe was used. The oligonucleotide sequence was

5'- CGA TCG CGA CAT TCC ATC GTG CCC GAT CGA AAA A-3'

The stock oligonucleotide concentration was 100nmoles. For the purpose of this experiment, the molar concentration of oligonucleotides taken is 10μM i.e. 3.3 nmoles of oligonucleotides.

Three test tubes labeled 1, 2 and 3 were taken and 33 μL of stock oligonucleotide + 297μL of buffer were added into each tube into each tube

Test Tube 1: The condition in this test tube was decided to be 100eq SFB/ eq Oligonucleotide-NH_2.

Test Tube 2: The condition in this test tube was decided to be 30eq SFB/ eq Oligonucleotide-NH_2.

Test Tube 3: The condition in this test tube was decided to be 10eq SFB/ eq Oligonucleotide-NH_2.

Molecular weight of SFB = 247.1

Table 4.12 Molar Ratio of SFB Activation

Condition	Molar Ratio of eq FB/ eq PMP-NH_2	Vol Oligonucleotide + Buffer (μL)
1	1 eq /1 eq	330
2	30 eq/ 1eq	330
3	100 eq/ eq	330

According to solulink chemistry, dissolve 4.8mg SFB in 100μL of DMSO. For activation of 1eq oligonucleotide-NH_2 with 100eq SFB we need 0.17μL of SFB. A stock solution for 100eq SFB/eq oligonucleotide-NH_2 was prepared by mixing 1mg SFB in 20.8μL of DMSO. A Dilution series from this stock solution of SFB + DMSO was prepared for 30eq SFB/eq oligonucleotide-NH_2 and 10eq SFB/eq oligonucleotide-NH_2.

Table 4.13 SFB Dilutions for Oligonucleotide Activation

Tube #	Condition	Volume SFB Stock	Volume DMSO
X	100eq SFB/eq oligonucleotide	2μL	0μL
Y	30eq SFB/eq oligonucleotide	3μL	7μL
Z	10eq SFB/eq oligonucleotide	5μL	42μL

These SFB dilutions were added to the test tubes 1, 2 and 3 according to table 4.12

Table 4.14 Addition of SFB to Oligonucleotide's

Tube #	Volume of Oligonucleotide + Buffer	Volume of SFB
1	330	1.7μL of X
2	330	1.7μL of Y
3	330	1.7μL of Z

The total reaction conditions were 330μL of (oligonucleotide + buffer) + 1.7μL of (DMSO + variable SFB). Test tubes 1, 2, and 3 were incubated at room temperature for 2.5 hours.

Purification of Oligonucleotide's after aldehyde modification: The SFB–oligonucleotide reaction was terminated after 2.3 hours. The total reaction volume of oligonucleotide+ SFB was about 330μL containing 3.3nmoles of oligonucleotide. The oligonucleotides were purified by using a NAP5 desalting column from Amersham Biosciences. Three NAP5 columns were used for purifying the three levels of oligonucleotide activation with 330μL of oligonucleotide in each column. The columns were first washed three times in pH 6.0 PO_4 buffer by eluting and discarding elute. The

column was allowed to drain completely and 330μL of oligonucleotide was carefully added to the column. After the oligonucleotide had penetrated into the column, 340μL of buffer was added. The first elute was discarded. The column was carefully watched to see the traveling of the oligonucleotide's to the tip of the NAP5 coulmn.660μL of buffer was again added to the column to elute the oligonucleotide band. Total volume of collected oligonucleotide was 660μL in pH 6.0. The oligonucleotide was collected in a test tubes, capped and stored in dark at 2° - 8°C. After purification, the oligonucleotide concentration becomes 5μM.

Purification of particles: The SANH activated particles were removed from the shaker after 2.5 hours. The particles were washed three times in pH 6.0 buffer. After final wash, the particles were resuspended in 400μL of pH 6.0 buffer. The particles were at a concentration of 25mg/ml.

Conjugation reaction (Immobilization of SFB activated oligonucleotide to SANH activated particle): The hydrazine modified particles in test tubes A, B and C was resuspended to 400μL after the final wash. Nine Test tubes were taken and labeled 1A, 1B, 1C, 2A, 2B, 2C, 3A, 3B and 3C. An optimization matrix was designed to compare the low, middle, high concentrations of hydrazine modified paramagnetic particles times the low, middle, high concentrations of aldehyde modified oligonucleotides.

80μL of activated PMP was added to the test tubes according to their designed condition. The test tubes were placed in a magnetic rack. With the particles separated to the sides of the tube, 21μL of the activated oligonucleotide was added according to table 4.13. The mixtures in test tubes 1A through 3C were vortexed and placed again placed in a magnetic rack to separate the particles. After the particles were fully separated to the

side, 22μL of supernatant were removed from each PMP + oligonucleotide mixture and diluted in 198μL of pH 6.0 buffer. The purpose of taking the supernatants were to get the input counts of fluorescence at time 0 of the conjugation reaction. The immobilization efficiency can be determined by calculating the % uptake of fluorescent oligonucleotide from input and output supernatant counts. The supernatants were stored in properly labeled centrifuge tubes in dark at room temperature. The test tubes with particles + oligonucleotide were incubated overnight at room temperature in a shaker.

Table 4.15 Conditions of Activation Levels in Test Tubes

Test tube #	PMP condition (80μL)	Oligonucleotide condition (210μL)
1A	0.3eq SANH/eq PMP-NH2	100eq SFB/eq oligonucleotide-NH2
1B	1.0eq SANH/eq PMP-NH2	30eq SFB/eq oligonucleotide-NH2
1C	3.0eq SANH/eq PMP-NH2	10eq SFB/eq oligonucleotide-NH2
2A	0.3eq SANH/eq PMP-NH2	100eq SFB/eq oligonucleotide-NH2
2B	1.0eq SANH/eq PMP-NH2	30eq SFB/eq oligonucleotide-NH2
2C	3.0eq SANH/eq PMP-NH2	10eq SFB/eq oligonucleotide-NH2
3A	0.3eq SANH/eq PMP-NH2	100eq SFB/eq oligonucleotide-NH2
3B	1.0eq SANH/eq PMP-NH2	30eq SFB/eq oligonucleotide-NH2
3C	3.0eq SANH/eq PMP-NH2	10eq SFB/eq oligonucleotide-NH2

Post Conjugation steps: After incubating the particles for about 16 hours, the particles were taken out from the shaker and place on a magnetic rack. The particles were allowed to separate to the side of the test tubes. After about five minutes of separation, 350μL of supernatant was withdrawn carefully to give the output counts after incubation. The particles were then washed three times in PBS Azide + Tween wash buffer and then

resuspended to 200μL in Immunoassay (PBS Azide + 2mg BSA) buffer. The final concentration of the particles was calculated to 10mg/ml.

A protocol was designed to read the input and output counts from the supernatants (in triplicates). 50μL of input and 50μL of output was pipetted into a 96 well microtiter plate and read in a Fluorolite 1000 microtiter plate reader.

Another protocol was designed to read the counts for dilutions at each particle preparation (in duplicates) at concentration ranging from 2000μg/ml to 10μg/ml. A dilution series for each particle was done and 100μL of each dilution was pipetted into the wells of a microtiter plate according to the designed protocol. The readings were taken using a Fluorolite 1000 microtiter plate reader.

Results

The readings of input and output counts suggest that the maximum uptake was in condition 1A. There was about 40% uptake. This condition had the least SANH activation and maximum SFB activation. At higher concentrations of SANH activation, we notice very less uptake. This could be because for activating using Hi levels of SANH we require more amount of DMSO and this could damage the particles. Since the amount of DMSO added to the oligonucleotides is negligible even at hi concentrations of SFB activation, we don't see the effect of DMSO at high SFB activation levels.

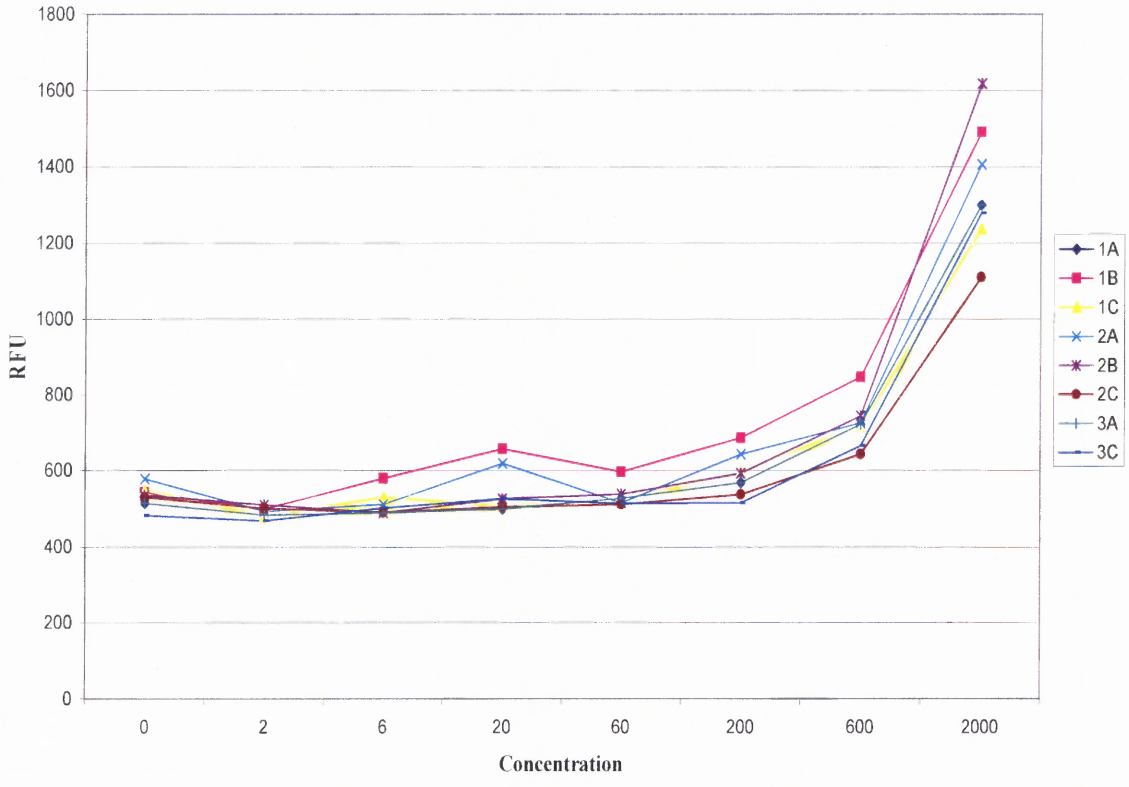


Figure 4.3 Titration curve of Particles with FTBProbe using Different Activation Levels of SANH and SFB.

Table 4.16 I/P & O/P of Biomag Plus at three different eq of SANH & SFB

	1	2	3	4	5	6	7	8	9	10	11	12	**Protocol** Dilution
A	0	0	0	0	0	0	0	0	0	0	0	0	A= 0.3eqSANH eq.PNP-NH_2
B	0	C013 0054	C3 2107	3B 3440	3A 3694	2C 2204	2B 2983	2A 3582	1C 1519	1B 2177	1A 2902	0	B= 1.0eqSANH eq.PNP-NH_2
O/P **C**	0	C013 0053	C3 2088	3B 3335	3A 3743	2C 2235	2B 3163	2A 3593	1C 1459	1B 2215	1A 2852	0	C= 3.0eqSANH eq.PNP-NH_2
D	0	C012 0050	C3 2198	3B 3383	3A 3765	2C 2309	2B 3215	2A 3949	1C 1536	1B 2244	1A 2960	0	1= 100eqSFB eq
E	0	C012 0045	C3 339	3B 340	3A 336	2C 316	2B 323	2A 364	1C 258	1B 266	1A 266	0	2= 30eqSFB eq
F I/P	0	C011 0033	C3 331	3B 334	3A 342	2C 330	2B 327	2A 364	1C 250	1B 267	1A 272	0	3= 10eqSFB eq
G	0	C011 0033	C3 339	3B 354	3A 338	2C 343	2B 347	2A 271	1C 256	1B 264	1A 287	0	
H	0	0	0	0	0	0	0	0	0	0	0	0	
Reading	VOLTAGE = 3.8 V LAMP COMP. APPLIED												

Table 4.17 Titration Curve of Particles at Different SFB and SANH Activation

	1	2	3	4	5	6	7	8	9	10	11	12	**Protocol** Dilution
A	1567	1492	1236	1407	1617	1109	1300	1616	1278	-	-	568	A= 0.3eqSANH / eq.PNP-NH_2
B	850	847	717	726	745	644	723	830	666	-	-	561	B= 1.0eqSANH / eq.PNP-NH_2
C	663	687	599	644	593	538	568	599	515	-	-	525	C= 3.0eqSANH / eq.PNP-NH_2
D	644	597	519	515	539	512	528	539	514	-	-	553	1= 100eqSFB / eq
E	697	657	508	619	526	504	499	548	526	-	-	535	2= 30eqSFB / eq
F	684	580	530	512	489	491	490	481	502	-	-	0839	3= 10eqSFB / eq
G	580	499	481	493	511	500	483	465	468	-	-	0547	
H	541	542	549	578	535	530	514	560	482	-	-	0552	
Reading	1A	1B	1C	2A	2B	2C	3A	3B	3C				

4.3.2 Experiment (ii):

Objective : To Determine the relative amount of fluorescently tagged oligonucleotide-FTBProbe immobilized to the particles at varying probe and activation levels (From (i)) as measured by direct fluorescent readings of particles at variable concentrations. The performance of the fluorescent DNA probe assay is being tested by using quencher labeled targets.

Materials:

1. Oligonucleotide immobilized particles from experiment (i)
2. Dilution buffer: 0.1M PO_4 pH 6.0
3. Target with DABCYL quencher

Experimental Procedure:

1. Dilution series of PMP-FTBProbe was prepared as shown in table 4.14.
2. 100µl of appropriate dilution of PMP-FTBProbe was pipeted into the wells of a 96 well microtiter plate. Dilution series of target oligonucleotide with DABCYL quencher was prepared according to table 4.15.
3. 100µl of appropriate quencher or buffer was into the wells of the plate according to the designed protocol.
4. The sides of the microtiter plate were tapped gently to mix the solutions in the well.
5. The microtiter plate was wrapped securely and the plate was placed in a shaker containing water. The temperature of the shaker is maintained at about 45 – 48°C.
6. The plates were incubated in the shaker overnight.
7. After overnight incubation, the plates were read in a microtiter plate.

Table 4.18 Dilution Series for FTBProbe Immobilized Particles

Tube #	Volume of immobilizes PMP µl	Buffer Volume ml	Total Volume ml
A (1000 µg/ml)	18	162	180
B(300 µg/ml)	42	98	140
C(100 µg/ml)	14	126	140
D(30 µg/ml)	14	126	140
E(30 µg/ml)	14	126	140
F(10 µg/ml)	14	126	140
G(1 µg/ml)	14	126	140
H(0 µg/ml)	0	140	140

Table 4.19 Dilution Series for Quencher:

Tube number (condition)	Volume of Quencher (µl)	Volume of Buffer µl	Total Volume ml
A (10 µM)	166µl of stock	1584	1750
B (3 µM)	420µl A	980	1400
C (1 µM)	140µl B	1260	1400
D (0.3 µM)	140µl C	1260	1400
F (0.1 µM)	140µl D	1260	1400
G (0.03 µM)	140µl E	1260	1400
H (0.0 µM)	0	1400	1400

Results: The data from this experiment confirms FTBProbe uptake from supernatant after immobilization. The particle titration shows dose dependent fluorescence as a function of particle concentration.

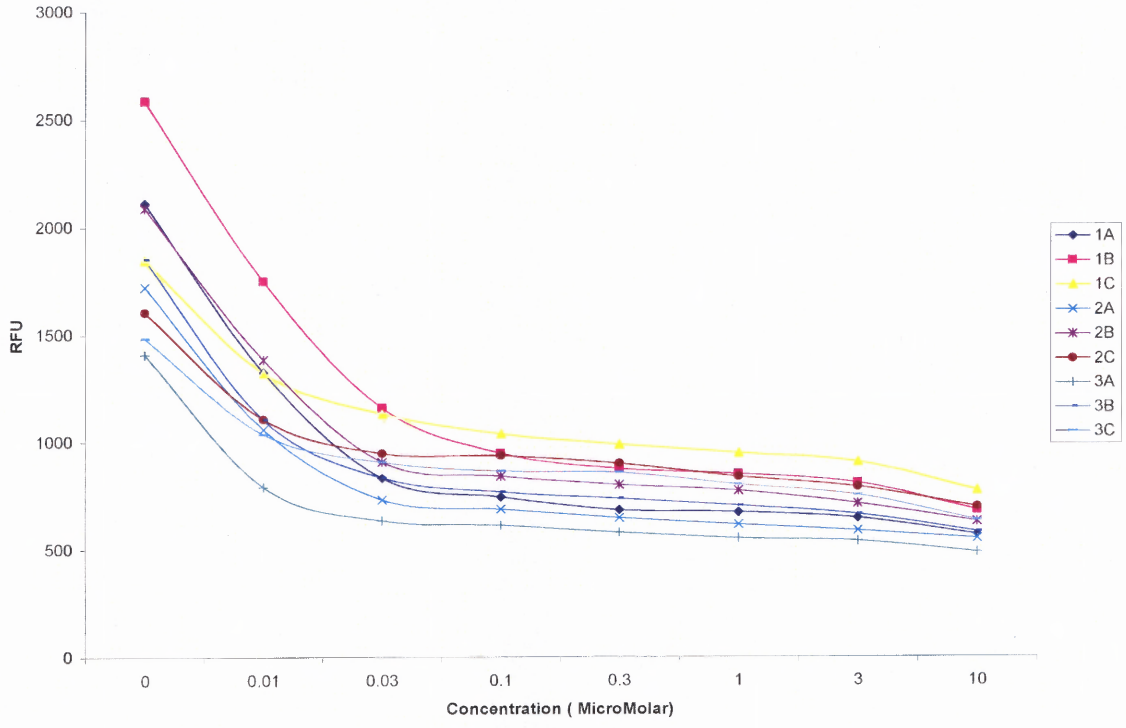


Figure 4.4 Quench curve of Particles with FTBProbe using Different Activation Levels of SANH and SFB.

Table 4.20 Hybridization Curve For Probe Immobilized Particles Using Solulink Chemistry

	1	2	3	4	5	6	7	8	9	10	11	12	Protocol
A	0456	415	569	683	774	550	629	697	487	581	634	415	
B	0444	427	645	809	905	587	713	790	540	604	752	456	100µL [QT] in each well
C	0457	445	674	851	948	616	773	838	552	704	801	458	100µL [PMP] in each well
D	0448	429	685	877	988	648	801	900	597	737	858	471	Total Volume in each well=200µl
E	0460	452	745	947	1038	688	842	936	612	769	866	452	
F	0440	454	833	1160	1130	731	907	948	634	835	908	481	
G	0471	457	1327	1748	1323	1060	1384	1107	790	1104	1038	463	
H	0472	0475	2111	2584	1846	1722	2088	1604	1409	1851	1484	305	
Reading					VOLTAGE = 7.5 V TEMP - 45^0 bath								

4.3.4 Immobilization of Oligonucleotide's to Particles of Different Sizes

Objective: To immobilize oligonucleotide probe to particles of different sizes using solulink chemistry.

Materials:

1. Paramagnetic particles from Spherotech of sizes 1.27 micron, 3.63 micron, 8.5 micron. Particles of 1 micron from Polyscience were taken as a control. Sample particles from Polyscience of size 1 micron, 1.6 micron and 9.88 micron.

2. Solulink S-9002 HydralinK

3. FTBProbe :
   5'- CGA TCG CGA CAT TCC ATC GTG CCC GAT CGA AAA A-3'

4. Sodium Phosphate/ Sodium Chloride buffer pH 7.8

5. PBS Azide/ Tween buffer pH 7.4

6. Sodium phosphate buffer pH6.0

7. PBS Azide buffer pH 7.4 + 2mg BSA

Experimental Procedure:

Particle preparation: Seven test tubes were taken and labeled 1, 2, 3, 4, 5, 6, 7

Test Tube 1: 1.27 micron particle from Spherotech. The particles were at a concentration 25mg/ml. To get 2mg of particles, 80µl of stock solution was pipetted it into 500µl of pH 7.8 phosphate buffer.

Test Tube 2: 3.63 micron particles from Spherotech. The particles were at a concentration 25 mg/ml.

Test Tube 3: 8.5 micron particles from Spherotech. The particles were at a concentration 10mg/ml. To get 2mg of particles, 200µl of stock solution was pipetted it into 500µl of pH 7.8 phosphate buffer.

Test Tube 4: 1 micron particles from Polyscience. The particles were at a concentration of 50mg/ml. To get 2mg of particles, 40µl of stock solution was pipetted it into 500µl of pH 7.8 phosphate buffer.

Test Tube 5: 1.6 micron particles from Polyscience. The particles were at a concentration of 50mg/ml. To get 2mg of particles, 40µl of stock solution was pipetted it into 500µl of pH 7.8 phosphate buffer.

Test Tube 4: 9.6 micron particles from Polyscience. The particles were at a concentration of 50mg/ml. To get 2 mg of particles, 40µl of stock solution was pipetted it into 500µl of pH 7.8 phosphate buffer.

All the particles were washed three times in pH 6.0 buffer by vortexing, magnetically separating and decanting. After the final wash, test tubes 1, 2, 3, 4, 5, 6 and 7 were resuspended in 100µl of pH 7.8 phosphate buffer. Final concentration for all particles before mixing of the oligonucleotide's is 20mg/ml

Activation of particles: The particles were modified with a hydrazine linker using SANH. From the previous experiment (experiment 3) results it was decided to use 0.3eq SANH/ eq PMP. It was noticed that the amine density on Spherotech particles is far less than the amine density on biomag plus particles. Therefore the lowest effective SANH to PMP ratio was used to ensure minimal use of DMSO. SANH was to be prepared at 5.5mg of SANH/ 100µl of DMSO. 0.378µl SANH/mg of PMP is needed. 0 .62mg of SANH was added to 56.4µl of DMSO (This is at a 1/5 dilution). Care was taken to ensure that most of the SANH goes into solution. 4.0µl of the resulting solution was then added to the test tubes 1, 2, 3, 4, 5, 6 and 7 which is at a PMP concentration of 20mg/ml. Therefore per test tube we had 4µl of SANH + DMSO and 8µl of PMP + PH 7.8 buffer. The test tubes were then incubated on a shaker at room temperature (20° – 25° C) for 2.5 hours.

Oligonucleotide activation: F- TBprobe-080803 oligonucleotide probe was used. The oligonucleotide sequence was 5'- CGA TCG CGA CAT TCC ATC GTG CCC GAT CGA AAA A-3'. The stock oligonucleotide concentration was 100 nmoles. The needed mass of oligonucleotide was 7.5nmoles. 75µl of stock oligonucleotide and put it in 675µl

of buffer pH 7.8. The concentration of SFB required for activation is 4.8mg/100µl of DMSO. From previous experimental data it was decided to use 100eq SFB/ eq oligonucleotide. 0.48mg of SFB was weighed and dissolve it 10µl of DMSO. 3.94µl of the resulting SFB solution was added to the oligonucleotides. The concentration of oligonucleotides is 10µM. The oligonucleotide + SFB were incubated for 2.5 hours at room temperature.

Purification of Oligonucleotides after aldehyde modification: The SFB – oligonucleotide reaction was terminated after 2.30 hours. The total reaction volume of oligonucleotide+ SFB was about 720µl containing 7.5 nmoles. The oligonucleotides were purified by using a NAP5 desalting column. Two NAP 5 columns were used for purifying 375µl of oligonucleotide in each column. The column was first washed 3 times in pH 6.0 PO_4 buffer by eluting and discarding elute. 375µl of oligonucleotide was carefully added to the column and after the oligonucleotide had penetrated into the column, 375µl of buffer was added. The first elute was discarded. 750µl of buffer was again added to the column to elute the oligonucleotide band. Total volume of collected oligonucleotide was 1.5ml. The oligonucleotide was collected in test tubes, capped and stored in dark at 2° - 8°C. After purification, the oligonucleotide concentration becomes 5µM.

Purification of particles: The SANH activated particles was removed from the shaker after 2.5 hours. The particles were washed three times in pH 6.0 buffer. After final wash, the particles were resuspended in 80µl of pH 6.0 buffer. The particles were at a concentration of 25mg.

Conjugation reaction (Immobilization of SFB activated oligonucleotide to SANH activated particle): The hydrazine modified particles in test tubes 1, 2, 3, 4, 5, 6 and 7 was resuspended to 80µl after the final wash. 200µl of aldehyde modified oligonucleotides were added to each of the PMP preparations. The mixtures in test tubes 1, 2, 3, 4, 5, 6 and 7 were vortexed and placed in a magnetic rack to separate the particles. After the particles were fully separated to the side, 22µl of supernatant were removed from each PMP preparation and diluted in 198µl of pH 6.0 buffer. The purpose of taking the supernatants were to get the input counts of fluorescence at time 0 of the conjugation reaction. The supernatants were stored in properly labeled centrifuge tubes in dark at room temperature. The test tubes with particles + oligonucleotide were incubated overnight at room temperature in a shaker.

Post Conjugation steps:

After incubating the particles for about 16 hours, the particles were taken out from the shaker and place on a magnetic rack. The particles were allowed to separate to the side of the test tubes. After about five minutes of separation, 220µl of supernatant was withdrawn carefully to give the output counts after incubation. The particles were then washed three times in PBS Azide + Tween wash buffer and then resuspended to 200µl in Immunoassay (PBS Azide + 2mg BSA) buffer. The final concentration of the particles was calculated to 10mg/ml.

Microtiter Plate Assay

A protocol was designed to read the input and output counts from the supernatants (in triplicates) and look at the dilutions of each particle preparation (in duplicates) at concentration ranging from 2000µg/ml to 10µg/ml. A dilution series for each particle was

done and 100µl of each dilution was pipetted into the wells of a microtiter plate according to the designed protocol. The input and output supernatants were pipetted into the wells at 50µl of respective input/output + 50µl of buffer. The readings were taken using a Fluorolite 1000 microtiter plate reader.

Table 4.21 Dilution Series for Particles with Different Sizes for Titration of Fluorescence.

Tube #	Concentration	Stock	Buffer (pH 6.0)	Total Volume
A	2000µg/ml	90µl stock activated oligonucleotide	360µl	450µl
B	1000µg/ml	120µl A	120µl	240µl
C	500µg/ml	60µl A	180µl	240µl
D	200µg/ml	24µl A	216µl	240µl
E	100µg/ml	24µl B	216µl	240µl
F	50µg/ml	24µl C	216µl	240µl
G	20µg/ml	24µl D	216µl	240µl
H	0µg/ml	0µl	240µl	240µl

Experimental Results:

1. Spherotech Particles: The tiratrion curves for the Spherotech particles show no variation in counts with increase in concentration of oligonucleotide immobilized particles. Also no change in oligonucleotide immobilization with respect to particle size was seen. The BiomagPlus control particles show a nice titration curve.

2. BiomagPlus particles:
   The titration curve for the BiomagPlus particles show the maximum counts for 6.0 micron particles. The least counts are for the 1 micron particles. This is counterintuitive to our hypothesis.

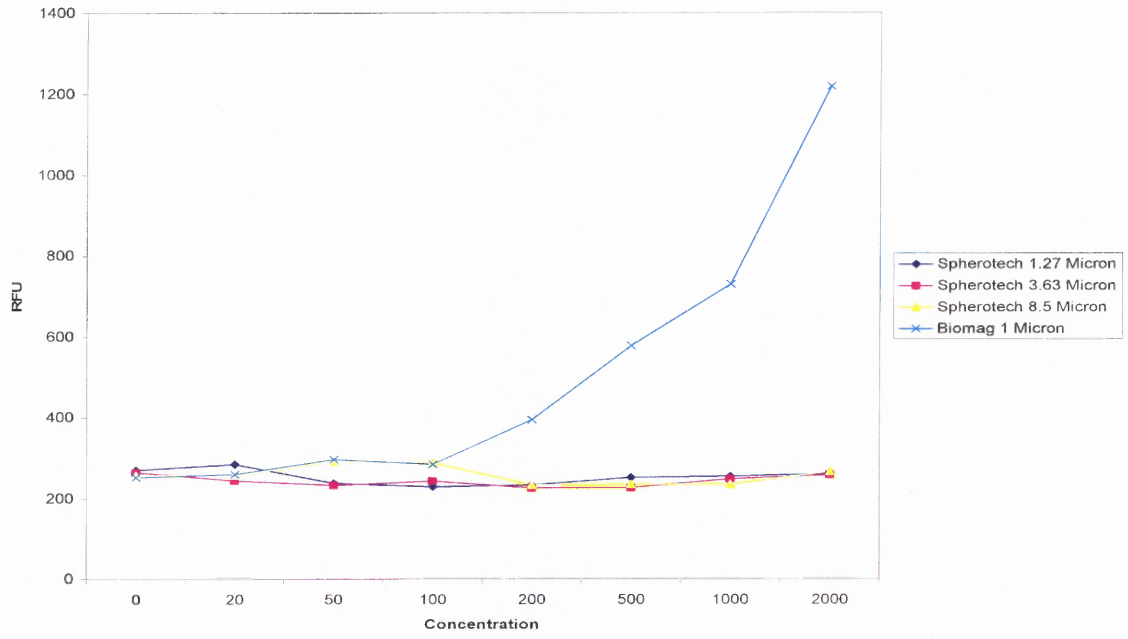


Figure 4.5 Titration curve for Spherotech Particles and Control Particles.

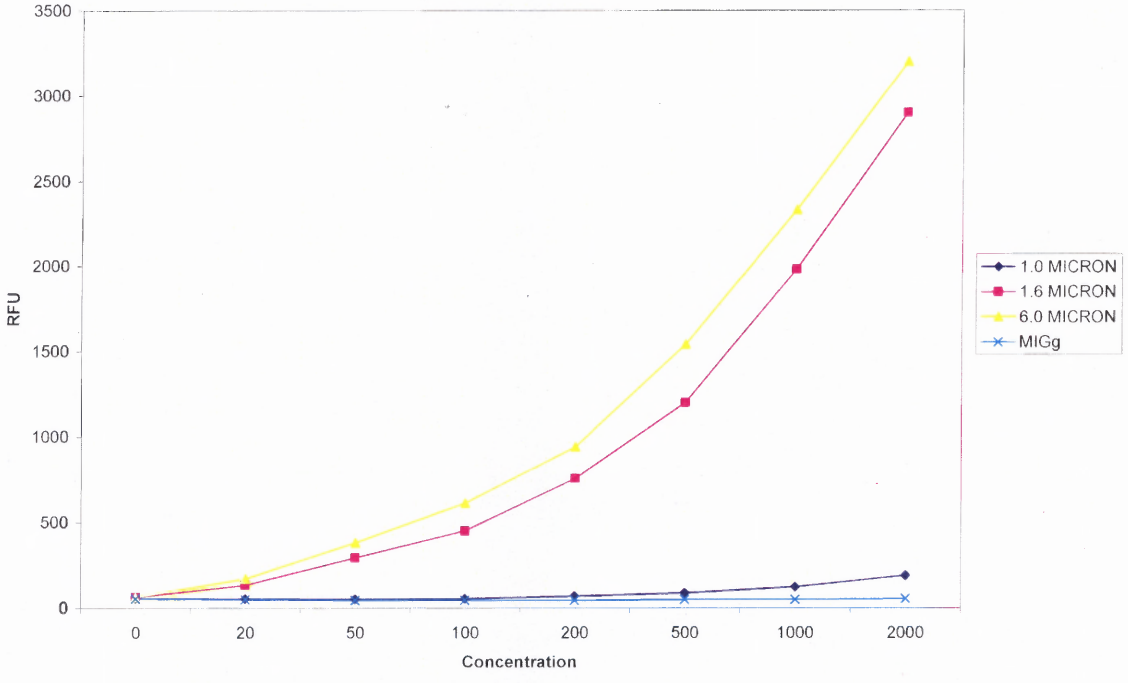


Figure 4.6 Titration Curve for BiomagPlus Particles.

Experiment Discussion:

The readings from the microtiter plate 1 give us the following conclusions

1. The input supernatant shows lower counts than the output supernatant.
   The speculated reasons for this kind of a result could be (a) An effect of pH of the dilutions of oligonucleotide with time (b) "Soaking" up of oligonucleotides at time t = 0 which gives us low input counts when we take the supernatants at time t = 0 (c) Some undetermined source of oligonucleotide contamination or photobleaching?

2. The dilutions series of the oligonucleotide + Spherotech PMP shows that there is no effective intake of oligonucleotide with change in PMP size and the counts are comparable to the background, while the oligonucleotide + Polyscience PMP control shows good variation in counts with dilutions. The Spherotech particles are prepared by coating a layer of magnetite and polystyrene onto monodispersed (i.e. uniform sized) polystyrene core particles. During the experimental procedures, we noticed that there was particle loss during washing processes, the particles did not separate well, and we could see films in the particle solution after activation and oligonucleotide immobilization which seemed to look like "coatings" of the particle. This raises the following question: (a) Has the amine coating or magnetic layer of the particle been stripped off during the activation or oligonucleotide immobilization. If yes then why? (b) Does pH have an effect on the magnetite coating or amine termination of the Spherotech particle?

The readings from microtiter plate 2 shows the following results

1. As observed with the Spherotech particles, the i/p counts are much lower than the o/p counts. The reason could be same as that speculated with Spherotech particles i/p and o/p counts.

2. The Polyscience biomag particles are not spherical in shape. They are irregular, comet shaped iron oxide particles. The dilution series done for the Polysciences particles show a nice titration curve in contrast to the Spherotech particles. But the data obtained is converse to the hypothesis. According to our hypothesis, we would expect the 1 micron particles of Polyscience to show more fluorescent oligonucleotide uptake than the 9.8 micron particles. But the results show that 9.8 micron particles have markedly more fluorescently labeled oligonucleotide's immobilized to them than the 1 micron particle. The reasons could be

   (a) As the particle size is decreasing, is some surface property of the particle preventing immobilization of oligonucleotide to activated amine groups even though there are more number of amine groups per volume on the 1 micron particle than the 9.8 micron particle.

(b) Effect of diffusion kinetics. We need to find out if there are more number of binding events taking place inside the pores of the one micron particles than on the 9.8 micron particles. If this is the case, then we will not be able to detect the fluorescent oligonucleotides bound inside the pores.

(c) There is more number of particles per unit volume in the 1 micron particles than in the 9.8 micron particle. As the particle size increases, the number of particles per unit mass should decrease at least 1000 times (as with the case of spherotech particles). Since there is more number of particles per unit volume or mass, there could be more quenching of the fluorescence emitted by the fluorescently tagged oligonucleotide than with the 9.8 micron. That is the particle density per unit volume is going to contribute to signal quenching.

The sensitivity of the microtiter plate reader may not be good enough to detect the signals emitted by the immobilized particles. The best way to detect the number of oligonucleotides immobilized per surface area or per particle will be with AFM or with flow cytometry. In order to find out why the data shows lesser number of input counts than output counts, a time course study of fluorescence was conducted.

Table 4.22 Plate I – Spherotech + Polyscience 1 micron

	1	2	3	4	5	6	7	8	9	10	11	12	**Protocol** Dilution
A	I/P S1.27 256	I/P S3.5 235	I/P S8.5 238	I/P P1 247	1238	1199	287	244	273	262	255	266	2000 µg/ml
B	186	188	234	176	742	720	238	237	259	244	254	256	1000 µg/ml
C	208	257	183	194	596	561	243	229	220	233	250	255	500 µg/ml
D	200	181	176	188	391	399	237	231	226	226	239	228	200 µg/ml
E	O/P S1.27 2171	O/P S3.5 2128	O/P S8.5 2026	O/P P1 2118	431	339	288	403	237	248	231	229	100 µg/ml
F	2250	1989	2020	2708	297	287	336	249	233	233	239	236	50 µg/ml
G	2432	2296	2217	2414	370	254	248	267	239	249	252	318	20 µg/ml
H	2221	2880	2241	2397	254	251	264	251	267	263	296	246	0 µg/ml
Reading	4.0Volts				B1 0		S8.5 3		S3.63 2		S1.27 1		50 µl / Well
									100 µl / Well 9.4 Volts				

Table 4.23 Plate II – Polyscience

	1	2	3	4	5	6	7	8	9	10	11	12	**Protocol** Dilution
A	I/P P1 146	I/P P1.6 22	I/P P6.0 52	S1 Bkd 18	55	54	3246	3124	2896	2979	191	192	2000 µg/ml
B	I/P P1 201	I/P P1.6 25	I/P P6.0 57	14	50	49	2342	2323	1989	1964	120	126	1000 µg/ml
C	I/P P1 166	I/P P1.6 27	I/P P6.0 51	S2 Bkd 15	54	46	1554	1531	1199	1276	88	89	500 µg/ml
D	I/P P1 180	I/P P1.6 29	I/P P6.0 55	16	43	45	965	929	758	754	71	72	200 µg/ml
E	O/P P1 191.4	O/P P1.6 344	O/P P6.0 439	P1 Bkd 12	46	43	678	609	461	436	56	54	100 µg/ml
F	O/P P1 2155	O/P P1.6 417	O/P P6.0 530	11	44	46	392	376	294	292	52	55	50 µg/ml
G	O/P P1 1928	O/P P1.6 331	O/P P6.0 461	P2 Bkd 12	53	43	201	158	139	128	61	50	20 µg/ml
H	O/P P1 3079	O/P P1.6 384	O/P P6.0 478	15	62	54	64	62	62	64	54	56	0 µg/ml
Reading	4.1Volts				MIgG 7		P6.0 6		P1.6 5		P1.0 4		100 µl / Well
					6.0 Volts								

4.3.5 Oligonucleotide Immobilization Time Course Study

Experimental Objective: To study the immobilization of oligonucleotides onto the paramagnetic particles during different time points.

Materials:

1. Particles of 1 micron from Polyscience.
2. Solulink S-9002 HydralinK
3. FTBProbe :
   5'- CGA TCG CGA CAT TCC ATC GTG CCC GAT CGA AAA A-3'
4. Sodium Phosphate/ Sodium Chloride buffer pH 7.8
5. PBS Azide/ Tween buffer pH 7.4
6. Sodium phosphate buffer pH6.0
7. PBS Azide buffer pH 7.4 + 2mg BSA

Experimental Procedure:

Activation of 1 μ Biomag plus particles

Particle Preparation: Stock PMP @ 50 mg/ml

50mg particles were activated. Therefore 1 ml of the stock PMP was taken and pipetted into 3ml of 0.1M pH 7.8 buffer. The particles were washed three times in 5ml of 0.1M pH 7.8 buffer. After washing, the particles were resuspended in 2.5ml of pH 7.8 0.1M buffer to 20mg/ml.

1. **Particle activation using SANH:**
   Condition of activation = 1eq SANH / 1eq PMP = 1.26μl SANH / mg particle
   63μl of SANH / 50mg PMP is needed.
   Solulink protocol: 5.5mg SANH / 100μl DMSO. Therefore 4.4mg SANH was dissolved in 80μl of DMSO
   63μl of the prepared solution was added to the washed 20 mg/ml particles and incubated in the shaker at room temperature for 2.5 hours.

Post Incubation Steps: After Incubation, the particles were removed from shaker. The particles were washed four times in pH 6.0 buffer and resuspend to 25 mg/ml i.e. in 2 ml of pH 6.0 buffer.

2. **FTBProbe Activation using SFB**

   Probe: 5'- CGA TCG CGA CAT TCC ATC GTG CCC GAT CGA AAA A – 3'
   Stock probe concentration = 100μM.
   1 / 5 dilution of the oligonucleotide stock i.e. 20μM was made by adding 300μl of stock oligonucleotide to 1200μl of buffer. The total volume of the resulting oligonucleotide solution is 1500μl. (20μM x 1500μl = 30 nmoles).
   100eq SFB/ eq oligonucleotide – NH_2 ratio was chosen for oligonucleotide activation.
   30 nmoles x 100eq SFB / eq oligonucleotide – NH_2 = 3000 nmoles SFB. To activate oligonucleotides with 3000 nmoles of SFB 15.78 μl of SFB is needed.
   Solulink ratio: 4.8 mg SFB/ 100μl DMSO.
   0 .88 mg SFB was weighed and dissolved in 20μl DMSO. 15.78 μl prepared SFB solution was added to 750μl prepared oligonucleotide solution. The oligonucleotide's activated with SFB was incubated in room temperature for 2.5 hours.

After incubation, the oligonucleotides are purified using NAP5 columns. For 1.5 ml of activated oligonucleotides, after purification through the desalting column, 3.0ml of purified activated oligonucleotides is obtained. The resulting molar concentration will be 10μM.

3. **SANH + SFB reaction**

   For the immobilization reaction, 8mg of activate particles is activated with 4 nmoles of purified activated oligonucleotides. The concentration of the activated particles is 25mg/ml and the concentration of the activated oligonucleotides is 10μM.
   Volume of activated PMP in the reaction = 32μl
   Volume of activated oligonucleotide in the reaction = 400μl
   Total reaction volume = 720μl
   Concentration of oligonucleotide in the mixture = $\frac{4\text{nmoles}}{720\mu\text{l}}$ = 5.55μM

   Concentration of PMP in the mixture = $\frac{8\text{mg}}{720}$ = 15.38g/l

Time Point Fluorescence Analysis:

The time points chosen were t = 0, t =10 minutes, t = 30 minutes, t = 2 hour, t = O/N

At each point two sample points from the master mixture tube is taken.

Sample 1 - To give reading of fluorescence of PMP + Oligonucleotide

Volume – 33µl

Dilution – 33µl of sample + 297µl of pH 6.0 buffer

Sample 2 – To give reading of amount of oligonucleotides in the supernatant amount of oligonucleotide's immobilized to the particle

Volume – 33µl

The sample was separated in a magnetic rack. 30µl of supernatant was carefully withdrawn.

For (i) the supernatant withdrawn was diluted in 270µl of pH 6.0 buffer

For (ii) the residual volume of particles + supernatant after removal of 30µl of oligonucleotide is diluted in 270µl of pH 6.0 buffer.

After samples are taken till time point t=2 hours, the PMP + Oligonucleotide master mixture is incubated overnight. After incubation O/N, the master mixture is separated in a magnetic rack and 600µl of supernatant is withdrawn. This would give us the output counts.

The particles were washed three times in PBS Azide + Tween wash buffer and then resuspended in Immunoassay (PBS Azide + 2mg BSA) buffer so that the final concentration of is particles 10mg/ml. A dilution series for the oligonucleotide immobilized particle as shown in the table below and read at a constant voltage the fluorescence count using fluorometer.

Table 4.24 Dilution Series for Particles with Different Sizes for Titration of Fluorescence

Tube #	Concentration	Stock	Buffer (pH 6.0)	Total Volume
A	2000µg/ml	90µl stock activated oligonucleotide	360µl	450µl
B	1000µg/ml	120µl A	120µl	240µl
C	500µg/ml	60µl A	180µl	240µl
D	200µg/ml	24µl A	216µl	240µl
E	100µg/ml	24µl B	216µl	240µl
F	50µg/ml	24µl C	216µl	240µl
G	20µg/ml	24µl D	216µl	240µl
H	0µg/ml	0µl	240µl	240µl

Results of Time course:

1/10 dilution of offered oligonucleotide's = 3268.8

Table 4.25 Results from Time Course Experiment

Time	Oligonucleotide + PMP	PMP	Supernatant
T=0	1370	685	1186.3
T=30 min	1227	211	1365.3
T=1 hrs	725.6	135.3	743
T=2 hrs	408	43.6	497
T = O/N	-	-	1212.39

Percentage of uptake during time courses:

(1) Offered Oligonucleotide's and time t = 0
Percentage uptake = 63.7%

(2) Offered Oligonucleotide's and t = 30 min
Percentage uptake = 58.2%

(3) Offered Oligonucleotide's and t = 1 hr
Percentage uptake = 77.2%

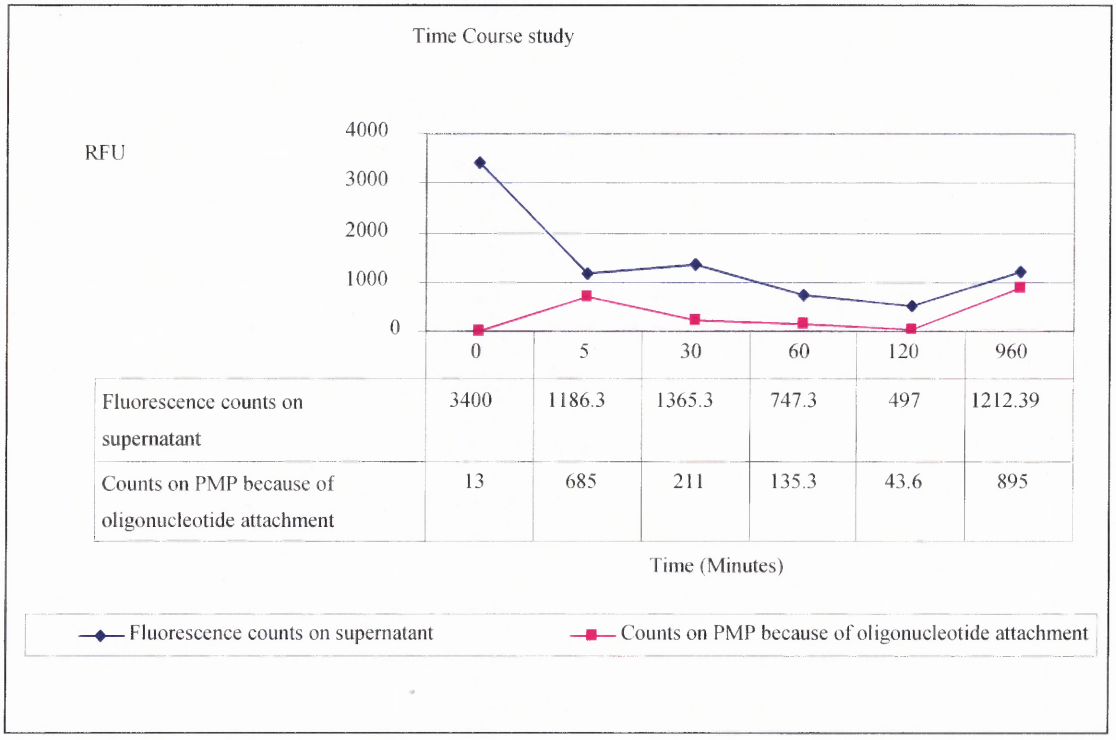
(4) Offered Oligonucleotide's and t = 2 hr
Percentage uptake = 84.79%

(5) Offered Oligonucleotide's and t = O/N
Percentage uptake = 62.9%

(6) Difference between input counts at time t=0 and time t = O/N

$$\text{Percentage uptake} = \frac{1186.3 - 1212.39}{1186.3} = -2.11\%$$

The results show very low input counts compared to the output counts as seen with the previous experiments. The sharp decrease in supernatant counts could be because of the following reason: Physical adsorption - At pH 6.0, the pyridine ring of the SANH should be protonated (PKa ~ 10). The oligonucleotide nucleotides have a phosphorus back bone which will have a slight negative charge. These positively charged groups on the particle maybe allowing the separation of phase of oligonucleotides from solution by means of electrostatic interaction with the phosphate backbone of the nucleic acid.



	0	5	30	60	120	960
Fluorescence counts on supernatant	3400	1186.3	1365.3	747.3	497	1212.39
Counts on PMP because of oligonucleotide attachment	13	685	211	135.3	43.6	895

Figure 4.7 Time Course Study of Supernatants.

CHAPTER 5

CONCLUSIONS AND FUTURE RECOMMENDATIONS

Investigation of substrates and chemistries for performing biomolecular binding assays reveal.

1. PMP is superior to microtiter plate.
2. Solulink Chemistry superior to glutaraldehyde chemistry for immobilization.
3. Surprisingly, the relationship between specific binding capacity and performance as measured by fluorescence binding capacity or performance labeling did not correlate inversely with particle sizes at fixed particle mass as originally postulated. The understanding of the mechanism of the reasons for this result requires carefully controlled experiments to be conducted.

The readings from the microtiter plate assay show increased immobilization with increase in particle size. Mathematical calculations and data from the particle vendors show a larger number of amine terminated groups available for binding per unit volume is much higher for the 1 micron particles than the 8.5 micron particles.

The reasons for this disparity can be speculated as (i) Since there are no: of particles per unit volume for the 1 micron particle than the 9.7 micron particle there will be greater quenching of fluorescence by the 1 micron particles than the 9.8 micron particles, (ii) Two dimensional detection technique has been used for detection in this research.

An experiment intended to be done as the next step is to hybridize labeled target sequence to the oligonucleotide probe immobilized particles of varying sizes from Spherotech and Polyscience Inc. After hybridization, the particles should be thoroughly washed so that only the probe – target complexes remain on the particle. The next step will be to cleave the target from the probe. An assay of the cleaved label target will allow

us to determine the number of targets hybridized to the probes and subsequently the number of probes immobilized to the particles.

Magnetic nanoparticles and microparticles have been proposed for use as biomedical purposes to a large extent for several years. In recent years, nanotechnology has developed to a stage that makes it possible to produce, characterize and specifically tailor the functional properties of nanoparticles for clinical applications. Many challenges clearly remain before magnetic nanoparticles achieve their full potential in the biomedical arena.

This thesis research has shown that in order to build an in vitro biological, toxic and chemical detection system or sensor, we need to search for new or improve on previous techniques to produce reliable particles, labels and detectors with the correct characteristics suitable not only for a particular application but extendable to other applications. The thesis required an understanding and application of various areas of discipline like molecular biology, chemistry, material science, physics and electronics.

For the realization of a robust, reliable detection system for either an in vitro sensor or an in vivo sensor, adoption of an interdisciplinary team is necessary. An interaction between cell biologists, molecular biologists, chemists, physicists, electronics engineers, and materials scientists is invaluable for rapid progress in this area of research. With this combined expertise, a robust, sensitive and rapid molecular scale sensing devices will soon become a reality.

APPENDIX A

BUFFER PREPARATIONS

This appendix gives the amount of reagents necessary for preparing the buffers used in the experimental procedure

1. **PBS Azide Wash Buffer pH 7.1 (1.0L)**
   Stock: 10X concentrated mix of PBS Azide (10mM NaPO4, 150mM NaCl, 0.25% NaN3). To make 1/10 dilution of stock, take 100ml of stock + 900ml of DI water.

2. **1M Na_2HPO_4 Dibasic Buffer (100ml)**
   Weigh 26.81gm of Na_2HPO_4 (Formula Weight = 268.1). Dissolve it in water to make up 100ml.

3. **0.1M Na_2HPO_4 Dibasic Buffer (250ml)**
   Weigh 6.7gm of Na_2HPO_4 (Formula Weight = 268.1). Dissolve it in water to make up 250ml.

4. **0.1M NaH_2PO_4 Monobasic Buffer (200ml)**
   Weigh 27.6gm of NaH_2PO_4 (Formula Weight = 138.0). Dissolve it in water to make up 200ml

5. **10mM $NaPO_4$ pH 8.0 Buffer (500ml)**
   Stock: 0.1M dibasic Na_2HPO_4 (pH 8.0 – 8.5), 0.1M monobasic NaH_2PO_4 (pH 4.5-5.0). To 400ml of DI water add 46.6ml of 0.1M Na_2HPO_4 and 3.4ml of 0.1M NaH_2PO_4. Test the pH of the resulting buffer. Adjust the pH to 8.0 by adding NaOH or HCl or DI water. Bring the total buffer volume to 500ml.

6. **10 mM $NaPO_4$ pH 7.4 Buffer (250ml)**
   Stock: 0.1M dibasic Na_2HPO_4 (pH 8.0 – 8.5), 0.1M monobasic NaH_2PO_4 (pH 4.5-5.0). To 200ml of DI water add 19.35ml of 0.1M Na2HPO4 and 5.65ml of 0.1M NaH_2PO_4. Test the pH of the resulting buffer. Adjust the pH to 7.4 by adding NaOH or HCl or DI water. Bring the total buffer volume to 250ml.

7. **Coating Buffer - 10mM $NaPO_4$ pH 7.0 (250ml)**
   Stock: 0.1M dibasic Na_2HPO_4 (pH 8.0 – 8.5), 0.1M monobasic NaH_2PO_4 (pH 4.5-5.0). To 200ml of DI water add 14.425ml of 0.1M Na_2HPO_4 and 10.575ml of 0.1M NaH_2PO_4. Test the pH of the resulting buffer. Adjust the pH to 7.0 by adding NaOH or HCl or DI water. Bring the total buffer volume to 250ml.

8. **Blocking Buffer** = PBS Azide + 2mg/ml BSA (No Tween)(250ml)
   Mix 500mg of BSA in 240ml of PBS Azide buffer. Adjust the pH to 7.1 and then bring up the volume to 250ml.

9. **Immunoassay Wash Buffer** = 10mM $NaPO_4$ (pH 7.0) +150mM NaCl + 0.025% NaN_3 + 0.05% Tween 20. (pH 7.1)
   Preparation of 1% Tween 20 spiking stock: Take 15ml centrifuge tube and pour 10 ml of DI water into the tube. Pipette 100µl of stock Tween 20 into the 10 ml DI water.
   Wash buffer: PBS azide + 0.05% Tween 20.
   To 570ml of PBS Azide buffer, add 30ml of 1% spiking Tween 20 stock. Mix well.

10. **Assay Buffer** = Immunoassay wash buffer + 2mg/ml BSA (pH 7.1)(250ml)
    Mix 500mg of BSA in 240ml of Wash buffer (PBS Azide + Tween). Adjust the pH to 7.1 and then bring up the volume to 250ml

11. **Reading Buffer** = 10mM $NaPO_4$ (pH 7.42)

12. **10mM Tris Buffer pH 7.5 (1.0L)**
    Add 1.211g of Trizma Base (Tris [hydromethyl] amino methane, Formula Weight = 121.1) to 900ml of DI water. Mix it well and pH to 8.5. Add 35.064gm of NaCl (Formula Weight = 58.44) to it. Mix well and pH to10.0. This gives 10mM Trizma Base + 0.6M NaCl. Titrate the pH down to 7.5 by adding HCl.

13. **Hybridisation Buffer**
    Stock: 10mM Trizma Base + 0.6M NaCl (pH 7.5), 2.5% Azide (2.5gm/100cc), 1% BSA (1gm/100cc), 0.25% Tween 20. Mix 148ml of 10mM Trizma Base + 0.6M NaCl (pH 7.5), 1.5ml of Azide, 1.5gm of BSA and 0.375 ml of Tween 20.

14. **Sodium Phosphate/ Sodium Chloride buffer PH 7.8**
    0.29 grams of 100mM NaCl added to 50 cc 100mM $NaPO_4$ pH 8.0

APPENDIX B

MATERIALS AND REAGENTS USED

This appendix gives the details of the materials and reagents used for the experiments conducted for this research.

1. Bovine Serum Albumin(BSA): Fraction V, Minimum 96%; Sigma P No# A2153-50G; Batch #063K0778

2. BioMag Plus Amine coupling Kit; Polysciences
   a) BioMagPlus Amine Terminated Particles (50mg/ml in 1mM sodium EDTA, pH 7.0) 25ml PNo# 86001-25 Lot#530096

   b) Glutaraldehyde (EM Grade, 25%) PNo# 01909A; Lot#527456
   Stock Glutaraldehyde (GA) was aliquoted for long time storage at -20° C in 1.5ml eppendorff tubes. 19 aliquotes 0.5 ml and 1 aliquot at 200 µl.

3. MIgG [MIgG] = 10mg at 6.3mg/ml + 0.1%NaN_3
   Lot # 500-70035, Code# M8-G10

4. Oligonucleotide Sequences

   a) FTBProbe-080803
   5'- CGA TCG CGA CAT TCC ATC GTG CCC GAT CGA AAA A-3'
   --- STEM: CGA TCG CGA TCG
   --- SEQUENCE: CGA CAT TCC ATC GTG CC
   --- SPACER: AA AA
   Length: 34 -mer
   Modifications:
   5' FLU
   3' Amino C7
   Amount: 35.6 OD, 1060µg, 95.3nmol
   Molecular Weight: 11126g/mol
   Volume for 100pmol/µl = 953µl

   b) Q-TBtarget-080803
   5'- CGA TCG GGC ACG ATG GAA TGT CGC GAT CG-3'
   --- STEM: CGA TCG CGA TCG
   --- SEQUENCE: GGC ACG ATG GAA TGT CG
   Length: 29-mer

Modification: 3' Dabcyl
Amount: 34.7 OD, 1026μg, 108.6nmol
Molecular Weight: 9447g/mol
Volume for 100pmol/μl = 1086μl

c) TBtarget-080803
5'- CGA TCG GGC ACG ATG GAA TGT CGC GAT CG-3'
--- STEM: CGA TCG CGA TCG
--- SEQUENCE: GGC ACG ATG GAA TGT CG
Length: 29-mer
Modification: None
Amount: 22.6 OD, 635μg, 70.7nmol
Volume for 100 pmol/μl = 707μl
Molecular Weight: 8984g/mol

d) TBtarget-NH_2-080803
5'- CGA TCG GGC ACG ATG GAA TGT CGC GAT CG-3'
--- STEM: CGA TCG CGA TCG
--- SEQUENCE: GGC ACG ATG GAA TGT CG
Length: 34-mer
Modification: 3' Amino C7
Amount: 10.3 OD, 279 μg, 26.0nmol
Volume for 100pmol/μl = 260μl
Molecular Weight: 10760g/mol
Note: Reconstitute to 50pmol/μl = 50μM. The stock TBtarget-NH_2-080803 is reconstituted in 520μl of DI Water.

APPENDIX C

EQUIPMENT AND INSTRUMENTS USED

This appendix lists the equipments and instruments used for the research.

1. Beckman DU 520 General purpose UV/Vi Spectrophotometer
2. Fluorolite 1000 Microtiter Plate reader
3. PH Meter
4. Mettler H20 Weight balance
5. Vortex- Genie; Scientific Industries Model: k550G
6. 96 well plate Magnet

APPENDIX D

GLOSSARY

This appendix lists the meaning of some of the terms used in this document.

1. **Affinity.** Affinity is a measure of the intrinsic binding strength of the ligand binding reaction. The intrinsic attractiveness of the binder for the ligand is typically expressed as the equilibrium constant (Ka) of the reaction. The equilibrium constant Ka = [Ligand-Binder]/[Ligand][Binder], where [ ] represents the molar concentration of the material at equilibrium.

2. **Analyte.** An analyte is the compound being measured.

3. **Assay.** A method to analyze or quantify a substance in a sample. An assay is an analysis done to determine: 1. The presence of a substance and the amount of that substance. 2. The functionality of the substance. An assay can also be defined as an instrument, kit or test system which applies one or more technical procedures or one or more analytical methods, using a defined and characterized protocol, to determine measure or otherwise describe the presence or absence of an analyte.

4. **Binding Site:** The active region of a receptor; any site at which a chemical species of interest tends to bind. The process by which a molecule (or ligand) becomes bound, that is, confined in position (and often orientation) with respect to a receptor. Confinement occurs because structural features of the receptor create a potential well for the ligand; Van der Waals and electrostatic interactions commonly contribute.

5. **Buffer.** The buffer is the defined media in which the ligand-binder reaction takes place. Many of the components in the buffer solution influence the avidity of the reaction.

6. **Concentration.** The concentration of an analyte is a measurement of the mass of the analyte per unit volume. The mass may be expressed in grams, moles, international units (IU), or other units. The liquid volume may be expressed in milliliters or other units of volume.

7. **Covalent bond:** the molecular force holding two atoms together that share a pair of electrons.

8. **Detector.** A detector is an analytical instrument which is capable of measuring the amount of tracer label in an immunoassay sample, such as scintillation counters, microplate readers, and automated immunoassay analyzers.

9. **Dilution.** A dilution is the ratio of the volume of pure specimen to the total volume of specimen plus a diluent such as buffer. A dilution of 1:10 contains one part pure specimen and nine parts diluent. A dilution factor is the total number of parts with the volume of pure specimen being one part.

10. **Emission Spectrum.** The emission spectrum of a substance such as a fluorophore is the intensity of the electromagnetic radiation from the substance as a function of the excitation wavelength of the absorbed light.

11. **Excitation Wavelength.** Excitation wavelength in fluorometry is the wavelength of radiant energy that is absorbed by a fluorophore molecule. This incident light excites the molecule and results in the emission of radiation at a higher wavelength (the emission wavelength).

12. **Emission Wavelength.** the wavelength of the light emitted by a fluorescent reporter molecule when stimulated by a laser at its characteristic excitation wavelength.

13. **Fluorescent Label.** A fluorescent label is either a fluorescent molecule, or an enzyme system that generates a fluorescent product, which is linked to a ligand or binder tracer. The tracer is quantified by measuring the amount of light emitted by the tracer at a specific wavelength after exciting the tracer with incident light of a shorter wavelength. Fluorescent molecules include fluorescein and lanthanide chelates such as europium, samarium, and terbium.

14. **Fluorescence.** Fluorescence is the property of certain molecules, or fluorophores, to absorb light at one wavelength and emit a light at a longer wavelength. The incident light excites the molecule to a higher level of vibrational energy. As the molecule returns to the ground state, the excited fluorophore emits a photon. This photon is the fluorescence emission. If the molecule returns to the ground state through an intermediate excited triple state, there is a delay in the emission of the photon. This delayed photon is termed a phosphorescence emission.

15. **Hybridization:** The formation of stable duplexes of two DNA and/ or RNA (complementary) strands via Watson- Crick base pairing used for locating or identifying nucleotide sequences and to establish the effective transfer of nucleic acid material to a new host.

16. **Ligand:** A molecule which binds to a receptor molecule, producing a response.

17. **Micron:** micrometer, one-millionth of a meter.

18. **Mole:** A number of instances of something (typically a molecular species) equaling ~6.022 X 10e23. *Mole* ordinarily means gram-mole; a kilogram-mole is ~6.022X 10e26.

19. **Nanometer:** one-billionth of a meter (from the Greek word for "dwarf").

20. **Nucleotide**: A molecule consisting of a nitrogenous base (A, G, T or C in DNA; A, G, U or C in RNA), a phosphate moiety and a sugar group (deoxyribose in DNA and ribose in RNA). Thousands of nucleotides are linked to form a DNA or RNA molecule.

21. **Oligonucleotide**: A short nucleic acid molecule (usually 2-50 bases), either obtained from an organism or synthesized chemically.

22. **Probe**: a labeled nucleic acid molecule for detecting complementary sequences. A DNA Probe is a small piece of nucleic acid that has been labeled with a radioactive isotope, dye or enzyme and is used to locate a particular nucleotide sequence or gene on a DNA molecule.

23. **Reaction:** A process that transforms one or more chemical species into others. Typical reactions make or break bonds; others change the state of ionization or other properties taken to distinguish chemical species.

24. **Reagents:** A chemical species that undergoes change as a result of a chemical reaction.

25. **Receptor**: A molecule which produces a response when another molecule ("ligand") is bound to it.

26. **Sensitivity.** The sensitivity of a test method is the lowest concentration that can be reliably measured using that test method. It is the ability of a test to detect all true cases of the condition being tested for; absence of false-negative results.

27. **Specificity.** The specificity of a binder is the ability of its binding site to distinguish between the ligand to which the binder is specific and other compounds. Specificity can also be defined as the ability of a test to correctly yield a negative result when the condition being detected is absent; absence of false-positive results.

28. **Specific binding**: It is the measurable and reversible or quasireversible, or saturable interaction between a ligand or a receptor. Ligands include e.g.: Drugs, hormones, antigens, proteins, sugars, analyte, neurotransmitter and nucleic acid probes. Receptors include hormone receptors, drug receptors, transmitter receptors, cellular receptors, antibodies and nucleic acid targets.

REFERENCES

1. Zeenia Kaul, Tomoko Yaguchi, Sunil C Kaul, Takashi Hirano, Renu Wadhwa, Kazunari Taira. Mortalin imaging in normal and cancer cells with quantum dot immuno-conjugates. Cell Research 2003; 13(6):503-507.

2. The Sixth Annual BCC Conference - Nanoparticles 2003 (http://www.bccresearch.com/nano2003/introduction.html). Retrieved from world wide web on 04/20/04.

3. R. Martin, David T. Mitchell. Nanomaterials in Analytical Chemistry. Analytical Chemistry News & Features.1998 May1; 322 A-327 A.

4. Running JA, and Urdea MS. A Procedure for Coupling of Synthetic Oligonucleotides to Polystyrene Microtiter Wells for Hybridization Capture. BioTechniques 1990; 8:276-277.

5. Todd Strother, Robert J. Hamers and Lloyd M. Smith. Covalent attachment of oligonucleotidedeoxyribonucleotides to amine-modified Si (001) surfaces. Nucleic Acids Research 2000; 28(18); 3535-3541.

6. Jill K. Veilleux and Lise W. Duran. Covalent immobilization of bio-molecules to preactivated surfaces. *IVD* Technology 1996; 2: 26-31.

7. Q A Pankhurst, J Connolly, S K Jones and J Dobson. Applications of magnetic nanoparticles in biomedicine. Journal of Physics D: Applied Physics 2003; 36:R167-R181.

8. Paula Gould. Nanoparticles probe biosystems. Materials today. 2004 February; 36-43.

9. H. A. Ferreira, D. L. Graham, P. P. Freitas, and J. M. S. Cabral. Biodetection using magnetically labeled biomolecules and arrays of spin valve sensors. Journal of Applied Physics. May 15, 2003 ; 93(10):7281-7286.

10. Willner I, Willner B. Biomaterials integrated with electronic elements: en route to bioelectronics. Trends Biotechnology. 2001 Jun; 19(6):222-30.

11. Joseph Wang. From DNA biosensors to gene chips. Nucleic Acids Research, 2000; 28(16): 3011-3016.

12. Jain K.K. Current Status of Molecular Biosensors. Medical Device Technology; May 1, 2003.

13. Riccelli P.V et al. Hybridization of single-stranded DNA targets to immobilized complementary DNA probes: comparison of hairpin versus linear capture probes .Nucleic Acids Research, 2001; 29(4): 996-1004.

14. Schwartz, David. Bioconjugation. [Document posted on Web site of Solulink Inc]. Retrieved May 1, 2004 from the World Wide Web: http://www.solulink.com/.

15. Molecular beacons: probes that fluoresce upon hybridization. Tyagi, S. and Kramer, F.R. Nat. Biotechnol. 1996; 14:303-308.

16. Marras S, Kramer F, Tyagi S, Multiplex detection of single-nucleotide variations using molecular beacons. Genetic Analysis: Biomolecular Engineering 1999; 14:151-156.

17. Piatek, A.S. et al. Molecular beacon sequence analysis for detecting drug resistance in Mycobacterium tuberculosis. Nat. Biotechnol. 1998; 1:359-363.

18. Elghanian R, Storhoff JJ, Mucic RC, Letsinger RL, Mirkin CA. Selective colorimetric detection of polynucleotides based on the distance-dependent optical properties of gold nanoparticles. Science. 1997 Aug 22; 277(5329):1078-81.

19. Ferguson, J.A., Boles, T.C., Adams, C.P., Walt, D.R. A fiber-optic DNA biosensor microarray for the analysis of gene expression. Nature Biotechnology , 1996; 14:1681-1684.

20. Jacobson, Bruce. Biosensors and Other Medical and Environmental Probes. [Document posted on Web site of Oak Ridge National Laboratory Review]. Retrieved May 1, 2004 from the World Wide Web: http://www.ornl.gov/info/ornlreview/rev29_3/text/biosens.htm.