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ABSTRACT

BIOMIMETIC SENSOR FOR IN-VIVO APPLICATIONS

by

Daya I. Sooryadas

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 agents has been an active area of research. A molecular sensor is a biomolecule or biomimetic molecule that performs two distinct functions in detecting target molecules. The sensor molecule recognizes a target molecule and specifically binds to it. This specific binding generates a signal in response to the event of target binding that can be then detected. The long term vision of this thesis research work is to develop a specific recognition ligand for detecting proteins in living cell. A molecular sensor for non-nucleic acid analytes (e.g., proteins, peptides, drugs, hormones) can be designed and demonstrated using molecular recognition and design principles analogous in some ways to the molecular switch principles used in Molecular Beacons invented by Dr. Kramer and Dr. Tyagi.

Conventionally, people have looked at nucleic acids and antibodies used as nucleic acid recognizing nucleic acid and antigen recognizing antibody. Nucleic acids have protein binding capabilities i.e. they can act as aptamers having same design construct as molecular beacons. Aptamer nucleic acids act as antibodies which can detect antigens. This thesis work focuses on the development of some basic tools and concepts for the development of aptamer beacons, like immobilization of molecules on substrates and to determine the performance of specific binding assays.

BIOMIMETIC SENSOR FOR IN-VIVO APPLICATIONS

by

Daya I. Sooryadas

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

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APPROVAL PAGE

BIOMIMETIC SENSOR FOR IN-VIVO APPLICATIONS

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Dedicated to my beloved family & friends *With Love.... Words are small, they don't tell all The things I feel and mean But folks like you are very few And very far between*

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CHAPTER 1

INTRODUCTION

Systems/device manufacture at the molecular level is a multidisciplinary scientific field undergoing explosive development. Miniaturization provides cost effective and more rapidly functioning mechanical, chemical and biological components. 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. A biosensor can be said to be comprised of five components: a biological sensing element, a transducer, a signal conditioner, a data processor, and a signal generator. The essential component must produce a signal that is related to the concentration of a specific chemical or biological substance in complex systems. This component takes advantage of the ability of a biomolecule, such as an antibody or enzyme, to specifically recognize the target substance. One of the most significant problems in developing reliable sensors is controlled and reproducible attachment of macromolecules, particularly biomolecules to inorganic substrates/materials. This research aims to develop tools that allow characterization and detection of single cells, single molecules and single molecular events, like protein-protein interactions.

Antibodies have been extensively used to detect protein targets, but, so far, it has proven difficult to adapt antibodies to chip-based formats for proteome analysis. In particular, large-scale biosensor arrays necessitate:

a) the development of sensors that can directly transduce molecular recognition into an easily acquired signal and

b) methods for the high-throughput production of biosensors.

Effective protein recognition mechanisms are thus necessary, especially those to monitor proteins in real time and in homogeneous solutions. Real time protein detection is important for two major reasons: clinical diagnosis in homogeneous solution and protein studies in living specimen like living cells. There is an increasing demand for the development of new molecular probes for real-time protein recognition in solution and in living specimens.

The use of aptamers facilitates the detection of small and large molecular weight analytes by means of specific recognition technology using labeled ligands. There should be a means to generate a signal without washing. One technology currently in use is molecular beacons, used for the detection of nuclei acids. The purpose of this research is to develop method for designing and engineering nucleic acid molecules, aptamer molecules that are capable of functioning like molecular beacons, except that they respond to non-nucleic acid molecules. The aptamer beacon concept can be generalized to the detection of different types of molecules/proteins such as small molecules (e.g. glucose) or large molecules.

There has been a small amount of work conducted with aptamer beacons [6, 7]. A thrombin aptamer is one of the best characterized aptamers. This can be modified so that it acts like a beacon. The purpose of this research is to design beacons that respond to other proteins that are not as well characterized as thrombin.

Preliminary data [6, 7] is there to suggest that one can modify an aptamer in such a way that it can act like a molecular beacon. So it is valuable to attempt to perform this kind of work to detect other molecules. There is also recently published data that molecular beacon can be used to detect mRNAs inside single living cells [9]. They developed a general method for directly visualizing endogenous mRNAs in living cells by using hybridization probes called "molecular beacons," which generate fluorescence signals only when they hybridize to complementary nucleic acid target sequences.

CHAPTER 2

BACKGROUND

2.1 Surfaces for Attachment of Sensors

Surfaces suitable for the immobilization of proteins have become an increasingly important biological tool in recent years. 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 [12].

Common surfaces used for immobilization of DNA include latex beads, polystyrene, carbon electrodes, gold and oxidized silicon or glass [13]. Each has unique surface characteristics that can affect the performance of an assay. 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 [13]:

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. The particle selected for the purpose of this research is paramagnetic particles. Magnetic particles offer some attractive possibilities in biomedical engineering [14]:

- 1. They have controllable sizes ranging from a few nanometers up to tens of nanometer, which places them at dimensions that are smaller than or comparable to those of a cell $(10-100\mu m)$, a virus (20-450 nm), a protein (5-50 nm) or a gene (2 nm 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.
- 2. 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.
- 3. 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.

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 Fe_2O_3 or Fe_3O_4 . These particles are available with diameters ranging from ~300 nm to less than 10 nm for nanoscale

applications. They exhibit super paramagnetic 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, each paramagnetic particle (PMP) should also have surface properties that allow antibodies or other biological recognition units to be linked to the particles.

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. On 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.



Figure 2.1 Schematic diagram of a functionalized magnetic nanoparticle showing a core/shell structure with a shell of silica, SiO_2 , and functional groups attached to the shell.

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.

2.2 Labeling

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 or the target has to be labeled.

There are two types of probe labels: radiolabels and non-radioactive labels:

- 1. Probe nucleic acid can be labeled using radioactive isotopes, *e.g.* ³²P, ³⁵S, ¹²⁵I, ³H. Detection is by autoradiography or Geiger-Muller counters. Radio labeled probes are the most sensitive, *e.g.* ³²P labeled probes can detect single-copy genes in only 0.5μg of DNA. High sensitivity means that low concentrations of probe-target hybrid can be detected.
- 2. Non-radioactive labels are safer than radiolabels and do not require dedicated rooms, glassware and equipment or staff monitoring, *etc.* but they are not generally as sensitive.

Some examples are

- a) Biotin: This label can be detected using avidin or streptavidin which have high affinities for biotin.
- b) Enzymes: The enzyme is attached to the probe and its presence usually detected by reaction with a substrate that changes color. Used in this way the enzyme is sometimes referred to as a "reporter group". Examples of enzymes used include alkaline phosphatase and horseradish peroxidase.
- c) Chemiluminescence: In this method chemiluminescent chemicals attached to the probe are detected by their light emission using a luminometer.

- d) Fluorescence: Chemicals attached to probes fluoresce under light. This type of label is especially useful for the direct examination of microbiological or cytological specimens under the microscope a technique known as fluorescent *in situ* hybridization (FISH).
- e) 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.

Phycobilisomes (PBXL): PBXL dye is used as label in the immunoassays in this research. Chemically stabilized phycobilisomes are complexes of phycobiliproteins and colorless polypeptides that function as the major light harvesting antennae in blue-green and red algae [24]. The major criterion for the functional integrity of these complexes is the demonstration that they exhibit highly efficient transfer of energy between component phycobiliproteins, for example, in Porphyridium cruentum phycobilisomes from phycoerythrin (PE) to phycocyanin (PC) and finally to allophycocyanin (APC). The colorless polypeptides are involved in the assembly and positioning of the phycobiliproteins within the phycobilisomes for proper stability and energy transfer.

Isolated phycobilisomes readily dissociate into free phycobiliproteins and a variety of phycobiliprotein complexes under all but the most favorable conditions. Low to moderate ionic strength (<0.5M phosphate), low phycobilisome concentration (<1 mg/ml), and low temperatures lead to dissociation of phycobilisomes. Morphologically, phycobilisomes are complex assemblies of oligomeric phycobiliprotein discs arranged in ordered stacks referred to as "rods". In general, several arm-like rods radiate out from a core assembly, also comprised of rods. Phycobilisomes from different organisms are morphologically and stoichiometrically diverse, having different numbers and types of constituent phycobiliproteins and rods. In general, peripheral rods are comprised of

phycoerythrocyanin, phycoerythrin, and/or phycocyanin, and the core is comprised of allophycocyanin and associated linker proteins.

Isolated phycobiliproteins, the component fluorescent proteins of phycobilisomes, have been used as labels in immunoassays [25]. Macromolecular assemblies of phycobilisomes could be similarly utilized. The signal which phycobilisomes can provide is theoretically so much larger than isolated phycobiliproteins. So they can be used as detectable markers for a host of assays and other applications. Note that fluorescence intensity (detection limit) of phycobiliproteins (e.g., phycoerythrin) is about 50-200 fold greater than fluorescein. Phycobiliproteins are 1000-5000 folds brighter than fluorescein.

2.3 Molecular Beacons

Molecular beacon (MB) is a novel probe for real time detection of nucleic acids and proteins. It is composed of hairpin-shaped oligonucleotide with a fluorophore and a non-fluorescent quencher moiety at opposite ends of the oligonucleotide. The ends of the oligo are designed to be complementary to each other and form an arm (stem) structure, while the intervening loop (probe) is complementary to a sequence within the amplified product. In the absence of a sequence target, beacons are designed to form a stem-loop structure with a fluorophore on one end of the stem and a quencher on the other end. In this structure, the fluorophore is quenched by energy transfer to the quencher in close proximity [1].



Molecular Beacon

Figure 2.2 Molecular Beacon - Schematic drawing of mechanism of signaling by molecular beacons.





The loop sequence contains a sequence that is complementary to the target nucleic acid. If the complementary target is present, the molecular beacon will spontaneously bind to the target and a duplex is formed between the molecular beacon and the target nucleic acid. When this occurs, the stem is broken, which causes a conformational reorganization in the probe that places the fluorophore and quencher far enough apart so that the fluorophore is no longer quenched, and the molecular beacon fluoresces. Molecular beacons can be readily designed to directly detect and quantitative different nucleic acid targets.

The signal transduction mechanism for molecular recognition is based on fluorescence resonance energy transfer (FRET) and the conformational change of a MB. The MB acts like a switch that is normally closed to bring the fluorophore/quencher pair together to turn fluorescence "off". When binding to a target biomolecule, it undergoes a conformational change that opens the hairpin structure and separates the fluorophore and the quencher, thus turning "on" the fluorescence. This feature makes MB an extremely useful probe in a variety of applications such as methods for detecting and quantitating PCR products, the real time monitoring of PCR processes and the detection of mRNA in living cells [2]. 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 [3], for monitoring the synthesis of specific nucleic acids in sealed reaction vessels [2], for homogenous one-tube assays for genotyping single-nucleotide variations in DNA [4, 5] and for multiplex PCRs for the detection of four different pathogenic retroviruses.

Although the original goal for developing the MB is for nucleic acids detection, recently MB has found promising applications in the homogeneous detection of DNAbinding protein. The MBs can be used for non-specific protein-DNA binding studies, which open a new approach to detect protein in homogeneous solution with high sensitivity, excellent reproducibility, fast speed and convenience. However, there is one apparent and critical disadvantage of MBs in protein recognition: the lack of sequence specificity in protein binding.

Molecular Beacons versus Linear Probes: While other systems use fluorescence to detect the accumulation of PCR product, molecular beacons add another level of specificity due to the presence of a distinct probe apart from the primers. Molecular beacons only report complementary PCR product; other fluorescence-based real-time systems that use DNA binding dyes do not. In addition, the stem-probe structure of a molecular beacon makes it better able to discriminate single base-pair mismatches (compared to linear probes) because the hairpin makes mismatched hybrids less thermally stable than hybrids between the corresponding linear probes and their mismatched target. Furthermore, unlike linear hydrolysis probes, quenching of molecular beacons has been shown to occur through a direct transfer of energy from fluorophore to quencher. Consequently, a common quencher molecule can be used, increasing the number of possible fluorophores that can easily be used as reporters. This is an important advantage when designing PCR experiments in which several molecular beacons with different colored fluorophores are used to detect multiple targets in the same tube (multiplexing).

2.4 Aptamers

Aptamers, derived from the latin *aptus*, meaning, 'to fit', first reported in 1990 [8], are attracting interest in the areas of therapeutics and diagnostics and offer themselves as ideal candidates for use as biocomponents in biosensors (aptasensors), possessing many advantages over state of the art affinity sensors. Aptamers are a new class of molecules that have the potential to revolutionize the diagnosis and treatment of disease. Aptamers are single-stranded nucleic acids that form well-defined three-dimensional shapes, binding target molecules in a manner conceptually similar to antibodies. Like antibodies, aptamers can be used for both therapeutic and diagnostic applications. For these applications, aptamers have a number of desirable characteristics, including high specificity, rapid discovery and development, and production through a reliable and scalable chemical process. As therapeutic agents, aptamers have clinically demonstrated biological efficacy and have excellent, tunable pharmacokinetic properties. The chemical stability and high specificity of aptamers make them ideal diagnostic reagents.

Aptamers have shown to be an effective approach for protein recognition. Aptamers are oligonucleotides that have been selected for specific binding to a variety of molecular targets, ranging from small organics to proteins. Nucleic acid libraries are easily obtained via combinatorial chemistry synthesis. Aptamers are screened from combinatorial libraries of synthetic DNA/RNA sequences for their ability to bind with desired molecular targets, by an iterative process of adsorption, recovery and reamplification. The screening process mimics natural selection and therefore, theoretically, it is possible to obtain aptamers that can recognize virtually any target molecules with high affinity and specificity. These nucleic acid-binding species can consist of RNA, DNA, or modified nucleotides and are typically 15 to 60 nucleotides long. Their binding affinities range from *K*d's of 1pM to 1mM, with most in the 1–10nM interval. Aptamer's are selected using a relatively rapid *in vitro* selection process and can be inexpensively synthesized. Most importantly, aptamers can be engineered using standard nucleic acid techniques to incorporate radioisotope, fluorescent, or other reporters.

As outlined in Table 1, antibody generation [8], particularly for use in biosensors, has several fallbacks that are addressed by aptamers and it can be envisaged that aptasensors, using already developed or novel transduction platforms, will be increasingly exploited in the coming years. If molecular beacon techniques could be generalized to protein detection it might prove possible to more readily develop proteomics chips.

	Antibodies	Aptamers
a.	Limitations against target representing	Toxins as well as molecules that
	constituents of the body and toxic	do not elicit good immune
	substances.	response.
b.	Kinetic parameters of Ab-Ag	Kinetic parameters such as on/off
	interactions cannot be changed on	rates can be changed on demand.
	demand.	
c.	Antibodies have limited shelf life and	Denatured aptamers can be
	are sensitive to temperature and may	regenerated within minutes;
	undergo denaturation.	aptamer's are stable to long term
		storage and can be transported at
		ambient temperature.
d.	Identification of antibodies that	Selection conditions can be
	recognize targets under conditions other	manipulated to obtain aptamers
	than physiological is not feasible.	with properties desirable for in
		vitro assay e.g. non-physiological
		buffer/T.
e.	Antibodies often suffer from batch to	Aptamers are produced by
	batch variation.	chemical synthesis resulting in
		little or no batch to batch variation.
f.	Requires the use of animals.	Aptamers are identified through an
		in vitro process not requiring
		animals.
g.	Labeling of antibodies can cause loss in	Reporter molecules can be
	affinity.	attached to aptamers at precise
		locations not involved in binding.

 Table 2.1
 Advantages of Aptamers over Antibodies

2.5 Issues in Assay Development

2.5.1 Specificity

The specificity of an assay is dependent on the reaction between probe and target. Specificity of a target is a function of the affinity and cross-reactivity of the target and is a principal determinant for the quality of the assay. Factors such as the matrix in which the probe is tested may also influence specificity and sensitivity by interfering with the probe-target reaction. Other extrinsic factors may lead to false negative or false positive results depending on the immunoassay format. An example of this sort of problem is the false positive result that may be obtained with some fluorescent methods when testing biological samples that express high levels of endogenous fluorescent molecules. In addition to optimizing environmental parameters, it is also critical to optimize probe quality to ensure maximum specificity and sensitivity.

2.5.2 Sensitivity

Sensitivity, along with specificity, is also a defining characteristic for an immunoassay/DNA assay and is a direct function of the antibody/target used. Greater sensitivity is generally achieved with higher affinity antibodies/targets. Testing of detector antibodies/target against a panel of specific antigens/probe that has been carefully titrated is commonly performed to determine the lower limit of sensitivity for each potential detector antibody/target. However, since sensitivity is dependent on the ability to discriminate signal from background measurement at low analyte concentration, sensitivity may also depend on the assay format and instrumentation used for detection. An assay using a format and/or instrumentation that has a high background signal in the

absence of probe/antigen is inherently less sensitive compared to an assay using a format or instrumentation with a lower background signal.

2.5.3 Cross-Reactivity

The cross-reactivity of an antibody plays a central role in the overall quality of an immunoassay. The obvious preference is to construct an assay around high affinity antibodies that exhibit minimal cross-reactivity. In practice, it may be difficult if not impossible to achieve this specification, despite the advances in antibody production techniques. At the molecular level, there are two models for cross-reactivity to be considered when evaluating antibody specificity and immunoassay results. The first is cross-reactivity resulting from an antibody that binds to structurally distinct but similar epitopes present on different antigens. The second is cross-reactivity resulting from an antibody that binds to structurally distinct but similar undesirable, a cross-reactive antibody may still provide useful diagnostic information.

2.5.4 Signal Detection

Assay sensitivity is also dependent on the signal used for measurement. Depending on the nature of the signal, the reactants may be detected visually, electronically, chemically, or physically, and a wide range of instruments can detect the presence of these labels with a high degree of sensitivity. Enzymes are effective labels because they catalyze chemical reactions, which can produce a signal. Because a single enzyme molecule can catalyze many chemical reactions without being consumed in the reaction, these labels are effective at amplifying assay signals. Most enzyme-substrate reactions used for immunoassays utilize chromogenic, chemiluminescent, or fluorescent substrates that

produce a signal detectable with the naked eye, a spectrophotometer, luminometer, fluorometer or X-ray film. A disadvantage of enzyme-based assays is that both the enzymes and substrates may be unstable and require specialized storage to maintain activity.

Fluorescent dyes and other organic and inorganic molecules capable of generating luminescent signals are also commonly used labels in immunoassays. Assays using these molecules are often more sensitive than enzyme-based immunoassays but require specialized instrumentation and often suffer from high background contamination due to the intrinsic fluorescent and luminescent qualities of some proteins and light-scattering effects. Signals for assays with these types of labels are amplified by integrating light signals over time and cyclic generation of photons. Other commonly used labels include gold, and magnetic or paramagnetic particles. All are quite stable under a variety of environmental conditions and can be detected by visual inspection or instruments.

However, these labels are essentially inert and therefore do not produce an amplified signal. Signal amplification is useful and desirable because it results in increased assay sensitivity. Increased signal strength can be attained by using amplifiable labels as described above or by using molecules capable of forming multiple bonds. These molecules can produce more complex lattices of signal generating compounds or molecules. Biotin and avidin are examples of molecules exhibiting these characteristics. They have very high affinities for each other, developing almost irreversible bonds (Kd = 10-15 M). In addition, avidin can bind as many as four biotin molecules, increasing the size of the complex. If biotin is bound to a signal-generating molecule or compound, the strength of the signal increases proportionally.

2.5.5 Optimization

Temperature, time, reagent concentration, kinetics, and reagent quality are five key parameters affecting the performance of immunoassays. Successful assay development requires that each parameter be optimized. The most common optimization method is an empiric approach where one or two parameters are varied between experiments using a matrix format. Depending on the number of steps involved in an assay, this process can be time consuming and complicated. However, for a given assay platform, many of the parameters such as time and temperature need only be optimized for a single assay. Subsequent assays will generally function well within the specification identified for the first assay. For many immunodiagnostic assays, diffusion of molecules within an assay matrix is the rate-limiting step. Increasing the assay temperature generally increases the rate of diffusion. However, antibody affinity decreases as temperature increases, so assay speed and sensitivity must be balanced. Again, because diffusion is a rate-limiting step, increasing the incubation time for critical assay steps often results in greater assay sensitivity.

The duration of key assay steps is more critical for non-equilibrium techniques, in which background signals may increase above acceptable levels if such steps are allowed to continue too long. Whit Some assays, this effect can be minimized by adding compounds at the end of the reaction period that inhibit enzymatic reactions or inactivate the reactants. Another concern is reagent concentration, especially in development of homogeneous assays, where incorrect concentrations of antibody can lead to pro-zone effects, where signals are artificially low due to the stoichiometry of the reactions. In general, reagent concentration drives the sensitivity of the assay, but it can also affect the

rates of false positive and false negative results and requires careful optimization. The efficiency of each step of the assay cascade is governed in part by the assay environment. This environment includes pH, ionic strength, and the presence or absence of additives, such as carrier proteins, detergents, enzyme inhibitors, and preservatives in the assay buffers. Each of these parameters must be examined and optimized for the desired application and requirements of the assay.

2.6 Literature Review

Tyagi S *et al.* [2] have shown that multicolor molecular beacons can be used for allele discrimination. They found that hairpin conformation of molecular beacon enables the use of a wide variety of differently colored fluorophores. Using several molecular beacons, each designed to recognize a different target and each labeled with a different fluorophore, they demonstrated that multiple targets can be distinguished in the same solution, even if they differ from one another by as little as a single nucleotide. A comparison of "hairpin probes" with corresponding "linear probes" confirms that the presence of the hairpin stem in molecular beacons significantly enhances their specificity.

In another study by Hamaguchi *et al.* [7] they designed a new class of molecules, which they term aptamer beacons, for detecting a wide range of ligands. Similar to molecular beacons, aptamer beacons can adopt two or more conformations, one of which allows ligand binding. A fluorescence-quenching pair is used to report changes in conformation induced by ligand binding. In this study they hypothesized that additional sequences could be added to an aptamer that would form a stable stem loop and destabilize the native, binding structure. Analogous to molecular beacons, the aptamer beacon would then exist in a quenched stem-loop structure in the absence of a target

molecule. Formation of the aptamer beacon: target molecule complex would alter the equilibrium between quenched, nonnative and unquenched, native structures, concomitantly generating change in observed fluorescence intensity.

An anti-thrombin aptamer was engineered into an aptamer beacon by adding nucleotides to the 5'-end which are complementary to nucleotides at the 3'-end of the aptamer. In the absence of thrombin, the added nucleotides will form a duplex with the 3'-end, forcing the aptamer beacon into a stem-loop structure. In the presence of thrombin, the aptamer beacon forms the ligand-binding structure. This conformational change causes a change in the distance between a fluorophore attached to the 5'-end and a quencher attached to the 3'-end. An Aptamer beacon can be a sensitive tool for detecting proteins and other chemical compounds.

In a research by Jianwei J. Li *et al.* molecular aptamer beacons for real-time protein recognition was studied [6]. Unlike MB, aptamers have the advantage in binding specificity and also generality, but lack the built-in mechanism for signal transduction, and the capability of detection without separation. It is thus desirable to combine the binding specificity and generality of aptamers with the excellent signal transduction capability of MB to develop novel protein-reporting probes for quantization in homogeneous solutions and for real time protein monitoring. Such a probe is called molecular aptamer beacon (MAB).

The basic concept of MBs can be adopted and broadened for the construction of MABs for proteins. First, both direct quenching (between fluorophore and quencher) and FRET (between donor and acceptor) can be used for signal transduction. Second, any conformational change, both opening and closing, may change the quenching efficiency

or FRET efficiency. Finally, both signal increase and decrease can be used to report protein-binding events. Combining all these requirements, a general working principle for MABs can be summarized in two points. Binding of target proteins forces the MABs to undergo a conformational change; the conformational change rearranges the relative positions between the fluorophore and the quencher, leading to an increase or a decrease in fluorescent signal. Apparently, whether there is a conformational change in an aptamer is the basic requirement for the MAB. It is known that aptamers usually experience a conformational change upon target binding. In addition, conformational change as large as that by a MB can be created in an aptamer by changing the aptamer's structure. Therefore, a general strategy for designing MABs can be briefly stated as how to transform conformational change to fluorescent signal change. Since energy transfer is highly dependent on distance between the two moieties, this problem can then be simplified as where and how to conjugate the moiety pair on the MAB in such a way to generate the largest possible fluorescent signal change upon protein binding [6].

There is no easy way to monitor proteins in real time and with living specimen. MAB approach has the potential to accomplish these challenging and significant tasks. Here the signal transduction is based on the conformational change of the aptamer. Because conformational change is usually accompanied by protein binding, this approach should be generally useful for the construction of other MBAs based on other protein binding aptamers. As molecular beacons, these MAB probes can be used in situations where it is either not possible or desirable to isolate the probe-target complex from an excess of the probes. The usefulness of detection without separation for protein studies cannot be overemphasized in diagnostic imaging and in living specimen studies. MABs are expected to function in real-time as ideal *in vivo* protein probes with high sensitivity and excellent selectivity. The development of MAB represents one important and promising step in the efforts for the elucidation of protein function and for efficient protein determination in homogeneous solutions.

In this they detailed the study of thrombin binding MAB. Two types of MABs have been constructed for thrombin binding studies. One is a quenching type MAB, which is labeled with a fluorophore-quencher pair. The other one is a FRET-type MAB, which is labeled with two fluorophores (a donor-acceptor pair), showing an increased fluorescent signal in the presence of target protein. Real-time observations of protein-aptamer recognition, protein quantization in homogeneous solution, and ratio metric imaging of proteins are tested using these MABs.

There have been three reports on the development of using aptamers for protein recognition with a similar signal transduction as that used in MBs. The first report was on the preliminary result on the construction of a DNA MAB for protein thrombin detection [25]. In the second report, Yamamoto *et al.* developed an aptamer to detect Tat protein of HIV-1 [26]. A duplex RNA aptamer was split into two portions, with one portion transformed into a DNA probe, and with the other portion partly complementary to the DNA probe. The two portions could not hybridize until Tat protein was present, and the hybridization caused restoration in the otherwise quenched fluorescence. In the third report, a thrombin-binding aptamer was constructed by Hamaguchi *et al.* [7]. A half stem sequence was added to the thrombin aptamer so as to change the aptamer into a MB form. Protein binding changes the MB form back to an aptamer restoring the quenched fluorescence.
Currently, the method of choice for mapping the intracellular distribution of mRNAs is *in situ* hybridization with labeled nucleic acid probes [10]. In this method, the cells are fixed, and excess probes are removed by washing to detect the hybrids. Fixing leads to the denaturation and cross linking of proteins and causes cell death, precluding the study of dynamic processes such as intracellular trafficking. To circumvent this problem, green fluorescent protein fused to bacteriophage MS2 RNA coat protein has been used to image engineered RNAs containing MS2 coat protein-binding sites in living cells. However, because naturally occurring mRNAs are processed and "dressed" with protein factors before being sent on their journey, it is important to be able to detect native mRNAs in live cells.

Recently Tyagi *et al.* [9] performed a study visualizing the distribution and transport of native mRNAs in living cells. They developed a general method for directly visualizing endogenous mRNAs in living cells by using molecular beacons. Because unbound molecular beacons are nonfluorescent, it is not necessary to remove excess probes to detect the hybrids. Moreover, these probes bind to their targets spontaneously at physiological temperatures. Therefore, the introduction of molecular beacons into cells is sufficient to illuminate a target mRNA. Furthermore, it is possible to detect different mRNAs simultaneously in the same cell by using molecular beacons that are specific for different targets and that are labeled with differently colored fluorophores. Ideal probes for targets in living cells should be stable inside the cell, should not induce the destruction or perturbation of their target, and should signal only the presence of their target when (and where) the target is present.

Molecular beacons constructed from natural deoxyribonucleotides are not suitable for this purpose, because cellular nucleases can digest them and cellular ribonuclease H can digest the target RNA in the region where the probe is bound. The former process leads to the generation of nonspecific fluorescence, and the latter process leads to destruction of the targets and loss of signal due to reformation of the molecular beacon stems. So Tyagi *et al.* modified the backbone of molecular beacons by substituting an oxymethyl group for the hydrogen located at the second position of the ribose sugar in each nucleotide. Although this modification does not adversely affect the spontaneity of their fluorogenic interaction with targets, it enables the molecular beacons to resist endonucleolytic cleavage. Furthermore, the mRNA in the hybrids that they form is not digested by ribonuclease H.

Using nuclease-resistant molecular beacons, they imaged the transport and localization of oskar mRNA in Drosophila melanogaster oocytes. When the localization pattern was altered by genetic manipulation of the mRNA's 3' untranslated region, or by chemical perturbation of the intracellular tubulin network, the distribution of the fluorescence signals changed accordingly. The migration of oskar mRNA in real time was tracked from the nurse cells where it is produced to the posterior cortex of the oocyte where it is localized. Their observations revealed the presence of a transient, and heretofore elusive, stage in the transport of oskar mRNA.

Real-time monitoring of the transport of *oskar* mRNA clarified the mechanism of its transport. Similar analyses of the movements of other mRNAs in living cells will improve the understanding of how they reach their destinations. By monitoring the transport of several different mRNAs at the same time, it will be possible to study how

the transport of various mRNAs is coordinated. The different mRNAs can be distinguished from one another by using molecular beacons that are specific for different mRNAs and are labeled with differently colored fluorophores, and that are microinjected together into oocytes or nurse cells. Direct visualization of specific mRNAs in living cells with molecular beacons will accelerate studies of intracellular RNA trafficking and localization, just as the use of green fluorescent protein has stimulated the study of specific proteins in-vivo.

CHAPTER 3

RESEARCH OBJECTIVE

Long term goal of this research is to develop a specific recognition ligand for detecting proteins in living cell. The purpose of this thesis is to develop some basic tools and concepts for combining emerging capabilities in aptamer and molecular beacon and intracellular detection technologies with the end goal of detecting and quantifying proteins in single living cell.

The basic tools and concepts developed during the course of this thesis research are:

- a. Immobilize ligands on to different substrates and evaluate the performance on different substrates.
- b. Perform immunoassay and evaluate specific binding of ligands to receptors.
- c. Demonstrate dose dependent immobility of proteins to different substrates (microtiter plate non-covalent adsorption and paramagnetic particles covalent adsorption) and dose dependence of specific binding with the conjugate.

The work of developing an antibody thrombin immunoassay and an aptamer thrombin binding assay that behaves substantially equivalent to an antibody thrombin assay is in progress.

Also the short term research steps to extend this work for the development of aptamer beacons has been laid out. The general long term path is to develop sensing and monitoring of molecular events inside single living cells. The results of this research can be applied to the development of new methods and devices. Critical challenges include:

- 1) Designed engineering of aptamer beacons for generalized detection i.e. homogeneous aptamer beacon capability
- 2) Placing sensors inside the cell, a process which is non-trivial.
- 3) Detection system with adequate sensitivity inside the cell
- 4) Immobilization of the aptamer beacon on a nanosubstrates so that it can be inserted into the living cell. Since more beacons can be clustered on nano-substrates by immobilization it is possible to get more signal when binding event happens inside the cell.

CHAPTER 4

EXPERIMENTS AND RESULTS

For the experiments Mouse Immunoglobulin (MIgG) and solution phase labeled Goat anti-Mouse (GAM- PBXL) model system is used. This system is chosen because the performance of the MIgG-GAM is well known. PBXL is used as label for detection because it yields substantially greater fluorescence than conventional fluorophore. The fluorescence intensity (detection limit) of phycobilisomes (PBXL) is greater than fluorescein. Also it has sufficient intensity for detection in microtiter plate format even with the low binding capacity of microtiter plate. As solid phase substrates black polystyrene microtiter plate and 1 micron amine terminated paramagnetic particles (BioMagPlus) from Polysciences Inc. are used.

4.1 Immobilization of FTB Probes to Microtiter Plate

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

Reagents

- 1. Coating Buffer = $NaPO_4$ pH 7.0
- Wash buffer = PBS Azide 10mM NaPO₄, 150mM NaCl, 0.25% NaN₃ pH 7.1 (No Tween, No Salt)
- 3. Bovine Serum Albumin (BSA)
- 4. FTB Probe: 5'- CGA TCG CGA CAT TCC ATC GTG CCC GAT CGA AAA A-3'

- 5. Glutaraldehyde (GA).
- 6. Glycine.

Protocol

- 1. Coat the microtiter plate with 200µl of varying concentration of BSA/well.
- 2. Stock = 16.47mg/ml.

Table 4.1 Dilution Series for BSA

Tube #	[BSA]	Stock BSA	Coating Buffer	Total Volume
	µg/ml		ml	ml
А	20.0	12.1µl	10.0	10.012
В	10.0	2.0ml	2.0	4.0
С	5.0	1.0ml	3.0	4.0
D	2.0	0.4ml	3.6	4.0
Е	1.0	0.4ml	3.6	4.0
F	0	0	4.0	4.0

- 3. Cover with Saran wrap and incubate overnight at $2^{\circ}C 8^{\circ}C$. (4°C).
- 4. Wash the BSA coated plates after incubation overnight three times in wash buffer.
- 5. Prepare the dilution series for Glutaraldehyde. Transfer the Glutaraldehyde from one of the aliquots of 25% GA stock for the dilution series.

Table 4.2	Dilution	Series	for	Glutaraldehyde
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Tube #	[GA]	Stock GA	Buffer	Total Volume
	%	μΙ	ml	ml
A	5	660	2.54	2.60
В	1	550	2.0	2.55
С	0.2	100	2.4	2.5
D	0	0	2.5	2.5

- 6. Add GA dilutions at 100µl/well on the microtiter plate after the wash
- 7. Incubate overnight at $2^{\circ}C 8^{\circ}C$. (4°C)

- 8. After overnight incubation, wash the GA from plates using PBS Azide wash buffer
- 9. Stock = 100μ M

 Table 4.3
 Dilution Series of FTB Probe

Tube #	[FTB probe]	F-TB stock	Coating Buffer	Total Volume
	μΜ	μl	ml	ml
A	10	200	1.80	2.0
В	3	450A	1.05	1.5
С	1	140A	1.26	1.4
D	0.3	140B	1.26	1.4

- 10. Add 50µl of the prepared probe dilutions according to designed protocol.
- 11. Incubate plate overnight at $2^{\circ}C 8^{\circ}C$. (4°C).
- 12. Wash plates three times in PBS Azide wash buffer.
- 13. Prepare 1M glycine in NaPO₄ 10mM (pH 8.0) buffer. Glycine is added to quench the unreacted aldehyde with the amine group of the acid. Add 200µl of Glycine/well and incubate at room temperature for 1 hour. Wash three times with PBS Azide (pH 7.1)
- 14. Read microtiter plate at 5.0 volts in Fluorolite 1000 microtiter plate reader

Results

The reading from the microtiter plate reader suggests that there is no significant immobilization of FTB oligo probe to the microtiter plate. This could be because of the low surface area offered for coating and binding by the microtiter plate well. Also the fluorescence intensity (detection limit) of fluorescein is very less to be detected in the microtiter plate format. So the phycobilisomes (PBXL) is chosen as label for immunoassay which is much brighter than fluorescein.

4.2 Immunoassay in Microtiter Plate Format

Experimental Objective: Co-titrate Mouse Immunoglobulin (MIgG) and solution phase

labeled Goat anti-Mouse (GAM- PBXL) (conjugate) in microtiter plate format.

Buffers

- a) Coating buffer = 10mM NaPO₄ (pH 7.0)
- b) Wash buffer = 10mM NaPO₄ (pH 7.0) +150mM NaCl + 0.025% NaN₃ + 0.05%Tween 20.
- c) Blocking Buffer = PBS Azide + 2mg/ml BSA (No Tween).
- d) Assay Buffer = Wash buffer + 2mg/ml BSA (pH 7.1).
- e) Reading Buffer = 10mM NaPO₄ (pH 7.42)

Protocol

1. Stock [MIgG] = 6.3 mg/ml

Table 4.4 Dilution Series for MIgG

Tube #	[MIgG]	Stock MIgG	Coating Buffer	Total Volume
	µg/ml	μl	ml	ml
Α	20	20.63	6.4793	6.5
В	10	1750A	1.750	3.5
С	5	875A	2.625	3.5
D	2	350A	3.150	3.5
Е	1	350B	3.150	3.5
F	0	0	3.50	3.5

- 2. Coat the microtiter plate with 200µl/well at the dilutions of MIgG in 10mM NaPO₄ pH 7.0 buffer. 16 wells at each concentration of MIgG.
- 3. Incubate overnight at $2^{\circ}C 8^{\circ}C$. (4°C)
- 4. After incubation wash the plate three times with wash buffer.

- 5. Decant and then block by exposing it to 200µl of PBS Azide blocking buffer pH 7.1. The purpose of blocking buffer is to adsorb proteins to hydrophobic sites of plastic to avoid non-specific reagent binding during assay. Keep for 15 minutes to 30 minutes at room temperature to give the plastic a start in absorbing proteins and time for the proteins to bind to the plate.
- 6. After blocking wash with the assay buffer three times.
- Prepare the Dilution Series for GAM-PBXL. Stock [GAM-PBXL] = 1mg/ml

Table 4.5Dilution Series for GAM-PBXL

Tube #	[GAM-PBXL]	Stock GAM-PBXL µl	Assay Buffer	Total Volume
	μg/ml		ml	ml
A	100	242	2.178	2.42
В	50	700A	0.7	1.40
С	20	280A	1.12	1.40
D	10	140A	1.26	1.30
E	5	130C	1.17	1.30
F	2	130B	1.17	1.30
G	1	130A	1.17	1.30
Н	0	0	1.3	1.3

- 8. Then add 100µl of labeled GAM dilutions in assay buffer and incubate the plate for two hours in room temperature ($25^{\circ}C 30^{\circ}C$).
- 9. After two hours wash the microtiter plates three times with the PBS Azide pH 7.1 (No protein and No Tween) and blot.
- 10. Re-suspend the wells with 100µl of reading buffer pH 7.42.
- 11. Read the plate in the Fluorolite 1000 microtiter plate reader using long pass filter.

Result and Discussion

The results show that the binding is higher for higher concentration of conjugate and MIgG offered.

Since the surface area of the microtiter plate is relatively small it is not possible to get good adsorption of MIgG on to the wells of the microtiter plate. Therefore to get a higher concentration of MIgG for the assay (higher surface area), the substrate for immobilization was changed to paramagnetic particles (paramagnetic particles were chosen as substrate for immobilizing the antigen).

	1	2	3	4	5	6	7	8	9	10	11	12	Protocol Dilution [GAM- PBXL] 100µl / Well
Α	225	203	126	114	125	160	62	59	62	63	56	59	100µg/ml
В	178	207	125	124	104	106	59	63	71	63	66	71	50µg/ml
C	98	97	97	83	90	85	53	62	56	62	61	62	20µg/ml
D	67	64	67	67	68	68	57	59	59	60	62	63	l0μg/ml
E	62	54	54	58	57	78	54	53	55	55	53	63	5µg/ml
F	52	53	73	47	47	47	56	58	63	58	55	54	2µg/ml
G	54	55	47	59	49	56	56	67	65	56	67	61	lμg/ml
н	46	49	64	58	55	55	59	51	85	58	50	49	0µg/ml
Read [MIg 200µ Volt	Reading[MIgG] $\rightarrow 20\mu g/ml$ $10\mu g/ml$ $5\mu g/ml$ $2\mu g/ml$ $1\mu g/ml$ $0\mu g/ml$ $200\mu l/Well$ Voltage: 6.0 Volts Emission filter: Long Pass Filter (>510nm)Excitation Filter: 475nmPlate: Dynex												

Table 4.6 Co-titration of MIgG with GAM-PBXL in Microtiter Plate Format



Figure 4.1 Co-titration of MIgG with GAM-PBXL in Microtiter Plate Format – Fixed [MIgG].



Figure 4.2 Specific Binding (background subtracted) of GAM-PBXL to MIgG as Determined by Titration of GAM-PBXL at Fixed MIgG in Microtiter Plate Format.

4.3 Immobilization of MIgG on to BioMagPlus Particles

Research Objective: Immobilization of MIgG onto BioMagPlus particles

Reagents

- 1. Wash Buffer = 10mM NaPO₄ pH 7.2 (no salt)
- 2. Reading Buffer = 10mM NaPO₄ pH 7.4.
- 3. Glutaraldehyde (GA).

Protocol

- 1. Wash 10mg of BioMagPlus particles three times in wash buffer (no salt).
- 2. Resuspended the particles in 5%GA at a particle concentration of 10mg/ml.
- 3. Incubate in the shaker for three to five hours at room temperature $(25^{\circ}C 30^{\circ}C)$
- 4. After incubation wash the particles thoroughly (more than five times until the smell of GA is undetectable).
- 5. Add MIgG at a concentration of 5mg/ml (concentration greater than 1mg/ml) to a total ratio of about 160µl MIgG/ 1mg of particle.
- Vortex the mixture and immediately separate the mixture with the magnet and withdraw 50µl of supernatant for measuring input counts in the Spectrometer (OD 280).
- 7. Incubate in the shaker overnight at room temperature (25°C 30°C).
- 8. After incubation vortex it and separate in magnetic separator.
- 9. Withdraw all of supernatant to measure the output count.
- 10. Wash the mixture in wash buffer.
- 11. Resuspended the final preparation with PBS Azide + Tween + BSA buffer to 10 mg/ml and store at $2^{\circ}\text{C} 8^{\circ}\text{C}$ (4°C).
- 12. Measure the input supernatant and output supernatant at a 1/20 dilution (50µl of supernatant and 950µl of reading buffer) in the Beckman DU 520 General

purpose UV/Vi Spectrophotometer at OD 280 to see how much protein has been taken by the particles.

Result and Discussion

Absorbance reading of output supernatant = 0.104

Absorbance Unit of MIgG is 0.104*20 = 2.08 AU

Extinction coefficient of MIgG = 1.42

Concentration of MIgG = 2.08/1.42 = 1.4mg/ml in suspension.

The amount of 1 mg/ml offered MIgG in $100 \mu \text{l} + 100 \mu \text{g}$ BioMag = 5 mg/ml

Net amount of MIgG on particles is 5mg/ml - 1.4mg/ml = 3.6mg/ml

Therefore immobilization efficiency of MIgG = 3.6/5.0 * 100 = 72%

4.4 Co-titration of BioMag Immobilized MIgG and

GAM-PBXL in Microtiter Plate Format

Research Objective: Co-titrate BioMag immobilized Mouse Immunoglobulin (MIgG) and solution phase labeled Goat anti-Mouse (GAM- PBXL) (conjugate) in microtiter plate format.

Variables

- a) [MIgG] to coat the plates.
- b) Labeled [GAM] GAM-PBXL to bind to MIgG.
- c) BioMagPlus Amine Terminated Particles (50mg/ml in 1mM sodium EDTA, pH 7.0)

Buffers

- a) Coating buffer = 10mM NaPO₄ (pH 7.0)
- b) Wash buffer = 10mM NaPO₄ (pH 7.0) +150mM NaCl+ 0.025% NaN₃ + 0.05% Tween 20.
- c) Blocking Buffer = PBS Azide + 2mg/ml BSA (No Tween).
- d) Assay Buffer = Wash buffer + 2mg/ml BSA (pH 7.1).
- e) Reading Buffer = 10mM NaPO₄ (pH 7.42)

Dilution Series of Variables

a) [BioMag-MIgG] - 10µl, 5µl, 2µl, 0µl

Tube #	[BioMag-MIgG]	Stock BioMag-MIgG	Coating Buffer	Total Volume
	µg/ml	μΙ	ml	ml
A	10	4.5	4.955	4.5
В	5	1300A	1.3	2.6
С	2	600A	2.4	2.5
D	0	0	2.5	2.5

 Table 4.7 Dilution Series for BioMag-MIgG

b) [GAM-PBXL] - 20µg/ml, 10µg/ml, 5µg/ml, 1µg/ml, 0.5µg/ml, 0.2µg/ml and 0µg/ml.

Fable 4.8	Dilution	Series for	GAM-PBXL
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Tube #	[GAM-PBXL]	Stock GAM-PBXL	Assay Buffer	Total Volume
	μg/ml	μΙ	ml	ml
A	20	52	2.58	2.6
В	10	700A	700	1.4
С	5	400A	1.2	1.4
D	2	150A	1.35	1.5
E	1	130A	1.17	1.3
F	0.5	130B	1.17	1.3
G	0.2	130C	1.17	1.3
Н	0	130D	1.17	1.3

Standard Protocol for the Microtiter Plate Assay

- 1. First coat the 96 wells of the microtiter plate with 200μ /well at 10μ l, 5μ l, 2μ l, and 0μ l concentrations of BioMag-MIgG with 10mM NaPO₄ (pH 7.0) coating buffer.
- 2. Incubate the plate overnight at 2-8°C (4°C).

- 3. After incubation wash the plate with wash buffer.
- 4. Decant and then block by exposing it to 200µl of PBS Azide blocking buffer pH 7.1.
- 5. After blocking wash with the assay buffer three times.
- 6. Final wash is with wash buffer (No protein and No Tween).
- 7. Prepare the dilutions for GAM-PBXL.
- 8. Then add 100µl of labeled GAM dilutions in assay buffer and incubate the plate for two hours in room temperature ($25^{\circ}C 30^{\circ}C$).
- 9. After two hours wash the microtiter plates three times with the PBS Azide pH 7.1 and blot it.
- 10. Re-suspend the wells with $100\mu l$ of reading buffer pH 7.42.
- 11. Read the plate in the Fluorolite 1000 microtiter plate reader.

Result and Discussion

The result shows that for higher concentration of conjugate and MIgG there is about 10%

binding. The results were as expected since the surface area on the plastic wells is less.

4.5 Specific Binding of GAM-PBXL and BioMag-MIgG

Research Objective: Evaluate specific binding of GAM-PBXL and BioMag-MIgG by

co titrating reagents in the presence or absence of excess soluble MIgG.

Buffers

- a) Wash buffer = 10mM NaPO₄ (pH 7.0) +150mM NaCl+ 0.025% NaN₃ + 0.05% Tween 20.
- b) Assay Buffer = Wash buffer + 2mg/ml BSA.
- c) Reading Buffer = 10mM PBS Azide (pH 7.1)

Variables & Particles

- a) [MIgG] to coat the plates.
- b) Labeled [GAM] GAM-PBXL to bind to MIgG.
- c) BioMagPlus Amine Terminated Particles (50mg/ml in 1mM sodium EDTA, pH 7.0)

Dilution Series

- a) MIgG Dilution 100mg/ml from stock of 10mg/ml.
- b) GAM-PBXL Dilution 80µg/ml, 20µg/ml, 5µg/ml, and 0µg/ml.

 Table 4.9 Dilution Series for Conjugate GAM-PBXL

Tube #	[GAM-PBXL]	Stock GAM-PBXL	Assay Buffer	Total Volume
	μg/ml	μΙ	ml	ml
A	80	152	1.748	1.9
В	20	350	1.05	1.4
С	5	100	1.5	1.4
D	0	0	1.4	1.4

c) BioMag-MIgG - 1000µg/ml, 200µg/ml and 40µg/ml

Stock 10mg/ml

Table 4.10 Dilution Series for BioMag-MIgG

Tube #	[BioMag-MIgG]	Stock BioMag-MIgG	Assay Buffer	Total Volume
	μg/ml	μΙ	ml	ml
A	1000	250	2.375	2.625
В	200	400A	1.6	2.0
С	40	50A	1.92	2.42

Protocol

- 1. Prepare 20µg/ml of MIgG in assay buffer to make the solution phase MIgG.
- 2. Take 48 tubes in the magnetic holder and label them according to the following protocol.

Table 4.11 Magnetic Holder Template

-MIgG									40µg/ml
+MIgG									
-MlgG									200µg/ml
+MIgG									
-MIgG									1000µg/ml
+MIgG									
[GAM- PBXL] →	0μg /1	ml	5με	;/ml	20µ	g/ml	80µ	g/ml	↑ [BioMag- MIgG]

- 3. Add 50µl of solution phase MIgG to the alternate rows tubes +MIgG
- 4. Add 100µl of varying concentration of GAM-PBXL conjugate to the solution phase MIgG in alternate rows and 150µl to the other set of rows.
- 5. Incubate this for about 30 minutes at room temperature $(25^{\circ}C 30^{\circ}C)$.
- 6. Add 100µl of varying concentration of the washed BioMag-MIgG to all the rows.
- 7. Incubate for 30 to 60 minutes at room temperature (25°C 30°C). Vortex it once in a while.

- 8. After incubation separate it using magnet. Withdraw 125 μ l of supernatant (i.e. half of the reaction volume, total reaction volume is 100 μ l+ 50 μ l+ 100 μ l = 250 μ l) and transfer it to one half of the wells of the plates for reading.
- 9. Decant the rest of the solution in the tube in magnetic holder
- 10. Wash it once with 500µl of wash buffer (pH 7.1). Separate in magnetic holder, decant and blot.
- 11. Wash again with 500µl of PBS Azide. Separate in magnetic holder, decant and blot.
- 12. Resuspend in 250µl of reading buffer.
- 13. Vortex the tube and transfer $125\mu l$ of that to other half of the plate
- 14. Read the plate.

Result and Discussion

The experiment was successful and gave good specific binding. A large excess of soluble target (analyte MIgG) into the solution will eliminate the binding of the conjugate onto the solid phase target stuck on the particles. This gives the non-specific binding. Comparing this with the total binding that includes the solid phase target (MIgG + Particles), solution phase target and conjugate (This has specific and non-specific binding) then the specific binding is the difference between the total binding and non-specific binding.

In solution there are GAM-PBXL and MIgG/ no MIgG forming:

- a) GAM-PBXL-MIgG complex → +MIgG (Completion of Binding Non-specific Binding)
- b) Only GAM-PBXL \rightarrow MIgG (No solution phase antigen Total Binding) Specific Binding = Total Binding – Non-Specific Binding (Conjugate binding to the MIgG stuck on to particles instead of onto the solution phase MIgG).

Good binding is the result of the increased surface area of the BioMag particles than the microtiter plate surface.

Benefits of BioMagPlus particles compared to microtiter plates:

- a) Huge surface area compared to microtiter plate.
- b) They are already coated with silane amine group.
- c) Another advantage is that we can put high concentrations of MIgG on the surface of BioMagPlus particles.

	1 MIgG+	2 MIgG-	3 MIgG+	4 MIgG-	5 MIgG+	6 MIgG-	7 MIgG+	8 MIgG-	9 MIgG+	10 MIgG-	11 MIgG+	12 MIgG-	Protocol Dilution [GAM- PBXL]
A	3468	2209	3816	3511	4047	3834	0042	0355	0022	0172	0020	0057	
В	3492	2189	3812	3422	4048	3749	0029	0352	0022	0146	0016	0052	80μg/ml
С	0837	0386	0887	0667	0858	0843	0023	0146	0019	0079	0016	0038	
D	0787	0367	0847	0635	0903	0835	0024	0121	0019	0078	0017	0035	20µg/ml
E	0251	0106	0266	0176	0274	0239	0018	0046	0015	0040	0020	0025	5ug/ml
F	0249	0105	0259	0185	0275	0234	0018	0053	0016	0041	0018	0028	βµg/m
G	0037	0034	0028	0029	0031	0029	0017	0014	0017	0018	0015	0015	
н	0035	0035	0029	0029	0031	0033	0014	0014	0013	0014	0013	0012	ομg/ml
Read [Bio! Volta	Reading800µg/ml200µg/ml40µg/ml800µg/ml200µg/ml40µg/ml[BioMag-MIgG]Voltage: 4.3 VoltsEmission filter: Long Pass Filter (>510nm)Excitation Filter: 475nmPlate: Dynex												

Table 4.12 Specific Binding of BioMag-MIgG with GAM-PBXL with MIgG and without MIgG Supernatant BioMag Particle



Figure 4.3 Specific Binding of BioMag-MIgG and GAM-PBXL with and without MIgG – Fixed [GAM-PBXL].



Figure 4.4 Specific Binding of BioMag-MIgG at 800µg/ml and varying [GAM-PBXL] with and without MIgG.



Figure 4.5 Specific Binding of BioMag-MIgG at 200µg/ml and varying [GAM-PBXL] with and without MIgG.



Figure 4.6 Specific Binding of BioMag-MIgG at 40µg/ml and varying [GAM-PBXL] with and without MIgG.

CHAPTER 5

CONCLUSIONS

The dose dependent immobility of proteins (MIgG) to microtiter plate by passive (noncovalent) adsorption has been demonstrated. Successful attachment of antigen to plastic and paramagnetic surfaces and specific binding of labeled antibodies (GAM-PBXL) to antigens (MIgG) immobilized to solid phase has also been experimentally determined. Dose dependent specific binding immunoassays have been performed wherein the dose dependence includes

- varying concentration of MIgG to substrates
- varying concentration of labeled conjugate (GAM-PBXL) per assay.

In this thesis research it is demonstrated that:

- (1) Paramagnetic particles serve as better substrates for attachment than microtiter plates,
- (2) High specific binding occurs with higher concentration of offered antigen probe and antibody target, and
- (3) PBXL dyes have sufficient intensity for detection in microtiter plate format even with the low binding capacity of microtiter plate.

It is possible to design and engineer nucleic acid molecules, aptamer sensors that

are capable of functioning like molecular beacons, except that they respond to nonnucleic acid molecules, and to immobilize them to nanosubstrates to make for insertion into the living cell to detect non nucleic acid target sequences.

CHAPTER 6

FUTURE PLANS

The long term goal of this research is to develop specific recognition ligands for detecting proteins in living cell. Problems encountered inside living cells include:

- a) To apply specific binding of immunoassay to inside living cells the antibody or beacon should be designed in such a way to give off signal as soon as it binds to the antigen without any further manipulation like washing etc.
- b) Also in very small amount such as few pico liters it is difficult to detect the signal. A high intensity emitting fluorophore is needed for better signal inside cell than that is used in a binding assay. It is neither possible to amplify the target inside cells nor to do signal amplification. So there is need for an extremely sensitive detection system.

The concept of nucleic acids and antibodies has been conventionally used as nucleic acid recognizing nucleic acid and antigen recognizing antibody. Nucliec acids have protein binding capabilities i.e. they can act as aptamers. Aptamer nucleic acids act as antibodies which can detect antigens. These aptamers has same design construct as molecular beacons.

Short term plans to continue this research includes:

- a) Demonstrate the antibody assay and the aptamer binding assay.
- b) Optimize the aptamer assay.
- c) Compare the aptamer binding assay with the antibody assay.
- d) Reproduce the aptamer beacon work done by Ellington et al. [7].
- e) Design and demonstrate generalized aptamer beacon assay.
- f) Use the aptamer beacons for detection inside living cells

Immobilization of an antigen (MIgG) to paramagnetic particles and detection by cotitrating labeled antibody (GAM-PBXL) has been experimentally determined in this thesis work. With this background, it is possible to develop an antibody immunoassay in which an antigen (Thrombin) is immobilized on to the particles and by cotitrating corresponding antibody (Mouse anti-thrombin) followed by labeled anti-antibody (Goat anti-mouse anti-thrombin) the amount of antigen immobilized on to the particles can be determined. It is then possible to demonstrate an aptamer thrombin binding assay that behaves substantially equivalent to an antibody thrombin assay. Immobilization of antigen to particles can be done using efficient immobilization chemistry from Solulink Inc.

6.1 Immobilization using Solulink Chemistry

Bioconjugation is defined as the linking of biomolecules to other biomolecules, polymers, small molecules, surfaces or metals. HydralinK is a novel bioconjugation system for the conjugation and immobilization of peptides, proteins, carbohydrates and DNA/RNA [28]. 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 maleimide/thiol and avidin biotin. The technique can be readily engineered to link small molecules (peptides, fluorophores), biomolecules (antibodies, proteins, DNA, RNA) or other molecules to solid surfaces (glass, plastic, latex, silica beads), for application in proteomics, genomics, drug discovery, therapeutics and diagnostics. The implementation of the technology is extremely simple. The technology is based on the reaction of a 2-hydrazinopyridyl moiety with benzaldehdye moiety to yield a stable bisaromatic hydrazone. Benzaldehyde moiety on surfaces is affected by treatment with SFB (succinimidyl 4-formylbenzoate). 2-hydrazinopyridine moiety is introduced by using SANH (succinimidyl 6-hydrazinonicotinate acetone hydrazone).



Figure 6.1 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 6.2 Bioconjugation reaction between Hydrazine and Aldehyde modified moieties.

Advantages of HydralinK Bioconjugation System

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.

6.2 Short Term Experiment Plans

6.2.1 Immobilization of Thrombin to BioMagPlus Particles.

Experimental Objective: Immobilization of thrombin to BioMagPlus particles using

Solulink chemistry.

Stock

- 1. Thrombin from human plasma: Lyophilized 1.4mg solid, 184units/mg solid, 566units/mg protein (Biuret)
- 2. Stock BioMagPlus 1 micron particle @ 50mg/ml

Buffers

- 1. Sodium phosphate buffer pH 6.0
- 2. Sodium Phosphate/ Sodium Chloride buffer pH 7.8
- 3. PBS Azide/ Tween buffer pH 7.4
- 4. PBS Azide buffer pH 7.4 + 2mg BSA

BioMagPlus 1 micron Particle Activation

- 1. For activating 50mg of particles take 1ml of the stock PMP and add it to 3ml of 0.1M pH 7.8 buffer.
- 2. Decant, blot and wash three times in 0.1M pH 7.8 buffer.
- 3. The particles are then resuspended to 20mg/ml.

4. Particle activation using SANH Condition of activation = 1eq SANH / 1eq PMP = 1.26µl SANH / mg particle For 50mg particle we need 50 x 1.26µl = 63µl of SANH / 50mg PMP

SANH solution preparation: According to Solulink protocol: 5.5mg SANH / 100µl DMSO Dissolve 5.5mg SANH in 100µl of DMSO Add 63µl of the prepared solution to the washed 20mg/ml particles. Incubate in the shaker at room temperature for 2.5 hours.

- Post Incubation steps: After Incubation, remove from shaker. Separate, decant and blot. Wash four times in pH 6.0 buffer. Resuspend to 25mg/ml.
- 2mg particle/condition of SFB-Thrombin is needed Therefore from 25mg/ml SANH-PMP 250µl of SANH activated particles is prepared.

Thrombin Activation using SFB

1. SFB Activation

Molecular weight of SFB = 247.1g/mole According to Solulink chemistry: 4.8mgSFB/100µl DMSO Prepare three different concentrations of SFB

- a) Condition A: 30eq SFB / eq of thrombin For 1nmole of thrombin 30nmoles SFB/ eq of thrombin is needed.
- **b)** Condition B: 10eq SFB / eq of thrombin For 1nmole of thrombin 10nmoles SFB/ eq of thrombin is needed
- c) Condition C: 3eq SFB / eq of thrombin For 1nmole of thrombin 3nmoles SFB/ eq of thrombin is needed

Preparation of the stock solution of (SFB + DMSO): Weigh out 1mg of SFB and dissolve it in 20.83μ l of DMSO – This gives the stock solution **X**.

Since the volume of (SFB + DMSO) solution that is needed to be added to each condition of SFB is very less Prepare a 1/100 dilution of the stock solution X: 2µl of (SFB + DMSO) solution + 198µl DMSO.

Table 6.1	Dilution	Series	of SFB
-----------	----------	--------	--------

Tube #	Condition	Volume of Stock 1/100 of X (SFB + DMSO)	Volume of DMSO
А	30eq SFB / eq of thrombin	15.5µl	0
В	10eq SFB / eq of thrombin	5.15µl	0
С	3eq SFB / eq of thrombin	1.55µl	0

- 2. Take 480µg of thrombin from stock, i.e. 160µg/condition of SFB or 1nmole/condition of SFB.
- 3. Add thrombin to each condition of SFB and incubate for 2.5 hours.
- 4. Post Incubation steps:

Purify the Thrombin-SFB using NAP5 desalting column from Amersham Biosciences. Take three columns for each condition of SFB. Equilibrate the columns with $0.1M \text{ pH } 6.0 \text{ PO}_4$ buffer. The columns are first washed three times in pH 6.0 PO₄ buffer by eluting and discarding elute. Add the SFB activated thrombin in each condition to each column.

Conjugation Reaction (Immobilization of SFB activated thrombin to SANH activated particle): SANH + SFB reaction

- 1. Add 250µl of SANH-PMP to each condition of SFB activated Thrombin.
- 2. Separate the sample in a magnetic rack and withdraw input supernatants from each condition.
- 3. Incubate overnight in shaker at room temperature $(25^{\circ}C 30^{\circ}C)$.
- 4. After incubation overnight the master mixture is separated in a magnetic rack and output supernatant is withdrawn. This should give the output counts.
- 5. The particles is then 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.

6. Make a dilution series for the thrombin immobilized particle and read at a constant voltage the fluorescence count using fluorometer.

6.2.2 Antibody Immunoassay

Experimental objective: Co-titrate Mouse anti-thrombin antibody to immobilized thrombin followed by labeled anti-antibody (Goat anti-mouse anti-thrombin) to determine the amount of antigen immobilized on to the particles.

Materials

- Thrombin from human plasma T-7572 250 units Sigma Lot#102K7775, EC No. 232-648-7 Lyophilized 1.4mg solid, 184 units/mg solid, 566units/mg protein (Biuret)
- 2. Mouse Anti- Human Thrombin US Biologicals Product # 75045 100µg @ 5mg/ml K = 1.4 * 10e-8 IgG1 in 50% glycerol

Protocol

- 1. Wash the immobilized thrombin.
- 2. Add four different concentration of primary antibody, Mouse anti-thrombin.
- 3. Incubate overnight.
- 4. After incubation wash it.
- 5. Add three different concentration of labeled secondary antibody, Goat anti-mouse anti-thrombin.
- 6. Incubate overnight.
- 7. After incubation wash it thoroughly.
- 8. Pipette it onto the microtiter plate and read it with the Fluorolite plate reader.
6.2.3 Aptamer Binding Assay

a) Experimental objective: Thrombin Aptamer binding assay using secondary label (Biotin-Avidin system).

Materials

- APT-N-031304
 GGT TGG TGT GGT TGG TTT TT-amino-3'
 Length: 20mer
 Modifications: 3' amino
- APT-B-031304
 GGT TGG TGT GGT TGG TTT TT-biotin-3' Length: 20mer
 Modifications: 3' biotin
- ANTI-APT-Q 031304
 5'-CCA ACC ACA CCA ACC A-dabcyl-3' Length: 16mer Modifications: 3' DABCYL

Protocol

- 1. Immobilize thrombin on to BioMag particles using Solulink chemistry.
- 2. Wash the immobilized thrombin.
- 3. Add biotin labeled primary aptamer to the immobilized thrombin.
- 4. Incubate overnight.
- 5. Wash it after incubation.
- 6. Add the Avidin labeled secondary aptamer to it.
- 7. Incubate overnight.
- 8. After incubation wash thoroughly.
- 9. Pipette it to microtiter plate and read using plate reader.

b) Experimental objective: Thrombin Aptamer binding assay using primary fluorescein labeled aptamer.

Materials

 FLU-APT-N-031304
 5' FLU-TGG TTG GTG TGG TTG GTT TTT-amino-3' Length: 21mer Modifications:
 -5' FLU
 -3' amino

Protocol

- 1. Immobilize thrombin on to BioMagPlus particles using Solulink chemistry.
- 2. Wash the immobilized thrombin.
- 3. Add the fluorescein labeled aptamer.
- 4. Incubate overnight.
- 5. After incubation wash thoroughly.
- 6. Pipette it to microtiter plate and read using plate reader.

APPENDIX A

BUFFER PREPARATIONS

This appendix contains preparation of buffers used in this research.

 PBS Azide Wash Buffer pH 7.1 (1.0L) Stock: 10 times 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 Na₂HPO₄ 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 Na₂HPO₄ 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 NaH₂PO₄ Monobasic Buffer (200ml)

Weigh 27.6gm of NaH_2PO_4 (Formula Weight = 138.0). Dissolve it in water to make up 200ml

5. 10mM NaPO₄ pH 8.0 Buffer (500ml)

Stock: 0.1M dibasic Na₂HPO₄ (pH 8.0 - 8.5), 0.1M monobasic NaH₂PO₄ (pH 4.5-5.0)

To 400ml of DI water add 46.6ml of $0.1M \text{ Na}_2\text{HPO}_4$ and 3.4ml of $0.1M \text{ Na}_2\text{PO}_4$. 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. 10mM NaPO₄ pH 7.4 Buffer (250ml)

Stock: 0.1M dibasic Na₂HPO₄ (pH 8.0 - 8.5), 0.1M monobasic NaH₂PO₄ (pH 4.5-5.0)

To 200ml of DI water add 19.35ml of $0.1M \text{ Na}_2\text{HPO}_4$ and 5.65ml of $0.1M \text{ Na}_2\text{PO}_4$. 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 NaPO₄ pH 7.0 (250ml)

Stock: 0.1M dibasic Na₂HPO₄ (pH 8.0 - 8.5), 0.1M monobasic NaH₂PO₄ (pH 4.5-5.0)

To 200ml of DI water add 14.425ml of $0.1M \text{ Na}_2\text{HPO}_4$ and 10.575ml of $0.1M \text{ Na}_2\text{PO}_4$. 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 NaPO₄ (pH 7.0) +150mM NaCl + 0.025% NaN₃ + 0.05% Tween 20. (pH 7.1) Preparation of 1% Tween 20 spiking stock: Take 15ml centrifuge tube and pour 10ml of DI water into the tube. Pipette 100µl of stock Tween 20 into the 10ml 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 NaPO₄ (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.

13. Hybridization 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 0.375ml of Tween 20.

APPENDIX B

MATERIALS AND REAGENTS USED

This appendix contains the materials and reagents used for this research.

- 1. Bovine Serum Albumin(BSA): Fraction V, Minimum 96%; Sigma P No# A2153-50G; Batch #063K0778
- 2. BioMagPlus Amine Coupling Kit; Polysciences Inc.
 - 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 aliquots 0.5ml and 1 aliquot at 200µl.
- 3. MIgG [MIgG] = 10mg at 6.3mg/ml + 0.1%NaN₃ Lot # 500-70035, Code# M8-G10
- 4. Goat Anti-Mouse IgG Sensilight PBXL-1 [GAM-PBXL] = 1mg/ml, Lot#CS11MF01
- 5. Thrombin from human plasma T-7572 250 units Sigma Lot#102K7775, EC No. 232-648-7 Lyophilized 1.4mg solid, 184units/mg solid, 566units/mg protein (Biuret)
- 6. Mouse Anti- Human Thrombin US Biologicals Product # 75045 100µg @ 5mg/ml K = 1.4 * 10e-8 IgG1 in 50% glycerol

7. FTB Probe-080803

5'- CGA TCG CGA CAT TCC ATC GTG CCC GAT CGA AAA A-3' Length: 34mer Modifications: -5' FLU -3' Amino C7

8. FLU-APT-N-031304

5' FLU-TGG TTG GTG TGG TTG GTT TTT-amino-3' Length: 21mer Modifications: -5' FLU -3' amino

9. APT-N-031304

GGT TGG TGT GGT TGG TTT TT-amino-3' Length: 20mer Modifications: 3' amino

10. APT-B-031304

GGT TGG TGT GGT TGG TTT TT-biotin-3' Length: 20mer Modifications: 3' biotin

11. ANTI-APT-Q 031304

5'-CCA ACC ACA CCA ACC A-dabcyl-3' Length: 16mer Modifications: 3' DABCYL

APPENDIX C

EQUIPMENT AND INSTRUMENTS USED

This appendix contains the instruments used in this research.

- 1) Beckman DU 520 General purpose UV/Vi Spectrophotometer
- 2) Fluorite 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 contains the glossary of the terms used in this thesis report.

- 1. Affinity: The tendency of a molecule to associate with another. The affinity of a drug is its ability to bind to its biological target (receptor, enzyme, transport system, etc.) For pharmacological receptors it can be thought of as the frequency with which the drug, when brought into the proximity of a receptor by diffusion, will reside at a position of minimum free energy within the force field of that receptor.
- 2. Antibody: A protein (immunoglobulin) produced by the immune system of an organism in response to exposure to a foreign molecule (antigen) and characterized by its specific binding to a site of that molecule (antigenic determinant or epitope).
- **3.** Antigen: A compound (protein, polysaccharide, microorganism, virus) foreign to the body that induces the production of specific antibodies.
- 4. Antibody affinity: A measure of the binding strength between antibody and a simple hapten or antigen determinant. It depends on the closeness of stereochemical fit between antibody combining sites and antigen determinants, on the size of the area of contact between them, and on the distribution of charged and hydrophobic groups. It includes the concept of "avidity," which refers to the strength of the antigen- antibody bond after formation of reversible complexes.
- 5. Aptamer: A synthetic, specially designed oligonucleotide with the ability to recognize and bind a protein ligand molecule or molecules with high affinity and specificity.
- 6. Assay: A set of operations having the object of determining the value of a quantity.
- 7. Binding site: A specific region (or atom) in a molecular entity that is capable of entering into a stabilizing interaction with another molecular entity.

- 8. Bioassay: A procedure for determining the concentration or biological activity of a substance (e.g. vitamin, hormone, plant growth factor, antibiotic, enzyme) by measuring its effect compared with a standard preparation.
- **9.** Enzymes: Macromolecules, mostly of protein nature, that functions as (bio) catalysts by increasing the reaction rates. In general, an enzyme catalyses only one reaction type (reaction specificity) and operates on only one type of substrate (substrate specificity). A substance (usually a protein) that speeds up, or catalyzes, a chemical reaction without being permanently altered or consumed.
- **10. 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.
- 11. Hydrophobic: Repels water.
- 12. Immunoassay: A ligand- binding assay that uses a specific antigen or antibody, capable of binding to the analyte, to identify and quantify substances. The antibody can be linked to a radioisotope, or to an enzyme which catalyses an easily monitored reaction (enzyme- linked immunosorbent assay, *ELISA*), or to a highly fluorescent compound by which the location of an antigen can be visualized (immunofluorescence). It is the only method possible for small molecular weight antigens, such as steroids, drugs, lipids, and peptides. There are three basic components in any immunoassay, the antigen to be detected and/or quantified, a specific antibody to this antigen, and a system to measure the amount of the antigen in the sample. The separation at the end of the immunological reaction uses a microplate.
- **13. Immunoglobulin, Ig:** A protein of the globulin- type found in serum or other body fluids that possesses antibody activity. An individual Ig molecule is built up from two light (L) and two heavy (H) polypeptide chains linked together by disulfide bonds. Igs are divided into five classes based on antigenic and structural differences in the H chains.
- 14. Ligand: A molecule which binds to a receptor protein. Any molecule that binds to the surface of another molecule such as an immune cell receptor.

- **15. Molarity:** The molar unit is probably the most commonly used chemical unit of measurement. Molarity is the number of moles of a solute dissolved in a liter of solvent.
- 16. Molecular beacons: Oligonucleotide probes that can report the presence of specific nucleic acids in homogeneous solutions (Tyagi and Kramer 1996). They are useful in situations where it is either not possible or desirable to isolate the probe-target hybrids from an excess of the hybridization probes, such as in real- time monitoring of polymerase chain reactions in sealed tubes or in detection of RNAs within living cells. Molecular beacons are hairpin- shaped molecules with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid.
- 17. Nucleic acid probes: Nucleic acid which complements a specific mRNA or DNA molecule or fragment thereof; used for hybridization studies in order to identify microorganisms and for genetic studies.
- **18.** Polymerase Chain Reaction(PCR): The most sensitive technique to amplify and detect low abundance mRNA and minute amounts of DNA.
- **19. Probes:** A specific DNA or RNA sequence which has been labeled by radioactivity, fluorescence labels or chemiluminescence labels and which is used to detect complementary sequences by hybridization techniques, such as blotting or colony hybridization.
- **20. Peptides:** Amides derived from two or more amino carboxylic acid molecules (the same or different) by formation of a covalent bond from the carbonyl carbon of one to the nitrogen atom of another with formal loss of water. Typically a peptide consists of less than 50 amino acids while a protein has greater than 50 amino acids. Peptides are ubiquitous and are found in most biological systems. In general, peptides are synthesized by chemical techniques.
- **21. Proteins:** Naturally occurring and synthetic polypeptides having molecular weights greater than about 10,000 (the limit is not precise). Polymers of amino acids linked by peptide bonds. The specific sequence of amino acids determines the shape and function of the protein. Proteins provide the critical link between genes and disease, and as such are the key to understanding of basic biological processes including disease pathology, diagnosis, and treatment.

22. Receptor: A protein or a protein complex in or on a cell that specifically recognizes and binds to a compound acting as a molecular messenger. In a broader sense, the term receptor is often used as a synonym for any specific (as opposed to non-specific such as binding to plasma proteins) drug binding site, also including nucleic acids such as DNA.

APPENDIX E

DATA SHEETS

This appendix contains the data sheets for the BioMagPlus particles used as substrates from the Polysciences Inc. and the data sheet for Goat Anti-Mouse.

E.1 BioMagPlus Technical Data Sheet

sciences, Inc.

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TECHNICAL DATA SHEET 617

BioMag[®] Plus Amine Terminated Particles (Cat. No. 86001-10) BioMag[®] Plus Amine Particle Protein Coupling Starter Kit (Cat. No. 86000-1)

Introduction

BioMagPlus superparamagnetic microparticles are utilized in the magnetic separation of cells, organelles, proteins, immunoglobulins, nucleic acids, and many other types of molecules in biological and non-biological systems. The irregular shape of the BioMagPlus particle allows for much greater surface area 20 to 30 times that of the same size spherical particle. This large surface area results in high binding capacities, allowing efficient target capture with minimal amounts of particles.

BioMagPlus particles are approximately one micron superparamagnetic particles consisting of an iron oxide core with a silane coating. BioMagPlus particles are similar to conventional BioMag particles with the important distinctions of being much more uniform in size and more magnetically responsive. The more uniform size results in a more predictable and consistent behavior during capture and magnetic separation steps. Greater magnetic responsiveness results in faster magnetic separations especially on high throughput automated platforms.

BioMagPlus Amine particles offer a high level of amine functionality on magnetically responsive particles. Typically the primary amine groups on the surface of the particles are activated by glutaraldehyde, allowing amine groups on proteins to be covalently attached.



Polysciences offers the BioMagPlus Amine Particle Protein Coupling Starter Kit for covalently coupling proteins to BioMagPlus superparamagnetic particles. The contents of the kit are sufficient for five coupling reactions. To use the kit for smaller orlarger samples adjust all volumes in a proportional manner.

Materials Supplied With Kit

Quantity	Kit Component	Volume
1	BioMagPlus Amine Terminated Particles (50mg/ml in 1mM sodium EDTA, pH 7.0) (Dilute to 5% with PWB before use)	25ml
2	Glutaraldehyde (EM Grade, 25%) (Dilute to 5% before use with PWB)	10ml
5	50ml conical centrifuge tubes	N/A
1	MultiSep Magnetic Separator (P/N 85200-1)	N/A
2	Pyridine Wash Buffer (PWB) 0.1M (Dilute 1:10 and adjust pH to 6.0 before use)	125ml
2	Quenching Solution (1M Glycine, pH 8.0)	125ml
1	Wash Buffer (Dilute 1:10 and adjust pH to 7.4 - 7.6 before use)	125ml

Coupling Procedure

BioMagPlus Activation (Perform all activation and coupling reactions in a ventilated

chemical fume hood.)

*Note: Dilute Pyridine Wash Buffer (PWB) and Wash Buffer 1:10 with water

before using. Dilute 25% Glutaraldehyde to 5% with PWB before using.

BioMagPlus Activation

- 1. Transfer 5ml of BioMagPlus Amine Terminated Particles (equivalent to 250mg) to a 50ml conical centrifuge tube.
- 2. Add 40ml of Pyridine Wash Buffer (PWB) to the flask and shake well to mix. Magnetically separate the particles until the supernatant is clear. Aspirate the supernatant and discard.
- 3. Repeat step #2 three times for a total of four washes.
- 4. If not done already dilute 25% glutaraldehyde to 5% with PWB. Add 20m I of 5% glutaraldehyde to the particles and shake vigorously to mix.
- 5. Place the flask on a non-magnetic mixing device (rotator) for 3 hours at room temperature. *Note: The particles should not be allowed to settle during mixing.*
- 6. Magnetically separate the activated particles until the supernatant is clear. Aspirate the supernatant and discard.
- 7. Repeat Step #2 four times.

Protein Coupling

- Calculate the amount of protein, antibody, or other ligand required for coupling. Generally, total protein concentrations of 50 to 500pg should be used for each milligram of activated BioMagPlus Amine Terminated Particles. Carrier proteins such as Bovine Serum Albumin (BSA) Fraction V (PIN 23854-10) may be added to increase total protein concentration, blocking, and provide proper orientation of specific ligand binding for covalent attachment.
- 2. Dissolve the protein in 20ml PWB.

- 3. Remove 50pl of diluted protein solution and add it to 950pl of PWB. This is a 1:20 dilution. If further dilution is necessary dilute accordingly. Set aside in a sealed tube for coupling efficiency determination (Pre-Coupling sample for Coupling Efficiency Determination below.)
- 4. Add the remaining protein solution to the activated particles and shake vigorously to mix. Place the flask on a mixing device for 16 to 24 hours at room temperature.
- 5. Magnetically separate the coupled particles until the supernatant is clear, remove, and set aside for coupling efficiency determination. (Post-Coupling sample for Coupling Efficiency Determination below.) If necessary dilute accordingly.
- 6. Resuspend the particles in 40ml of PWB.
- 7. Add 20ml of Quenching Solution. Shake vigorously to mix. Place the flask on a mixing device for 30 minutes at room temperature.
- 8. Magnetically separate the particles until the supernatant is clear.' Aspirate the supernatant and discard.

Washing and Diluting Coupled BioMagPlus Particles

- 1. Add 40ml of Wash Buffer and shake vigorously to mix. Magnetically separate the particles until the supernatant is 'clear. Aspirate the supernatant and discard.
- 2. Repeat Step #1 three more times.
- 3. Resuspend the particles to 50ml in wash buffer. Particle concentration is now approximately 5mg/ml. Store the coupled BioMagPlus particles at 4°C as a suspension in Wash Buffer or in a buffer that is compatible with the attached protein.

Coupling Efficiency Determination

- 1. Set up a UV/Vis spectrophotometer to measure at 280nm. Fill both cuvettes with PWB and blank the spectrophotometer.
- 2. Measure the absorbance of the Pre-Coupling and Post-Coupling Protein samples. Note: Further dilutions may be necessary to read the absorbance within the linear range of the instrument.

- 3. Calculate the coupling efficiency, expressed as the % Protein Uptake as follows: (OD280nm Pre-Coupling Sample x Dilution) - (OD280nm Post-Coupling Sample x Dilution) x 100 (OD280nm Pre-Coupling Solution x Dilution)
- 4. Acceptable values of % Protein Uptake are 60% to 100%.

Notes:

- A. Phosphate Buffer (0.01 M, pH 7.0) can be used as a Coupling Buffer, but with reduced coupling efficiency compared to the recommended pyridine buffer. The polyvalent, negative phosphate ions clump the positively charged amine support. Do not use primary amines, ammonium ion, or other strong nucleophiles in the coupling buffer. All Coupling Buffers should be used at minimal ionic strengths. Buffers containing amines (e.g. Tris) or phosphate buffers (e.g. PBS) can be used as Wash Buffers. Ionic strength has little or no effect on BioMagPlus particles once protein is attached.
- B. Some noncovalent adsorption invariably accompanies covalent coupling to particulate supports. Noncovalent adsorption is controlled by the washing procedure used after covalent protein attachment. The degree of noncovalent adsorption varies with each application and the washing procedure may have to be adjusted for individual applications. Additional washes to reduce non-covalently adsorbed protein can include high salt (1M NaCI), mildly acidic or basic media, mildly elevated temperatures, or increased time of exposure to the Wash Buffer. Dissociation of active, non-covalently adsorbed molecules from BioMagPlus particles can make magnetic materials appear unstable in some applications.
- C. Prolonged vigorous shaking or sonication should be used to resuspend BioMagPlus particles after magnetic separation or settling with gravity.

Storage and Handling Information

Freezing, drying, or centrifuging BioMagPlus particles result in extensive aggregation and loss of binding activity. DO NOT FREEZE OR DRY BioMagPlus Particles. Store BioMagPlus particles and the liquid contents of the Coupling Kit at 4°C.

Ordering Information

Cat. #	Description	Size
86001-10	BioMag [®] Plus Amine Terminated Particles	10 ml
86000-1	BioMag*Plus Amine Particle Protein Coupling Starter Kit	kit
86011-10	BioMag*Plus Carboxyl Terminated Particles	10 ml
86010-1	BioMag [®] Plus Carboxyl Particle Protein Coupling Starter Kit	kit
86031-10	BioMag*Plus Streptavidin Particles	10 ml
86030-1	BioMag [®] Plus Streptavidin Particle Biotin Binding Starter Kit	kit
86021-50	BioMag [®] Plus Goat Anti-Mouse IgG Particles	50 ml
86020-1	BioMag [®] Plus Goat Anti-Mouse IgG Particle Antibody Coupling Starter Kit	kit
86041-10	BioMag*Plus Protein A Particles	10 ml
86040-1	BioMag*Plus Protein A Particle Antibody Isolation Starter Kit	kit
86051-10	BioMag [®] Plus Protein G Particles	10 ml
86050-1	BioMag [®] Plus Protein G Particle Antibody Isolation Starter Kit	kit

Researchers are advised to optimize the use of BioMagPlus particles in any application as

procedures designed by other manufacturers may not be ideal.

For research use only, not intended for use in humans or *in vitro* diagnostics use.

To Order:

In The U.S. Call: 1-800-523-2575 or 215-343-6484

In The U.S. FAX: 1-800-343-3291 or 215-343-0214

E.2 Goat Anti-Mouse Data Sheet



Goat anti-Mouse IgG: SensiLight[™] PBXL-1

Catalog # 50725 Lot # CS11MF01

Description:

- Supplied as a lyophilized powder, which contains 1 mg of SensiLight™PBXL-1 (a powerful red emitting fluorescent dye), conjugated to goat anti-mouse IgG (with heavy and light specificity).
- Upon reconstitution with 1.0 ml distilled, deionized water, the resulting solution is 100 mM sodium phosphate (pH 7.4), 150 mM sodium chloride. 200 mM sucrose, 0.1% BSA, and 0.05% sodium azide.
- Upon receipt, store hypohilized material in the dark at 4°C. Product should be used within 4 weeks of reconstitution and stored at 4°C. Do not freeze.

Spectral Characteristics

♦ Visible absorption max
 ♦ Emission max
 545>566>624>650 nm, 498 (sh) nm
 ♦ Emission max
 666-668>573 nm

>2.5

- Stokes shift 119 nm
- Emission 666/573



Excitation versus emission spectra of GAM:SensiLight™ PBXL-1 in PBS (pH 7.2) + 0.05% sodium azide

Quality control analysis run on lot #CS11MF01 and this product passed all designated specifications

Analyzed by Saigh Date: 10/15/00



6480 Dobbin Road Columbia: MD 21645 (410) 740-008) fax (410) 740-2985 fluors*ia* martkebio com

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