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Michael J. Sepaniak, Major Professor

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DEVELOPMENT AND PRELIMINARY EVALUATION OF A FIBER OPTIC-BASED MICROSCALE BIOSENSOR FOR PERFORMING REMOTE COMPETITIVE-BINDING FLUOROIMMUNOASSAYS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Shannon L. Gerhardt

December 1991

DEDICATION

This thesis is dedicated to my parents
Reid and Nancy Gerhardt
and to
Oz
for their invaluable support and love.

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Michael Sepaniak, for his guidance and for his patience when I had none. I would also like to thank the other committee members, Dr. Michael Keene, for his enthusiasm and encouragement, and Dr. Earl Wehry for his helpful comments. Thanks to the other members of the Sepaniak research group for their patience and assistance in the laboratory.

I also thank the University of Tennessee Department of Chemistry for financial assistance, in the form of a teaching assistantship, during the length of my graduate career. I would also like to thank the Science Alliance Summer Research Program which initiated my involvement in graduate research.

ABSTRACT

Certain complex samples require repetitive *in situ* analysis; however, those samples might be toxic or inaccessible. Fiber optic sensors can analyze remote samples because the flexible fibers are small, and able to transport information over substantial distances. Fiber optic-based biosensors combine the sensitivity of laser-based fluorescence detection with the selectivity of affinityreagents. Our group developed a novel biosensor that delivers fluoroimmunoreagents (solid/liquid phase) to and from a remote sample site through capillary columns, and retains the reacted complex near the fiber with a porous frit. I examined a prototype of this sensor, the Microscale Regenerable Biosensor (MRB), regarding its operational characteristics as they relate to performing competitive-binding fluoroimmunoassay (FIA). I employed a "model" assay of protein A as an affinity reagent for rabbit IgG.

I evaluated the MRB's physical capacities and its response to the model FIA. I found the MRB able to: 1) collect sample, 2) deliver appropriate amounts of liquid/solid reagent phase to the sample reproducibly, 3) rinse away possible interferents, and 4) measure the signal from the reacted complex--all without removing the sensing tip from the sample. I also found the MRB capable of measuring assay parameters such as: amount of total antibody-antigen binding, various analyte:labeled analyte ratios, and specific/nonspecific antibody binding. However, when liquid reagent flows over solid reagent (during MRB operation), I found that the interaction between the model reagents was inadequate to allow a demonstration of a sequential competitive-binding FIA.

These findings suggest that with further development, the MRB could allow 1) repetitive competitive-binding assays for near-continuous monitoring, and 2) multiple analyses within a single remote sample by employing alternate reagents.

PREFACE

I wrote this thesis for many reasons, the most obvious one being to satisfy the "partial fulfillment of the requirements " for a degree in chemistry. However, while science is a worthy pursuit, it is useless until scientists communicate their ideas and discoveries effectively. This is why I chose to include technical communications in my graduate education program.

Scientific writing is traditionally delivered in the third person. Unfortunately, this forces scientists to write in the passive voice when describing their own work. For example, I could present a portion of this thesis like this:

> When the bundle had hardened, the heat shrink was removed, and the bundle was successively polished to a smooth surface using 32-, 15-, 3-, and 0.3-μm lapping film. Then, using a microscope, a 5-mm section of $^{1}/_{16}$" o.d. stainless steel tubing was placed over the bundle like a sleeve. The tubing was epoxied into place while it was extended 0.5 mm beyond the fiber bundle. All gaps between the inside of the tubing and the outside of the bundle were filled.

Ken Porter * describes four benefits of passive voice use:

- Statements sound official and authoritative (but no one can tell who is responsible for the statements, and the reader can only assume they are made by God).
- It provides an opportunity to create longer and more obscure sentences, thus confusing the reader more easily.

---

* Ken Porter, "Usage of the Passive Voice," *Tech. Commun.* 38 (1), (1991): 87-88.

- Statements become forgettable. He provides the example of worked-over Shakespeare, "'Friends, Romans, countrymen, your ears should be lent to me. It is intended that Caesar be buried, not praised.'"
- It bores the reader to tears.

Therefore, I wrote this thesis in a somewhat untraditional manner, using the first person when necessary to avoid the passive voice. I also employed a decimal heading system that is useful in technical documents.

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LIST OF ABBREVIATIONS

Antibody	Ab
Antigen (labeled)	Ag*
Antibody-Antigen Complex	Ab-Ag
Antibody..-Antigen Complex (labeled)	Ab-Ag*
Enzyme Immunoassay	EIA
Fluorescence Polarization Immunoassay	FPI
Fluorescent Excitation Transfer Immunoassay	FETI
Fluorescein isothiocyanate	FITC
Fluoroimmunoassay	FIA
Fiber Optic Chemical Sensor	FOCS
Microscale Regenerable Biosensor	MRB
Natural Antigen (analyte)	Ag
Phosphate Buffered Saline	PBS
Photomultiplier Tube	PMT
Radioimmunoassay	RIA
Time-Resolved Fluoroimmunoassay	TR-FIA

CHAPTER 1

INTRODUCTION AND SPECIFIC AIMS

Monitoring the presence and movement of chemical substances is a fundamental need of science, medicine, and industry. In many instances a single sample source can vary over an extended period of time, and monitoring the presence of compounds requires frequent analysis. For example, food industries must sample a process stream often to ensure quality control. Similarly, certain conditions in a variety of industries are prone to change, such as factory discharge or waste container integrity, evoking a need for repetitive environmental tests.

These analysis situations generally require continuous monitoring; thus, a sensing device that is remote, continuous, and sensitive would be highly useful. Many sensing devices are capable of monitoring various chemical and physical properties within a sample, using a variety of detection methods (1, 2). One family of sensors uses affinity assay techniques. These sensors are called biosensors not for the type of sample they monitor, but for the type of reagent they employ. The "bio" in relation to this work describes biologically-based immunoassay reagent(s). Several texts and reviews describe various immunoassay techniques (3-5), and others address biosensors of this variety (6-8).

Immunoassay techniques can quantitatively or qualitatively indicate the presence of specific drugs, hormones, or toxic compounds, often within small sample volumes. Yalow and Berson developed the first immunoassay in 1959 for the measurement of insulin in plasma (9). The development of immunoassays has progressed steadily since then as demonstrated in the fields of biochemistry and clinical medicine, and in recent years other scientific disciplines such as forensic science, agriculture, and environmental protection have begun to appreciate the immense usefulness of this technique (10).

1.1 Immunoassays

A variety of techniques use immunoreactions as a tool for analytical determinations of many significant molecules. These techniques are called immunoassays and they vary by reaction type and detection method. Immunoassays capitalize on the antibody's affinity for antigens (i. e. analyte). To date there is no distinct system of nomenclature that applies to the various immunoassays, but they can be classified by two characteristics: homogeneity or protocol. One particular class of immunoassays uses a fluorescent molecular label to trace the immunoreaction: these assays are called fluoroimmunoassays (FIAs).

1.1.1 *Antibodies and Antigens*

A major class of immunoassays use antibodies as highly selective reagents. The most well-known antibodies are the serum immunoglobulin proteins belonging to the subclass immunoglobulin G (IgG). These immunoglobulins are more effective reagents in the monoclonal form rather than the polyclonal mixture (see following discussion).

Reaction. Antigens are molecules that elicit immune responses when introduced into the body of an animal. The immune response forms a protein molecule (MW approximately 160,000) called an antibody (Ab) that has specific attraction to the antigen (Ag) that evoked its synthesis. The antibody-antigen interaction is highly selective and forms an immune complex that involves multiple, non-covalent, antibody-antigen bonds. The reaction is an equilibrium,

$$Ab + Ag \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AbAg \qquad [1]$$

where $K_a = k_1/k_2$ is the affinity constant. Typical K_a values range from 10^6 to 10^{12} M^{-1} depending upon the Ab-Ag system, values for k_1 range from 10^7-10^9 $M^{-1}s^{-1}$ and typical k_2

values range between 10^2-10^{-4} s^{-1} (11, 12). Mass-transport essentially controls the forward reaction due to these large k_1 values.

Immunoglobulin G. The body produces several classes of antibodies including immunoglobulin M (IgM) and immunoglobulin G (IgG). Immunoglobulin G is widely used for immunoassay techniques. Immunoglobulin G antibodies are Y-shaped macromolecules, having antigen-specific sites at the two upper ends. If the molecule is cleaved at the joint, the resulting two upper portions are identical. These are called Fab fragments (ab denotes antibody-binding). The antigen combines with the antibody at the Fab segments; therefore, each antibody has two binding sites at which an antigen may bind. The single lower section is called the Fc portion and is generally constant from antibody to antibody (13). Several procedures call for the immobilization of the Fc portion of the antibody onto a solid phase support (see section 1.1.2.2).

Monoclonal Antibodies. Because several types of cells contribute to the immune response, the body produces a heterogeneous antibody population after immunization, even within a single class of immunoglobulins. Another contribution to this heterogeneity is that the immune system can produce Fab sections specific for different epitopes (a group within the antigen recognized by the antibody) of the same antigen (14). The resulting mixture is polyclonal; it contains a number of antibody molecules, each having its own affinity and recognition site for the antigen. The K_a of a polyclonal mixture is the average of all the affinities within that mixture (13).

A pure strain of identical antibodies is called monoclonal antibody and has a single affinity constant. When employed as a reagent in an assay, monoclonal antibody responds uniformLy to an antigen, whereas a polyclonal mixture will not (15). Monoclonals are produced by fusing a normal antibody-producing cell to a myeloma cell to create a hybridoma. The myeloma cell is an antibody-producing cell gone awry with cancer. The

myeloma disorder causes the cell to divide rapidly and produce antibodies (13). These hybridoma cells supply identical antibodies.

1.1.2 *Assays*

There are many ways to describe immunoassays. They can be either homogeneous or heterogeneous. They can also be classified according to their reaction protocol, as either a direct, competitive binding, or sandwich assay.

Homogeneity. All types of homogeneous immunoassays compare some parameter (such as conformation change, reaction inhibition, or one of many fluorescent properties) before and after the immunoreaction. Assays based on measuring changes in this parameter may become difficult when the relative change is small.

A homogeneous assay takes advantage of some property that varies between the Ab-Ag complex and the unbound Ag, thus eliminating the need to physically separate interfering or excess reagents before detection. This lack of a separation step simplifies the analysis; however, it also allows excess unbound Ag, as well as other potential interferents, to remain during detection. For example, a molecule (other than the antigen) that bonds non-specifically to the antibody interferes with the measurement property since it creates a Ab-interference complex.

Conversely, heterogeneous assays physically separate the complex and free antigen. There are many ways to achieve separation, but the method must be compatible with the reaction and the detection conditions. One separation method immobilizes the antibody (ideally the Fc portion) onto a solid phase such as beads, a tube, or a plate, then the rinsing step removes the free components. Other separation methods include affinity chromatography, ion exchange, and gel filtration (16).

While heterogeneous assays are operationally more complex than homogeneous assays due to the extra step(s) of separation, it is this step that removes interferents. The

heterogeneous assay can also demonstrate a "concentrating effect" on the antigen by collecting the antigen from a larger sample size. These effects in turn provide greater sensitivity.

Another reason for the superior sensitivity of the heterogeneous method arises from the nature of the signal. A homogeneous assay measures the difference between two signals (i. e. the signal before and after the addition of the sample), and the heterogeneous assay measures the absolute signal derived from the addition of the sample. Consequently, a heterogeneous method using a fluorescent label can exploit the "fluorescence advantage" that results from the direct relationship between fluorescence signal and the incident power, described by the following equation

$$P_F = \Phi_F P_0 \varepsilon b c \qquad [2]$$

where P_F is the radiant power of fluorescence, Φ_F is the quantum yield of fluorescence, P_0 is the radiant power incident on the sample, ε is the molar absorptivity of the compound, b is the path length, and c is the concentration (17). Thus a heterogeneous assay can exploit this advantage, while the homogeneous method must rely on the difference between two signals for its dynamic range.

Protocols. Three common FIA techniques are the direct assay, the sandwich assay, and the competitive-binding assay. Both homogeneous and heterogeneous procedures are possible for each technique.

The direct assay (see Figure 1a) measures the sample's intrinsic fluorescence and is only useful for naturally fluorescent molecules. The antibody binds the fluorescent antigen, resulting in a detectable complex. The signal is proportional to the amount of fluorescent antigen present in the sample.

The sandwich assay (see Figure 1b) requires two antibodies (labeled and unlabeled) that are specific for separate epitopes of the antigen structure (see section 1.1.1.3). This technique generally provides for the immobilization of the first antibody onto a solid phase which facilitates the subsequent rinsing steps typical of a heterogeneous assay. The assay consists of two steps in which an unlabeled antibody reacts with the analyte to form a complex, then a second, labeled antibody reacts with the complex, sandwiching the antigen between the two antibodies.

A variation of the sandwich assay allows both labeled and unlabeled antibodies to simultaneously react with the sample antigen. Both the sequential and the simultaneous sandwich assay give a signal response that is directly proportional to the amount of analyte and usually employ a separation step to remove the excess antibodies from the sandwich complex. These sandwich assays are useful for the detection and quantitation of large (MW > 5,000), nonfluorescent antigens that have at least two distinct binding sites (16).

Figure 1c demonstrates the steps of the competitive-binding assay. This heterogeneous assay involves the addition of labeled antigen instead of labeled antibody, where labeled (known concentration) and unlabeled (analyte) antigens compete for a limited number of antibody binding sites. The post-reaction mixture contains labeled and unlabeled complexes and excess free components (also labeled and unlabeled). In the most commonly used heterogeneous procedure, rinsing removes the excess free components, and the resulting signal is inversely proportional to the amount of naturally occurring (non-fluorescent) analyte.

The competitive-binding assay can employ a sequential procedure variation, just as the sandwich assay. First, a limited amount of antibody reacts with the naturally occurring non-fluorescent analyte. If the assay procedure controls the incubation time properly, some of the antibody sites remain free from antigen, and other antibody sites become filled,

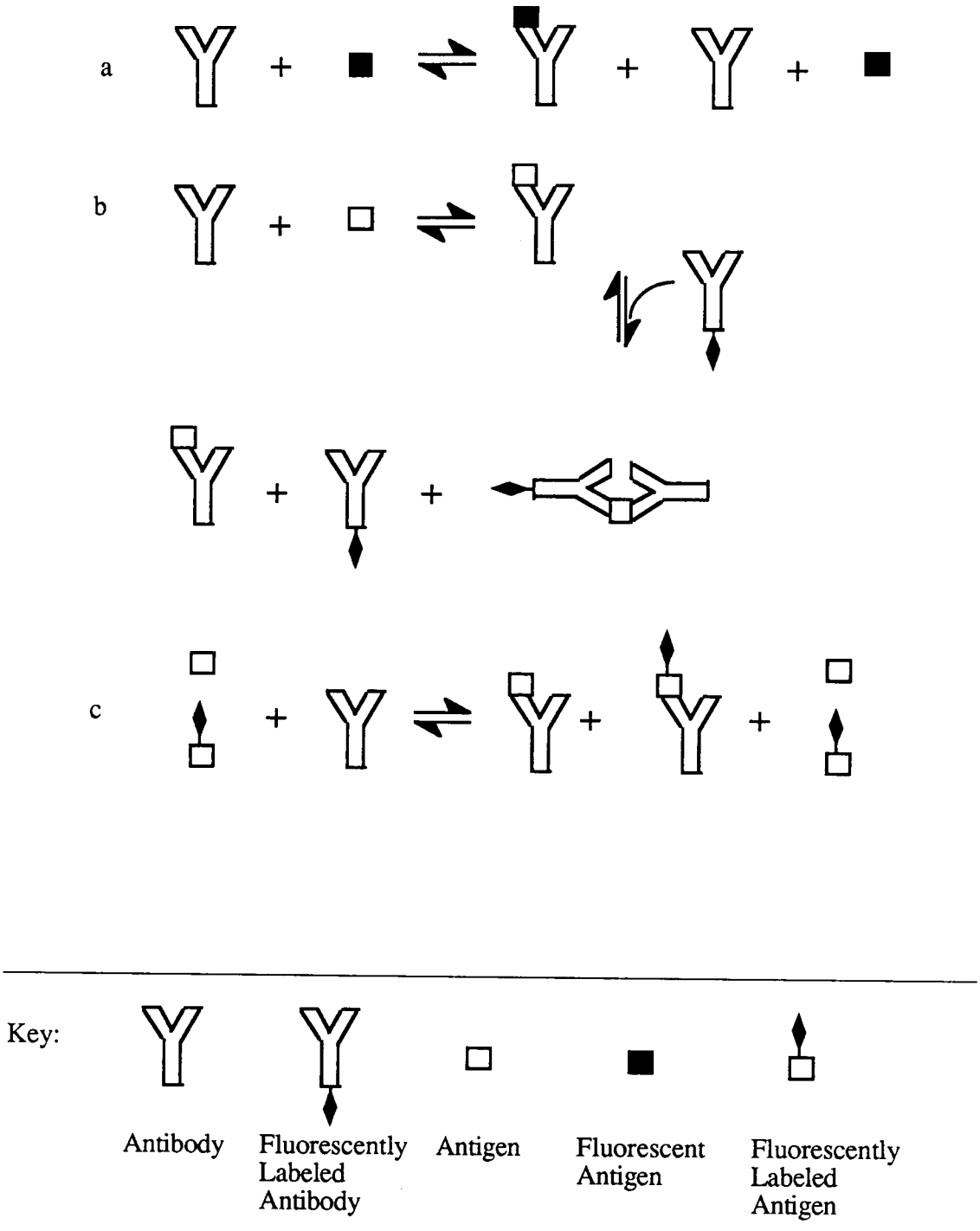


Figure 1. Fluoroimmunoassay Protocols. (a) Direct FIA, (b) Sandwich FIA, (c) Competitive-binding FIA. Adapted from General Principles of Immunoassay; Chan, D. W.; in Immunoassay: A Practical Guide; Chan, D. W., Ed.; Academic: New York, 1987; pp 1-23.

creating a complex. A separation step removes the excess analyte, then the remaining antibody reacts with a known amount of labeled antigen, filling the remaining available binding sites. A separation step removes the excess labeled antigen, and the signal resulting from the complex mixture indicates the amount of analyte in an inversely proportional relationship.

1.1.3 *Fluoroimmunoassay*

There are many ways to exploit the specificity of an immunoassay, but the reaction must be monitored in some way. The common methods of immunoassay, radioimmunoassay (RIA), enzyme immunoassay (EIA), and fluoroimmunoassay (FIA), use characteristic labels; radioisotopes, enzymes, and fluorescent molecules, respectively. Fluoroimmunoassay methods have several advantages over RIA and EIA techniques, and the family of FIA approaches allows this method to be useful for a large number of applications (18, 19).

Attributes and Limitations. Radioimmunoassay was the first technique developed to take advantage of the selective reaction between antibodies and antigens and to monitor that reaction with a label. Later, enzymes and fluorescent molecules became available as alternative labels.

Fluoroimmunoassay techniques offer solutions to many of the problems associated with RIAs and EIAs (20, 21). For example:

- When stored properly, the shelf-life of fluorescent labels is years rather than months.
- The safety hazards and regulations of radioactive materials do not apply.

- Fluorescent labels are stable (unlike some EIA labels) and, for the most part, do not alter the immunoreactivity of the reagents (unlike some RIA labels).
- Most FIAs are quick (unlike some EIAs) and do not need special equipment.
- Fluoroimmunoassays exploit the fluorescence advantage, allowing excellent sensitivity.
- Fluoroimmunoassays make homogeneous assays possible (see section 1.1.2).

Some disadvantages of FIA technique include:

- Certain assay condtitions such as pH (22), temperature (17), or binding position on a substrate (23) can alter the fluorescence of a labeling molecule.
- Many fluorescent molecules are subject to quenching by certain biological substances (such as bilirubin); however, fluorescein isothiocyanate seems to be a practical, soluble label that avoids most biological interferences. It also binds to proteins readily (23).

Approaches. Fluoroimmunoassays indicate an antibody-antigen interaction by supplying comparative fluorescence signals before and after a sample antigen is added to the system. Procedures generally call for the addition of some combination of antibody (one or more) and antigen, of which at least one is fluorescently labeled. The sample antigen interacts with these added components producing a fluorescence signal that is different from the signal when no sample is present. Some FIA approaches include:

fluorescence enhancement, fluorescence quenching, fluorescence energy transfer, time-resolved fluorescence, fluorescence polarization, and others (24).

Fluorescence enhancement uses a fluorescent derivative of the antigen. The fluorescence of the derivative increases when bound to the antibody; therefore, the degree of enhancement is inversely proportional to the concentration of sample antigen (25).

Fluorescence quenching is the opposite of fluorescence enhancement. In this approach, the antibody quenches the antigen's fluorescent label when binding occurs, while the free labeled antigen retains its full signal. When sample antigen is added, it occupies a portion of available antibody sites, thus releasing more unbound labeled antigen and the fluorescence signal increases accordingly in comparison to pre-sample conditions. This technique is most appropriate for small antigens because the label must be in close proximity to the epitope for quenching to occur. An indirect method employing a second label-specific reagent is useful for larger molecules (25).

An approach related to quenching is a fluorescent energy transfer immunoassay, or FETI. It involves a nonradiative transfer of energy between two molecules through a dipole-dipole coupling (26). Energy transfers from a fluorescence-donating molecule, a labeled antigen, to a florescence-accepting molecule, a labeled Ab. This transfer quenches the labeled antigen's fluorescence. When sample unlabeled antigen is present, some of the labeled Ab reacts with it, thus displacing some labeled antigen and reducing the total amount of energy transfer between the donor and acceptor. The resulting signal is inversely proportional to the amount of sample antigen (25).

Time-resolved fluoroimmunoassay (TR-FIA) solves many spectral interference problems by using a label with a long fluorescent lifetime, usually a lanthanide metal chelate. There is essentially no background fluorescence at the time of measurement because the intense fluorescence of the metal chelate lasts longer (10μs-100μs) than the

background fluorescence. With this approach the signal is directly proportional to the concentration of natural antigen (27).

Another approach is called fluorescence polarization immunoassay (FPIA). When plane polarized light excites a fluorophore, the rotational motion of the molecule occurring between absorption and emission can cause the loss of polarization information (28). This loss of information depends upon the size of the fluorescent molecule; small molecules rotate quickly, losing more polarization signal than a larger, more slowly rotating molecule. Therefore, an FPIA using a competitive-binding assay shows an increase in polarization signal due to the resulting large labeled-antigen-antibody complex. Thus a natural (unlabeled) antigen displaces labeled antigen in complex formation and decreases the degree of polarization relative to an assay in which all antigen is labeled (29).

The MRB's (Microscale Regenerable Biosensor) approach is somewhat less complicated--it measures fluorescence intensity of a sample assay compared to an assay containing no analyte. The MRB's physical separation (see section 1.4) of bound and unbound components allows a direct measurement of fluorescence intensity that is inversely proportional to the amount of sample antigen.

1.2 Optical Biosensors

Most sensors monitor properties such as electrochemical, acoustical, thermal, or optical characteristics (1). Advanced sensors use an "indicating reaction" to measure chemical concentrations that cannot be measured directly. Many such sensors use electrochemical or optical transduction elements (8, 30).

While electrochemical sensors are plentiful (6), there are several major advantages of fiber optic sensing over electrochemical sensing in remote sample situations. One advantage is that fiber optics can deliver multiple wavelengths (thus multiple information)

along a single fiber. Since fiber optic sensors measure power, the reference element becomes the measured power when no analyte is present, according to Beer's law:

$$A = \log P_x / P_O \qquad [3]$$

Therefore, the reference element and the sensing element are the same fiber under differing conditions.

1.2.1 *Optical Sensors*

Optical sensors indicate the concentration of an analyte by measuring a change in some optical property. Optical sensors most often use an optical fiber to transport the spectroscopic information generated by a reaction between the analyte and some reagent phase. Some call these sensors FOCS, or fiber optic chemical sensors (30).

Optical Fibers. An optical fiber generally consists of a cylindrical, transparent core made of glass or quartz, surrounded by a cladding having a lower index of refraction (n) than the core. See Figure 2. Optical fibers transmit electromagnetic radiation through total internal reflection, where light entering the fiber reflects repeatedly down the length of the fiber according to its angle of incidence. The angle at which the light enters the fiber determines whether the beam will propagate through the fiber or not. The critical angle Θ_C (see Figure 2) is defined as

$$\Theta_C = \arcsin\ (n_2/n_1) \qquad [4]$$

where n_1 = index of refraction of the core material and n_2 = the index of refraction of the cladding material. At the core-cladding interface, light rays encountering the cladding at

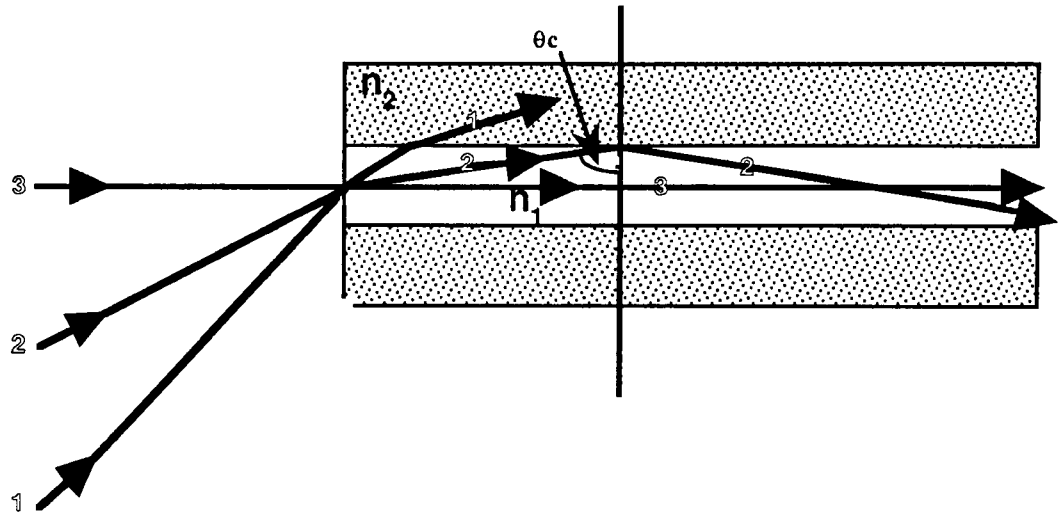


Figure 2. Fiber Optic Transmission. Adapted from Fiber Optic Affinity Sensors in Chemical Analysis; Sepaniak, M., J.; Tromberg, B., J.; Vo-Dinh, T.; in *Prog. Anal. Spectrosc.* **1988,** *11*, 484.

incident angles greater than Θc reflect back into the core allowing total internal reflection, while others are lost in the cladding. The fiber transmits light efficiently only when n_2 is smaller than n_1 (31).

A single optical fiber can transmit a range of wavelengths simultaneously. For example, the end of a fiber can transmit light of one wavelength while receiving light at another wavelength. Thus a single optical fiber can carry both the fluorescence excitation and emission radiation between a spectroscopic instrumentation and a remote sample.

Reagents. A fiber optic chemical sensor may employ an indicating reaction that aides in distinguishing the analyte from the sample matrix. In a major review of biosensors, Rudolf Seitz identifies a number of functions that the reagent phase can perform (7):

- *Reactant* to directly or indirectly react with analyte to form an optically detectable product

- *Adsorbent/extractant* to preconcentrate an optically detectable analyte in the field of view of an optical fiber (the adsorbate may be directly detectable optically or it may modify optical properties of the adsorbent such as its refractive index)
- *Catalyst* to accelerate the rate at which analyte is converted to an optically detectable product
- *Substrate* for the detection of catalysts.

Some of the more well-known applications of optical sensing include continuous *in vivo* measurements of pH, pCO_2, and pO_2. Optical sensors can also measure glucose, metal ions, antigens, enzyme activities, and albumin, *etc.* (1, 7, 8).

1.2.2 *Fiber Optic Affinity Sensors*

A fiber optic affinity sensor (FOAS) is a specialized FOCS that exploits the advantages of specialized biological reagents. An FOAS uses selective and sensitive biological reagents to identify the analyte, and then employs the unique properties of fiber optics to collect and transport the pertinent signal to a detector. This discussion focuses on antibody-based FOAS.

Reagents. An ideal biosensor may be defined several ways:

- A self-contained analytical device that responds selectively and reversibly to the concentration or activity of chemical species in biological samples.
- An analytical device which incorporates biologically active material (a bioreagent) in intimate contact with an appropriate transduction element for the purpose of detecting (reversibly and selectively) the concentration or

activity of chemical species in any type of sample (8).

- An instrument meeting both of the above requirements.

The first definition describes any device that detects within a biological sample, while the second definition distinguishes the reagent as a bioreagent. Arnold divides bioreagents into two groups: biocatalysts and molecular receptors (8). A biocatalyst initiates a chemical reaction with the analyte and produces some detectable change. Some examples of biocatalysts are: isolated enzymes, bacteria, sections of mammalian and plant tissues, subcellular fractions of bacteria and tissue materials, and artificial enzyme systems (8).

Although molecular receptors, for example antibodies, recognize the analyte strongly, they do not react chemically with the analyte after molecular recognition occurs. The affinity constant describes a particular antibody-antigen interaction, and is the quotient of the forward and reverse reaction rate constants (see Equation [1]). Consequently reagents with large affinity constants provide an FOAS with excellent sensitivity (see section 1.1.1) but do not respond in a rapid reversible manner. A reversible sensor's response will follow a decreasing or increasing analyte concentration and requires the immunoreaction to proceed in both the forward and reverse directions. For example, a molecular receptor, Ab, reacts to a large Ag concentration, by creating an Ab-Ag complex (forward reaction). If, subsequently, the sample concentration decreases, the Ab must respond by relinquishing an amount of Ag (reverse reaction).

Since a *large* affinity constant provides sensitivity, and *small* affinity constant provides reversible response, this creates a quandary concerning the suitability of any molecular receptor's affinity constant when employed in an FOAS. The MRB proposes a solution by providing a design that replenishes (regenerates) the reagent rather than reversing the reaction. The MRB's remote delivery system allows the advantages of both a large and small Ka receptor by

- employing a receptor that has a large K_a that gives the MRB excellent sensitivity
- replacing the "used" reagent rapidly (and repetitively), allowing a suitable response for a changing concentration.

The MRB uses this last approach because it allows unique control over reagent transport (see section 2.3.2). This approach also allows the use of reagents with high affinity constants for superior sensitivity. With these two advantages the MRB can meet the special requirements of *in situ* monitoring (see section 1.3.2).

To employ this approach, a sensor must retain the reagent phase near the fiber tip during traditional affinity operational steps such as rinsing, adding secondary reagents, etc. There are several ways to accomplish this, such as:

- immobilizing the Fc portion of the Ab directly onto the fiber
- immobilizing Ab onto a membrane that is attached to the fiber
- retaining the reagent solution with an analyte-permeable membrane
- incorporating the Ab into a membrane-entrapped polymer or gel (32)
- immobilizing the Ab onto beads and retaining the reagent-beads within a membrane or frit

Approaches. Several FOAS design approaches have appeared in the literature. Many affinity procedures involve labels that are convenient for laser-based detection--a technique which fully exploits the "fluorescence advantage." The laser provides coherent excitation radiation that is easily focused onto a fiber and, since fluorescence intensity is proportional to the incident power (see Equation [3]), increasing the laser power can

generally improve detection. Thus a laser source coupled with an optical fiber transducer becomes an excellent combination for remote sensing.

Tromberg *et. al.* devised a sensor capable of performing direct assays of a naturally fluorescent benzo(a)pyrene metabolite, benzopyrene-tetrol, or BPT (33). The sensor entraps anti-BPT within a membrane and detects the smaller BPT molecule as it diffuses into the fiber's viewing area. Incubating the sensor in PBS allows the interferents to diffuse out of the sensing chamber while retaining the membrane-permeable antibody-BPT complex. The resulting signal is proportional to the concentration of BPT within the sample. This sensor demonstrated excellent limits of detection (LOD) of 6×10^{-12} M BPT, but is limited to naturally fluorescent analytes.

Another sensor developed by Tromberg *et. al.* permits either sequential or simultaneous competitive-binding assay procedure (see section 1.1.2.2)(34). The sensor detects anti-rabbit IgG via the rabbit IgG immobilized onto its fiber tip. Either procedure presents signals that are inversely proportional to the amount of analyte present in the sample. Limits of detection varied with incubation time, sample size, and measurement conditions, but a typical value was 25 fmol of unlabeled antibody in a 20-minute incubation period. Since operators using the sequential procedure must develop the sensing tip outside the sample environment, this sensor does not detect *in situ*. The MRB proposes true measurements of the analyte within the sample environment.

Another sensor relevant to this work is a continuous competitive-binding sensor for phenytoin developed by Anderson and Miller (35). This sensor is based on a homogeneous FETI (see section 1.1.3). It consists of a mixture of B-phycoerythrin-labeled phenytoin and Texas Red-labeled antibody to phenytoin sealed inside a reaction chamber formed by dialysis tubing which is cemented to the end of an optical fiber. When the antigen reacts with the antibody, the resulting complex brings the two labels (one of the

antigen and the other of the antibody) close enough to facilitate a nonradiative energy transfer between them.

Both the labeled antigen and the labeled antibody are large enough to be held within the chamber, yet sample phenytoin is small enough to diffuse in and out freely. A fiber optic carries the excitation wavelength to the reaction site and returns the fluorescence from the B-phycoerythrin-labeled phenytoin for detection.

When the sensor contacts a solution containing phenytoin, the phenytoin permeates the reaction chamber, allowing the sample phenytoin to displace some of the B-phycoerythrin-labeled phenytoin from the antibody complex. Displacement causes a proportional decrease in energy transfer between the antigen label and the antibody label, thus increasing the amount of B-phycoerythrin fluorescence. Therefore, the observed fluorescence is directly proportional to the concentration of the phenytoin available in the sample. This sensor demonstrated a LOD of 5×10^{-6} M phenytoin, and typical response time of 5 to 15 minutes.

Co-workers recently developed two MRBs and employed anti-BPT to detect the naturally-fluorescent BPT antigen (36). These MRBs were similar to the one described in section 2.3; varying only by the means of reagent entrapment. One MRB employed a 10,000 M.W. cut-off cellulose membrane and allowed the sample to diffuse into the sensing chamber. The other MRB employed a cylindrical frit that was porous on all surfaces--the operator aspirated the sample into the sensing chamber. The diffusion-based MRB demonstrated an LOD of 3.8×10^{-9} M BPT, and the aspiration-based MRB showed a higher LOD, presumably due to the preliminary nature of the studies.

The work described in this thesis evaluates a similiar aspiration-based MRB that uses a different frit (see sections 2.1 and 2.3.1). These studies employ an immunochemical system designed to model a competitive-binding assay of a nonfluorescent antigen (see section 2.4).

1.3 Ideal Biosensor Characteristics

Besides the usual analytical figures of merit, there are several additional capacities unique to a biosensor. These include 1) remote operation, 2) in situ capabilities, and 3) continuous operation. A biosensor would also benefit from being adaptable to a variety of analyte types.

1.3.1 *Analytical Figures of Merit*

Reproducibility, limit of detection, sensitivity, and selectivity all describe various aspects of any analytical method. Biosensors must be able to detect very small amounts of analyte since many important substances such as drugs, hormones, toxins, and pollutants are present only in very small concentration ranges within a sample. Biosensors must display suitable sensitivity for a relevant concentration range (17) and be able to distinguish the analyte from similar molecules. Biosensors' special sensing requirements (see following section), such as remote and in situ operation, place a special burden on meeting these usual requirements.

1.3.2 *Special Sensing Requirements*

As an analytical tool, the biosensor is designed to provide certain unique characteristics. For instance, a biosensor should monitor conditions at a sample site that is removed from the main body of the instrument. This becomes important when a sample is in a hostile environment or is difficult to access. In fiber optic biosensors, the optical fiber transports information quickly and efficiently. Another advantage of fiber optics is their diminutive size. A small flexible sensor requires less sample and can access sample sites that are at best difficult to reach, for example, underground water near a waste burial site or human internal cavities and fluids.

A biosensor should monitor *in situ*. This mode of monitoring is superior to bench techniques where the analyte may be affected by its removal from the sample site or sample pretreatment procedures. For example, some analytes degrade during the time generally necessary to transport samples from site to laboratory. Other analytes exist only in a delicate equilibrium system. Tampering with that equilibrium will distort subsequent analyses of the sample.

A biosensor should also respond reversibly and in a continuous fashion. A reversible sensor responds to both increases and decreases in analyte concentration, regardless of the concentration at the previous measurement. To achieve this, most biosensors rely on the reversible nature of the molecular receptor-analyte reaction (35). However, reaction rates to some extent and mass transfer to a greater extent also affect the response rate of a biosensor. With this delay, a truly continuous biosensor is not possible; however, rapid, repetitive measurements can give a nearly continuous response that is quite useful. A suitable sensor response time arises from an appropriate balance between the affinity of the molecular receptor for the analyte and the delay time necessary to regenerate (or replenish) the receptor.

Ideally, a single biosensor would be useful for a variety of analyte types including large and small molecules, both fluorescent and non fluorescent. Since the specificity of biosensors depends on the immunoreagents (antibody) employed, simply using the appropriate antibody provides a wide range of applications. Advances within the immunochemical field have created immunoreagents specific for a smorgasbord of antigens, and other reagents are available through custom work (37). This widens biosensors' applications considerably. While biosensors use different reagents for specific applications, and some biosensors regenerate their reagent, only one design to date has the potential to do both without being removed from the sample site. The MRB's unique design may allow 1) repetitive analysis of a single analyte by replacing the used reagent, or

2) a series of analyses for several unrelated species by using various antibody reagents. Also, the MRB could perform a simultaneous multi-component analysis if noninterfering immunoreagents were employed .

1.4 Scope and Aims of This Project

The competitive-binding FIA is a very sensitive method; however, its application becomes complicated within the bounds of *in situ* monitoring. This project aims to evaluate a prototype MRB that is able to apply a competitive-binding FIA *in situ*.

The competitive-binding FIA will allow the MRB to monitor the concentration of nonfluorescent molecules and to increase the assay's sensitivity by exploiting the "fluorescence advantage." The MRB will also address the problem of true *in situ* monitoring more thoroughly than Tromberg's competitive-binding sensor by performing the entire assay at the sample site rather than developing the assay outside the sample.

Since the MRB replenishes the antibody reagent regularly, it does not require an antibody with an affinity suitable for a reversible reaction. Therefore, unlike Miller and Anderson's competitive-binding sensor, the MRB allows the maximum use of large affinity antibodies.

The MRB proposes to meet the special needs of *in situ* monitoring by replenishing the reagents for each assay measurement via a delivery system. This delivery system may also allow multi-assay capacity by simply applying antibody that is specific towards another antigen of interest.

This work's specific aims are to provide fundamental information about a prototype MRB concerning

- its ability to physically perform the various steps of its proposed competitive binding operation (see section 2.5).
- its response to some common FIA conditions during operation.

The evaluations described in this thesis employ a model for a competitive-binding FIA. This model consists of protein A and rabbit IgG to fulfill the antibody and antigen roles respectively. This model represents any antibody-antigen pair available for use as immunoreagents (37).

As mentioned previously, these studies evaluate a prototype MRB. Other workers have endeavored to improve the MRB design during the time concurrent with this work (38). Since all of these studies are preliminary, other studies are necessary to complete the MRB's characterization, such as flow dynamics and assay optimization.

CHAPTER 2

EXPERIMENTAL

The following sections describe the materials, instrumentation, and methods used to fabricate and evaluate the MRB.

2.1 Materials

To construct the MRB I used: 400 μm core diameter, plastic clad fused silica fiber (QSF-400) from General Fiber Optics, Cedar Grove, NJ; 200 μm i.d. silica capillary columns from Polymicro Technologies, Inc., Phoenix, Arizona; 5 minute epoxy manufactured by Devcon Corp., Danvers, MA; and standard 16 gauge luer lock syringe needles. In house services crafted a "T" union and a fiber optic/column template from Plexiglass. From Alltech Assoc., Deerfield, IL, I obtained: a Model 7010 Rheodyne HPLC injection valve; luer lock syringe valves (Mininert syringe valves, catalog number 654051); $^1/_{16}$" o.d., $^1/_{32}$" thick stainless steel frits of 2-μm porosity; and 7-μm diameter protein A silica beads. I used a fiber optic cutter and lapping film (32-, 15-, 3-, and 0.3-μm) from Math Associates Inc. Westbury, NY. The syringe pump (Model 341A) came from Sage Instruments, Cambridge, MA.

I obtained these reagents from Sigma Chemical Co., St. Louis, MO: fluorescein isothiocyanate (FITC); rabbit-immunoglobulin-G (IgG); rabbit-immunoglobulin-G-fluorescein-isothiocyanate conjugate (IgG*), fluorophore to protein ratio (F/P) 4.2; Fab fraction rabbit-immunoglobulin-G; and phosphate buffered saline (PBS), pH = 7.0.

Rob Thompson developed FITC-labeled 10 μm silica beads using fluoresceinthiocarbamyl ethylenediamine and pyridine (39).

2.2 Instrumentation

Figure 3 diagrams the instrument configuration for the MRB. An argon ion laser (Model 2001SL Cyonics/Uniphase, San Jose, CA) provided the 488 nm excitation wavelength (approximately 10 mW) for the fluorescein label used throughout the evaluations. A chopper (Model 9479 EG&G Ortec, Oak Ridge, TN) minimized the signal deterioration due to thermal degradation of the fluorophore. When emerging from the chopper, the laser radiation passed through a beam-sized hole in a round 40-mm diameter mirror (prepared in house). Then lens 1 (Corion, Holliston, MA) focused the beam onto a fiber positioner (NRC Model FP-1, Fountain Valley, CA) that contained the non-sensing terminus of the MRB's optical fiber.

The same fiber carried the 522 nm emission wavelength in the opposite direction through lens 1, back to the mirror. The angled mirror reflected the emission radiation to the side onto lens 2, through a narrow bandpass filter (518 nm peak, 20 nm FWHM) (both from Corion, Holliston, MA), and into a monochromator (Model H-10, Instruments SA, Metuchen, NJ) set at 522 nm (10 nm bandpass). The monochromator passed a portion of the emission light onto a PMT (Model R943-02 Hamamatsu, Middlesex, NJ) equipped with a cooled housing (Fact 50 MK III Thorn EMI Gencom Inc., Fairfield, NJ) and a high voltage power supply (Model 556, EG&G Ortec, Oak Ridge, TN). The autoranging picoammeter (Model 485 Keithley, Cleveland, OH) measured the electrical signal, and a strip chart (Model BD40 Kipp & Zonen, Holland, or a Model 8376-20 Cole-Parmer Instruments Co., Chicago, IL) recorded the signal.

2.3 Construction

The MRB's design (see Figures 4 and 5) uses the small dimensions of an optical fiber to transmit signal, while a unique microtubing system delivers reagents (both solid and liquid phases) to and from a tiny reaction chamber at the sensing tip.

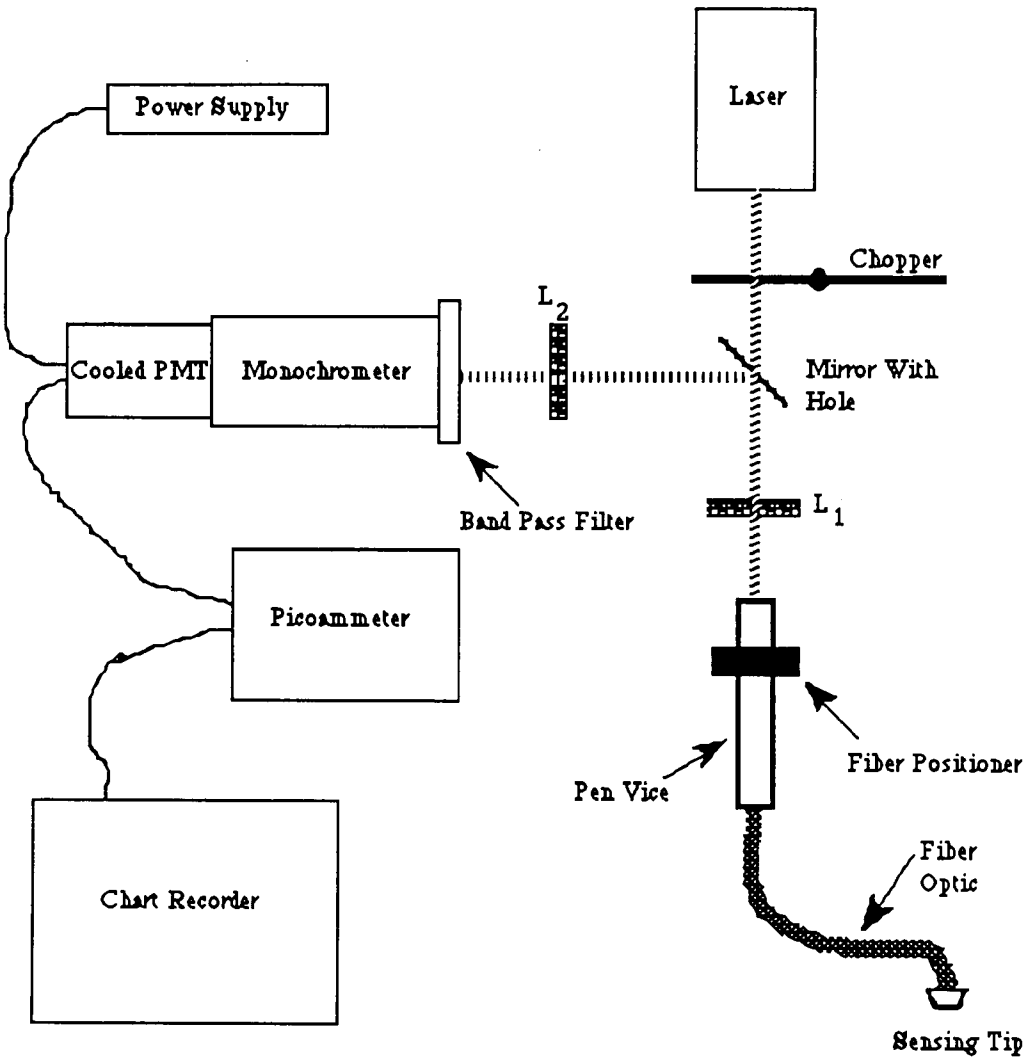


Figure 3. Instrument configuration for the MRB.

2.3.1 *Tip Design*

I prepared a length (approximately 0.5 m) of optical fiber by stripping a small section of the plastic cladding from the ends. Then I cleaved one end and positioned it into a pen vice to serve as the non-sensing terminus.

I epoxyed three pairs of capillary columns (approximately 50 cm long) into three syringe needles so that the columns could flow freely. I positioned the capillaries concentrically about an optical fiber with the help of a Plexiglass template, placing members of the same pair opposite each other (see Figure 4). I then epoxyed this arrangement along a 2-cm length, heated a section of heat shrink tubing over the wet epoxy, and allowed the bundle to harden.

When the bundle (approximately 1-mm diameter) had hardened, I removed the heat shrink tubing and polished the tip to a smooth surface using 32-, 15-, 3-, and 0.3-μm lapping film. Using a microscope, I positioned a 5-mm section of $^1/_{16}$" o.d. stainless steel tubing over the bundle like a sleeve. Then while extending the tubing approximately 0.5 mm beyond the smooth end of the bundle, I epoxyed it into place, filling all gaps between the inside of the tubing and the outside of the bundle.

I placed a brass ferrule onto a fitting and positioned a $^1/_{16}$" HPLC frit (2-μm porosity) inside the small end of the ferrule, making sure the edges of the frit and ferrule were flush. Then I tightened the ferrule onto the fitting, enclosing in the frit in the end of the ferrule (see Figure 4). I removed the ferrule-frit unit and epoxyed it onto the tubing-encased bundle, making sure that the tubing contacted the frit. This assembly created a small chamber between the frit and the fiber bundle. Typically, the inside tubing (r = 75 μm) at a height of approximately 0. 5 mm (500 μm), formed a volume of approximately 0.89 μL.

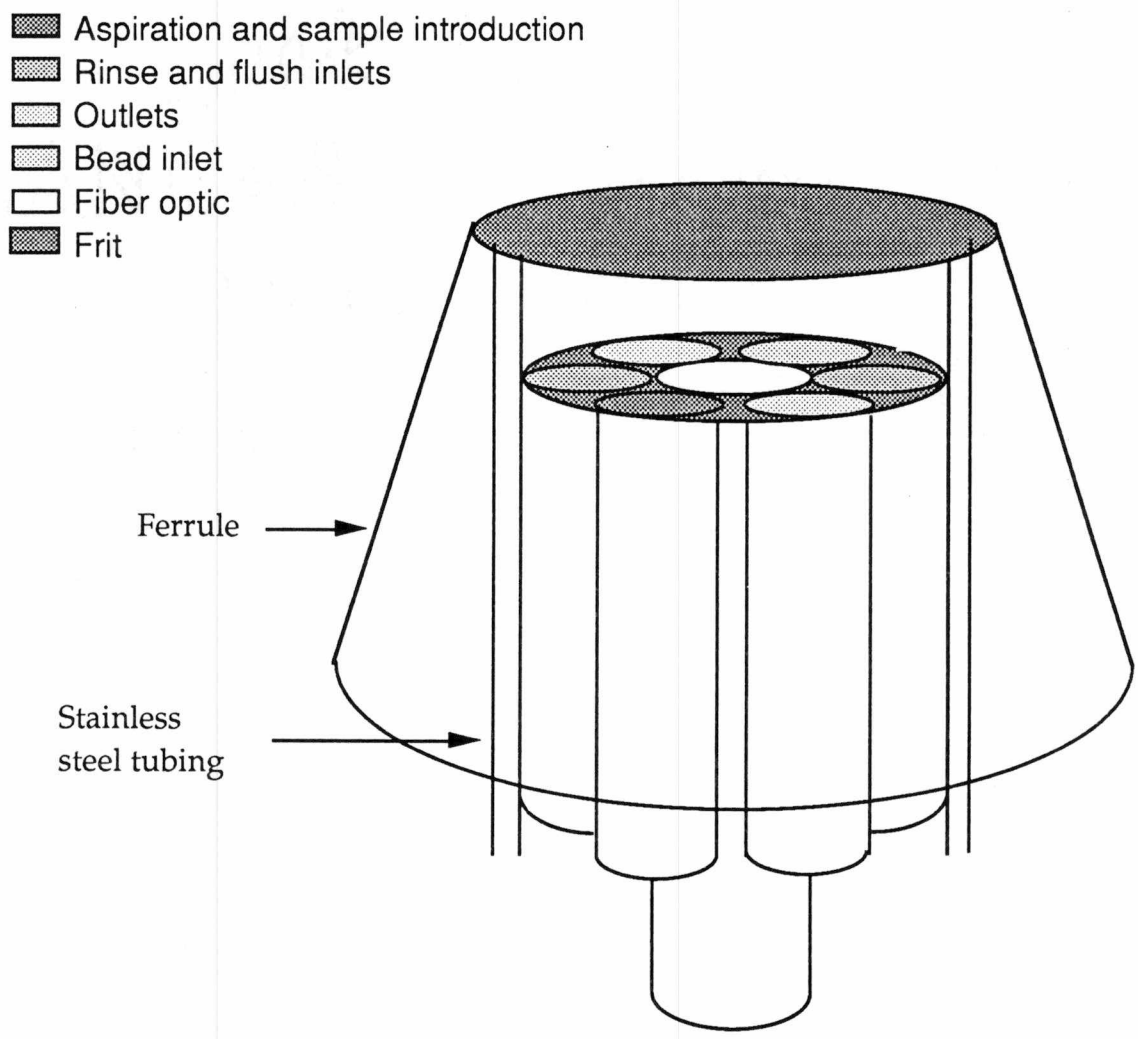


Figure 4. Microscale Regenerable Biosensor Tip Design. Adapted from Construction and Evaluation of a Regenerable Fluoroimmunochemical-Based Fibre Optic Biosensor; Bowyer, J. R.; Alarie, J. P.; Sepaniak, M. J.; in *Analyst*. **1991,** 116, p. 119.

2.3.2 *Transport Control*

A system consisting of a syringe pump, a "T" union, syringe valves, and an injection loop controls the transportation of materials within the MRB (see Figure 5). This system allows each capillary pair to direct materials to a specific location and to control the assay conditions without removing the sensing tip from the sample.

I designated the first pair of capillaries to serve as a general exit from the MRB. This pair terminates into a luer lock syringe valve. The second pair of capillaries delivers materials to the sensing tip. I connected this pair to the injection valve equipped with a 50-μL loop. The loop ensures that the MRB delivers a reproducible amount of reagent to the sensing tip. The syringe pump provides a nearly constant flow rate for any materials introduced by that capillary pair. The pump delivers materials to the sensor as well as forcing materials out of the sensor tip.

I modified the third set of capillaries to act as a sample reservoir by attaching a Plexiglass "T" union. The "T" holds the capillary termini in one arm and additional single capillaries (equipped with valves) in each of the remaining arms. The addition of the "T" union creates a constant length (and hence volume) from the sensing chamber to the "T" intersection. This segment of column functions as a holding chamber for sample during the MRB's operation (see section 2.5). The studies within the scope of this thesis do not employ this "T" union.

2.4 Preparation of Reagents

I chose the affinity binding between protein A and rabbit IgG to serve as a competitive-binding model for any number of significant antibody-antigen reagent pairs. With this immunochemical system, I used a prototype MRB to perform experiments that mimic competitive-binding FIAs.

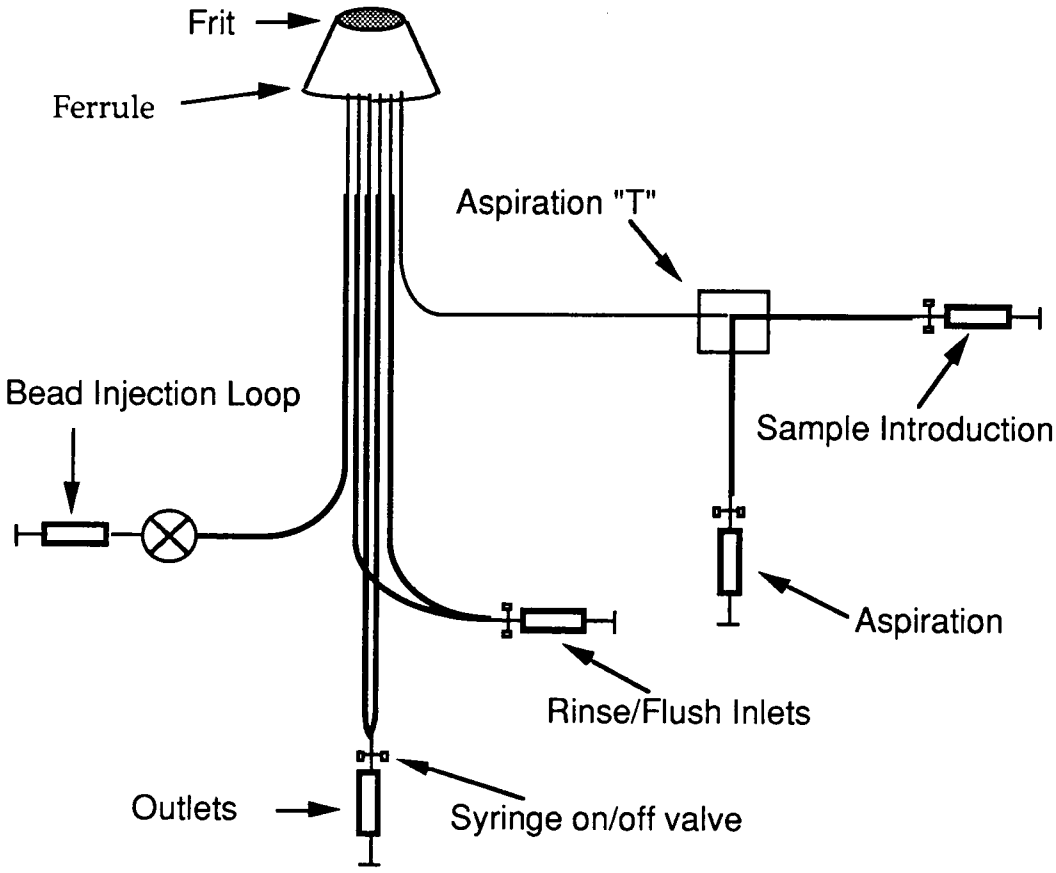


Figure 5. Transport Control System of the MRB. Adapted from Construction and Evaluation of a Regenerable Fluoroimmunochemical-Based Fibre Optic Biosensor; Bowyer, J. R.; Alarie, J. P.; Sepaniak, M. J.; in *Analyst*. **1991,** *116*, 119.

2.4.1 Protein A's Affinity for IgG

Protein A is a cell wall component of most strains of *Staphylococcus aureus* and binds to the Fc portion of immunoglobulins (40). Lancet et al. determined the binding constant of this interaction between protein A and rabbit IgG to be approximately 3×10^6 M^{-1} (41). This affinity interaction leaves the antibody-binding sites of the IgG available for antigen interaction. This special quality makes protein A a versatile immunoreagent with a variety of uses including: the purification of IgGs by affinity chromatography, the study of surface antigens on intact cells, isolation of cell-surface antigens and receptors, and others (42).

2.4.2 Reagent Roles in the MRB

As described above, the protein A - IgG interaction is not a true antibody-antigen reaction, but for the studies described in this thesis, their reaction serves as a mock antibody-antigen reaction. The protein A beads act as the solid phase immunoreagent (in most assays this would be the antibody), IgG represents the analyte, and IgG* represents the labeled antigen.

Although there are many methods suitable to immobilize a true antibody onto silica particles (including the use of protein A) (43), I chose commercially-prepared protein A affinity particles for convenience. The 7-μm diameter protein A beads are small enough to travel freely through the capillaries, yet too large to pass through the frit. This is indicative of how the MRB confines an antibody (molecular receptor) within the fiber's field of view by using silica particles as a reagent support.

2.4.3 Standard Bead Batches

For all experiments, I used an 8 mg/mL slurry of beads suspended in PBS. To make a "standard" batch of beads, I placed 8 mg of protein A beads and 0.25 mg of IgG* in a 1-mL volumetric tube and diluted to the mark with PBS. I incubated the mixture for 15

minutes, then rinsed the beads (using a centrifuge) with two 1-mL portions of PBS, and re-diluted to 1 mL with PBS. I prepared all "batch" beads in this same general manner with variations in reagent type, concentration, or incubation time as I indicate in the following sections.

2.5 Operation of the MRB

The proposed operation of the MRB requires the operator to perform the following steps:

1) Aspirate sample through the frit and into the capillaries, past the "T" intersection (See Figure 5).
2) Rinse excess sample from the tip chamber by delivering a small volume of solvent out through the frit.
3) Deliver a known amount of the Ab-beads to the tip from the injection loop.
4) Push sample into the tip from the other side of the "T" column. (Here the analyte fills as many sites of Ab as possible).
5) From the loop, add a known amount of labeled analyte to the tip and out the frit. (This fills the remaining Ab sites).
6) Rinse excess antigen (labeled and unlabeled) from the tip out through the frit.
7) Measure the fluorescence signal. (The resulting change in signal would be inversely proportional to the concentration of natural analyte in the sample.)
8) Flush all reagents from the tip out the exit columns.

2.6 Measurements

This thesis attempts to characterize a prototype MRB through two sets of experiments: evaluations of the MRB's physical capacities, and preliminary evaluations of the MRB's response to a model competitive-binding FIA.

2.6.1 *General Procedures*

To prepare the MRB for use, I filled all the capillaries and the sensor tip with phosphate buffered saline, PBS, and kept air bubbles out. I stored the sensing tip in distilled water at all times. In the following sections, I refer to flushing and rinsing the MRB. To clarify, *flushing* the MRB forces solvent to sweep reagents out of the sensor tip through the exit leads; *rinsing* with the MRB forces liquid (either solvent or liquid reagent) over the beads and out the sensor tip.

I performed all of the following experiments (except the last) in the batch mode; I prereacted an aliquot of beads with some quantity of IgG (or IgG*) to make an antibody-antigen complex. Then I delivered the beads (in 50-μL portions via the injection loop) to the sensing tip to evaluate either the protein A - IgG reaction or the MRB's functions and responses. In actual *in situ* operation, the reagents (antibody and antigen) would react in the sensing chamber (see section 2.5).

2.6.2 *Physical Evaluation of the MRB*

I performed the following sequence of experiments to provide information about various steps in the MRB's proposed operation (outlined in section 2.5). These physical evaluations characterize the transport of materials within the MRB and the exchange of materials between the sample and the MRB.

Aspirate Sample. The initial steps (1, 2, and 4) in the MRB's operation are to aspirate a small volume of sample into a side holding column above the reaction chamber,

sweep out excess sample from the chamber, and then deliver the discrete volume of sample back to the chamber from the side column. James Bowyer, a co-worker, has presented data demonstrating the MRB's sample aspiration reproducibility (38). I present his pertinent experiment here and in section 3.1.1 to complement the studies herein. While the MRB tip was in one one of three series of samples, 1) 10^{-4} M FITC; 2) 5 mg/mL IgG*, [FITC] = 1.4 x 10^{-4} M; 3) 5 mg/mL IgG* in human blood serum, [FITC] = 1.4 x 10^{-4} M, he pulled back slightly but firmLy on the syringe at one of the side arms at the "T" union. He retained the sample there while he swept away excess solution from the reaction chamber. Then, with all other leads closed, he re-introduced the sample volume to chamber, out the frit, and collected it in a 1-mL volumetric vial. He then diluted to the mark and measured the fluorescence using a fluorometer.

He prepared a calibration plot by diluting given volumes of FITC to a 1-mL volume and measuring the signals with the fluorometer. He then compared the signal of the sample volume to the calibration plot to find the volume of FITC aspirated by the MRB.

Deliver and Remove Beads. Step 3) in the MRB's operation is to deliver and retain solid reagent phase near the fiber, and step 8) is to remove the used reagents (solid phase). I performed these procedures using standard batch beads.

I closed all leads to the chamber except the inlet columns; the ones attached to a syringe pump that delivered PBS to the tip and out the frit. Via the injection loop, I introduced 50 μL of standard IgG* bead slurry into the PBS flow toward the tip. After the signal from the beads stabilized, I flushed the beads from the sensing chamber by opening the exit valve and increasing the syringe pump rate. Occasionally I used manual pulses on the syringe to aid the removal of beads. I alternately compared two batches of beads, one a standard batch, the other incubated with 0.25 mg of unlabeled IgG. I injected the standard IgG* beads seven times over two days and the IgG beads four times within the same period to evaluate the reproducibility of the bead delivery operation.

Adjust Bead Amount. The dimensions of MRB chambers vary slightly from one sensor to another since each MRB is made by hand. Therefore, the MRB operator must deliver an appropriate amount of beads to the sensing tip to ensure the fiber's view of the assay. I evaluated an MRB's chamber using two dilute beads slurries of FITC-labeled silica.

Using the flow sequence described in the last section, I delivered multiple portions of FITC-bead slurry (concentration unknown) without flushing between. When the signal from the first injection stabilized, I injected another portion, and so on, until the signal remained constant for two consecutive deliveries. Then I flushed all beads from the sensor tip and repeated the procedure with FITC-bead slurry that was twice as concentrated as the one above to evaluate the configuration of the MRB's chamber.

Deliver Solution. Step 5) in the MRB's operation is to deliver a known amount of labeled antigen to the sample chamber. I performed this operation using both a solution of FITC and a solution IgG*.

I delivered 50 μΛ of 2.0 x 10^{-6} M FITC (via injection loop) to the sensing chamber and out through the frit. In effect, this mimicked a delivery of a plug of antigen into the stream of PBS as it traveled to the sensing tip. I repeated this procedure with 0.4 mg/mL IgG*, and performed several deliveries of each "antigen" solution to determine this procedure's reproducibility.

Rinse Excess Analyte. Step 6) of the MRB's operation is to rinse excess antigen (labeled or unlabeled) from the chamber out through the frit, away from the bead-bound Ab-Ag complex. I performed this operation using standard bead slurries that contained excess unbound IgG*.

I compared the signals of several standard bead slurries containing 0.02, 0.04, and 0.08 mg of excess unbound IgG*. I delivered 50 μL of each slurry to the tip and followed with PBS rinse out the frit. A blank slurry contained no excess antigen.

2.6.3 *Assay Evaluation with the MRB*

This series of experiments attempts to describe the MRB's response to various assay conditions.

Calibration of Bead Coverage. I used the MRB to evaluate the protein A beads' capacity to bind antigen under various conditions. To do this, I created several batches of beads by adding different amounts of IgG* for various incubation times. To one portion of protein A beads I added 0.05 mg of IgG* and incubated for 20 min. I incubated a second portion with 0.25 mg IgG* for 10 min, and another with 0.25 mg IgG* for 20 min. I incubated a fourth portion of beads with 0.50 mg IgG* for 20 min. I delivered 50 μL of each slurry to the sensing tip and measured the MRB's signal response.

Batch Competitive Assays. I evaluated the MRB's response to several batch mode competitive-binding assays of various labeled/unlabeled antigen ratios. I prepared a series of bead batches, incubating each with a total of 0.050 mg IgG, but I adjusted the ratios of IgG* (labeled analyte) to IgG (natural analyte) to 0, 20, 40, 80, and 100%. I repeatedly delivered 50 μL of each batch of beads to the MRB tip, recorded the MRB's response, and removed the beads for the next delivery. I repeated the delivery of one slurry to obtain a relative standard deviation.

Batch Fab. Next, I tested the model antibody/antigen system's ability to specifically bind only the analyte, not closely related molecules. To do this, I prepared four batches of beads: the first was a standard batch, incubated with 0.25 mg/mL of IgG* as described previously; the second was incubated with a 50/50% mixture of 0.25 mg/mL IgG and 0.25 mg/mL IgG*; the third was incubated with a 50/50% mixture of 0.25 mg/mL IgG*and 0.25 mg/mL Fab portion of rabbit IgG (unlabeled); and the fourth was a blank, incubated with 0.25 mg/mL IgG. (Recall that protein A binds the Fc portion of an IgG, not the Fab sections.) I introduced a 50-μL portion of each of these bead slurries into the sample chamber, measured the signal, and removed the beads for the next injection.

Assay at the Sensing Tip. Finally, I evaluated how the MRB's reagent delivery system affects the assay's binding. To do this, I delivered reagents to the protein A beads while they were in the sensing tip rather than in the batch mode.

This procedure exposed the protein A beads to a sequence of material deliveries (within the MRB) that a solid phase immunoreagent would experience during an assay. For example, after aspirating a volume of sample, the operator would deliver the solid phase antibody to the sensing tip, flow sample (containing analyte) over the beads and out the tip, and then flow a labeled antigen over the beads and out the tip.

This experiment compares two sequential competitive-binding assays; the first delivers labeled antigen, 0.05 mg/mL, to the antibody (to fill some binding sites) and then follows with labeled antigen, 0.05 mg/mL, (to fill the remaining sites). The second assay delivers sample containing unlabeled antigen, 0.05 mg/mL, to the antibody (to fill some binding sites) then follows with labeled antigen, 0.05 mg/mL, (to fill the remaining sites). This should produce a signal for the first assay that is twice that for the second assay.

I delivered a 50-μL portion of protein A beads to the sensing tip and then (via the injection loop) delivered a 50-μL portion 0.05 mg/mL IgG* to the tip. When I saw a significant increase in signal indicating the IgG* had filled the chamber, I stopped the flow for an incubation period of 20 minutes and then resumed rinsing the IgG* over the beads and out the tip. I repeated this procedure without replenishing the beads, and recorded the signal as the IgG* left the chamber through the frit. I flushed the MRB of all reagents to prepare it for a new assay. I repeated the above procedure substituting 0.05 mg/mL IgG (unlabeled) during the first incubation period, and compared the recorded signals.

CHAPTER 3

RESULTS AND DISCUSSION

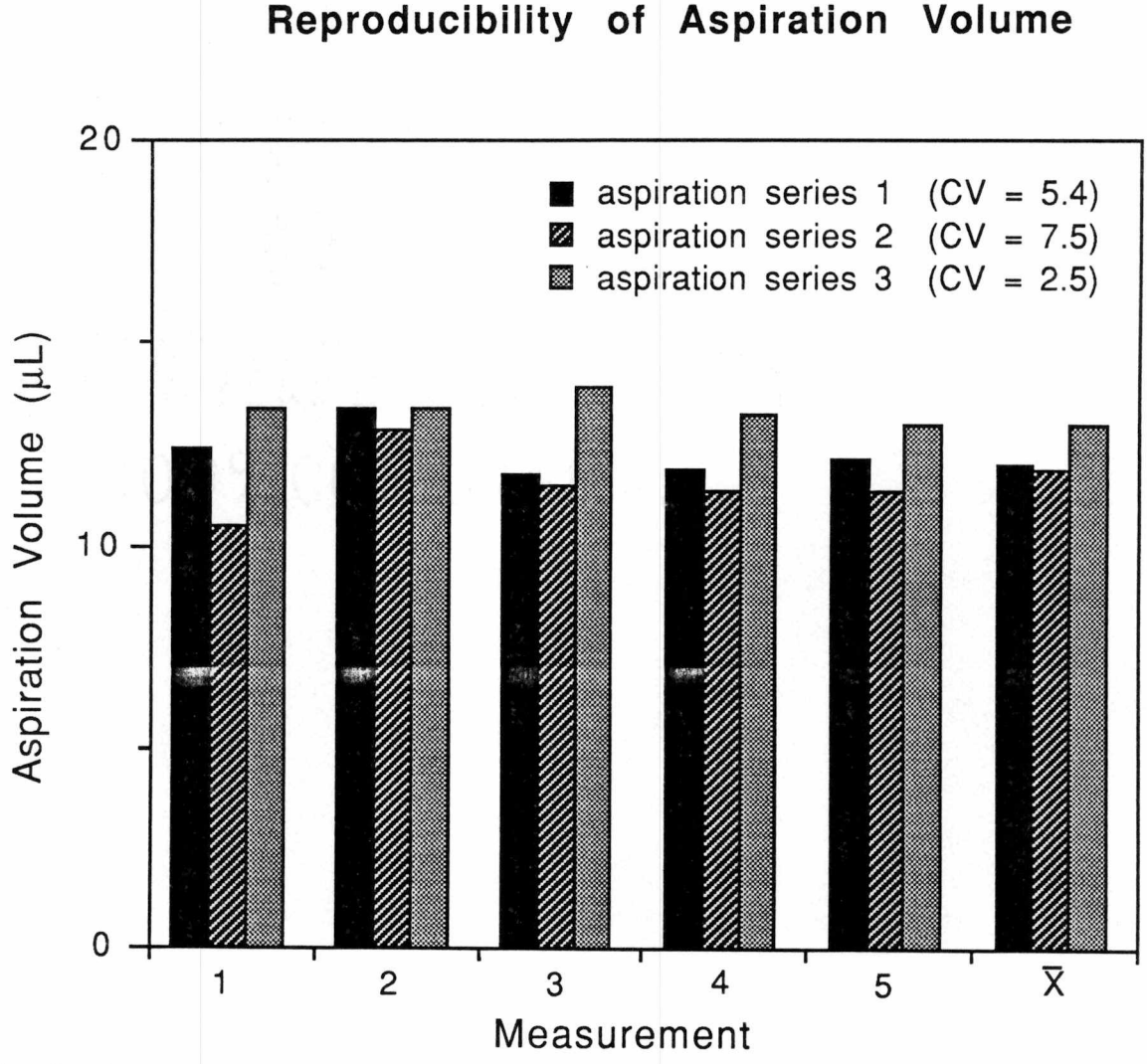
The following sections describe and discuss the results of the studies outlined in sections 2.6.2 (Physical Evaluation of the MRB) and 2.6.3 (Assay Evaluation with the MRB).

3.1 Physical Evaluation of the MRB

This section describes the results of the studies exploring the MRB's ability to perform the physical steps of an assay.

3.1.1 Aspirate Sample

Figure 6 demonstrates the MRB's capacity to aspirate a reproducible amount of sample into its side arm holding chamber. Bowyer described the coefficient of variation (CV) for five aspirations as less than 8.0% among three matrices with the biological matrix exhibiting the lowest CV. The differences in sampled volumes and the CVs are probably due to adsorption of the IgG onto the frit and columns, and the various matrix viscosities.



Aspiration of:
Series 1 10^{-4}M FITC
Series 2 5 mg/mL rab-IgG-FITC, FITC conc. = 1.4×10^{-4} M
Series 3 5 mg/mL rab-IgG-FITC in human blood serum,
FITC conc. = 1.4×10^{-4} M

Figure 6. Reproducibility of Aspiration Volume. Source: Construction and Evaluation of a Regenerable Fluoroimmunochemical-Based Fibre Optic Biosensor; Bowyer, J. R.; Alarie, J. P.; Sepaniak, M. J.; *Analyst*. **1991,** *116*, 119.

3.1.2 Deliver and Remove Beads

Figure 7 demonstrates the MRB's capacity to deliver solid phase reagents to the tip in a reproducible fashion. Although the background signal and the IgG* bead signals decreased by approximately 4 nA on day 2 compared to day 1, the CV for the delivery of IgG* beads over a two day period was 10.6%.

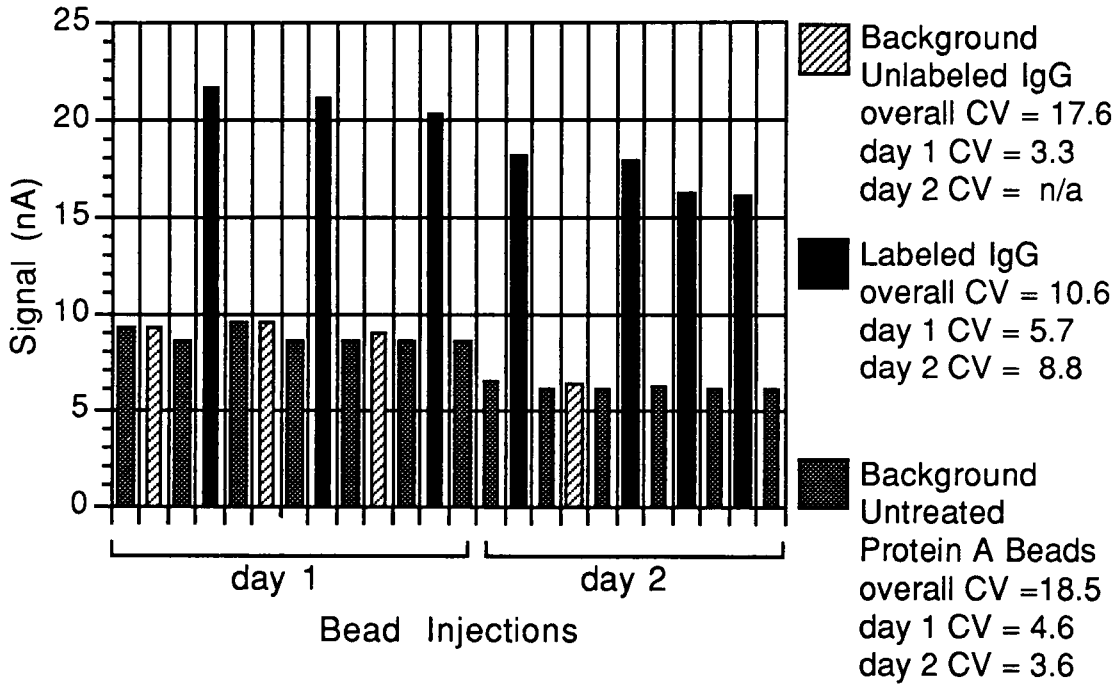


Figure 7. Delivery of Beads to the Sensing Tip.

When conducting these experiments, an initially large signal results from solid reagent phase, but then it levels out to a somewhat smaller value (see Figure 8). This is probably caused by beads "settling" to the bottom of the chamber, thus increasing the distance between the beads and the fiber face. The fiber probably "sees" the beads more easily as they come out of the columns (near the fiber) and their signal becomes less intense as the continuous solvent flow pushes the beads farther away from the fiber to rest on the frit at the bottom of the chamber.

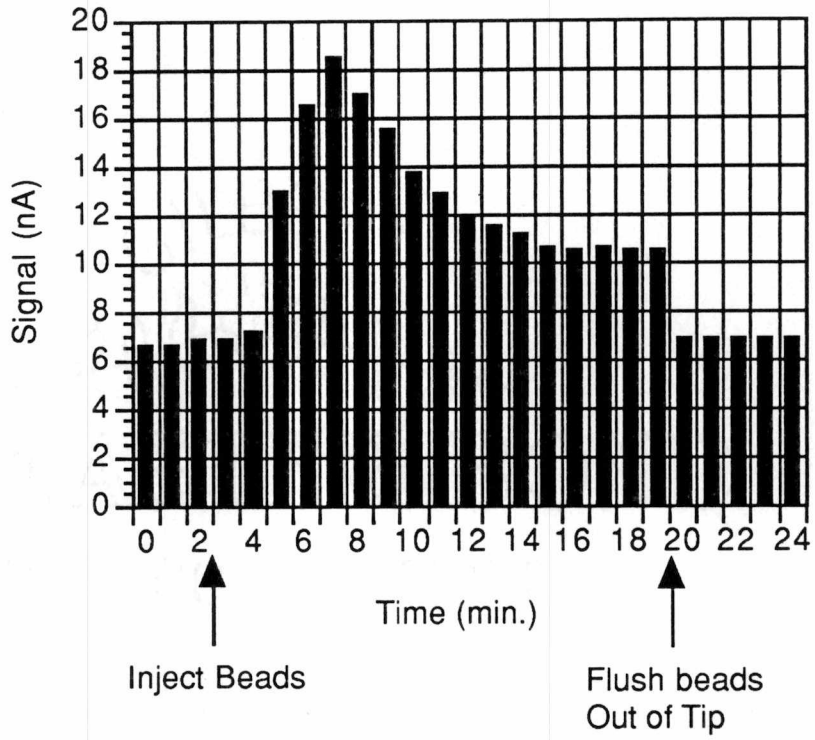


Figure 8. Signal Profile of Bead Delivery.

The later, more subtle signal decrease may be due to thermodegradation of the label, or to the force of the solvent flow slowly forcing the antigen (IgG*) off the mock antibody (protein A).

3.1.3 Adjust Bead Amount

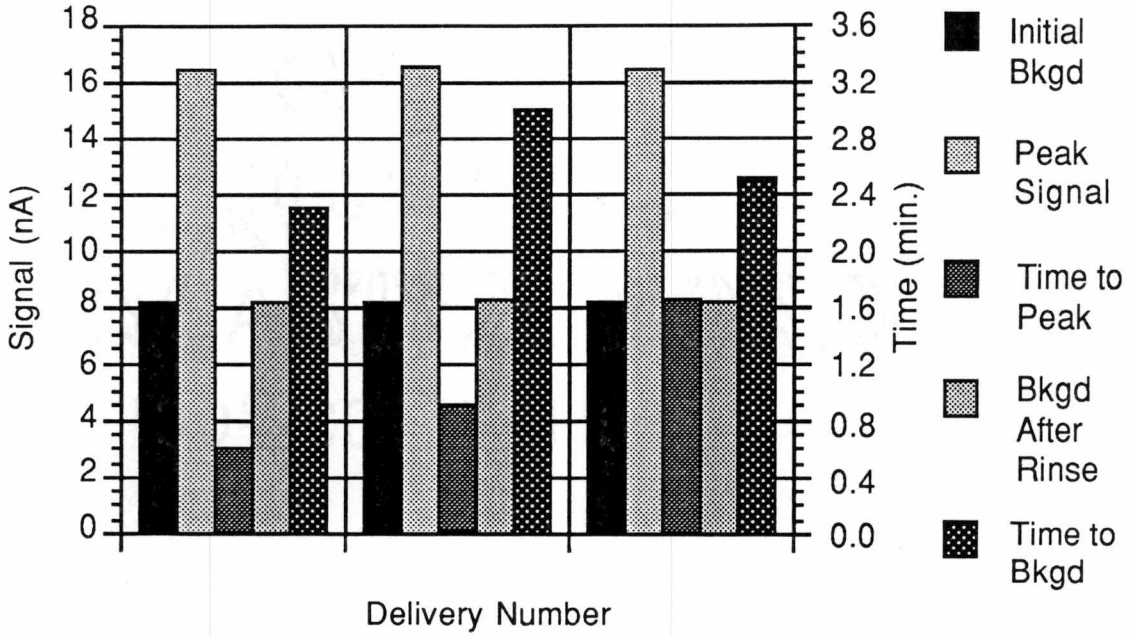
The internal dimensions vary slightly from one sensor to the next since each is fabricated manually. These variations are significant since slight change in dimensions can result in a large percentage of change in chamber volume. When using an MRB with a large chamber, I brought the beads into the fiber's view by either using a large-volume injection loop or "stacking" several portions of beads up to the fiber face.

Figure 9 demonstrates successful "stacking" at two bead slurry concentrations using a very large MRB chamber . A suitable MRB has desirable chamber volume and injection loop size to provide a single portion of beads that fall within the fiber's view. These particular experimental conditions (large chamber and low slurry densities) show several 50 mL injections of labeled beads before the signal became constant.

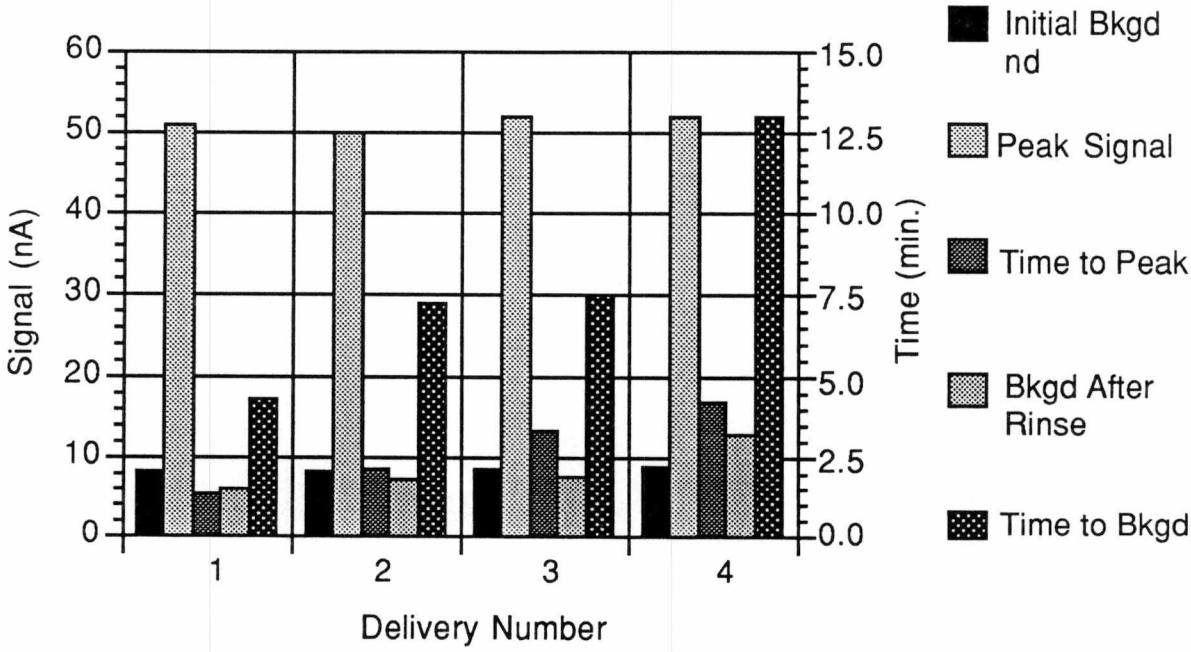
Figure 9. Multiple Bead Deliveries within the Sensing Tip.

3.1.4 Deliver Solution

Figures 10a-b show two examples of how the MRB operator can deliver liquid phase reagents to the sensing tip reproducibly. However, the amount of time the MRB requires to deliver the full injected volume of fluorophore and to force it out of the chamber (through the frit with rinsing) often increases with each successive use. I observed this effect while introducing and removing beads as well (see Figure 7). Overall, this effect seems to be related to variances in flow rate of solutions out the MRB's frit. Several factors affecting the rate may include:



(a.) FITC



b. Labeled IgG

Figure 10. Delivery of (a.) FITC; and (b.) Labeled IgG Solutions to the Sensing Tip.

- protein adsorbing onto the frit material
- fines in the bead slurry that lodge in the frit
- proteins adsorbing to the silica delivery columns (44).

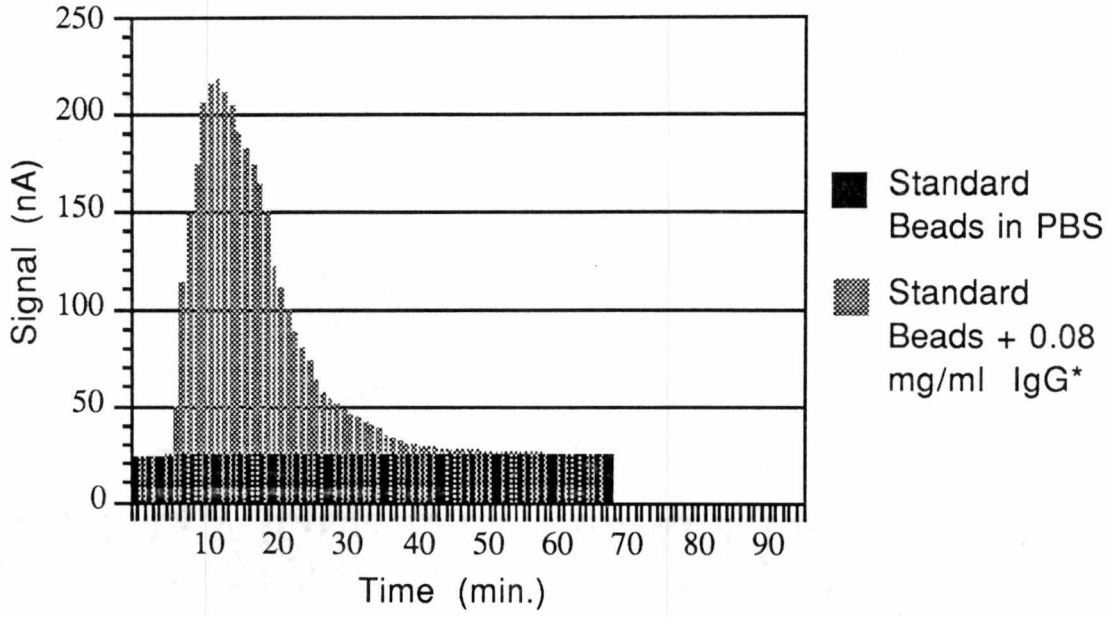
3.1.5 Rinse Excess Analyte

Figures 11a-c show how an MRB operator can rinse various amounts excess analyte (IgG*) out of the sensing chamber, leaving the Ab-Ag* complex behind. These figures overlay the independent signals of two bead slurries collected at different time frames. These signals compare a standard bead slurry and standard bead slurries containing excess labeled antigen.

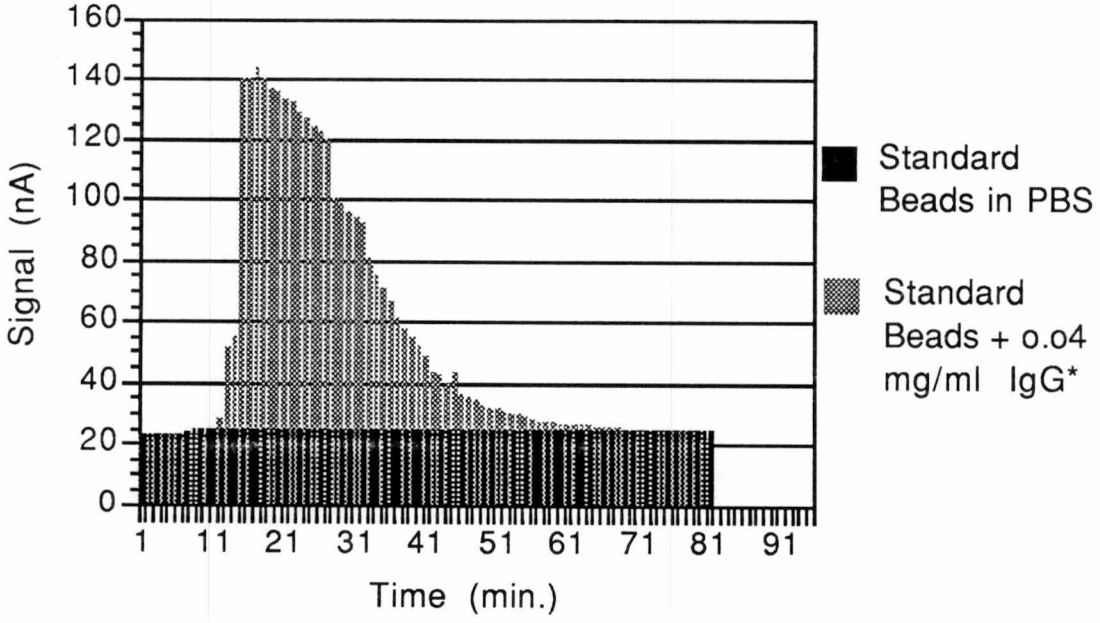
I often observed that a given signal would decrease slightly over several hours of continuous laser time (see section 3.1.2). Signals were not normalized to compensate for laser power variances; therefore, a portion of a bead slurry would give one signal early in the day, and an identical portion would give a somewhat lower signal as the day progressed. This might explain why the initial and final signals given by the standard bead slurry containing 0.02 mg/mL of excess IgG* (last in the sequence) fall below the signal given by the standard bead slurry (first in the sequence) alone. For this experiment, with these fluorophore concentrations, the length of time to rinse solution out the frit is too long to be of practical use. This consequently hinders the practical use of the MRB at this point in its development.

3.2 Assay Evaluation with the MRB

This section describes the results of the studies exploring the MRB's capacity to monitor certain assay conditions.

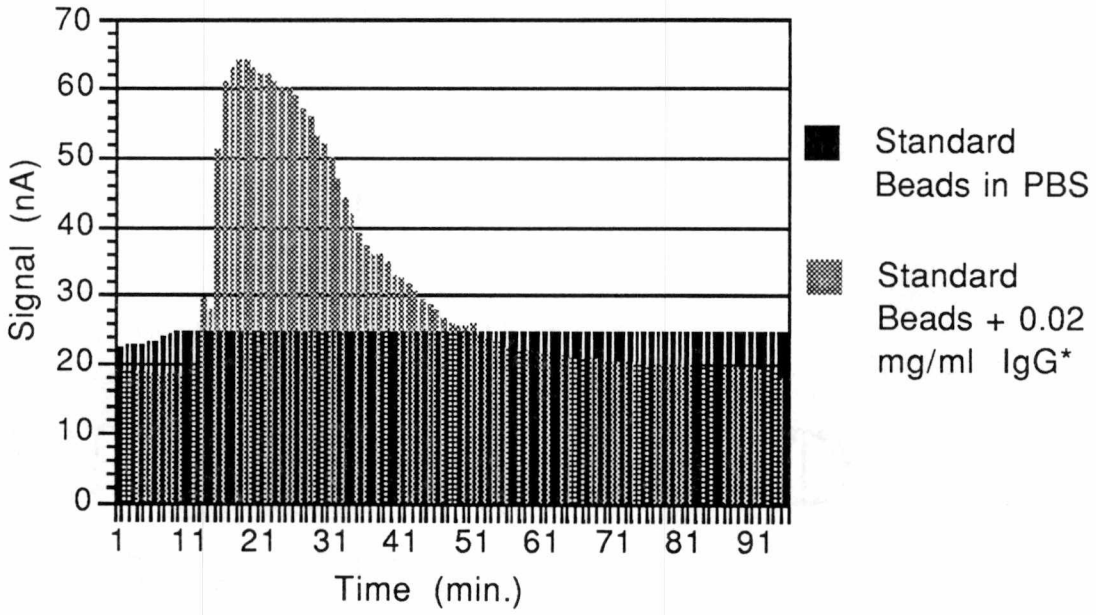


(a.) 0.08 mg/mL



(b.) 0.04 mg/mL

Figure 11. Rinse of (a.) 0.08; (b.) 0.04; (c.) 0.02 mg/mL Excess Analyte from Beads Supporting Ab -Ag*.



(c.) 0.02 mg/mL

Figure 11 (continued).

3.2.1 Calibration of Bead Coverage

Figure 12 shows the MRB's response to several bead slurries (supporting Ab-Ag* complex) assayed in the batch mode at room temperature with various antigen concentrations and two incubation periods.

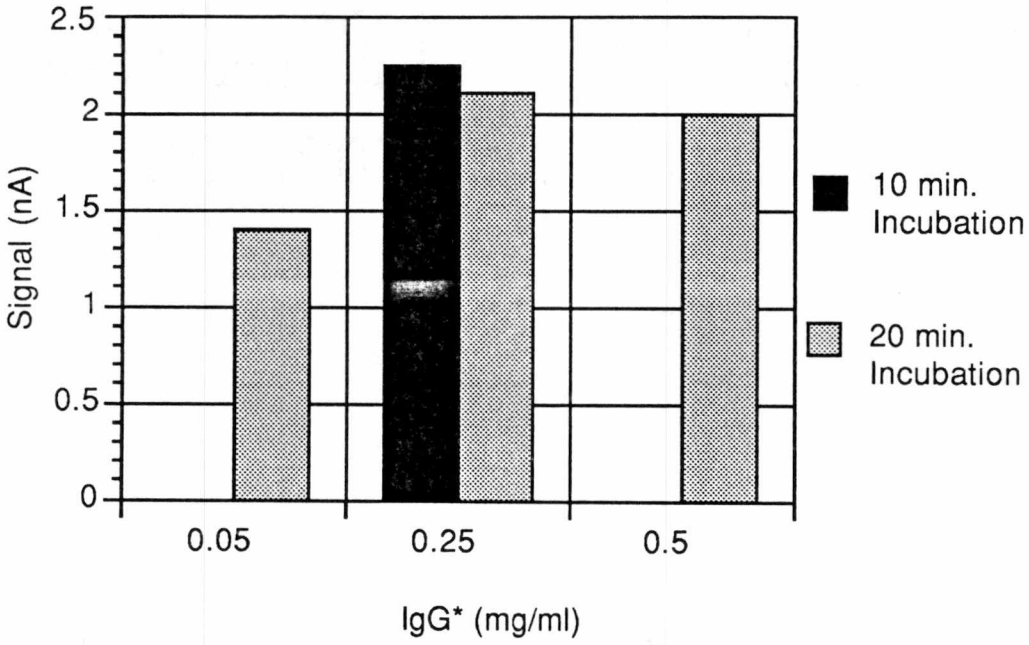


Figure 12. MRB Response to Several Batch Assays Under Various Antigen Concentration and Incubation Periods.

Concentration of IgG* appears to affect the protein A - IgG* interaction more closely than incubation time. However, Natali *et. al.* systematically investigated the effects of antibody concentration, and found that, at room temperature, the binding of antibody to protein A was almost complete in 15 minutes and was maximal in 30 minutes (45). Although I did not study reproducibility in this experiment, the unexpected slight decreases in signals (20 min. vs 10 min. at 0.25 mg/mL and 0.5 vs 0.25 mg/mL at 20 min.) may be due to slight changes in preparing or rinsing the batch beads.

3.2.2 Batch Competitive Assays

Figure 13 is a calibration plot indicating the MRB's average signal response to several competitive-binding assays (on solid phase) prepared in the batch mode. Each assay contained 0.0500 mg of total IgG; however, the ratio of IgG/IgG* varied at 0, 20, 40, 80, and 100% while the incubation time remained constant at 10 minutes for all batch assays.

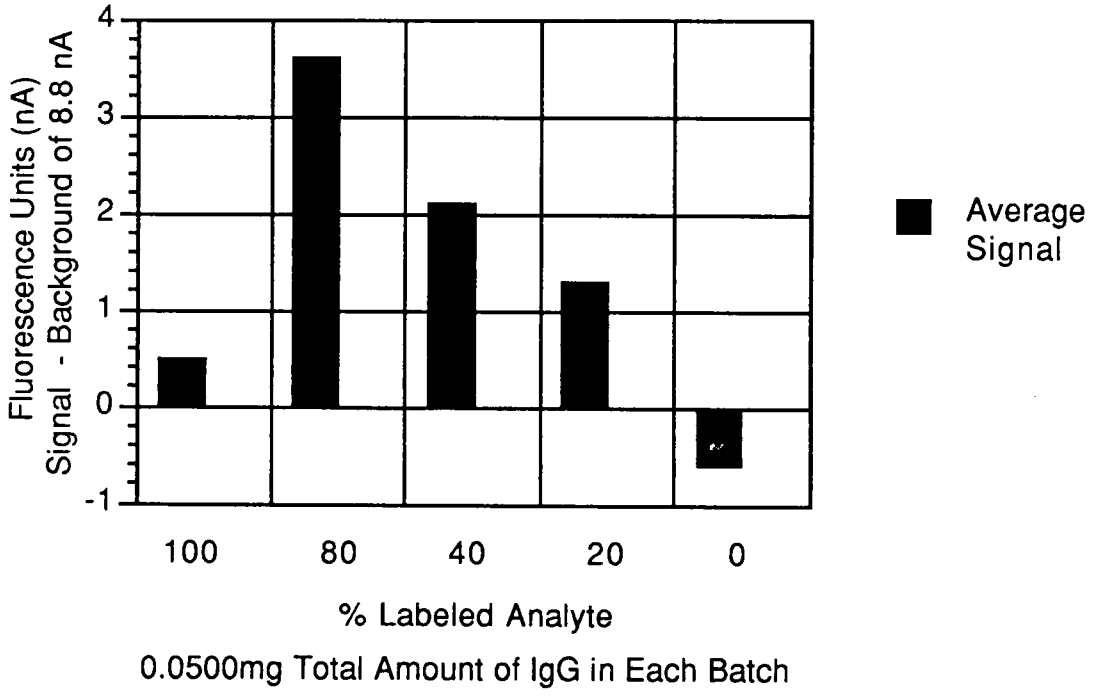


Figure 13. Batch Competitive-Binding Assays.

I delivered the slurry containing 80% labeled antigen five times and obtained a CV of 23%. The 100% labeled antigen beads gave a radically lower signal than expected. This could have been due to an error in rinsing during the assay. Nevertheless the 0 to 80% labeled analyte values produce a negatively sloped dose-response curve (for unlabeled analyte) characteristic of competitive-binding assays (33).

3.2.3 Batch Fab

Figure 14 shows how the MRB indicates the specific binding of a batch mode assay. The signals given by the MRB show that protein A beads bound the whole IgG molecule

(containing the Fc binding site) specifically over the Fab fragments which contain no binding sites for protein A. The slight signal difference between IgG* alone and IgG* + Fab fragment is probably due to variances in batch preparation or rinsing.

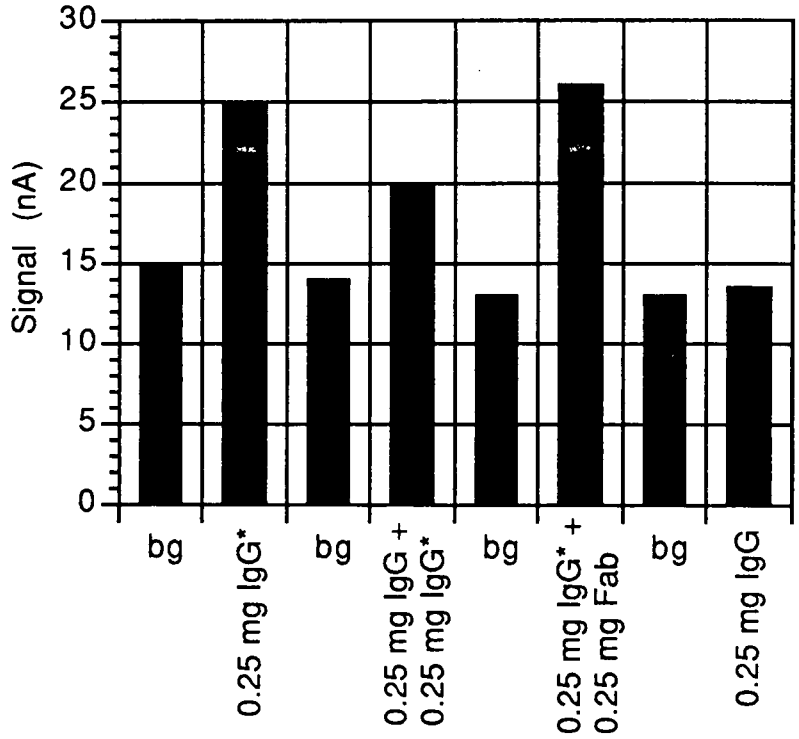


Figure 14. Batch Fab Competitive-Binding Assay.

3.2.4 Assay at the Sensing Tip

Figure 15 compares the signals of two assays, labeled antigen versus unlabeled antigen, during a modified sequential competitive-binding assay performed at the MRB's tip. Rather than sampling analyte into the aspiration column (see Figure 6), I introduced sample to the protein A beads with the injection loop. After I injected the antigen (labeled in Case 1 and unlabeled in Case 2) and rinsed it out through the frit, I then introduced another labeled antigen of the same concentration to fill the remaining antibody sites. Figure 15 records the fluorescence signal as I rinse the excess second, labeled antigen out through the frit.

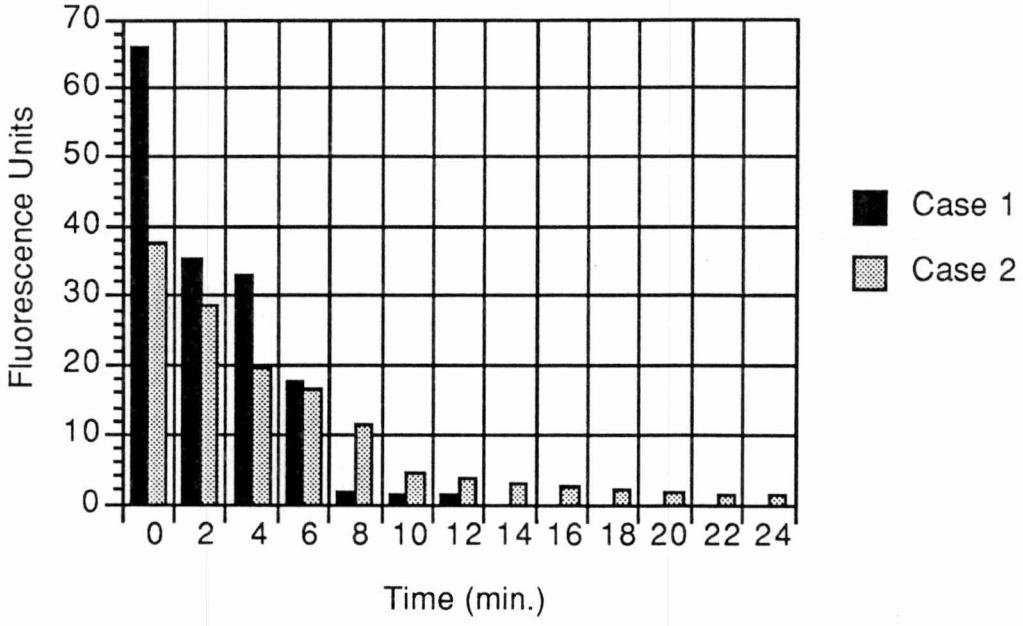


Figure 15. Assay at Sensing Tip Employing Reagent Flow.

For this experiment, I calculated the antibody binding sites available at the MRB tip (based on previous evaluations of protein A bead - IgG evaluations (43) and the volume of the tip) and adjusted the concentration of antigen (both IgG* and IgG) to 0.05 mg/mL so that one loop-full of antigen solution would provide enough antigen to fill all the sites. I assumed that the protein A would bind the antigen less effectively during reagent flow than it did during the batch reactions in a quiet test tube.

Another factor affecting the antibody-antigen interaction is incubation time (45). I included the 20 minute incubation periods to more closely imitate the batch mode conditions. However, since the sensing chamber volume is <1 μL, the incubation period exposed the antibody to a significantly smaller total amount of antigen than the injection loop contained.

This unsuccessful experiment depicted in Figure 15 indicates several experimental conditions concerning the assay and flow characteristics within the MRB tip. The assay of IgG* allowed the sequential IgG* to rinse out of the sensing tip almost twice as fast as the

assay of IgG. One explanation for this response is a change in frit permeability over time. I assayed IgG* first and then IgG, and I observed a decrease in the flow rate out the frit during the time required to perform the two assays. The most likely cause of the loss in frit permeability is the affinity of proteins for stainless steel. I initiated this same experiment using a more highly concentrated protein solution to incubate the antibody with an amount of antigen large enough to fill the antibody sites (see discussion above). The flow of solution out the MRB frit ceased as soon as the concentrated IgG* reached the sensing chamber. Therefore the protein solutions seem to coat and eventually plug the frit pores.

There is, perhaps, another factor contributing to the MRB's inability to detect the assay binding under the conditions of reagent flow. The affinity constant of protein A for rabbit IgG ($K_a = 3 \pm 0.5 \times 10^6\ M^{-1}$) is rather small compared to that for true antibody-antigen immunoreagent pairs, which typically fall within the range of 10^8 - $10^{12}\ M^{-1}$ (12). However, the affinity constant is the quotient of the forward and reverse reaction rates of a given reaction at equilibrium (see equation [1]). Perhaps the forward rate (k_1) for protein A - rabbit IgG affinity reagent pair is too small to allow measurements under true flow conditions. Since this convenient and readily available system only mocked a true Ab-Ag competitive-binding assay situation, there perhaps there would be more value in pursuing these experiments ausing a system with a somewhat higher k_2 value.

CHAPTER 4

CONCLUSIONS

The data leading to this thesis suggest several conclusions concerning the MRB's design and the operational conditions necessary for a successful assay. These studies suggest several improvements within these areas.

4.1 Problems

While the MRB allows its operator to perform the 8 separate steps involved in the physical transport of liquid and solid phase reagents, the MRB's construction materials and its mode of assay execution hinder the completion of a meaningful FIA. The experiments exploring the rinse of excess reagents (see section 2.6.2) and the assay at the sensing tip (see section 2.6.3) show how the stainless steel tortuous-path frit loses its permeability with continued use of a moderately concentrated protein solution, and it loses all permeability when employing a concentrated protein solution.

The major finding of these studies reveals that the MRB's operation must be altered before it can perform an assay in a reagent flow situation (as prescribed by current operation procedure). The MRB, employing these particular immunochemicals, cannot observe the affinity reaction adequately. Perhaps as the antigen (IgG) flows past the antibody (protein A), interaction may take place, but the following solvent rinse probably removes the antigen. The low K_a for protein A and rabbit IgG (relative to true antibody-antigen systems) may be a major factor in this situation. In addition, the MRB operation procedure does not allow the antibody to incubate with a practical amount of sample for a period of time suitable for significant reaction. The current procedure requires a highly concentrated sample to provide enough analyte (within the tiny volume of the chamber) to

allow detection. To assay reasonably dilute samples, the MRB must allow the antibody to incubate with a significant volume of sample.

4.2 Improvements

There are several possible remedies for the frit's loss of permeability within the MRB. For example, a non-tortuous path frit made of a less-protein-loving material such as titanium might improve the stability of the MRB's flow rate. Another solution might involve a suitable wash that would expunge proteins without disrupting the epoxy. Still another possibility might be to employ a construction material, other than epoxy, that is more water insoluble and can withstand a severe chemical wash.

One solution to the low K_a of the immunoreagents is to eliminate the model reagents and employ a true antibody-antigen with a higher K_a. The other problem of suitable incubation volume and time might be remedied by modifying the MRB so that the larger volume side arm holding chamber could be used as an incubator. This modification would involve installing a frit at the "T" union, and adjusting the side arm to an appropriate length (volume).

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VITA

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