To the Graduate Council:

I am submitting herewith a thesis written by Jean Pierre Alarie entitled "Development of a Regenerable Fiber Optic Biosensor." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemistry.

Michael J. Sepaniak,
Major Professor

We have read this thesis and
recommend its acceptance

Accepted for the Council:

Vice Provost and Dean of
The Graduate School

DEVELOPMENT OF A REGENERABLE FIBER-OPTIC BIOSENSOR

A Thesis

Presented for the

Master of Science

Degree

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Jean Pierre Alarie

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This work is dedicated to my parents

Micheline and Yves

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ABSTRACT

Fiber-optic chemical sensors have been developed that employ immunochemicals to perform fluoroimmunoassays. We have developed a regenerable fiber-optic sensor with a capillary delivery system to perform repetitive assays using antibodies immobilized to beads, "immunobeads." The sensitivity of these sensors is directly proportional to the amount of antibody present. For the immunobeads, the amount immobilized (loaded) and the ability of the immobilized antibody to maintain antibody recognition (activity) are two criteria which affect the sensitivity of the sensors. This study evaluates four reagents [1,1' carbonyldiimidazole (CDI), Protein A, glycidoxypropyltrimethoxysilane (GOPS), and 2-fluoro-1-methylpyridinium toluene-4-sulfonate (FMP)] using these two criteria.

Two antibody/antigen systems were employed to investigate the four procedures. The first system is a polyclonal rabbit anti-human IgG with a $F(ab)'_2$ fragment human IgG as the antigen. This represents a polyvalent, large-antigen case whereas the second system is a monoclonal mouse anti-benzo(a)pyrene tetraol (BPT) IgG with BPT as the antigen. This represents a monovalent, small hapten (small antigens with M.W. < 5000 are usually called haptens) case.

The regenerable sensor, termed a "microscale regenerable biosensor" (MRB) is evaluated using immunobeads for the direct assays of BPT. Both a diffusion-based and an aspiration-based MRB are evaluated based on their precision, dynamic range, limit of detection, and selectivity. The main emphasis of the study is to

show that a direct assay of BPT is feasible and not to optimize all operational parameters.

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CHAPTER 1

INTRODUCTION

Remote, spectroscopic-based measurements of physical parameters and chemical concentrations has been made possible with the advent of small diameter optical fibers that are capable of efficiently transmitting radiation over relatively long distances. By providing an optical link between the sample located in its native environment and the spectroscopic instrumentation located in the laboratory, many analytical advantages can be potentially realized. Recently, fiber-optic chemical sensors (FOCS) have been the focus of considerable research activity [1-3]. Spectroscopic signals from FOCS result from interactions between analyte and a reagent phase that is immobilized at the sensing terminus of the fiber-optic via various methods including direct covalent attachment to the fiber [4], entrapment within a gel [5], or entrapment within a chamber that includes an analyte permeable membrane [6]. These interactions can involve either the formation of fluorescent adducts or the quenching of fluorescent reagent phases by analytes such as O_2. FOCS have been most often used to measure small analytes such as H^+, CO_2, O_2 [7-10]. However, the FOCS measurement of large molecules has also been accomplished through the use of bioaffinity reagent phases [6,11-13]. These reagent phases include an assortment of enzymes and antibodies. The specificity of immune complexes with the use of monoclonal antibodies combined with the sensitivity of

fluorescence has made fluoroimmunoassays (FIAs) prominent technique in the area of bioanalysis.

Immunoassays, including FIA, can be broadly classified into either heterogeneous or homogeneous assays depending on whether a separation step is required to distinguish antibody-bound antigen from free antigen. The heterogeneous assay requires a separation step, either by centrifugation or by washing the antibody-antigen complex, to remove unbound antigen from the sensing region. Homogeneous assays involve the measurement of a chemical or physical property that changes upon complex formation and therefore does not require a separation step. One example is the fluorescence polarization immunoassay (FPIA) where the sample is excited with polarized light after the immune complex is formed. The polarization degree of the fluorescence signal from the antibody-bound antigen molecules decreases (as compared with the free antigens fluorescence polarization) due to a decrease of rotational movement of bound antigen. Of the two classifications, heterogeneous assays tend to be more specific and have increased sensitivity as a result of the separation step.

Immunoassay measurement strategies can be divided into three categories: competitive, direct, and sandwich assays. In competitive assays, labeled antigen competes with unlabeled antigen for a limited number of antibody binding sites. Antibody-bound antigen (labeled and unlabeled) is separated from the free antigen and the fluorescence of either phase is measured. The sensitivity in the bound phase is inversely proportional to the concentration of the unlabeled antigen. The higher the fluorescent

signal, the lower the unlabeled antigen concentration. Direct assays are performed by simply incubating the antigen with an excess amount of antibody. The antigen is a natural fluorophor and can be measured directly either before or after a separation step. Sensitivity of direct assays is directly proportional to the amount of antibody present and response proportional to the amount of antigen present. Sandwich assays are performed by incubating the antigen with a primary antibody present in excess in the same manner as the direct assay. The antibody-antigen complex is then incubated with a second antibody labeled with a fluorophor. Unbound labeled antibody is rinsed away and the bound labeled antibody is measured. Sensitivity is again proportional to the amount of primary antibody present with response proportional to the antigen concentration. One problem with sandwich assays is the fact that the antigen must be large enough to have two binding sites so the second antibody may still recognize it.

We have successfully used immunoassay procedures to develop FOCS based on competitive and direct assay principles [4,6]. However, these FOCS along with many of the bioaffinity FOCSs reported in the literature might be better referred to as probes, not sensors. That is, they are essentially "single-measurement" devices and are removed from the sample between measurements either for a wash or regeneration of the reagent phase. A sensor, however, does not have to be removed from the sample in order to provide more than a single-measurement. For a FOCS to be considered a true sensor, one of the following criteria must be satisfied: (1) the interaction between analyte and reagent must be reversible and

rapid, (2) there must be sufficient reagent such that it is not depleted over long periods of time, or (3) there must be some means of regenerating the reagent phase, preferably in situ. Most of the bioaffinity FOCS developed so far do not meet these criteria partly due to the large equilibrium constants associated with most affinity reactions, which render affinity reagent-analyte interactions highly selective but, adversely, not very reversible (i.e. they react very slowly to changes in analyte concentration).

The competitive assay FOCSs utilized Rabbit IgG immobilized at the distal end of an optical fiber [12]. This fiber was either incubated in a mixture of labeled and unlabeled anti-rabbit IgG or incubated first in unlabeled ligand then in labeled ligand. Measurements were performed following rinsing in 1 mL fresh phosphate buffered saline (PBS). This FOCS proved efficient in measuring small-sample volumes but attempts to regenerate the fiber was not completely successful. The optical fiber bound antibody was incubated with 3 M KSCN to remove bound ligand but this failed to restore the signals to original levels upon reincubation with antigen. In fact, after 3 rinses of KSCN, the fibers failed to bind an appreciable amount of antigen. Because of this failure, a new optical fiber is required for each measurement.

Another example of an affinity FOCS is the direct sensor we developed for the measurement of benzo(a)pyrene tetraol (BPT) which is a fluorescent by-product of the interaction of the polynuclear aromatic compound benzo(a)pyrene with DNA [6]. The FOCS employed a reagent phase that included either a chamber entrapped solution of anti-BPT antibodies or anti-BPT immobilized

to silica beads via Protein A. Operation was based on diffusion of BPT into the sensing chamber where it was bound by the entrapped antibody. This process concentrated the BPT within the sensing region and enhanced signals permitting the detection of subnanomolar concentrations of BPT (attomole quantities in the low μL samples that could be analyzed with this diminutive sensor). Because of the moderately large amount of antibody in the chamber, criterion 2 stated above is marginally satisfied and true sensing is possible over a short (about 2 hr) interval. Nevertheless, this sensor is basically a "single-measurement" device. Significant analytical selectivity was also exhibited, although this required removing the sensor from the sample and dialyzing to remove interferences (again diminishing the true sensing characteristics of the device).

We recently demonstrated how a single fiber FOCS could be combined with a capillary delivery system to permit a variety of affinity assay procedures (e.g. adding reagents, rinsing to remove impurities, and recycling) to be performed at the sensing terminus of the FOCS [11]. The configuration of the microscale regenerable biosensor (MRB) is shown on page 30. It consists of a 200 μm core diameter fiber with six 200 μm i.d. fused silica capillary columns. The diameter of the fiber/6-column bundle is about 1 mm. The chamber at the sensing end of the bundle was comprised of a chamber wall (made of heat shrink tubing), which extends less than 0.5 mm beyond the fiber and is capped with the same membrane as the direct FOCS previously described. This sensor is operated by filling the sensor tip through the appropriate capillary with antibody solution and then placing it in the sample to be measured. The same

operating parameters which controlled the aforementioned direct FOCS applies here (diffusion through membrane, concentrating effect).

The preliminary experiments using the MRB for the direct assay of BPT illustrated that the sensor can be regenerated. The sensor was first filled with a rinse solution (distilled H_2O) and placed in $1.0x10^{-7}$ M BPT solution. The signal behaved as expected with equilibrium occurring in approximately 10 min. With all the outlets closed, the sensor was purged through the membrane via the rinse capillaries. The signal dropped to baseline and remained there after being placed in the rinse solution.

In the next experiment, the chamber was filled with $2.0x10^{-6}$ M anti-BPT solution and placed in the BPT solution. The signal increased at a faster rate than the case without antibody showing the concentration factor. The sensor was then rinsed in the same manner as the case without antibody with the signal dropping due to the movement of the immune complex to the membrane where the fiber is unable to "see" it. The signal, however, was larger than the no antibody case and the signal slowly increased (albeit not to its pre-rinse level) as the immune complex diffused away from the membrane back into the viewing region. The sensor was then regenerated by flushing the chamber with rinse solution with the outlet capillaries open.

There are several limitations associated with the direct assay sensors described above. First, the direct sensors are limited to natural fluorophors thereby ignoring the large number of non-fluorescent antigens that are detected by competitive or sandwich

assays. The affinity sensor cannot perform a sandwich assay since there is no possibility to introduce the second antibody. The MRB can introduce a second antibody but there is no way to remove unbound excess second antibody without removing the immune complex as well. A second limitation is sampling via diffusion, which can be prohibitively slow for large molecules. Another limitation is that the antigen must be substantially smaller than 160,000 M.W. in order to efficiently diffuse through the membrane and also to keep the antibody in the sensor. A final problem is that continuous, in situ operation as previously discussed for the affinity sensor is only possible over a short period. Memory effects associated with the membrane also limits the amount of time the sensors can be used.

The cellulose membrane, although effective for single-shot measurements, is the limiting factor when using the MRB. Three changes, one to the design, the others to the operation of the MRB, can reduce the limitations. The design change is to replace the membrane with a rigid hollowed-out stainless steel frit (see page 30). The frit eliminates the memory effect. The thickness of the frit does not readily lend itself to diffusion but the rigidity allows aspiration as the mode of sampling. This procedural change now permits large and small antigens to be pulled into the MRB, extending the range of antigens the sensor can measure by either direct or sandwich assays.

A third change is also required because of the frit. Unlike the membrane that is able to entrap the antibody, the frit's pores are large enough so the antibody will be rinsed away along with the

interferences. To ensure that the antibody remains within the sensor, immobilization to a solid support is necessary. The solid support chosen was silica beads based on its physical characteristics. They are rigid, do not swell or contract and therefore easily delivered and removed from the sensing chamber. This ability enables criterion 3 for being a true sensor to be fulfilled. Other important characteristics are their transparency to UV and visible light as well as their availability in a variety of controlled diameters and pore sizes.

Since sensitivity of the MRB is directly proportional to the amount of antibody, the immobilization procedure chosen is important for two main criteria. A high degree of immobilization of antibody is desired yet the percentage of the immobilized antibody must still be able to recognize antigen may dictate which procedure is better. For example, a procedure with a high degree of immobilization but low retention of antibody activity may have less active antibody than one with low loading but high retention of activity. Ideally, a high loading with high activity is desired.

This study evaluates four procedures for immobilizing antibodies to silica beads. The main criteria are loading of antibody as well as the activity of the loaded antibody. Of these four methods, the best method was chosen and a direct assay of BPT using a diffusion-based and aspiration-based MRB is performed. The emphasis of this preliminary MRB study is to show that a direct FIA of BPT is possible and not to optimize such parameters as bead density, flow rate, or sample volume.

CHAPTER 2

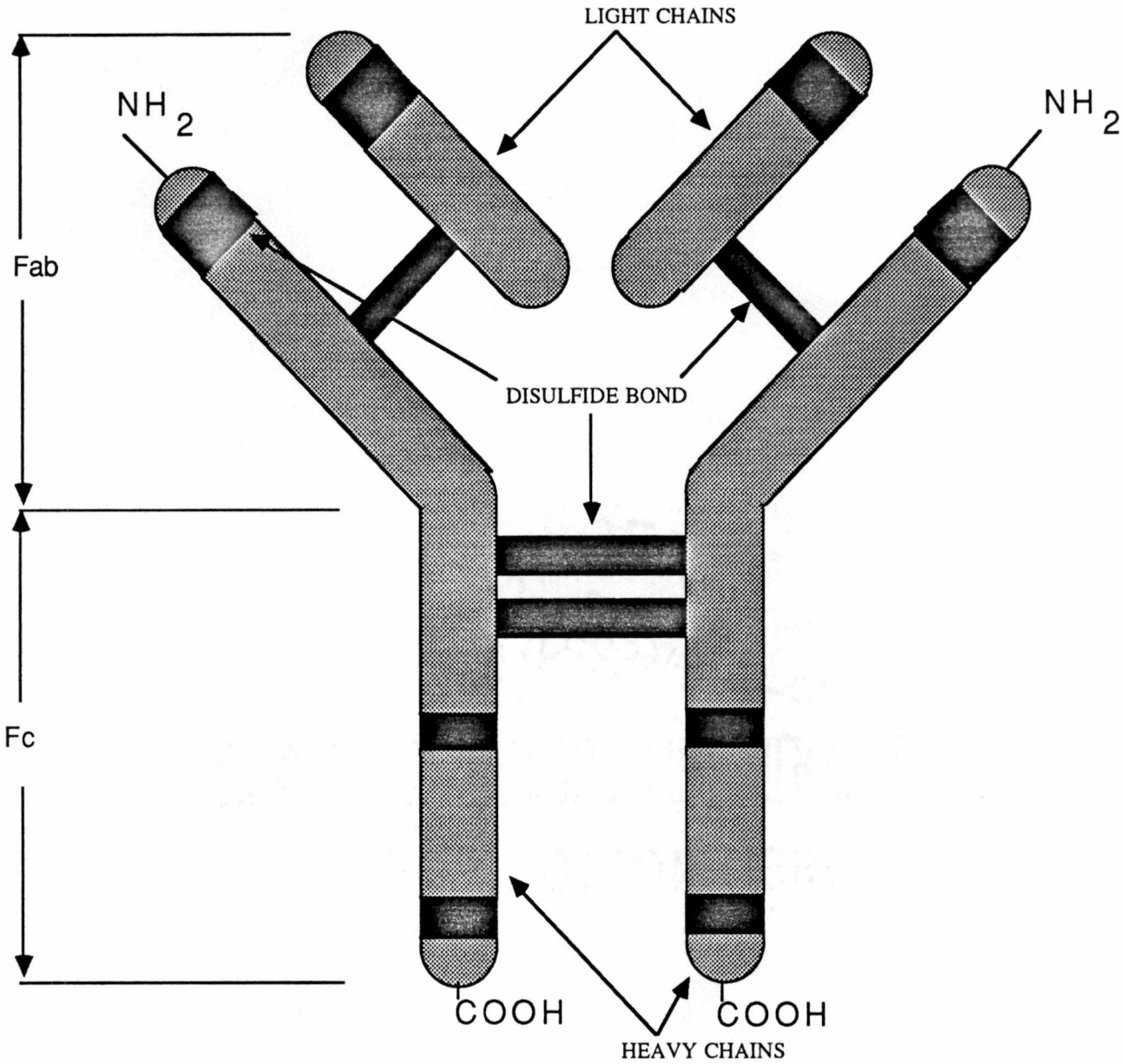
EVALUATION OF ANTIBODY IMMOBILIZATION TECHNIQUES FOR FIBER-OPTIC-BASED FLUOROIMMUNOSENSING.

There are numerous methods for immobilizing proteins to solid supports such as agarose, cellulose, and silica [14]. The primary purpose for these procedures is for the use in affinity chromatography. However, the criteria for use in the MRB and in affinity chromatography are quite different. As previously mentioned, evaluation of these immobilization procedures is based primarily on two criteria: (i) the amount of antibody that can be "loaded" onto the beads and (ii) the retention of antibody "activity" (i.e., the ability of the immobilized antibody to recognize its specific antigen after immobilization as determined by the ratio of the moles of antigen bound to moles antibody immobilized). These criteria are important because in direct and sandwich assays sensitivity is directly proportional to antibody loading [6,15]. In addition, the preservation of the antibody activity upon bonding to the solid support influences both sensitivity and selectivity. Added importance for these criteria is the small volume of the regenerable sensor. Unlike affinity chromatography, where a large amount of solid support (the equivalent of immunobeads) can compensate for low loading or high activity loss, the regenerable sensor with a volume of less than 1 mL can contain only minute amounts (< 0.5 mg) of immunobeads.

A cursory knowledge of antibody structure is helpful in understanding the effect of immobilization on antibody function. Most antibodies belong to the immunoglobulin G (IgG) class of blood proteins [16]. IgG is composed of two identical "heavy" and two identical "light" protein chains that are joined by disulfide bonds. The entire molecule is often depicted as being Y-shaped and has a M.W. of about 160,000 (see Figure 2.1). The common heavy chain end of the molecule is referred to as the Fc fragment and has a fixed structure while the two separate heavy-light chain combinations are referred to as the Fab (Fragment antigen binding) portion. The Fab fragment contains amino acid sequences that form tertiary structures which permit strong binding to a specific region, "epitope," of the antigen against which the antibody was generated. Immobilization through covalent attachment is most successful when access to antigen binding sites on the antibody is not hindered or disrupted and when the bonds to the solid support are stable but do not radically distort the antibody structure. Integrity of the antibody combining site geometry is critical to specific antigen recognition.

Two antibody/antigen combinations were evaluated in this study. The first combination, polyclonal rabbit anti-human IgG, $F(ab)'_2$ fragment specific/ fluorescein derivatized $F(ab)'_2$ fragment Human IgG (Accurate Chemical and Scientific Corporation, Westbury, New York), represents a polyvalent, large molecule antigen case. The second combination, monoclonal mouse anti-benzo(a)pyrene tetraol (BPT) IgG/BPT, represents a monovalent small molecule (hapten) case (anti-BPT was supplied by Dr. Regina Santella of Columbia

Figure 2.1 The IgG antibody molecular structure. The upper half consisting of both heavy and light chains is the Fab fragment. The lower half with just the heavy chains is the Fc fragment. The $F(ab)'_2$ fragment results from cleavage just below the two disulfide bonds linking the heavy chains. Reduction of these bonds results in two Fab' fragments.


LIGHT CHAINS
NH 2
NH 2
Fab
DISULFIDE BOND
Fc
COOH
COOH
HEAVY CHAINS

University Health Centers, New York, N.Y.). Small antigens (M.W. ≤ 5,000) are usually referred to as haptens.

It is likely that each new antibody/antigen combination would need to be optimized in terms of the immobilization procedure (linking reagent and reaction conditions). However, it was anticipated that a comparison of the four procedures mentioned above, using previously reported reaction conditions, for these two antibody/antigen combinations would provide fundamental information that could be applied to other systems.

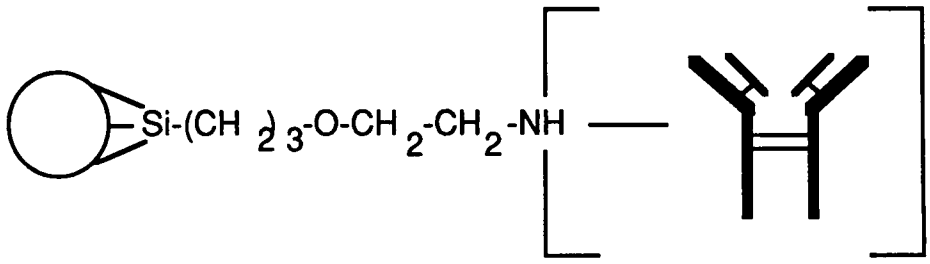
EXPERIMENTAL SECTION

Diol Silica: The silica beads used for the glycidoxypropyltrimethoxysilane (GOPS), 1,1' carbonyldiimidazole (CDI), and 2-fluoro-1-methylpyridinium toluene-4-sulfonate (FMP) immobilization procedures were prepared as previously described[11]. For our study, 0.5-1.0 g of 7-μm diameter, 500-Å pore size silica beads (Synchrom, Inc) are cleaned by suspending in hot 1-M HNO_3 for 15 min and rinsed with 100 mL H_2O. The washed silica beads are then suspended in 20 mL 10% (v/v) GOPS in H_2O and degassed for 10 min by ultrasonic vibration. The mixture is heated for 3 h at 90°C with the pH maintained at 3 using 1-M HCl. The diol silica beads are collected by filtration using a 0.45-μm nylon filter and washed with 100 mL H_2O. The filtered beads are dried overnight in vacuo at 105°C. At this point, the silica beads are referred to as diol silica beads.

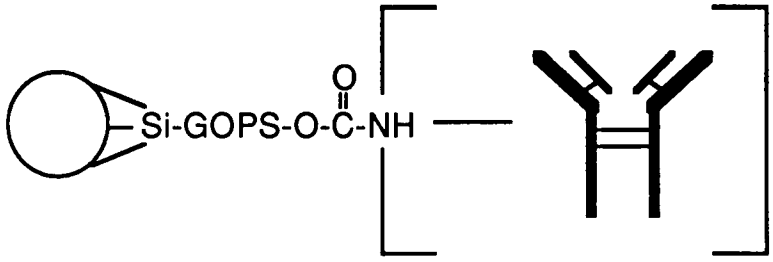
GOPS Immobilization Procedure: The activation of the diol silica beads for direct GOPS attachment proceeded as described previously [18]. In 5 mL HIO_4 (5 mg/mL in 80% (v/v) acetic acid), 25-40 mg diol silica beads are suspended and shaken at room temperature for 30 min followed by three 1 mL H_2O and two 1 mL Phosphate Buffered Saline (PBS, pH=7.3) washes (All washes are with PBS, pH=7.3 unless specified). The diol silica beads (aldehyde functional group) are then aliquotted into 10-15 mg portions in 1.5 mL microcentrifuge tubes (Eppendorf). To each tube, 100 µL of (1.0-1.7 mg/mL) antibody (in PBS) is added and shaken for 20 h at 4°C. Upon completion of this incubation step, the "immunobeads" are washed three times with PBS (all washes 1 mL). Solutions obtained from each wash are saved for fluorimetric measurements. The Schiff base formed at the linkage is reduced with 1 mL (20 mg/ml in H_2O) sodium cyanoborohydride for 30 min at 4°C. The immunobeads are washed twice with PBS after this step and are ready for antibody activity determinations. Figure 2.2a shows the final linkage.

CDI Immobilization Procedure: CDI activation of the diol silica beads is a modification of a previously described method [18]. Ninety mg of the diol silica beads are suspended in 2 mL acetonitrile with 320 mg CDI. The mixture is degassed for 15 min by ultrasonication and aspirator vacuum, then shaken for 30 min at room temperature. The activated diol silica beads are filtered, washed with acetonitrile and dried. The beads are washed with PBS after drying and aliquotted in the same manner as in the GOPS procedure. The PBS is removed by centrifugation and 100 µL of

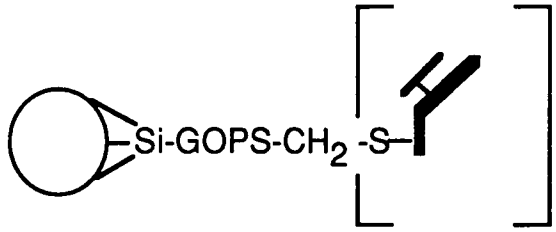
Figure 2.2 Structures formed when linking reagents are used to immobilize antibody to diol bonded silica.



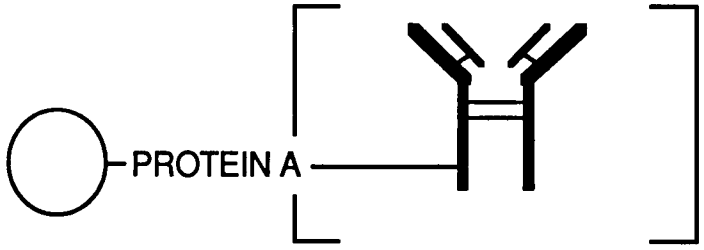
(a) Glycidoxypropyltrimethoxysilane (GOPS)



(b) 1,1' carbonyldiimidazole (CDI)



(c) 2-fluoro-1-methylpyridinum toluene-4-sulfonate (FMP)



(d) Protein A

(1.0-1.7 mg/mL) antibody is added to each vial and the volume subsequently brought up to 1 mL with H_2O. This antibody-bead mixture is incubated on a shaker for 6 days at 4°C. The immunobeads are washed three times with PBS and remain in PBS overnight to hydrolyze any unreacted sites after the incubation step. Each wash after the incubation was again saved for fluorimetric measurements. Figure 2.2b shows the final linkage.

FMP Immobilization Procedure: Activation of the diol silica again followed a modification of a previously reported method [19]. The diol silica beads (0.5 g) are washed with 50 mL volumes of the following H_2O:acetone ratios: 100:0, 75:25, 50:50, 25:75, 0:100. The beads are ultrasonicated for 2 min and filtered between each wash. After the final acetone wash, the beads are washed with acetonitrile and then suspended in 15 mL of acetonitrile. To this solution, 500 mg FMP dissolved in 150 mL acetonitrile and 0.5 mL triethylamine is added in 5 mL increments with vigorous stirring. The beads are washed with 50 mL of acetonitrile, filtered, and washed with acetone 30 min after the last addition of FMP solution. The FMP-activated beads are washed with 0.1-M PBS (pH=6.0) and degassed prior to addition of antibody.

Preparation of the Fab' fragments has been previously described [20]. The starting point for our study is the $F(ab)'_2$ fragment of the antibody. The $F(ab)'_2$ fragment is first dialyzed against 0.1-M PBS (pH=6.0) for 18 h at 4°C. To the dialyzed solution, 10 mg dithiothreitol is added and incubated for 1 h in the dark at room temperature. The Fab' fragments arising from reduction of the disulfide linkage are separated and collected from a Sephadex G-25

column using the 0.1-M PBS (pH=6.0), and then incubated with the activated beads at 4°C for 24 h [19]. The immunobeads are washed three times with 0.1-M PBS (pH=6.0) and placed in Tris buffer (pH=8.2) overnight to remove any unreacted sites. The beads are then ready for activity determinations.

Durosphere Protein A: Approximately 12 mg quantities of Durosphere Protein A beads (Alltech Assoc.) are added to microcentrifuge tubes followed by 100 μL of (1.0-1.7 mg/mL) anti-human IgG. The mixtures are allowed to incubate for 90 min at room temperature. Following incubation, the beads are washed three times with PBS (pH=7.3) and once with borate buffer (pH=8.0). The washes are saved for fluorimetric measurements. One mL dimethylsuberimidate (5 mg/mL in borate buffer) is added and incubated for 30 min to crosslink the antibody to the Protein A [21]. The beads are washed twice with PBS and incubated with 3-M KSCN overnight at 4°C to remove any noncrosslinked antibody. The chaotropic rinse was removed and the beads washed twice with PBS.

Immobilization of the anti-BPT proceeds by first precipitating the antibody with Na_2SO_4 and adjusting the concentration of Na_2SO_4 to 1 M with H_2O. The antibody is then immobilized and crosslinked in the same manner as the anti-human IgG. This sample preparation is required as Protein A exhibits species specific affinity constants. In the case of mouse antibodies, the optimal pH for immobilization is 9.1 [22] which is too basic for the Durosphere beads. It has been shown that mouse antibodies in 1.0-1.5-M Na_2SO_4 with no NaCl present will bind to Protein A at pH 7.3 more readily than in a PBS solution [23].

Blanks: Blanks were prepared by substituting a non-specific rabbit IgG for each antibody immobilization procedure described above.

Determination of Loading: The loading of antibody was determined by measuring the fluorescence of the antibody solutions prior to and after incubation (pre-post fluorescence) using a Perkin-Elmer Model MPF-43A spectrophotofluorimeter. The excitation wavelength was 288 nm with emission wavelength set at 335 nm. Slit widths were optimized for each set of antibody measurements. The accuracy of the pre-post measurements was checked by hydrolyzing the antibody from the GOPS beads and measuring the antibody removed from the beads. This verification has been done before with the different measurement procedures yielding similar results [17]. The results from our hydrolyzed antibody and pre-post measurements showed a maximum variation of 10%.

Determination of Activity: Activity of the immobilized antibodies was determined by incubation of the immunobeads with 1.0-1.7 mg/mL fluorescein-labelled $F(ab)'_2$ fragment Human IgG or 1.78×10^{-5} M BPT solution depending on the antibody. Each incubation was for 90 min at room temperature. After this incubation, the beads are washed three times with PBS with each wash saved for fluorimetric measurements in the same manner as the antibodies. Excitation for the fluorescein derivatives was 488 nm with 521 nm as the emission wavelength. BPT was measured at a 345 excitation wavelength and a 397 emission wavelength. The slits were optimized for each set of measurements and resulted in band passes in the 2-5 nm range. Since the CDI, GOPS, and Protein A procedures involve immobilizing bivalent antibody, the theoretical

activity (moles antigen/mole antibody) is 2. The FMP involves immobilizing the monovalent Fab' and has an activity limit of 1.

RESULTS AND DISCUSSION

Loading of Antibody: Table I shows the results of the loading and activity determinations for both antibody/antigen systems tested. CDI achieved the highest loading for both systems tested, which is consistent with previously reported results [19]. The anti-human IgG and blank antibodies were immobilized to a higher degree than were the anti-BPT antibodies. This is probably due to other proteins present in the ascitic fluid of the anti-BPT which were not present in the other incubation solutions. Since CDI reacts with amine groups, these proteins may interfere with the binding step resulting in the lower loading of the anti-BPT. Evidence of this can be seen in the Protein A data, as both antibodies are immobilized to the same degree. Since Protein A interacts with the Fc region, the effects of interfering protein are expected to be less severe than for the GOPS and CDI procedures. FMP showed the second highest loading of antibody, though this loading is not readily seen in terms of mg antibody per bead but rather in terms of antigen binding sites. FMP attaches to the SH group of the Fab' fragment (M.W.=56,000) which has one binding site (see Figures 2.1 and 2.2c) as compared with two binding sites for the whole molecule (M.W.=160000). The average number of binding sites for FMP is 1.09×10^{-4} mmoles/g of bead compared with 1.58×10^{-4} mmoles/g of bead for CDI. GOPS and

Table I: Results of antibody loading and activity

SAMPLE	mg Ab/g Bead	mg Ag/g Bead	mol Ag/mol Ab
		CDI	
Anti-BPT 1	9.8	4.2E-02	1.9
Anti-BPT 2	9.2	4.1E-02	2.0
Anti-BPT 3	9.4	3.5E-02	1.7
BLANK	12.3	8.7E-04	NA
Anti-IgG 1	12.5	9.5	1.1
Anti-IgG 2	12.7	9.5	1.1
Anti-IgG 3	12.7	9.7	1.1
BLANK	12.9	1.3	NA
		GOPS	
Anti-BPT 1	3.1	2.4E-03	0.4
Anti-BPT 2	4.2	2.3E-03	0.3
Anti-BPT 3	3.4	2.4E-03	0.4
BLANK	4.2	1.1E-04	NA
Anti-IgG 1	5.8	4.0	0.3
Anti-IgG 2	4.8	3.1	0.3
Anti-IgG 3	4.6	2.6	0.5
BLANK	3.9	2.1	NA
		PROTEIN A	
Anti-BPT 1	5.0	2.8E-02	2.8
Anti-BPT 2	8.2	3.4E-02	2.0
BLANK	6.1	4.5E-04	NA
Anti-IgG 1	5.7	7.4	2.0
Anti-IgG 2	5.7	7.9	2.1
Anti-IgG 3	4.3	7.3	2.6
Anti-IgG 4	4.6	7.2	2.4
Anti-IgG 5	3.9	7.3	2.8
BLANK	2.9	3.2	NA
		FMP	
Anti-IgG 1	5.3	5.9	0.5
Anti-IgG 2	6.8	6.8	0.5
Anti-IgG 3	6.3	8.3	0.8
BLANK	5.6	1.0	NA

Ag=Antigen Ab=Antibody

Protein A immobilized similar amounts of antibody. For the GOPS procedure, the anti-BPT had a slightly lower loading than the anti-human IgG in agreement with the CDI results. The results of the Protein A immobilization were obtained post crosslinkage. Since the crosslinking process resulted in some loss of antibody (15-40% in this work), the Protein A originally attached more antibody than the GOPS. Improvements in the crosslinking step would improve the loading for the Durosphere Protein A, which has been reported to be as high as 8 mg/g bead [24].

Activity of the immobilized antibody: Protein A demonstrated the greatest amount of activity for both antibody/antigen systems. This activity is expected as Protein A binds the Fc region of the antibody leaving the antigen binding sites free (see Figure 2.1, 2.2d) [25]. In both systems, the activity exceeds 100% of the theoretical limit. Possible explanations for this include non-specific interactions between the antibody and antigen as well as the Protein A and antigen. These interactions, along with the specific interactions, could explain the excess binding that was observed. The apparent excess binding may also be attributed to "statistical variations" due to the complexity of the sample workup, including the crosslinking procedure as well as the chaotropic rinse (see Blank Section, page 23, for further discussion).

CDI demonstrated high activity for the BPT system but only averaged about 60% of the theoretical limit for the IgG system. This difference between the two systems may be attributed to the size of the antigens. CDI attaches non-specifically to amine groups: therefore, linkage of the antibody to the silica beads can occur on

the Fab region as well as the Fc region (see Figure 2.2b). Presumably, the smaller BPT molecule can reach sterically hindered binding sites more readily than the larger $F(ab)'_2$ fragment antigen. The binding sites may further be hindered after binding the large antigen as well.

FMP retained from 55-80% of the Fab' fragment activity. The degree of activity loss was higher than expected as the binding sites should be free due to immobilization at the reduced disulfide bond (see Figure 2.2c). Loss of activity may be the result of other disulfide linkages being reduced. These reductions would take place in the Fab region which, by altering the 3-D structure of the antigen binding site, could reduce activity. Once the disrupted Fab' fragments were immobilized, they would have no antigen recognition.

GOPS was the least successful in retaining activity of the immobilized antibody. In the case of both antibodies, only 15-25% of the immobilized antibody remained active. Since GOPS randomly reacts with amine groups in the antibody, as does CDI, the same factors affecting CDI activity will also affect the GOPS immobilized antibodies. This feature does not explain the large difference between the two reagents. One possible reason for the large loss of antibody activity for GOPS, besides steric hindrance, could be the reduction of the Schiff base with cyanoborohydride. This step may also reduce sites on the antibody, altering its quaternary structure and thereby denaturing the antibody.

Blanks: The blanks were performed to assess any non-specific interactions between the antigen and the immunobeads. The use of

the non-specific antibody would recreate the same conditions seen for the specific antibodies except for the recognition of antigen. For each BPT blank, the amount adsorbed was 5% or less of the total BPT bound in the real samples. This indicates that there was little non-specific interaction between the BPT and the immunobeads. The $F(ab)'_2$ fragment antigen did show adsorption on the blank species. Some of this adsorption is due to protein-protein interactions between the immobilized non-specific antibody and antigen. Another cause of adsorption may be sites on the diol silica beads which were still active. Both the CDI and FMP procedures have deactivation steps and consequently exhibited the lowest adsorption. The GOPS surface was not specifically deactivated and this probably accounts for the large blank. The Protein A beads show the highest amount of adsorption. This is due in part to protein-protein interactions but also to the sample preparation steps as mentioned in the Protein A activity discussion. The crosslinking reagent may not have been totally deactivated and therefore may have reacted with the antigen non-specifically. A longer wash period to ensure hydrolysis should reduce this problem. The chaotropic reagent, KSCN, denatures the antibody and may also denature the Protein A. The antibodies and Protein A may not fully renature during the washes creating new adsorption sites not created in the other procedures. Incubation of the $F(ab)'_2$ fragment antigen with unreacted Durosphere Protein A beads did not produce any significant adsorption. Again, a longer wash period would allow the proteins to renature and decrease the amount of adsorption.

CHAPTER 3

FLUORESCENCE MONITORING OF BPT USING AN IMMUNOCHEMICAL BASED FIBER-OPTIC SENSOR

The use of immunochemical reagent phases in FOCS designs provides sensitive and selective measurements for a broad range of compounds. Their major drawback has been a "single-measurement" limitation due to large equilibrium constants which make the reactions highly selective but not very reversible. The development of the microscale regenerable biosensor has shown promise in being able to have the reagent phase regenerated in situ. The addition of the immunobeads and metal frit combined with the capillary delivery system enables the MRB to fulfill criterion 3 set out in Chapter One for being a true sensor.

There are a number of different parameters associated with the aspiration MRB which now help to determine selectivity and sensitivity. Among these are reproducibility of the aspirated sample, volume of the sample, the flow rate at which the sample is reintroduced to the sensing region, the volume of the sensing region, the immunobead density (i.e. the amount of beads) delivered to the sensing tip, and how long the immune complex should be rinsed in order to remove interferences.

Both the diffusion and aspiration MRB are employed for the direct assay of BPT. The diffusion MRB uses a liquid antibody solution entrapped in the sensing chamber while the aspiration MRB employs the immunobeads. For the selected operating parameters, the

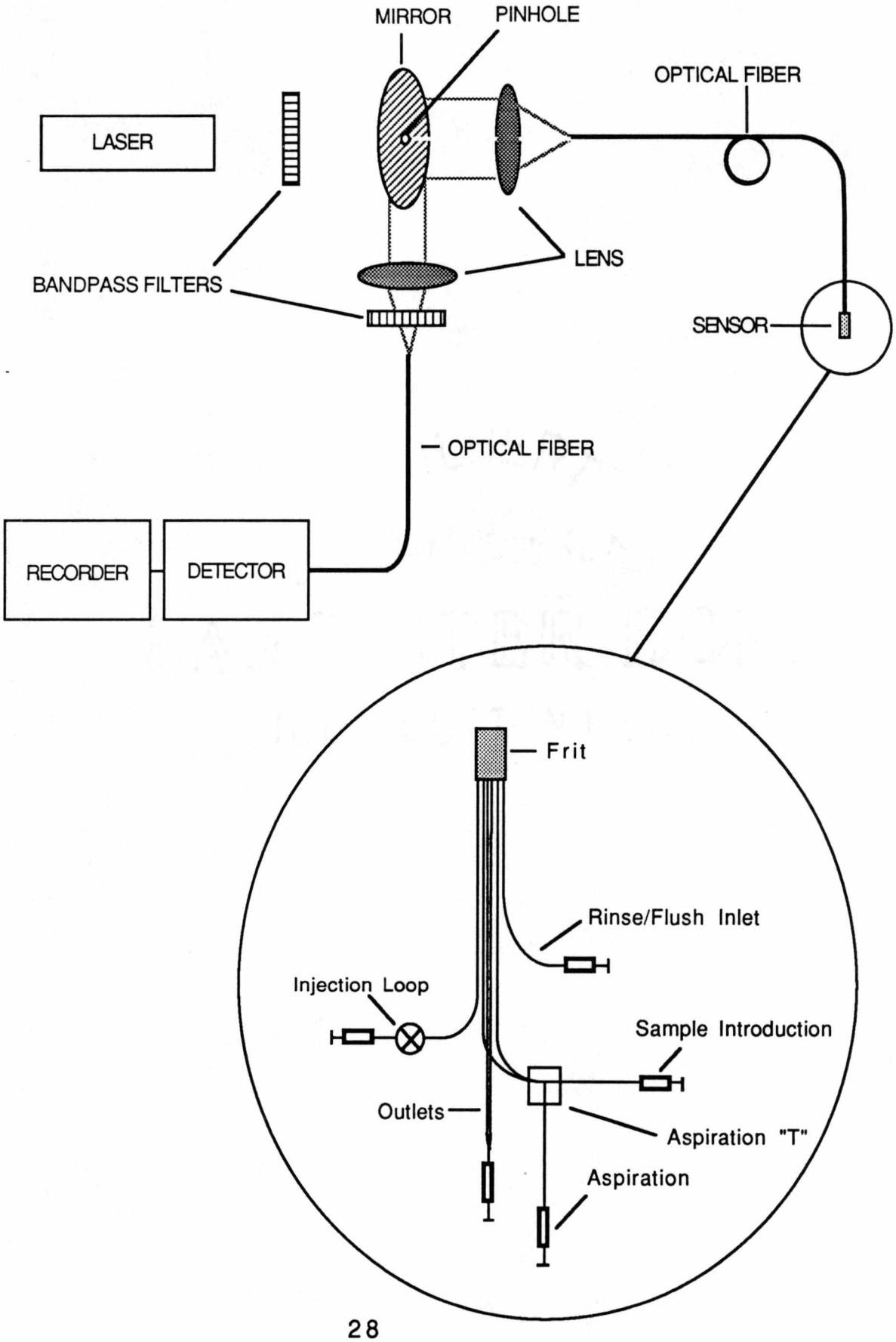
sensitivity, precision, dynamic range, and selectivity of both MRBs are reported. Although the conditions are far from optimal, they give an indication if regenerable, direct assays of BPT are feasible using the MRB design.

EXPERIMENTAL SECTION

Phosphate buffered saline (PBS), pH 7.3 was obtained from Sigma Chemical Co., Saint Louis, MO. Monoclonal anti-BPT was provided by Dr. Regina Santella, Columbia University, New York, NY. The results of an immunochemical characterization of this antibody has been reported [26]. BPT and chrysene were obtained from Midwest Research Institute (Kansas City, MO) and Aldrich Chemical Co. (Milwaukee, WI), respectively. The BPT, chrysene, and anti-BPT solutions used in this work were prepared by diluting to volume using PBS solution.

Optical configuration: A block diagram of the optical configuration employed in this work is shown in Figure 3.1. Radiation from an Omnichrome (Chino, CA) model 356 XM He-Cd laser (15 mW, 325 nm) is passed through a beamsplitter constructed by boring a 2 mm hole through the center of a 25 mm diameter mirror, and focused onto the incident end of a 200 or 400 μm core diameter plastic clad fused silica optical fiber or silica-silica fiber (General Fiber Optics, Cedar Grove, NJ). A laser line filter is used to isolate the 325 nm output line of the laser. The focusing lens has an f/number that is approximately equal to that of the fiber. Laser radiation that exits the fiber at its terminus can excite fluorophors

Figure 3.1 Optical configuration of the fiber-optic based sensor


MIRROR
PINHOLE
OPTICAL FIBER
LASER
LENS
BANDPASS FILTERS
SENSOR
OPTICAL FIBER
RECORDER
DETECTOR
Frit
Rinse/Flush Inlet
Injection Loop
Sample Introduction
Outlets
Aspiration "T"
Aspiration

in the sensing chamber. Some portion of the resulting fluorescence emission is collected by the fiber and transmitted back to the optical configuration. This radiation exits the fiber in a cone determined by the numerical aperture of the fiber, is collimated by the lens, reflected by the beamsplitter, and finally imaged through a secondary filter (a bandpass filter centered at 395 nm) onto the photocathode of a Hamamatsu model R760 photomultiplier tube. Signals are processed with a Keithley model 485 autoranging picoammeter. Photothermal complications are minimized by employing a Uniblitz Model SD-10 shutter (Vincent Associates, Rochester, NY) to provide 1 second laser exposure every 40-90 seconds.

Sensor construction: The sensing tips that were employed in this work are shown in Figure 3.2. They are constructed by surrounding a 400 μm core diameter fiber, with its protective coating removed, with 6 fused-silica capillary columns (200 μm i.d., 300 μm o.d., and about 50 cm in length) from Scientific Glass Engineering (Austin, TX). The fiber/capillary assembly is coated with epoxy, a piece of heat shrink tubing is placed around the assembly as the epoxy cures, the heat shrink tubing is removed, the tip is polished, and finally, the assembly is slipped into a cylindrical chamber. The diffusion controlled MRB chamber is formed using heat shrink tubing and is capped with a 10,000 M.W. cellulose membrane (reference number 10.17, Diachema AG, Zurich, Switzerland). In this work the chamber diameter and depth were approximately 1.1 mm and 0.5 mm, respectively, resulting in a volume of about 0.5 mL. Three pairs of capillary columns, oriented on opposite sides of the assembly, are

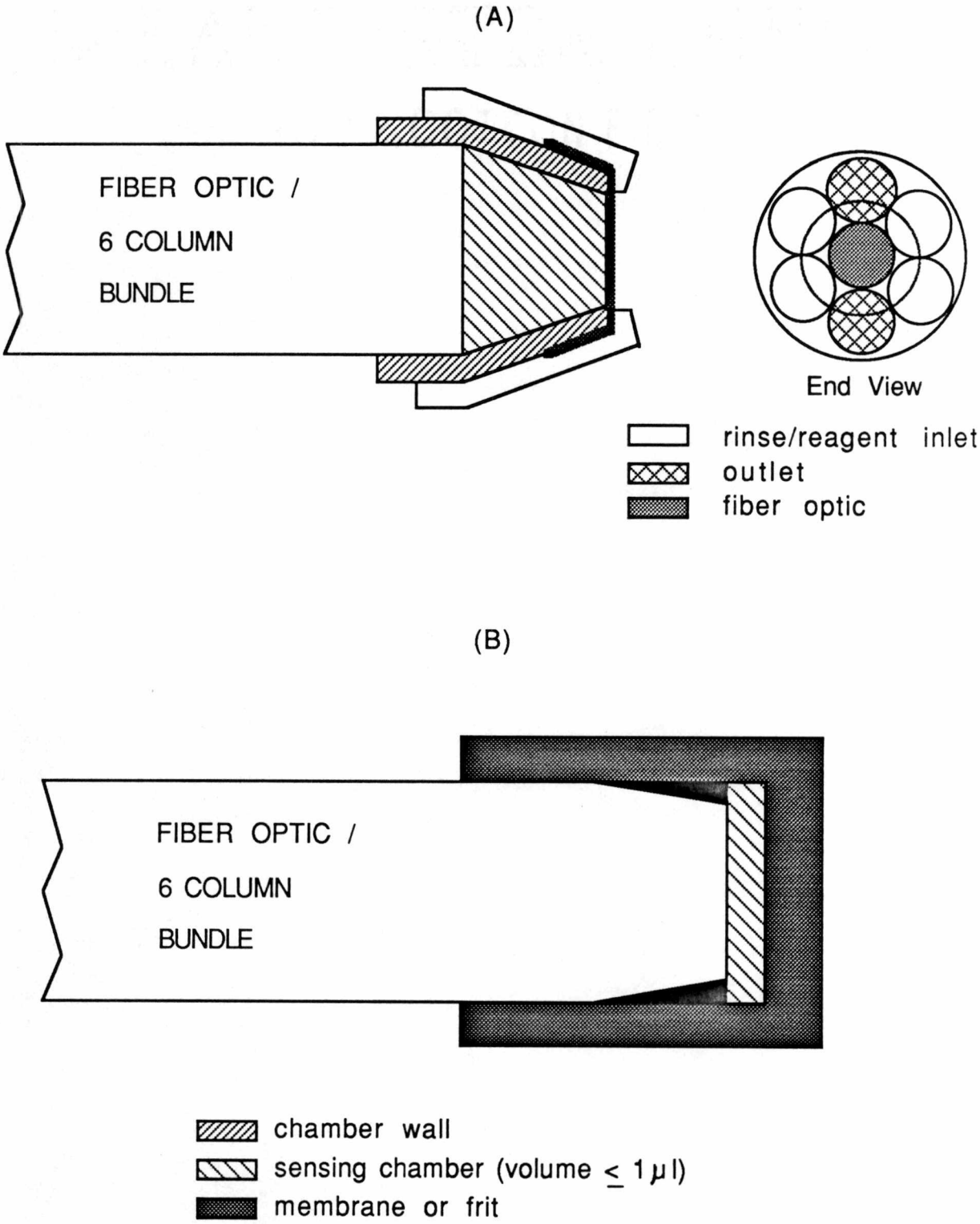


Figure 3.2 Terminal designs of MRB with membrane (A) and frit (B).

used to deliver anti-BPT solution, rinse solution and outlets. Each capillary pair is secured in a valved-luer adapter to facilitate connection to a syringe/syringe pump for reagent delivery. The entire MRB is purged with liquid in all applications, so as to be as bubble-free as possible. The aspiration-based MRB is constructed by afixing the hollowed stainless steel frit (Newmet Krebsoge, Terryville, CT) onto the end of the capillary assembly. The 0.5 μm porous frit was 4.8 mm long, 2.5 mm o.d. and was formed with a 4.0 mm deep, 1.5 mm diameter hole. Two capillary columns are attached to a 3-way luer lock stopcock and used to aspirate the solution and push the sample back through the sensing chamber to permit binding to the entrapped immunobeads. Two capillaries are used as outlets, one capillary is used for immunobead injection via an HPLC 6-port injection valve, and the remaining capillary is used for rinsing.

Sensor operation: Normal operation for the diffusion MRB direct assay involves the following steps: (1) the capillary columns are filled with the appropriate reagent solutions, (2) the sensor is placed in the sample, (3) anti-BPT solution is delivered to the sensing chamber and a baseline signal level is measured , (4) analyte is allowed to diffuse into the sensing chamber for 20 min, (5) signal (i.e., the increase in signal relative to the established baseline signal level) is measured, and (6) the sensor is recycled by flushing-out the used reagents and delivering fresh antibody solution to the sensing chamber. Ideally, the effects of non-specific interferents can be minimized by rinsing the antibody-BPT complex, with the outlets sealed, to expel interferents through the membrane. This procedure proved unsuccessful with this design and led to the

utilization of the aspiration-based MRB (see Results and Discussion Section).

The aspiration MRB is operated by (1) filling the capillaries with the appropriate solutions, (2) placing the sensor in the sample, (3) aspirating sample through the frit and into the aspiration capillaries, (4) injecting and delivering immunobeads into the sensing region through the HPLC injection valve, (5) slowly passing the aspirated sample through the immunobeads with the outlets sealed, (6) washing the chamber and immunobeads to remove interferents, (7) measuring the resulting signal and finally, (8) recycling. These steps can be performed in about ten minutes. The 3 way stopcock permits the delivery of reproducible volumes (about 100 μL in this case) of aspirated sample. The reproducibility of the delivery system of immunobeads is improved by use of a fixed-loop HPLC injection valve. In this work a 20μL volume of a 22 mg/mL immunobead slurry was introduced into the chamber. Anti-BPT was covalently attached to silica beads using 1,1' carbonyldiimidazole as previously described. The average bead coverage was 11.0 mg anti-BPT/ g bead.

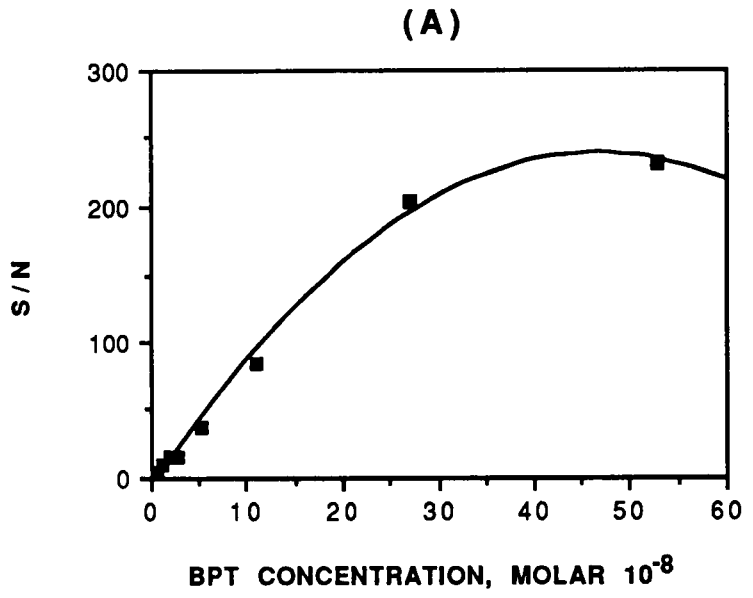
RESULTS AND DISCUSSION

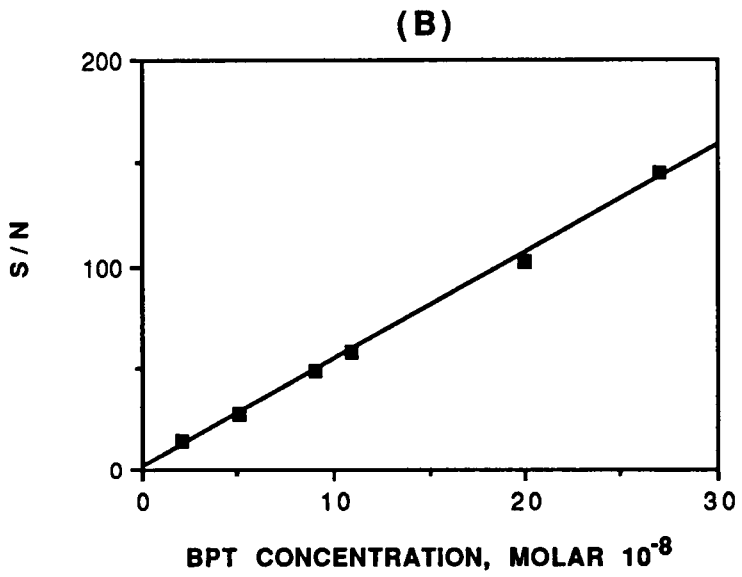
The sensors (MRBs) described in this report are designed to perform remote, in situ fluoroimmunoassays in a repetitive fashion. Although many assay protocols are possible, this work focused on the measurement of BPT, a natural fluorophor, which can be measured using a direct assay protocol (i.e., no fluorescent label is

required). The usual analytical figures of merit were evaluated. The principle feature of merit of the MRB is that it is capable of relatively rapid, repetitive, and selective (with appropriate rinsing) equilibrium measurements without being removed from the sample.

Diffusion-based MRB: The response of this sensor was evaluated by establishing dose response curves over the range of 5.3×10^{-9} M to 5.3×10^{-7} M BPT using the procedure described in the experimental section. The response (signal level over background after a 20 minute incubation with the investigated sample) is plotted in Figure 3.3a. The last data point in the curve exhibits considerable saturation (i.e., the 1.9×10^{-6} M solution of anti-BPT in the sensing chamber is significantly depleted via the information of immune complex for the highest concentration sample). Ignoring the last data point the correlation coefficient of the curve is 0.9996. The dynamic range can be extended, at the expense of sensitivity, by simply measuring signals after shorter incubation times. The baseline noise, the relative standard deviation (RSD) of the peak signals resulting from 1 second exposures to laser excitation, was approximately 1 nA. Extrapolation of the dose-response curve to a S/N level of 2 yields a limit of detection for these measurement conditions of 3.8×10^{-9} M or 1.2×10^{-10} g in a 100 µL sample. However, the detectability observed for this MRB is substantially enhanced by the concentrating effect of the anti-BPT [6]. For example, the S/N for a 1.1×10^{-7} M BPT solution was 16 and 84 without and with anti-BPT in the sensing chamber respectively.

Figure 3.3 Dose-response curves for (A) diffusion based MRB and (B) aspiration based MRB.


(A)
300
200
100
0
S / N
0
10
20
30
40
50
60
BPT CONCENTRATION, MOLAR 10-8


(B)
200
100
0
S / N
0
10
20
30
BPT CONCENTRATION, MOLAR 10-8

Measurement precision was evaluated by performing eight replicate measurements of a $2.7x10^{-8}$ M BPT solution. The mean S/N of 17.6 exhibited a RSD for these measurements of 10.1%. Membrane memory effects probably degrade precision. The BPT is very soluble in the thin (7μm) membrane and requires significant rinsing to completely remove. Nevertheless, by rinsing between measurements with anti-BPT solution (a costly procedure) and plotting increases in signal over background, that changed with use, reliable dose-response curves could be generated, even if standard solutions of decreasing BPT concentration were sequentially measured.

While the above experiments demonstrate the true sensing capabilities of this MRB, efforts to exploit the specificity of antibody-antigen binding to enhance analytical selectivity were unsuccessful. Large numerical aperture optical fibers have viewing depths of about two fiber diameters[1]. As one travels from the distal face of the fiber, the fraction of fluorescence emission hitting the distal face of the fiber decreases but a higher fraction of that experiences total internal reflection (TIR) within the fiber. The probability of TIR occurring is defined by taking the ratio of the volume of the acceptance cone to the volume of the corresponding sphere with a radius equal to the height of the cone (i.e. the diameter of a f/1 fiber). For a 200 μm, 0.45 numerical aperture fiber with the fluorescence species 2 fiber diameters away, the probability of the fluorescence emission being transmitted is 1.6%. This leads to the general rule of a 2 fiber diameter sensing depth for large numerical fibers. In order to restrict signals to the sensing chamber, the distance between the fiber terminus and the

transparent membrane used in this MRB was made relatively large. The process of rinsing the immune complex after incubation to eliminate interferents forces the complex to the membrane. Thus, signal levels were nearly eliminated and required long periods to recover following the rinsing step. Moreover, when the system was not completely purged of air bubbles, the signal often did not completely recover. These problems were largely eliminated with the implementation of the aspiration-based MRB that employs solid phase antibody and a nontransparent frit to isolate the antibody.

Aspiration-based MRB: The dose-response curve was established by evaluating the response of the MRB over the range of 2.1×10^{-8} M to 2.7×10^{-7} M BPT. The response is plotted in figure 3.3b. The correlation coefficient of the curve is 0.9989. The aspiration MRB does not appear to be as sensitive as the diffusion MRB. The sensitivity and dynamic range of the aspiration MRB may be improved by decreasing the rate of flow of sample through the immunobeads. A stop flow technique where the flow is stopped when the antigen peak is within the immunobeads would also increase sensitivity and dynamic range by increasing the interaction time for the antibody-antigen. Extrapolating the dose-response curve to an S/N of 2 yields a limit of detection of 1.4×10^{-9} M BPT or 4.5×10^{-11} g for the 100 µL volume sampled.

The aspiration-based MRB exhibited an RSD of 4.8% based on five measurements of 2.7×10^{-7} M BPT with a S/N average of 145. The improvement over the diffusion MRB may be attributed to the lack of memory effect observed by the stainless steel frit. The immunobeads may also align themselves more reproducibly in the

sensing chamber compared to the antibody solution where internal concentration gradients may effect the signal. The same improvement was seen when going from a liquid phase FOCS (RSD≈ 15%) to the same FOCS with a solid phase (RSD≈6%) [6].

Selectivity of the MRB was demonstrated by sampling BPT along with the polynuclear aromatic chrysene in the same solution. Figure 3.4 shows the fluorescence emission spectra (λ_{ex}=325 nm) of (a) BPT, (b) chrysene, and (c) BPT/chrysene mix showing that chrysene does interfere spectrally. The concentrations of the chrysene and BPT were 6.9×10^{-6} M and 2.7×10^{-7} M respectively. Figure 3.5 summarizes the results of the interference study. The aspiration signal for the mixture and the two individual compounds added together are essentially the same. The maximum signal is likewise essentially the same for the mixture and the two compounds. During the rinse period, the chrysene in the mixture is more effectively removed than when run alone. This may be due to the hydrophobicity of chrysene as it interacts with the BPT more than the aqueous solution and is rinsed out with the non-bound BPT. However, at 20 min, the mixture signal is the same as the BPT signal with the chrysene signal practically zero indicating that no chrysene has been retained.

Although conditions were not optimized in terms of flow rate, bead density, and sample volume, the aspiration-based MRB was capable of performing a sensitive, low volume repetitive direct assay of BPT. The sensing chamber configuration is another possible area where improvements could lead to increased sensitivity. As

Figure 3.4 Fluorescence emission spectrum of (A) BPT, (B) chrysene, and (C) BPT/chrysene mixture. (λ_{ex}=325 nm)

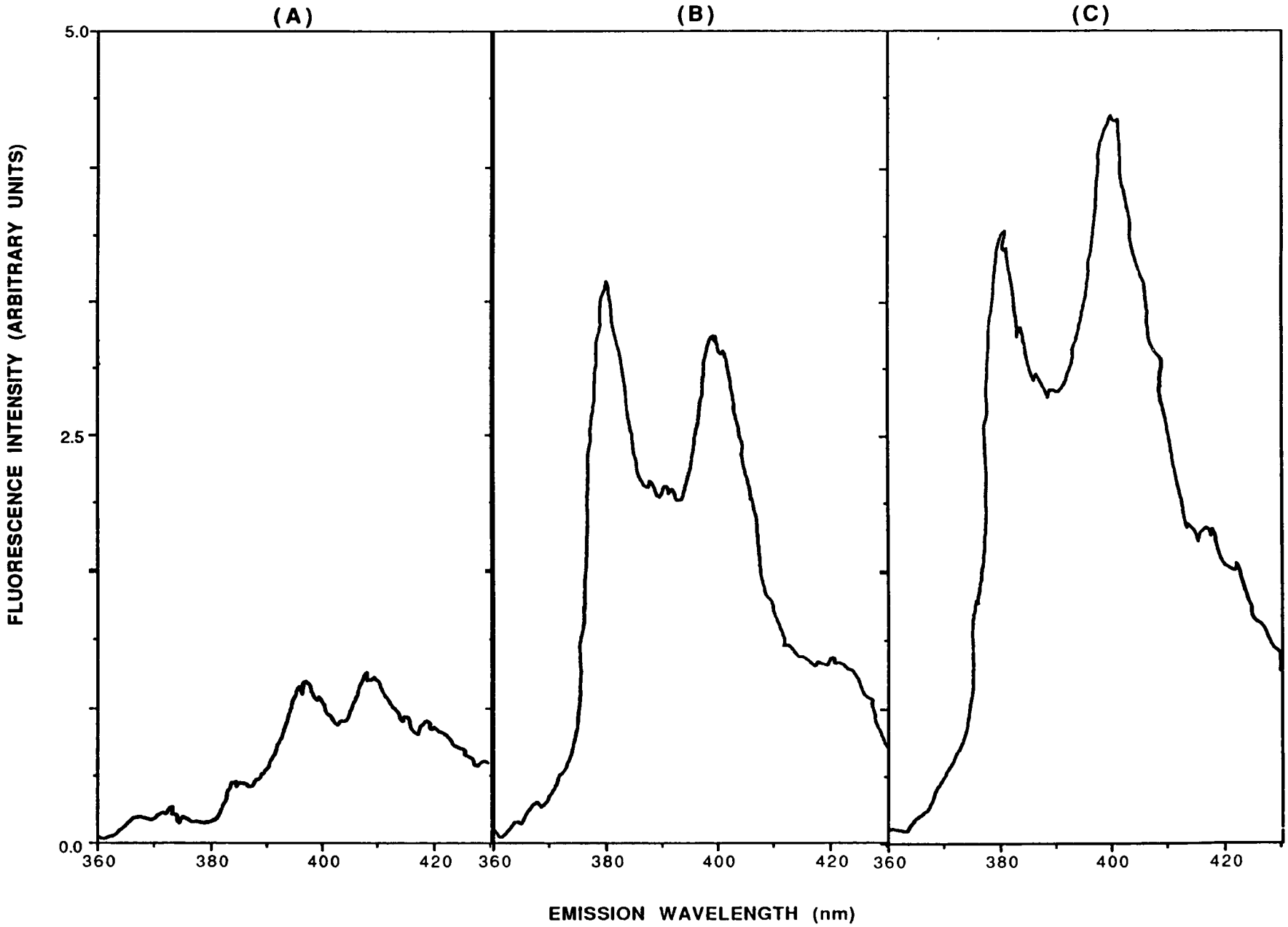
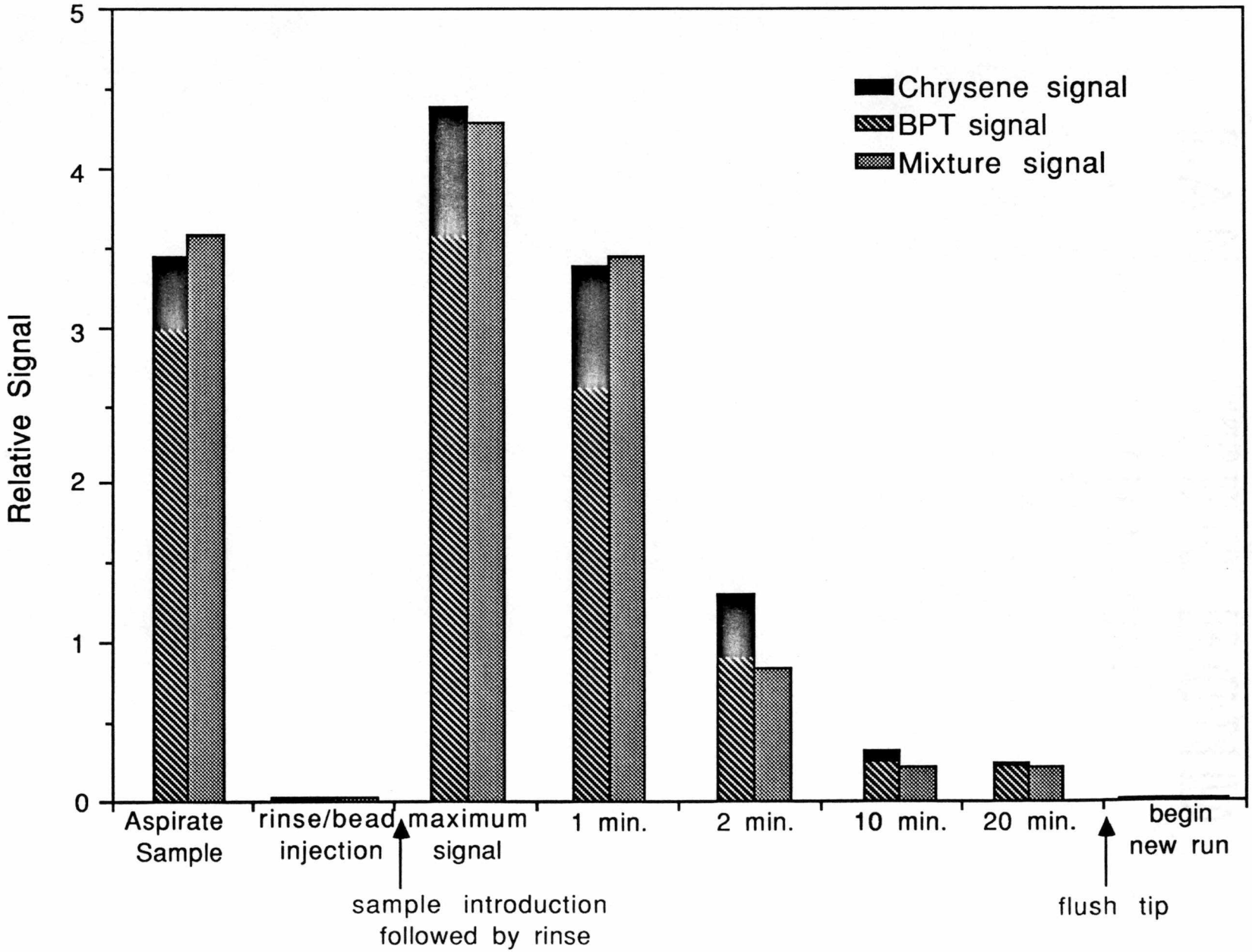

(A)
(B)
(C)
5.0
2.5
0.0
360
380
400
420
FLUORESCENCE INTENSITY (ARBITRARY UNITS)
EMISSION WAVELENGTH (nm)

Figure 3.5 Interference study in PBS using the PNA chrysene as a spectral interferent.


Relative Signal
5
4
3
2
1
0
Chrysene signal
BPT signal
Mixture signal
Aspirate Sample
rinse/bead injection
maximum signal
1 min.
2 min.
10 min.
20 min.
begin new run
sample introduction followed by rinse
flush tip

seen in figure 3.2 on page 30, tapering of the inside walls would help to hold more of the immunobeads in the center of the chamber and thus in the fibers field of view. Non-porous sides would also serve to hold the immunobeads in the field of view as the sample could not be flushed out the sides taking the immunobeads with it. This step may also increase the number of interactions between antigen and antibody as the sample would have to flow through the majority of immunobeads before diffusing through the end of the frit. Other possibilities for increased sensitivity include increasing the number of capillaries used to aspirate the sample which should increase mixing in the chamber when pushed back through the sensing chamber. The diffusion-based MRB however, was limited in its ability to rinse interferents from the sensing chamber, therefore limiting the usefulness as a sensor.

CHAPTER 4

CONCLUSIONS

In terms of potential antibody-based, regenerable FOCS applications, the two most important criteria for the evaluations of antibody immobilization techniques described herein are (1) the solid supports that are employed must possess physical properties that permit efficient delivery/removal to the sensing terminus of the fiber and optical properties that allow large signal levels and (2) large amounts of active antibody must be immobilized. With regard to the latter criteria, the CDI and Protein A immobilization approaches performed the best in these studies. For example, previous work with antibody-based, non-regenerable sensors, possessing somewhat similar sensing termini, resulted in LODs of 10^{-9} M (sub picomole amounts) for BPT [6]. This indicates that the amount of BPT that could be bound at saturation by the estimated 0.1-0.5 mg of CDI immunobeads that would be delivered to the regenerable sensing terminus (i.e., 10-50 pmole based on Table I data) should provide reasonably large S/N.

Care should be taken not to over-extrapolate these results. CDI binds antibody non-specifically and this could be more problematic with antibody/antigen systems other than the two investigated in this work. Moreover, the Protein A exhibited significant nonspecific adsorption, which could be more severe with other systems. Nevertheless, these studies provide a necessary framework for future antibody-based FOCS development.

The MRB design allowing reagents to be delivered and removed from the sensing terminus, permitting a variety of affinity assay procedures to be performed. The aspiration-based MRB shows more promise than the diffusion-based MRB because of the ability to more readily remove interferents. Further optimization of operating parameters should reduce the limit of detection as well as improve the dynamic range and sensitivity.

Since the majority of antigens are non-fluorescing, extension of the MRB to sandwich assays would be the next step in development. The operating parameters will be the same as direct assays up until the second labeled antibody is delivered to the sensing region. Once this step is accomplished, the majority of antigens should be detectable by either direct or sandwich assays using the MRB. Further versatility of the MRB would be that different antigens could be performed sequentially as the fluorescent label of the second antibody can remain the same and there would be no need to change the excitation source.

Using instrumentation developed for HPLC and LC systems, automation of the assay procedures involved with using the MRB is conceivable. The MRB would then be able to perform remote, low volume, regenerable assays continuously, with the only intervention needed being to replenish the reagents used in the assays.

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VITA

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