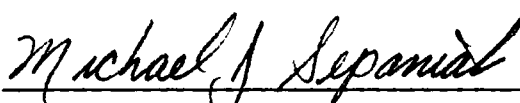
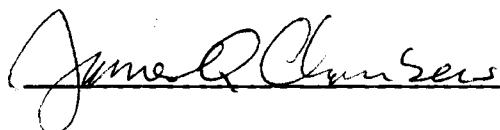


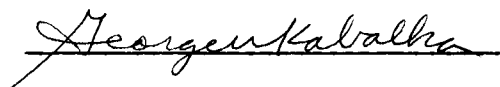
To the Graduate Council:

I am submitting herewith a thesis written by Randy Dell Petrea entitled "Fiber Optic-Based Time-Resolved Fluorimetry for Immunoassays." 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
Dean of the Graduate School

FIBER OPTIC-BASED TIME-RESOLVED FLUORIMETRY FOR IMMUNOASSAYS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Randy D. Petrea

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ABSTRACT

Rare earth metal chelate fluorescent labels for immunoassays possess extremely long fluorescence lifetimes and permit the effective use of time-resolved detection. This is shown to be very important in fiber optic-based fluorimetry, which is a technique that ordinarily exhibits large signal backgrounds due to backscatter radiation. Using time-resolved detection to reject the backscatter radiation, the fiber optic-based limit of detection for Eu-2-naphthoyltrifluoroacetone is 10^{-12} M; nearly three orders of magnitude lower than for the fiber optic-based measurement of the most common fluorescent label, fluorescein isothiocyanate. Commercially available Eu-chelate labeled reagents are used to demonstrate the potential utility of time-resolved fluorimetry in fiber optic-based immunoassays. Rabbit Immunoglobulin G (IgG) is covalently bonded to the distal end of quartz optical fibers prior to exposing to Eu-chelate labelled anti-rabbit IgG. The limit of detection for the assay is approximately 1×10^{-4} mg/mL.

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CHAPTER I

INTRODUCTION

A. Immunoassays

Immunoassay is based on the antigen-antibody reaction, which is the first step in the body's immune system(1). The presence of foreign matter in the body stimulates the production of antibody proteins, which when released in the bloodstream, specifically bind with the foreign material. Antibodies belong to the group of proteins known as immunoglobulins and it is the class of immunoglobulin G (IgG), which is used in immunoassay(1). These proteins, which have a molecular weight of about 160,000, contain a variable amino acid sequence that develops a complementary fit for the foreign matter (antigen). For a molecule to elicit an immunogenic response (cause the production of antibodies) it must be of molecular weight greater than 5000. Smaller molecules, haptens, when covalently attached to natural immunogenic "carrier" proteins, such as bovine serum albumin (BSA), can produce an immunogenic response. The antibody mixture that is produced will contain both carrier protein and hapten specific antibodies.

In order to obtain antibodies for use in immunoassay, an animal (e.g., mouse, sheep, or rabbit) is injected with an immunogen. After an appropriate time period, the animal is bled and the serum is collected. Since the antiserum contains not only specific, but also nonspecific proteins, it is necessary to isolate the specific antibodies from the other serum constituents. This can be achieved by the

immunoabsorbant technique, in which antigen that specifically binds with the desired antibody is attached to a solid support(2). The antibody may then be washed from the solid phase by adjusting the pH to levels where the antigen-antibody binding is disrupted without causing the antibody to denature. Better quality antibodies can be obtained using the monoclonal procedure(3). In this procedure, antibody-producing spleen cells of a laboratory animal are fused with laboratory grown tumor cells. The resulting hybrid cells (monoclones) produce large quantities of antibodies which are identical in chemical structure and binding affinity. In contrast, polyclonal antibodies (conventionally produced antibodies) are generally mixtures of antibodies that vary in antigen affinity and the binding sites on the antigen for which they are specific.

In immunoassay, an analyte is measured by exploiting the specificity of the antigen-antibody binding reaction in which the analyte is the antigen. The extent of the reaction is monitored by means of labelling either the antibody or antigen with a reagent that can be detected by some physical means. The assay determination then requires measuring either the bound form (antibody-antigen complex) or the free form (uncomplexed).

Labelled immunoassays were first performed by Yalow and Berson(1). There are two basic assay designs: (a) competitive assays (also called limited reagent) in which antigen and labelled antigen compete for a limited number of antibody binding sites, and (b) extraction assays (also called reagent excess and sandwich assays)(4). The most common

immunoassay procedure involves the competition within an incubation mixture of labelled antigen (Ag*) and sample antigen (Ag) for binding sites on a limited amount of antibody (Ab).

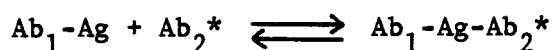


After a suitable incubation period, the free and bound fractions are separated, and the amount of antigen in the sample determined from measurement of the amount of labelled antigen in either the free or bound fraction. Comparison with a calibration plot (dose-response curve) yields the concentration of the antigen in the sample. The amount of labelled antigen that is bound is inversely related to the amount of antigen in the sample(4). In some assays, binding of labelled antigen modifies a property of the label and separation of the bound and free fractions is not necessary (nonseparation assay). For competitive assays, simultaneous addition of reagents and sample is generally most common, however, it has been reported that sequential addition sometimes improves the sensitivity(4). Also, the use of immobilized reagents simplifies separation of the bound and free fractions.

In extraction (sandwich) assays, reagents are present in excess. Antibody extracts antigen from the sample and then labelled reagent acts as a marker of the extracted antigen. In the one-site extraction process, immobilized antibody (Ab) extracts antigen from the sample, and in a second step, labelled antigen is bound to the remaining sites. The amount of antigen in a sample is inversely related to the amount of

bound labelled antigen(4). The two-site assay requires that the antigen has at least two combining sites with an antibody or antibody pairs. In this type assay, immobilized antibody (Ab) is specific for an antigenic determinant (binding side) on the antigen (Ag) and labelled antibody (Ab*) is specific for a different antigenic determinant of the antigen.

The immunoreactions can be depicted as follows:



Reagents can be either added simultaneously or sequentially(5). The amount of antigen in the sample is directly related to the amount of bound labelled antibody(4).

The type of label employed in an immunoassay is the major distinguishing factor between types of immunoassays. The label first used by Yalow and Berson(1) was a radioisotope and the term given to this assay was radioimmunoassay (RIA). RIA is still the most common immunoassay method being performed at this time.

In an RIA procedure, the antigen is labelled with a radioisotope. These labels are small in size and cause little change in the biological properties of the molecules being labelled. There are numerous radioisotopes that can be used as labels, however, iodine-125 has become the most popular(4).

Detection in RIA is achieved by either electrical measurement of ionization caused by radiation or by the measurement of visible light produced by the radiation arising from the decay of the radio-

isotope(4). Generally there are two types of instruments, the gamma counters (for gamma-emitting isotopes) and liquid scintillation counters (for gamma- or beta-emitting isotopes)(4,6).

Basically most RIA methods involve some kind of separation step in which the bound form is separated from the free form. Nonseparation RIA have been rarely used due to the difficulty in modulating the activity of the labelled material as a direct result of binding to an antibody or antigen(4). Therefore, most RIAs have been either competitive binding or extraction assays.

Despite the extreme sensitivity of these RIAs (detection limits of 1×10^{-17} moles for iodine-125 are common)(4), there exist certain disadvantages(1). Reagents prepared with iodine-125 have only a short effective shelf life ($t_{\frac{1}{2}} = 60$ days) and thus routinely performed assays must be supported by regular preparatory work. Also because the decay rate of the radiolabel is not altered when in the bound form, a separation is necessary. And lastly, due to public concerns on the safety of the use and disposal of radioactive material, radiation safety standards are likely to become more stringent in the future and thus hinder the use of RIA methods. Due to these disadvantages, researchers have been seeking alternate labels in order to perform nonradioactive immunoassays.

Among the alternate labels for immunoassays, fluorescent dyes have shown much promise. When the label on an antigen or antibody is a fluorescent material, the term given to the assay is fluoroimmunoassay (FIA). Fluorescent dyes, such as benzanthyl isocyanate, were

first used as labels for proteins by Creech and Jones(7). This work was later extended to antibodies labelled with benzanthyl isocyanate and other fluorescent dyes(8), and to antibody precipitation titrations(9). Subsequently, the use of fluorescent dyes as labels for antibodies and antigens has provided a plethora of immunoassay techniques, particularly in nonseparation techniques for which binding modulates the fluorescent signal of the label such that the bound and unbound labeled reagents can be spectroscopically distinguished.

As indicated above, FIAs can be separated into two classes, separation and nonseparation FIAs. The former group is subdivided into assays that are competitive and those that involve an extraction step. Nonseparation assays are subdivided according to the particular aspect of fluorescence that is modulated in the assay(4).

Because of the versatility of the fluorescence technique, a wide variety of separation and nonseparation assays have been developed. Almost all the separation techniques that have been applied in RIAs can be extended to FIA under the proper conditions. Separation of the bound and free form has been achieved by precipitation with neutral salts and by immobilizing a component of the assay (either antibody or antigen) onto a solid support(1). The types of solid supports employed include polyacrylamide beads(4), magnetizable cellulose particles(10), strips of polymethyl methacrylate(11), and quartz optical fibers(12).

Since the properties of fluorescent labels can be modulated when antigen binds with an antibody, numerous nonseparation techniques have been developed that could not be performed using radioactive labels.

Typical nonseparation techniques are quenching FIAs(13) (in which the fluorescent intensity of the label is diminished upon binding), enhancement FIA(14) (in which there is an increase in fluorescence of the label upon binding), polarization FIAs(15), and energy transfer FIAs(16). With the development of nonseparation techniques, the possibility of a continuous monitoring of antigen has been realized(17).

In order for a FIA analysis to be sensitive, the label must have high fluorescent intensity, which is distinguishable over background, and must not adversely alter the biological properties of the molecule being labelled. General requirements for fluorescent labels, reported by Soini and Hemmila(18), are as follows:

- 1) They should have a high fluorescent intensity; the molar absorptivity should be high ($>10^4 \text{ L mole}^{-1} \text{ cm}^{-1}$), and the quantum yield in the solvent used should approach unity.
- 2) In order to reduce the background fluorescence it desirable that the labels have a large Stokes' shift ($> 50 \text{ nm}$).
- 3) The label must be water soluble and form a stable covalent bond to the compound.
- 4) The conjugate must remain stable in storage and under measurement conditions.

There are numerous fluorescent dyes which have been used in FIAs(1), however, by far the most popular label has been fluorescein

isothiocyanate (FITC). Despite its small Stokes' shift (~30 nm), FITC has excellent spectroscopic properties. FITC possesses a high fluorescence efficiency, a high molar absorptivity, and a quantum yield approaching unity. It is readily excited in the violet and blue ranges of the spectrum by a variety of sources, and its emission at 520 nm, can be detected by common photomultipliers(1).

FITC attaches to the amino groups of the side chains of the amino acid in the labeled protein. The attachment of small groups of FITC onto proteins is generally not detrimental to the biological properties of the protein, however, due to the small Stokes' shift of FITC, simple spectral rejection of backscatter of radiation is not very efficient and large optical background levels have been observed(12), particularly when intense laser radiation was used for excitation.

Time-resolved fluoroimmunoassay (TR-FIA) provides a means to overcome these background problems. In TR-FIA, the difference in the lifetime of the signal of the label; relative to the nonspecific background (arising from sample matrix fluorescence), is used to temporally discriminate against the latter and thereby increase the sensitivity of the assay(19). In this type of assay, a fluorescent label is chosen whose lifetime (the time required for fluorescence emission to fall to $1/e$ of the initial intensity(4)) is significantly longer than the average background decay. Following excitation of the long-lived label with a short pulse of light, the backscatter and nonspecific background fluorescence decays relatively fast and, after a suitable delay period, the fluorescence signal collected is due solely to the fluorescence of

the label. Figure 1 illustrates the principles of time-resolved fluorescence.

The labels in TR-FIA must meet the same requirements as those of conventional FIA, but also meet the requirement that the radiative lifetime of the label must be at least 10-fold that of the background(18). Although pyridine derivatives meet these requirements, most fluorescent dyes do not.

An alternate type of fluorescent label, a rare earth metal chelate, offers unique and important characteristics for fluoroimmunoassays(18,20). The metal chelates which are used most often in FIAs are europium-(III) and terbium-(III) ions complexed to β -diketones and EDTA derivatives(19). Excitation of these labels involves absorption in the near UV spectral region by the ligand. This is followed by an energy transfer from the ligand's excited singlet state, through its triplet state, to the resonance levels of the rare earth ion. The metal ion emission exhibits a narrow band in the green to red visible spectral region. Concomitant with this energy transfer process is an unusually large Stokes' shift (typically 200-300 nm) and an extremely long emission lifetime (typically 0.1-1.0 ms). The former characteristic facilitates efficient spectral rejection of backscatter, while the latter characteristic permits the virtual elimination of optical background, including sample matrix fluorescence, via the use of time-resolved detection. Using the time-resolved technique, very sensitive fluoroimmunoassays of a variety of analytes have been performed(21-28). Unfortunately, assays using these metal chelate labels do have

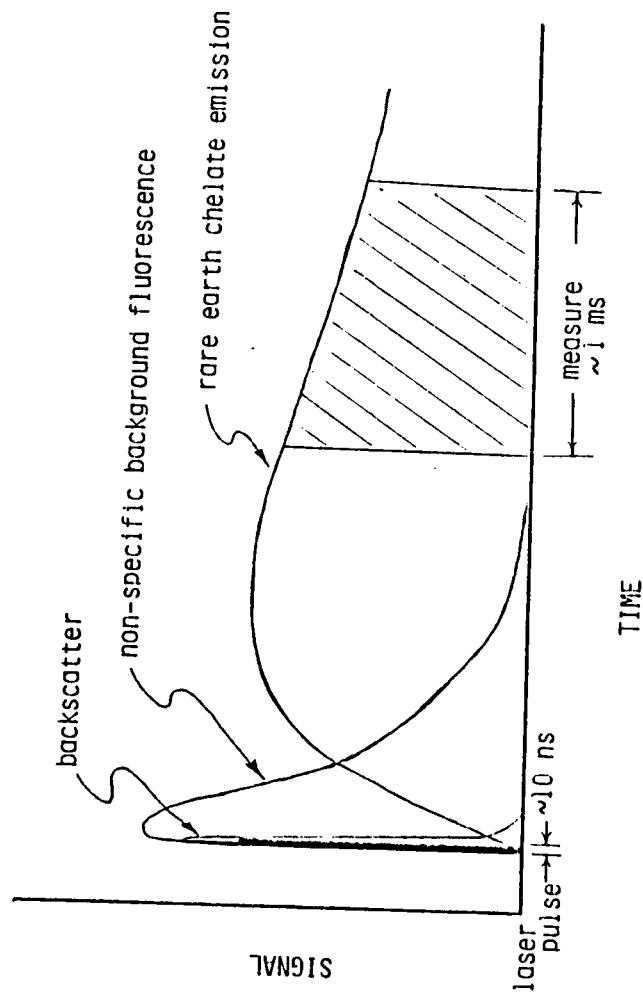


Figure 1: Principles of time resolved detection.

problems. Most significant are those of quenching by water in the solution sphere of the rare earth ion and the general instability of complexes of rare earth ions. These problems can necessitate fairly complicated assay protocols.

B. Fiber optic sensors

Technological developments and applications of fiber optics have increased dramatically in recent years. The impetus for this activity originates in the communications field. In order to fill the need to transmit light to and from remote places over long distances with minimal light loss, the communications field has developed extremely rugged fiber optics with high quality transmitting characteristics. With the development of these fibers, also came the realization of their potential use in spectroscopy and the field of medicine.

The concept of fiber optics is simple. Optical fibers operate on the principle of total internal reflectance. Fibers consist of a core (typically glass, quartz, or plastic) of refractive index n_1 , surrounded by a cladding (typically a polymer coating) of a lower refractive index, n_2 . Incident light that enters the core at an angle greater than the critical angle of the fiber is then transmitted through the fiber by multiple reflections at the core/cladding interface(29). (Figure 2). Light entering the fiber over an acceptance cone is transmitted. The acceptance cone half angle, a , is dependent upon both the refractive indices of the cladding and the core, as well as the refractive index of air, n_0 :

Figure 2: Optical fiber schematic. a = acceptance angle, n_1 and n_2 are the refractive indices for the core and the cladding interface, θ is the critical angle of total internal reflection which only occurs if light hits the interface at an angle θ or more.

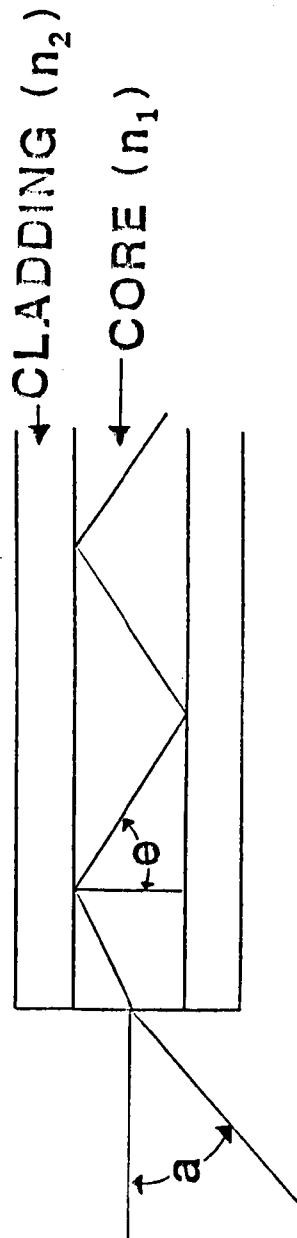


Figure 2:

$$\sin a = (n_1^2 - n_2^2)^{1/2} / n_0 \quad (1)$$

In the specific case of fiber optic sensors, light from a suitable source travels through the fiber to its terminus. Reflection, scattering or emission of radiation(i.e., the measured signal) is returned through the same fiber, or another fiber, to a light measuring device. At the sensing end of the fiber, the light may be reflected by transducers which alter the reflected light of interest, backscattered by the medium in which the fiber is inserted. In addition the returned light may be the emission of a luminous reagent at the end of the fiber that was excited by the incident light(31).

Various fiber optic applications have been developed for use in the field of medicine. For example, various fiber optic based endoscopes have been employed by physicians to view internal organs(32) Other medical applications include transillumination, surgical illumination, ophthalmoscopic instrumentation(33), photoinduced chemotherapy(31), and the placement of dosimetry tubes to detect small radiation doses within tissue(33).

There are basically three general types of fiber optic sensors: the optical, or bare ended fiber; the physical sensors, in which a transducer modifies a signal by some physical parameter of interest; and the chemical sensor, in which a reversible reagent at the end of the fiber provides some kind of spectrophotometric or fluorometric signal(31).

The oldest use of fiber optics as sensors involved the bare ended or optical sensors. Fiber optic bundles have been used to measure

oxygen saturation(34), circulatory activity(35), cardiac output(31), and the velocity of flowing blood in vessels(36). Fiber optic sensors have also been developed to measure the fluorescent signal from drugs possessing naturally fluorescent fluorophores(37), and for use in an absorbance probe(38).

Chemical sensors are the most recent type of fiber optic sensor to appear. Fiber optic chemical sensors (FOCS) have been developed which measure pH(39,40), pO_2 (41), and glucose(42). The advantage of fiber optic chemical sensors(FOCS), over their optical sensor counterparts, is that spectroscopic signals result from specific interactions between immobilized reagents and the analytes of interest. The interaction between the immobilized reagent and the analyte must be reversible if continuous monitoring is to take place. Immobilization is accomplished by a variety of methods, including trapping a reagent within a membrane(40,41,43), direct covalent bonding of reagent to the distal end of the fiber(44), and incorporating the reagent into a polymer phase that is covalently bonded to the fiber's distal end(45). These immobilization procedures are depicted in Figure 3. The method of immobilization and the amount of reagent phase can influence FOCS response characteristics(30,45,44).

In order to transmit light to and from the reagent phase, both bifurcated fiber optic (two or more fibers with a common end, Fig 4) and single fiber optic (Fig 5) designs have been employed. Bifurcated fiber design use separate fibers to transmit light to and from the reagent phase, whereas in the single fiber optic design, the excitation

Figure 3: Typical fiber optic sensor configurations.

- (a) Membrane consisting of reagent in a polymer phase.
- (b) Direct covalent bonding of reagent to the distal end of the fiber.
- (c) Reagent trapped within a membrane.

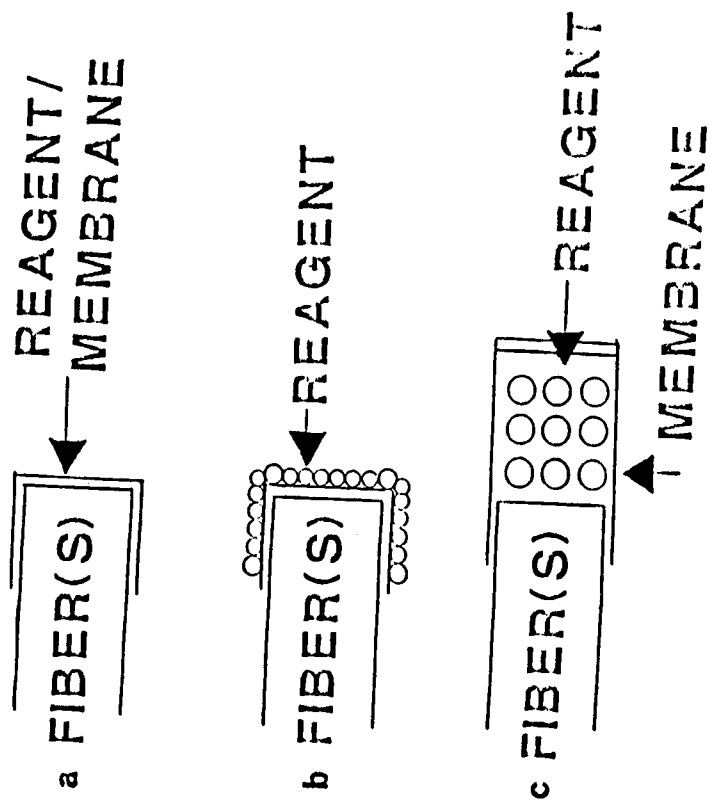


Figure 3:

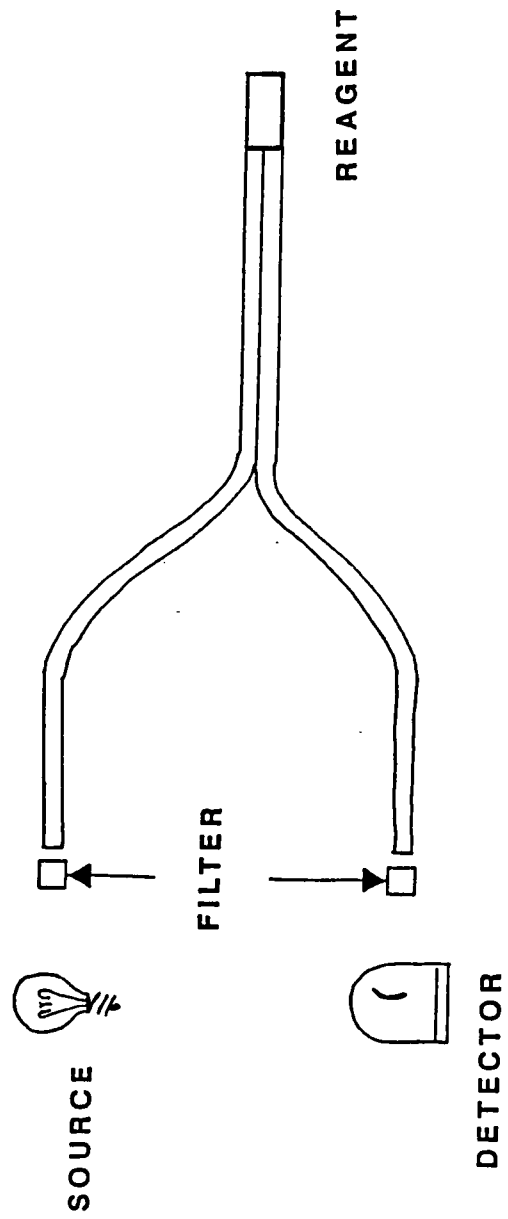


Figure 4: Typical fiber optic bundle design.

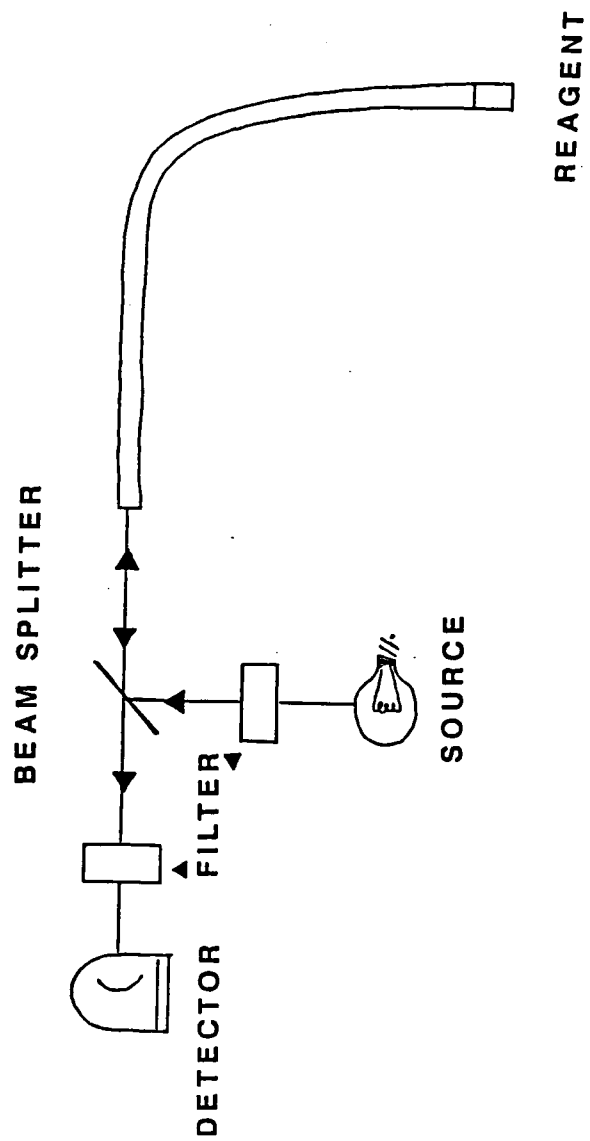


Figure 5: Typical single fiber optic design.

and emission radiation travels through the same fiber. The former design offers the advantage that the detection system is not subjected to the reflected radiation arising from the interfaces where the source radiation enters and exits the excitation fiber(29). This type design is extremely useful in fiber optic absorbance measurements since the measured signal is distinguished from the incident radiation by a change in intensity, rather than on the basis of a wavelength change. However, there are disadvantages in the use of bifurcated fiber optics in that (a) the sensors are larger and require twice as much fiber and (b) there is a certain volume at the terminus of the sensor which is not within the acceptance angle of both the excitation and emission fibers, and therefore is not viewed effectively(29).

When compared to bifurcated fiber designs, sensors that utilize a single fiber optic to transmit excitation radiation to the sample and signal radiation from the sample to the detector have advantages of better signal collection efficiency and smaller size. The latter attribute can be important in their eventual in vivo use. Unfortunately, single-fiber sensors are usually plagued by high optical background levels. This is due to the fact that a significant amount of excitation radiation is reflected at the fiber face and is not easily rejected by spatial filtering. Attempts to circumvent this backscatter radiation problem have included employing high rejection-ratio double monochromators(45), and grinding fiber faces at angles that minimize reflected optical background(46). Limitations to these

correction techniques include the low throughputs of double monochromators and the difficult, irreproducible nature of "angling" small quartz surfaces.

Recently the principles of solid-phase immunoassay have been applied to FOCS design(47-50). The fiber and immobilized receptors form a fluoroimmunosensor(FIS), that can be used to perform in situ fluoroimmunoassays. The FISs offer the potential for excellent analytical selectivity by exploiting the specificity of antibody-antigen reactions(19). FISs have been developed for direct assays of natural fluorophors(50) and for competitive binding assays(49). Furthermore, it should be possible to design FISs to perform "sandwich assays" and, using relatively low avidity antibodies so as to improve response times, for continuous monitoring.

In this thesis, an investigation of fiber-optic based measurements using europium-chelate labels and time-resolved fluorescence detection is reported. Temporal rejection of backscatter from the fiber optics, which is shown to be effective, allows for much more sensitive fiber optic-based measurement of Eu-2-naphthoyltrifluoroacetone than for FITC. Commercially available rare earth chelate labelled immunochemicals are used to demonstrate the potential utility of time-resolved fluorimetry in FIS measurements.

CHAPTER II

EXPERIMENTAL

A. Materials

Eu-Chelate labelled sheep anti-rabbit immunoglobulin G (IgG), enhancement solution, wash concentrate, and assay buffer were purchased from LKB Instruments, Inc., Gaithersburg, MA. The enhancement solution was composed of 1.0 g/L Triton X-100, 6.8 mmol/L potassium hydrogen phthalate, 100 μ mol/L acetic acid, 50 μ mol/L tri-n-octylphosphine oxide (TOPO), and 15 μ mol/L 2-naphthoyltrifluoroacetone. Rabbit IgG and ovalbumin were purchased from Cooper Biomedical Inc., Malvern, PA. 3-Glycidoxypyrpyltrimethoxysilane (GOPS) was acquired from Aldrich Chemical Co., Milwaukee, WI. All other reagents were supplied by Sigma Chemical Co., St. Louis, MO.

Multi-mode, fused silica, 600 μ m core diameter optical fibers (numerical aperture = 0.22) were obtained from Math Associates, Westbury, NY. These fibers were cut into 75 cm lengths and terminated at one end with an SMA connector (Math Associates). The ends were then successively polished with 15 μ m, 3 μ m and 0.3 μ m lapping film.

B. Apparatus

A Perkin Elmer 650-40 spectrofluorimeter was used to evaluate the IgG binding capacity of FIS sensors. Time resolved fluorescence measurements using fiber optics were performed with the apparatus shown in Figure 6. A Lambda Physik Model 50B excimer laser (emission

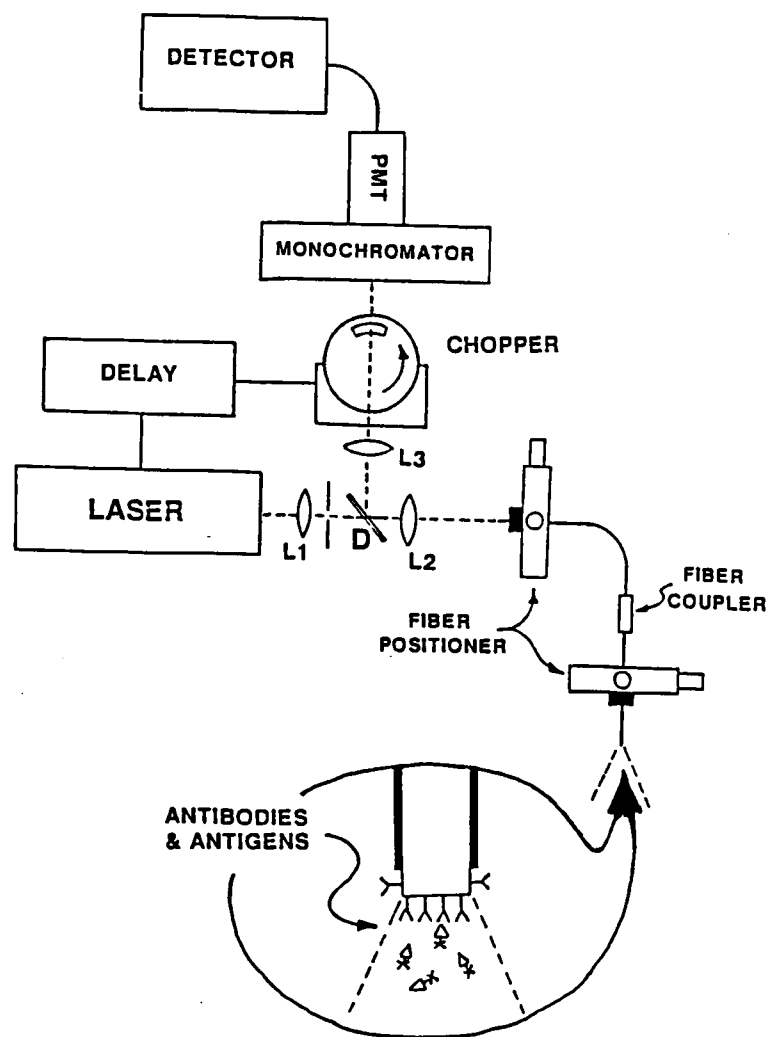


Figure 6: Diagram of apparatus used for FIS measurements.

wavelength 308 nm) was used as the excitation source for most of the time resolved fluorescence measurements. Alternatively, the excimer laser was used to pump a Lambda Physik Model FL2000 dye laser containing p-terphenyl laser dye, whose emission at 340 nm served as the excitation source in a determination of a limit of detection for Eu-2-naphthoyltrifluoroacetone. The excimer laser was operated in this work at 20 Hz and produced 10 nsec duration output pulses of approximately 20 mJ. The laser radiation was focused by a 38 mm diameter (f-number = 4.7) lens, L_1 , through an iris opening approximately 2 mm. The iris aperture served to limit the beam diameter and reduce the incident power to levels that generally did not damage the fiber optic. The radiation was then passed through a custom dichroic filter, D, (Newport Corporation, Fountain Valley, CA), which transmits 90% at 308 nm, and focused by a 25 mm diameter (f-number = 2) lens, L_2 , onto the incident end of the fiber optic. The Eu-chelate fluorescence ($\lambda_{\text{emission}} = 613$ nm) arising from excitation at the distal end of the fiber, was partially collected by the fiber, transmitted to and collimated by L_2 , and reflected by the dichroic filter (reflectivity = 60% at 613 nm). The collimated radiation was focused by a 25 mm diameter (f-number = 4) lens, L_3 , through a modified chopper (Ortec Model 9479) and onto the entrance slit of an Instruments SA, Model H-10, monochromator (f-number = 3.5). Photocurrents generated by a cooled (-25°C) RCA C31034B photomultiplier tube (PMT), operated at 1800 V, were measured by either a Pacific Precision Instruments, Model 125, quantum photometer in the current or photon count mode, or by an EG&G, Model 165,

boxcar detector. Data were collected and recorded on a stripchart recorder.

The chopper modification involved replacing the stock aperture wheel with one that contained a single 1/15th sector aperture. Thus, operation at 20 Hz resulted in a 3 ms optical gate. The synchronous output of the chopper was fed to a laboratory-built delay circuit, (Fig. 7), which then triggered the laser. The temporal relationship between the chopper gate and laser pulse was adjusted with the delay circuit. The delay timing was optimized by placing the distal end of the fiber alternately in Eu-chelate and solvent solutions and adjusting the delay to maximize signal to background levels. The size of the imaged signal at the chopper wheel was slightly less than 2 mm and this resulted in an effective gate opening time of about 100 μ sec. Detection with this gated system was ultimately limited by timing jitter, which was approximately a few hundred μ sec, as observed with an oscilloscope at the synchronous output of the laser.

C. Procedures

Fiber Silanization with 3-Glycidoxypropyltrimethoxysilane

The outer claddings of the fibers were stripped back 3 mm from one end in order to expose the quartz core. The stripped ends were then rinsed in hot nitric acid, then distilled water, and submerged in a 10% aqueous GOPS solution at 90 °C for 3 hours. The pH of the solution was maintained at 3 by addition of 1 M HCL. The GOPS fibers were then dried overnight at 105 °C.

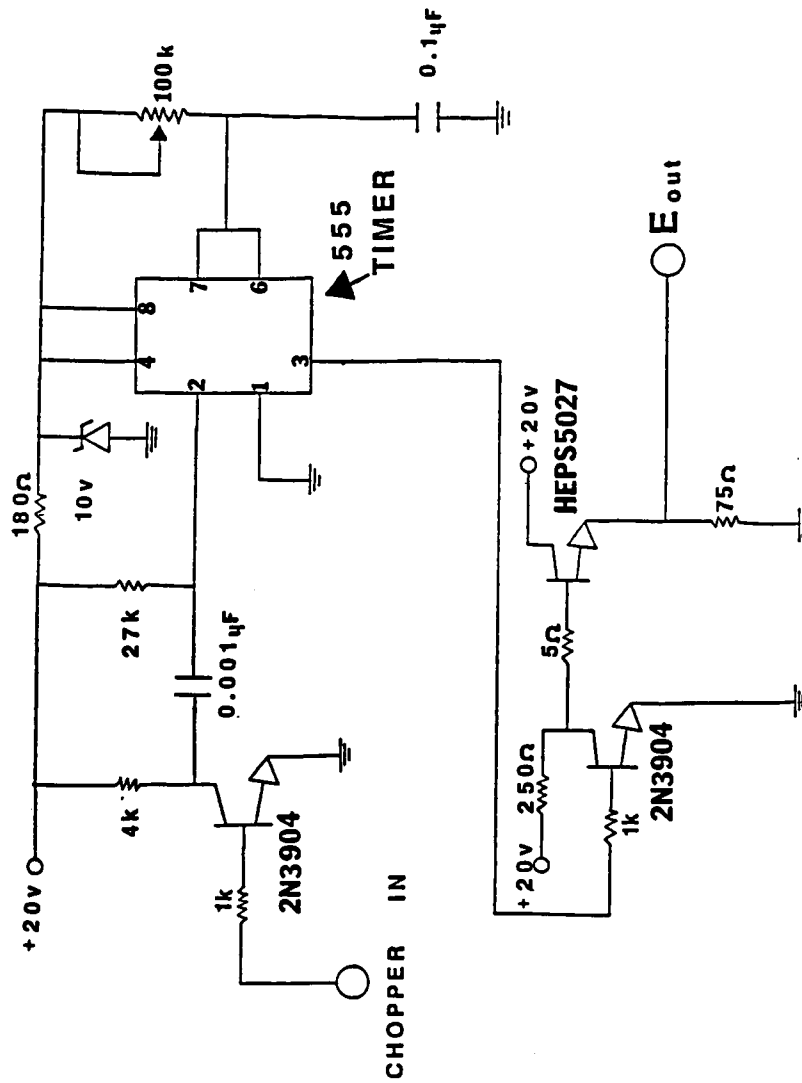


Figure 7: Schematic of the delay circuit.

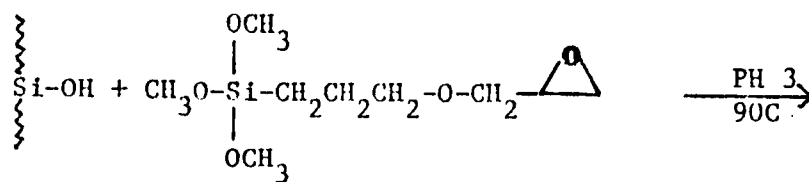
Fiber Activation and Protein Attachment

GOPS derivatized fiber were oxidized in 0.1 M periodic acid for 1 hour at room temperature and then washed with distilled water and phosphate buffered saline (PBS), pH 7.4, before being suspended in polypropylene vials. The vials contained 1 ml of 5 mg/ml rabbit IgG. Fiber bonding reactions were carried out for 24 hours at 4 °C. During this time, covalent bonds between the aldehydic groups of the oxidized GOPS and the amino groups of the protein were formed via Schiff base reactions. The fibers were then placed in 1 mL of PBS, and three 2 mg portions of NaBH_4 were added at 15 minute intervals, in order to reduce the Schiff bases. Fibers were stored in a 1% ovalbumin in PBS solution at 4 °C. The reaction sequence is shown in Figure 8.

D. Measurements

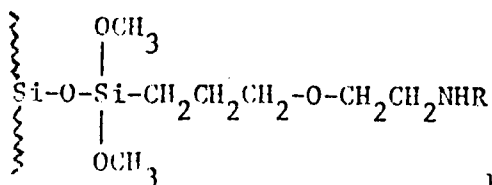
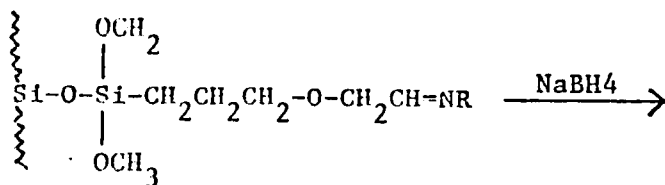
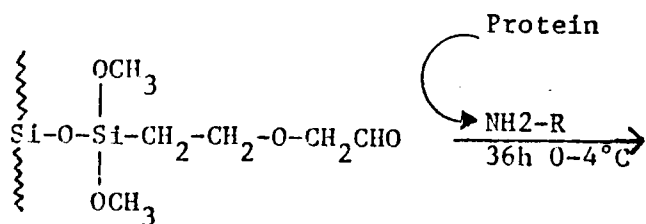
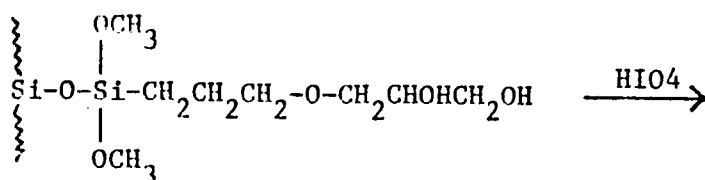
Determination of Fiber Binding Capacity

The plastic cladding on the optical fibers was stripped from the quartz core in order to expose 1 cm sections of quartz. Rabbit IgG was bonded to the sections using the procedure described above. After incubation of the sections in a 0.01 mg/ml solution of Eu-chelate labelled anti-rabbit IgG for 48 hours at 4 °C, the sections were rinsed with PBS and placed in 1.5 ml of the enhancement solution. It was assumed that this long incubation resulted in complete saturation of the fiber surface with anti-rabbit IgG. In the enhancement solution, the Eu ion dissociates from the antibody bound chelate and forms a highly fluorescent chelate with the 2-naphthoyltrifluoroacetone in the



QUARTZ
SURFACE

GOPS



R = Protein

Figure 8: Immobilization reaction sequence.

enhancement solution. The binding capacity was determined by measurement of the resulting Eu-2-naphthoyltrifluoroacetones fluorescence ($\lambda_{\text{ex}} = 337 \text{ nm}$, $\lambda_{\text{em}} = 613 \text{ nm}$) with the Perkin Elmer 650-40 spectrofluorimeter and comparison to a standard curve (Fig. 9). A value of $1 \mu\text{g}$ of labelled IgG/cm² of quartz fiber was obtained.

Comparison of Conventional FITC and Time-Resolved Eu-Chelate Limits of Detection.

Limits of detection for FITC were determined for measurements in a standard cuvette and for fiber optic-based measurements. Excitation was provided by a Spectra Physics, Model 171, Argon ion laser ($\lambda = 488 \text{ nm}$, 30 mW). For the cuvette measurements, the laser radiation was focused by a 25 mm diameter (f-number = 2.0) lens through a 1.0 cm cuvette. The FITC fluorescence ($\lambda_{\text{max}} = 523 \text{ nm}$) was collected at 90° with a 25 mm diameter (f-number = 4) lens and imaged onto the entrance slit of the monochromator. Photocurrents generated by an RCA 1P28 PMT, operated at 700 V, were measured with the photometer. The instrumental arrangement shown in Figure 6 and described previously was used for the fiber optic-based measurements, except that the laser, dichroic filter, lenses, and PMT (see above) were chosen for the measurement of FITC. Calibration plots, using standard solutions of FITC in borate buffer, pH = 9.3, were obtained and limits of detection were determined as the concentration yielding a signal that was twice the noise of a blank.

Using similar procedures, and the laser and optical components described previously, the limits of detection for Eu-2-naphthoyltrifluoroacetone were also determined for measurements in a standard

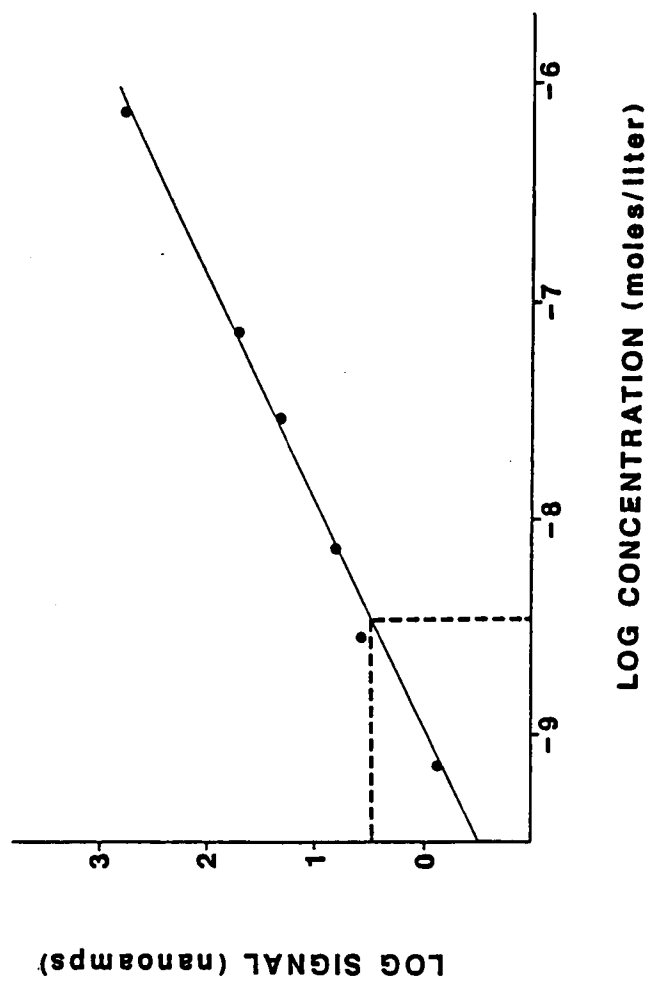


Figure 9: Standard curve for the determination of the binding capacity for the Eu-chelate.

cuvette and for fiber optic-based measurements. Three different forms of signal recovery were employed: (1) electronically gated detection with a boxcar, (2) optically gated detection with a photometer and (3) optically gated detection with a photon counter.

Time-Resolved Fluorescence Measurements Using FIS

FIS prepared in accordance to the aforementioned procedure, were incubated in varying concentrations of diazophenyl-EDTA-Eu-labelled anti-rabbit IgG for 14 hours (overnight) at 4 °C. The sensors were then rinsed with and stored in wash concentrate until ready for use. The sensors were then coupled using standard SMA connectors to the apparatus shown in Figure 6. The fibers were then translated at 1 mm into 1.5 mm diameter micro-wells, drilled in a Teflon pad. The micro-wells were filled with 10 µl of the enhancement solution. The fiber was left at this position for 1 minute, translated back to a pre-determined position, and fluorescence was measured after a 1 minute interval (Fig. 10). The fiber was then translated out of the micro-well and the fiber and micro-well were thoroughly washed with enhancement solution. The fiber was returned to the well for a measurement of the optical background.

- Figure 10: Diagram of the immunochemical measurement procedure.
- (a) Dissociation of Europium ion in enhancement solution;
Y - immobilized rabbit IgG; ● - Eu ion; —● chelate with Eu-ion; 4—● - Eu-chelate labelled antibody; O - 2-naphthoyltrifluoroacetone, forms highly fluorescent complex with Eu-ion.
 - (b) Measurement after translating fiber lmm.
● - Eu-2-naphthoyltrifluoroacetone, highly fluorescent complex; O-2-naphthoyltrifluoroacetone.

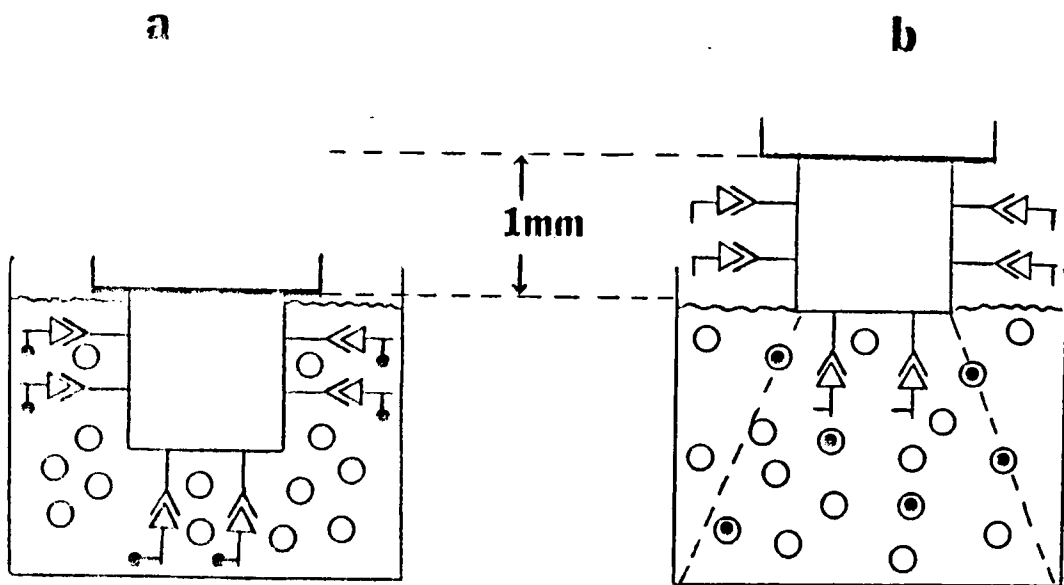


Figure 10:

CHAPTER III

RESULTS AND DISCUSSION

The data presented in Table I illustrates the efficacy of time resolved detection at improving the limit of detection in fiber optics-based fluorimetry. No immunochemicals were employed in that initial study. The limits of detection for cuvette measurements demonstrate the excellent sensitivity of laser fluorimetry. Although the optical systems (lasers, lens, etc.) and detectors were different for the FITC (conventional) and Eu-2-naphthoyltrifluoroacetone (time-resolved) measurements, the limits of detection are comparable and extremely low. The Eu-chelate molar absorptivity at the excimer laser output (308 nm) is approximately 40% of its λ_{max} value. Furthermore, the pulse to pulse energy reproducibility of the excimer is rather poor ($\pm 10\%$). If these factors are corrected, the time-resolved detection of the Eu-chelate might exhibit a lower limit of detection than FITC.

The time resolved cuvette measurements were performed using a boxcar detector. Considering the extremely long lifetime of the Eu-chelate it was a relatively simple matter to temporally reject the laser specular scatter and the Rayleigh and Raman scatter of the solvent. A 1 μsec delay and 1 μsec gate window were employed with the detector. Moreover, the specular scatter occurring at the cuvette walls was easily rejected spatially by imaging the path of the laser beam through the cuvette, onto the monochromator, such that the

TABLE I. Comparison of Limits of Detection

	FITC ^a	Eu-2-naphthoyletri- fluoroacetone ^b
Cuvette	4×10^{-14} M	8×10^{-14} <u>M</u> ^c
Fiberoptic	7×10^{-10} M	3×10^{-8} <u>M</u> ^c
		2×10^{-12} <u>M</u> ^d
		1×10^{-12} <u>M</u> ^e
		9×10^{-13} <u>M</u> ^{d,f}

NOTES:

^aExcitation - argon ion laser, 488 nm, 30 mW

Emission - 520 nm, monitored with photometer

^bExcitation - excimer laser, 308 nm, 20 Hz, ~20 mJ/pulse

Emission - 613 nm, monitored with (c) boxcar detector, (d) optical gating and photometer, or (e) optical gating and photon counter.

^fExcitation - dye laser containing p-terphenyl dye; 340 nm, 20 Hz, ~1mJ/pulse.

fluorescence passed through the entrance slit while the specular scatter was blocked.

As discussed previously spatial rejection of backscatter radiation is not easily accomplished when fiber optic measurements are performed. Thus a larger optical background was observed for the fiber optic case, which partially accounts for the much poorer limits of detection when measurements were performed by placing the distal end of an untreated fiber in the fluorophor solutions. The fluorescence collection efficiency is also poorer for the fiber optic case. It is interesting that the time-resolved measurements using the boxcar detector resulted in very poor detectability. Electronically gated detection using the boxcar is not effective in the fiber optic case because the intense backscatter radiation that is coincident with the excimer laser pulse is not spatially filtered and tends to "blind" the PMT. When the boxcar gate opens, the PMT response to the Eu-chelate fluorescence is severely dampened. Detectability is improved by several orders of magnitude, and is much better than for the fiber optic-based FITC measurement, when optical gating is performed using the chopper arrangement shown previously in Figure 6. Background levels were very low (approximately 100 cps for photon counting and 2 nA for the dc photocurrent measurement). Thus further electronic gating was not necessary and, due to the low radiation levels, slightly lower limits of detection were observed for the photon counting case.

Reducing the timing jitter for the optical gating should improve limits of detection. The low background levels given above were

achieved by adjusting the temporal position of the optical gate to totally block the backscatter radiation, which is coincident with the laser pulse. This results in a signal reduction since much of the fluorescence during the first few hundred μsec following laser pulses is blocked. Using more sophisticated mechanical shutters or electro-optical gating it should be possible to reduce the jitter and, thereby, improve detectability.

A standard curve was prepared for the fiber optic-based measurements of Eu-2-naphthoyltrifluoroacetone (Fig. 11). A correlation coefficient of 0.9998 was obtained over the range from the limit of detection to 7×10^{-7} M.

The excimer laser used in this work has several disadvantages in these measurements. The high powers (>1 MW at the output of the laser) associated with short duration pulses eventually damaged the optical fibers and, more critically, tended to photodegrade the samples. Measurements were performed by exposing the sample to the laser for 3 sec intervals so as to minimize the degradation. This made it necessary to use relatively short (1 sec) detector time constants. Noise levels for the non-immunochemical measurements discussed above were determined as the standard deviations for peak signals resulting from multiple laser exposures of the solvent. The high peak power and low repetition rate of the laser resulted in large peak signals with a low duty cycle ($\sim 2\%$). This contributed to the photometer saturation problem described above. Finally, the pulse to pulse and long term

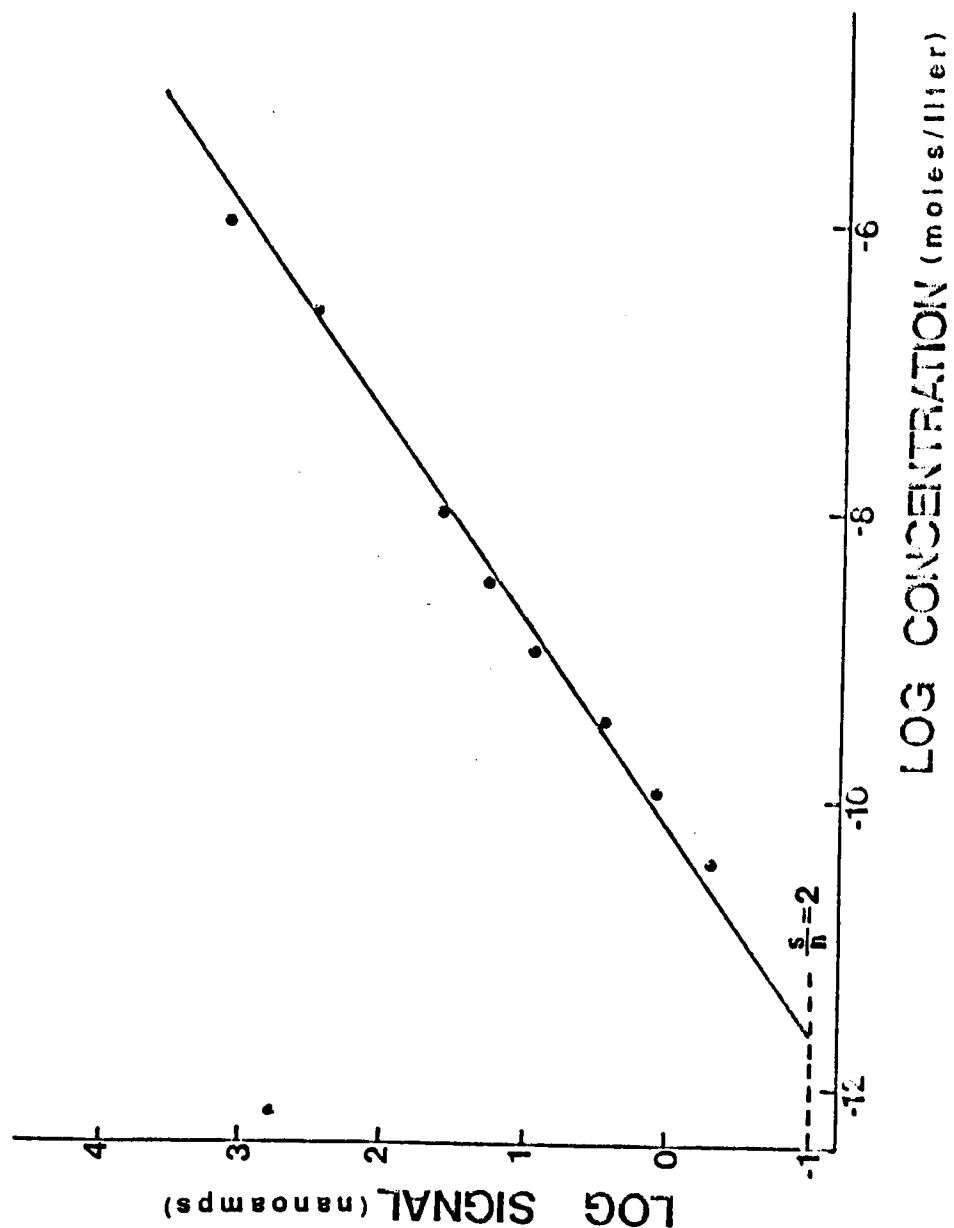


Figure 11: Standard curve for the fiber optic-based measurements of Eu-2-naphthoyl-trifluoroacetone. Limit of detection determined at the concentration which yielded a signal to noise cation (s/n) equal to two.

stability of the laser was poor, thereby contributing to noise and calibration problems.

In order to improve the detectability of the Eu-chelate measurement, limit of detection measurements were performed using the dye laser mentioned previously, and the optical gating arrangement shown previously in Figure 6. The dye laser possesses much better beam characteristics than the excimer laser, and the emission (340 nm) of the laser dye, p-terphenyl, is extremely close to the absorption maximum of the Eu-chelate. Although excitation of the Eu-chelate with the dye laser provided larger signals, the limit of detection was only slightly lower (9×10^{-13} M). The reproducibility of the measurements were ultimately limited by the instability in the dye laser beam due to power fluctuations in the excimer, as well as fluctuations resulting from the dye laser itself.

A cw helium-cadmium laser would be more appropriate for these measurements. Its 325 nm output is closer to the λ_{max} of the Eu-2-naphthoyltrifluoroacetone than the 308 nm output of the excimer laser. The helium-cadmium laser is characterized by moderate power (typically ~10 mW) and excellent stability. It could be synchronously modulated at a few hundred Hz to produce stable time-resolved signals with a fairly large duty cycle.

Several sandwich assay kits based on time-resolved fluorimetry are commercially available. In a solid phase sandwich assay a "first" antibody to the antigen of interest is immobilized onto a substrate, incubation with the antigen is effected, then a "second," fluorescently

labelled antibody is added. The second antibody is specific for a different antigenic determinant than the first and together the two antibodies sandwich the antigen (Fig 12). After appropriate washing steps, the fluorescence of the labelled second antibody is measured. The sandwich assay protocol is very useful. The major advantages in this process are that the sandwich assays could easily be adapted to FIS measurements, with the possibility of performing the initial incubation in vivo, and it doesn't require the antigen to be fluorescent.

For any given sandwich assay system, adaption to FIS measurements would require systematic optimization of the immobilization and incubation procedures. To avoid such complications a single antibody-antigen system was employed in the present study. It had previously been demonstrated that rabbit IgG could be covalently bounded to fibers using the aforementioned GOPS procedure, while maintaining significant activity for polyclonal, FITC labelled, anti-rabbit IgG(12). The binding capacity, $\sim 1 \mu\text{g}/\text{cm}^2$, of the Eu-chelate labelled immunochemicals used in this work (see experimental section for details) was comparable to that obtained in the previous work. This corresponds to approximately 10^{-13} moles of Eu-labelled antibody on the sensing portion of 600 μm fiber. As our measurements are performed (see experimental section), this corresponds to the antibody on the end and first mm of the sides of the fiber. Except for the fact that antigen, instead of the first antibody, was covalently bonded to the fiber, the protocol

Figure 12: Sandwich Assays Protocol.

- (a) Immobilize antibody 1, Y - antibody 1.
- (b) Incubate (in situ);  - antigen.
- (c) From "sandwich" using rare earth chelate labeled antibody 2.  - Europium ion, — chelate; —● chelate complexed with Europium ion; ● - rare earth chelate labeled antibody 2.
- (d) Dissociate Europium ion into the enhancement solution and perform the measurement. 0-2-naphthoyltrifluoroacetone forms highly fluorescent chelate with Europium ion; ● -Eu-2-naphthoyltrifluoroacetone, highly fluorescent complex.

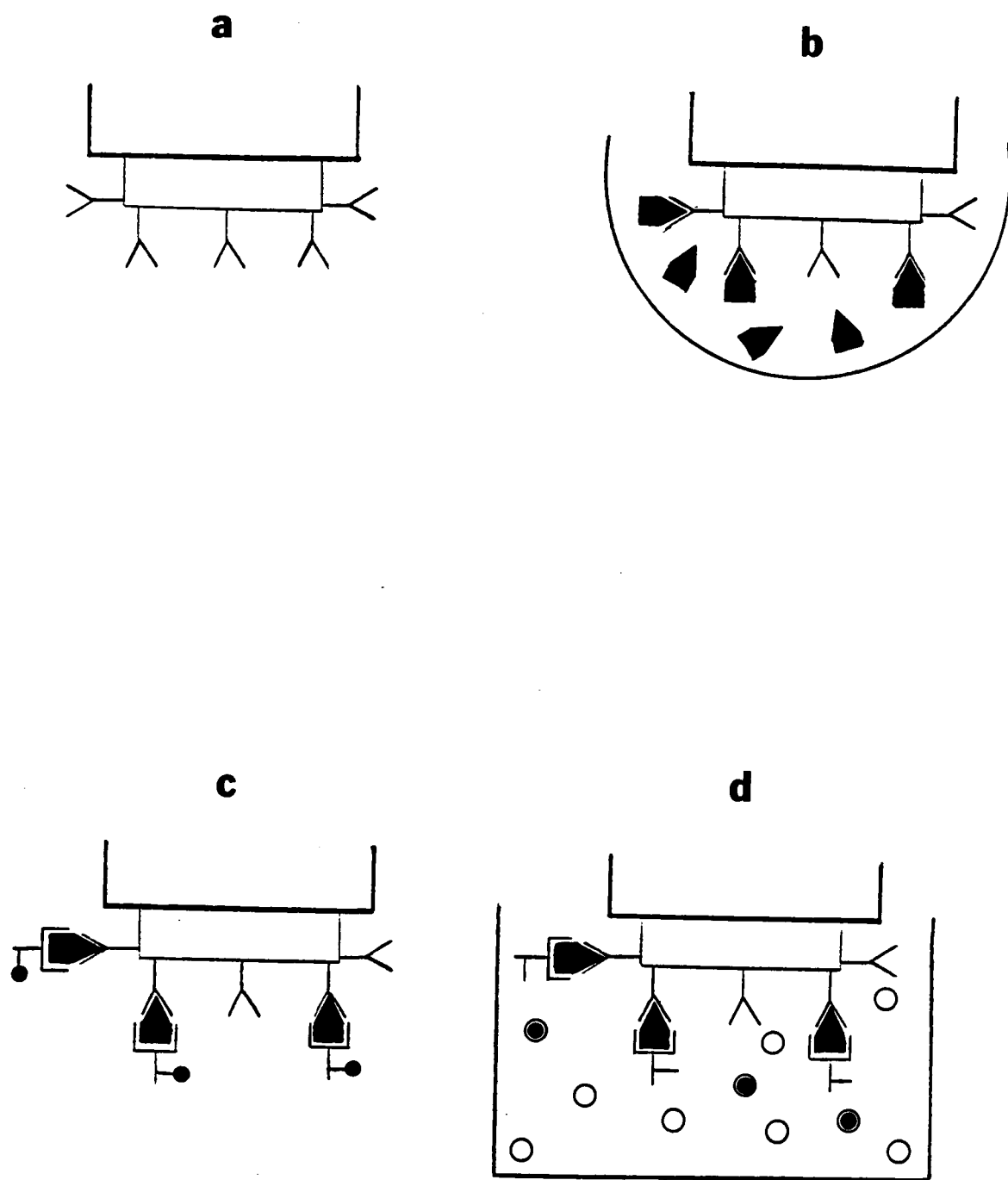


Figure 12:

for the time-resolved FIS measurements described herein is similar to that which would be employed in an actual sandwich FIS assay.

The analytical characteristics which are of greatest interest in this FIS development work are the sensitivity, reproducibility, and dynamic range of the FIS measurements. Our preliminary experiments were aimed at assessing these characteristics for the simple immunochemical system investigated.

The antibody bound Eu-chelate used in this work does not have a sufficiently large fluorescence quantum efficiency to be used directly for quantitation. Instead, after incubation, the Eu is released into an enhancement solution which contains another chelate, 2-naphthoyl-trifluoroacetone, as well as surfactant and other compounds which serve to exclude water from the Eu solvation sphere, and thereby increase fluorescence signals. Detection limits for Eu in this enhancement solution (see Table I) are much lower than reported for direct measurements that involve a single ligand for protein conjugation and for fluorescence measurement(24).

The amount of Eu released into enhancement solution depends on FIS experimental conditions, but is ultimately limited by the modest binding capacity of the fibers. Assuming eight Eu-chelates are bonded to each labelled protein(20), the amount of Eu at saturation is approximately 10^{-12} moles. For reasons of sensitivity it is advantageous to restrict the released Eu to a small volume of enhancement solution at the distal end of the fiber. Several approaches were investigated

before settling on the microwell technique described in the experimental section.

The possibility of performing the FIS measurements in a semi-rigid matrix, a gel composed of Knox jello dissolved in enhancement solution, was explored initially. It was expected that the gel would decrease the diffusion rate of the Eu and therefore minimize the dilution effect of the dissociation step. However, the inhomogeneity of the gel led to irreproducible measurements. The measurement was performed next in 700 μm diameter capillary tubes. A small volume of enhancement solution was injected into the tube and the FIS was then inserted into the tube, so that it just touched the meniscus of the fluid. Upon contact of the fiber tip with the liquid surface, capillary action caused the enhancement solution to rise up the walls of the tube, thus removing the Eu from the field of view of the fiber. Another measurement technique investigated was "wetting" of the fiber tip. The fiber was dipped into, and removed from, a small volume of enhancement solution. Upon removal, a drop of solution remained at the fiber tip, and the measurement was made in this drop. Although this procedure provided large signals, the reproducibility of the measurement was limited by the rapid evaporation of the drop from the fiber tip. Finally the micro-well technique was chosen for detailed study. The micro-well was constructed by drilling a small hole in a Teflon pad. The micro-well was nearly filled with 10 μL of enhancement solution. The release of 10^{-12} moles of Eu then results in the formation of a 10^{-7} M solution of Eu-2-naphthoyltrifluoroacetone. Saturated

fibers, when placed into micro-wells containing 10 μ L of enhancement solution yielded signal levels which were roughly equal to the 10^{-7} M Eu-chelate standard solutions used in the non-immunochemical work.

Reproducibility for time resolved FIS measurements was evaluated by incubating eight prepared fibers, overnight, with 0.005 mg/mL Eu-chelate labelled anti-rabbit IgG. The incubated fibers were sequentially aligned in the apparatus shown in Figure 6, the bound Eu released into 10 μ L of enhancement solutions, and the fluorescence signals measured using the photometer. When the signal measurement was complete, the fiber and micro-well were thoroughly washed with enhancement solution, the micro-well was filled with enhancement solution, and the background was measured. With the current arrangement it is difficult to center the fiber within the micro-well. To compensate for signal variances resulting from gradual reductions changes in laser power, fiber position, and other factors, we filled the micro-well with a dilute Eu-chelate standard solution after the background was determined and a normalization fluorescence signal was measured. Sample signals, S, were then determined using equation 2 where I_f , I_b , and I_n are the fluorescence,

$$S = \frac{I_f - I_b}{I_n} \quad (2)$$

blank, and normalization signals, respectively. The sample signal relative standard deviation for the eight fibers was 19%. Both chemical and physical factors contribute to noise in these

measurements. The chemical factors include the reproducibility of silanating and activating fibers, of bonding protein to fibers (quantity and condition of the bonded protein), and of the incubation step. The physical factors include the reproducibility of the laser output, of coupling fibers fitted with SMA connectors, of positioning fibers in the micro-wells, and the dispensing of small volumes of enhancement solution into the micro-well.

Utilization of FISs for actual assays will require the generation of standard curves. The standard curve shown in Figure 13 was obtained for incubations with standard solutions of Eu-chelate labelled anti-rabbit IgG over the range 1.0×10^{-4} to 2.0×10^{-2} mg/mL. The signal to noise ratio (S/N) for the lowest concentration standard (1.0×10^{-4} mg/mL) is approximately 2, with the noise being determined as the standard deviation of the blanks in the aforementioned reproducibility study. Although these measurements were performed using 300 μ L sample volumes, we have previously demonstrated the ability to perform competitive binding FIS measurements using sample volumes as small as 10 μ L(12). Working with 10 μ L sample volumes, the absolute limit of detection would be approximately 6 femtomoles. The actual amount of bound anti-rabibt IgG is probably considerable less than 6 femtomoles.

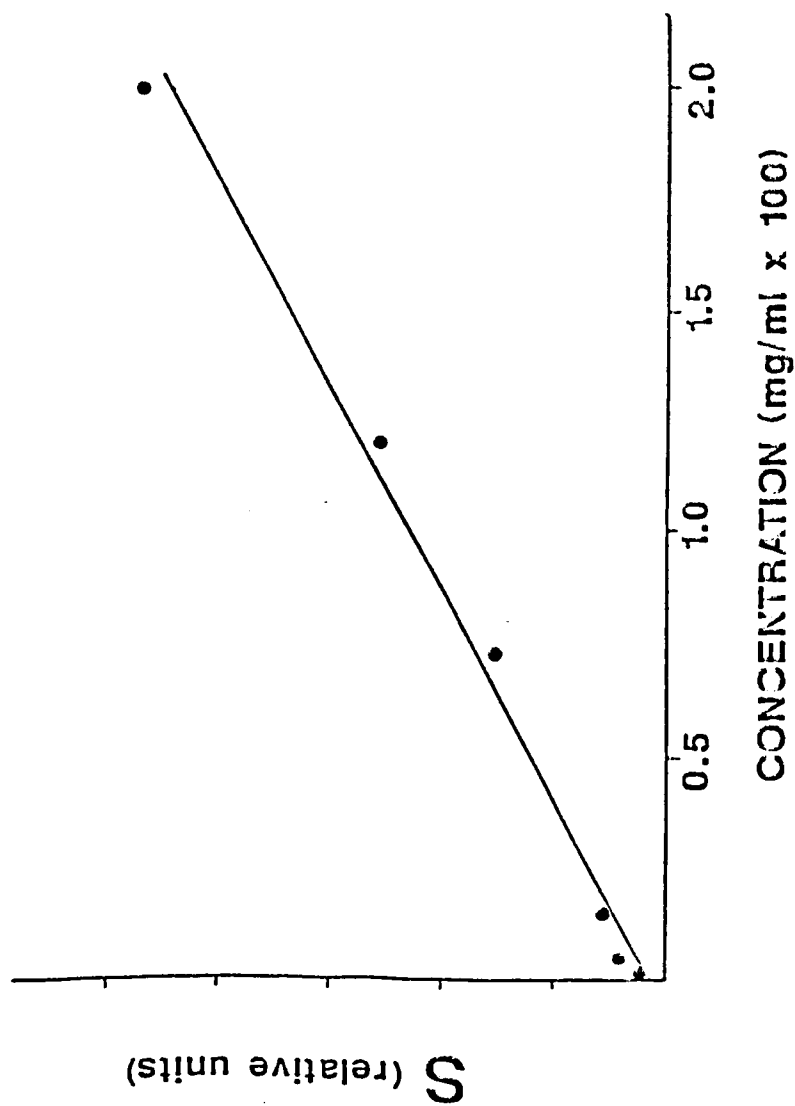


Figure 13: Standard curve for measurements of Eu-chelate labelled anti-rabbit IgG. Each data point corresponds to the average of four measurements.

CHAPTER IV

CONCLUSION

The time-resolved fluoroimmunosensor described herein offers the potential of being an extremely useful tool in both the clinical and scientific fields. The characteristics which are most important are:

- a) rejection of background arising from both the scatter from fiber interfaces, and sample matrix fluorescence,
- b) extremely small sample size requirements, and
- c) the potential of employing the sensor for direct in-vivo measurements.

The efficacy of time-resolved detection at improving detectability in fiber optics-based fluormetry has been demonstrated in this work. Rare earth metal chelates, with their extremely long fluorescent lifetime offer distinct advantages as fluorescent labels for immunoassays. With the advent of new rare earth metal chelates possessing both large quantum efficiencies and efficient antibody coupling characteristics, it should be possible to eliminate the europium ion dissociation step, and perform direct "on-fiber" measurements. This should improve both the sensitivity and the reproducibility of these immunochemical measurements, and provide for a wider range of uses.

The work presented in this thesis demonstrates that sensitive, low-volume, in situ immunoassays can be performed using rare earth metal chelate labels and fiber optic-based time resolved fluorimetry.

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