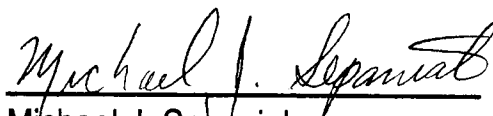


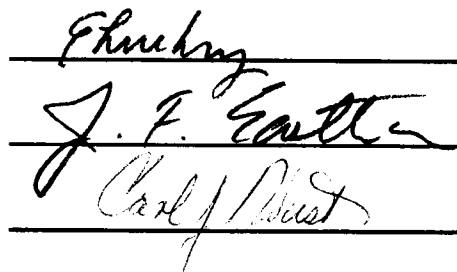
To the Graduate Council:

I am submitting herewith a dissertation written by Bruce Jason Tromberg entitled "Development of Antibody-Based Fiber Optic Sensors." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Chemistry.

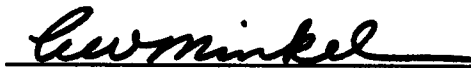


Michael J. Sepaniak,
Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:



Vice Provost and Dean of
The Graduate School

**DEVELOPMENT OF ANTIBODY-BASED
FIBER OPTIC SENSORS**

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Bruce Jason Tromberg
March 1988

This Work is Dedicated To My Wife and Best Friend

Patti

and My Parents

Jessie and Sheldon

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ABSTRACT

The speed and specificity characteristic of immunochemical complex formation has encouraged the development of numerous antibody-based analytical techniques. The scope and versatility of these established methods can be enhanced by combining the principles of conventional immunoassay with laser-based fiber-optic fluorimetry. This merger of spectroscopy and immunochemistry provides the framework for the construction of highly sensitive and selective fiber-optic devices (fluoroimmunosensors) capable of in-situ detection of drugs, toxins, and naturally occurring biochemicals.

Fluoroimmunosensors (FIS) employ an immobilized reagent phase at the sampling terminus of a single quartz optical fiber. Laser excitation of antibody-bound analyte produces a fluorescence signal which is either directly proportional (as in the case of natural fluorophor and "antibody sandwich" assays) or inversely proportional (as in the case of competitive-binding assays) to analyte concentration. Factors which influence analysis time, precision, linearity, and detection limits include the nature (solid or liquid) and amount of the reagent phase, the method of analyte delivery (passive diffusion, forced convection, etc.), and whether equilibrium or non-equilibrium assays are performed. Data will be presented for optical fibers whose

sensing termini utilize: 1) covalently-bound solid antibody reagent phases, and 2) membrane-entrapped liquid and solid antibody reagents. Assays for large-molecular weight proteins (antigens) and small-molecular weight, carcinogenic, polynuclear aromatics (haptens) will be considered. In this manner, the influence of a system's chemical characteristics and measurement requirements on sensor design, and the consequence of various sensor designs on analytical performance, will be illustrated.

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INTRODUCTION

Fiber optic sensors have been designed to provide measurements of a wide range of chemical and physical phenomena. They are distinguished from their electrical analogs primarily by their lack of a separate reference sensor and by the unique spectroscopic information they can supply. In addition, they are rugged, versatile, transparent over broad spectral regions, exhibit low power attenuation over long distances, and are immune from electromagnetic interferences. These features indicate that optical sensors can be useful alternatives to the more commonly used potentiometric and amperometric electrode sensors.

Fiber optic sensor instrumentation frequently evaluates the following properties: light intensity,^{1,2} displacement,³ temperature,⁴⁻⁶ pressure,⁷⁻⁹ rotation,¹⁰⁻¹² sound,^{12,13} strain,¹⁴ magnetic field,^{12,15} electric field,¹⁵⁻¹⁷ ionizing radiation,^{18,19} microwave power,²⁰ flow,²¹⁻²³ liquid level,²⁴⁻²⁶ particle size,^{27,28} vibration,²⁹ and chemical concentration.³⁰⁻⁸⁰ These analyses have been performed with sensors which, by virtue of the nature of the sensing element, have been categorized as either intrinsic or extrinsic devices.

Extrinsic sensors utilize a chemical or mechanical transducer external to the measurement fiber. Changes in the intensity of the observed optical signal occur as a function of the measured phenomenon. In the case of fiber optic chemical sensors (FOCSs), a

chemically selective reagent phase is immobilized at the fiber's sampling terminus. Detection of analyte is accomplished by observation of analyte-induced optical changes in the sensor's reagent phase. Because the reagent phase (or external transducer) recognizes the analyte, the specificity and sensitivity of the device is regulated by the transducer's fundamental characteristics. For FOCS these critical features may include: abundance, association constant (K_a), molar absorbtivity, and fluorescence quantum efficiency.³⁹⁻⁴³

The response of intrinsic sensors is determined by the extent to which the measured phenomenon produces physical changes in an optical fiber. These instruments typically utilize interferometric detection to compare the phase of light in a single-mode sensing fiber to that of an identical reference fiber.⁸¹ In practice, phase-dependent sensors are generally more accurate but significantly less versatile than intensity-dependent extrinsic devices.^{1, 12, 81}

In previous work, a sensor was developed for laser-excited fluorescence detection of the naturally fluorescent anti-tumor drug, doxorubicin.^{34,35} The device, known as a "flouoroprobe", was successful in performing analyses in whole blood, cerebral spinal fluid, and in the interstitial fluid of live tumors. Limitations to this device included interference effects and, during measurements in complex matrices, high levels of optical background. The extent of interference varied and was difficult to

determine since observation of doxorubicin's broad-band fluorescence emission was the sensor's primary selectivity parameter. Thus, during fluoroprobe measurements, doxorubicin was indistinguishable from its metabolites and naturally occurring spectral interferants.

In this work, fluoroprobe performance was improved by incorporating chemical selectivity into sensor design. In order to conduct simple, rapid, in-situ analyses of large molecules (M.W. > $\approx$ 200) in complex matrices, an extrinsic sensing configuration was adopted in conjunction with a highly selective reagent phase. Due to its sensitivity and outstanding coupling efficiency to small-diameter optical fibers, laser-excited fluorescence intensity was selected as the optical parameter to be measured. A number of FOCSs have been described which utilize fluorescence detection in conjunction with the specificity of ligand/binder reactions. Excellent sensitivity for naturally fluorescent and fluorophore-labeled molecules has been achieved.³⁹⁻⁴³

Many of these sensors have been used to detect pH,⁴⁴⁻⁴⁸ metal ions,⁴⁹⁻⁵³; and small molecules, e.g., CH₄,^{30,31} CO₂,^{46,54,55} NH₃,⁵⁶⁻⁵⁸ O₂,⁵⁹⁻⁶², and H₂.⁶³ In order to perform highly sensitive and selective detection of trace amounts of large molecules, the specificity of antibody:antigen (Ab:An) and antibody:hapten (Ab:Hn) ligand/binder reactions can successfully be applied to FOCS design.⁶⁸⁻⁷⁸ This approach was first described by Kronick and Little in their report of a total internal

reflection fluorescence (TIRF) immunoassay of dye-labeled antibodies bound to an antigen-coated planar waveguide.⁶⁸ Hirschfeld,⁶⁹ Sutherland and Dähne, *et al.*,⁷⁰ and Andrade *et al.*,⁷¹ have continued to pursue TIRF immunoassays on both planar and cylindrical waveguides.

TIRF sensors rely upon evanescent-wave excitation of surface-bound biomolecules. They are characterized by large exposed surface areas (to achieve sufficient sensitivity) and low evanescent wave penetration depths. Their primary limitation is the fact that the evanescent wave intensity and penetration depth varies considerably with the light source's incidence angle and waveguide's structure (e.g. planar or cylindrical).^{70-72,82,83} An additional drawback is the poor coupling efficiency of evanescently-excited fluorescence into the waveguide.⁷³ The practical consequence of these restrictions is that relatively short, large surface-area fibers must be used. This design prevents the loss of higher order modes (see Chapter 1) and maintains adequate interfacial excitation intensity. Unfortunately, the use of short fibers with large exposed surface areas limits the in-situ capability of these devices (see Chapter 1 for further discussion).

In an attempt to circumvent the limitations of TIRF designs, a fluoroimmunosensor (FIS) was fabricated which utilizes an immobilized immunochemical reagent phase located at the distal face of a single sensing fiber (distal-face sensors).^{73,74} By locating the reagent phase in this position, the sensing element

experiences maximum excitation light intensity and emission coupling efficiency. Either fiber bundles or single fibers can be used. Advantages to single-fiber designs include their superior fluorescence emission collection efficiencies^{73, 77} and their small size. Single-fiber devices ranging in diameter from 200 μm to 600 μm probing nL to low μL volumes have been reported.⁷³⁻⁷⁷ These features typically result in single-fiber sensors capable of femtomole limits of detection.^{75,76}

Unlike TIRF devices, distal-face sensors are capable of large sample penetration depths. This may cause difficulty in resolving reagent-bound analyte from free analyte and interferant. For continuous in-situ sensors which do not require removal from the sample (homogeneous devices), this problem has been addressed by recording binding-dependent reagent phase changes. These changes include the observation of spectral shifts,^{39-42,44-46, 48-51,54-58,61} luminescence quenching,^{39-42,46,52,59,60,62,} energy transfer,⁴⁷ and "field of view" detection of unbound indicator molecules.³³ All of these sensors are reversible and respond quickly to variations in bulk analyte concentration. Specificity, however, is generally poor due to the fact that their reagent phases are sensitive to broad classes of compounds.

Extrinsic devices which employ immunochemical reagent phases and distal-face excitation have the potential to enhance selectivity, sensitivity and versatility over TIRF immunosensors and extrinsic sensors employing conventional reagent phases. The abundance of commercially available immunochemicals and their

relative ease of manufacture should increase the number and type of sensor applications. The large affinity constants observed for Ab:An and Ab:Hn reactions allows an immunosensor to concentrate analyte within the sensor's observation zone. Depending upon the type of immunoassay, this may permit the sensor to function homogeneously, since the only observable contributor to the optical signal may be reagent-bound analyte.^{75,76} If this is not the case, the sensor can be removed from the measurement matrix after a pre-determined incubation period.^{73,74,77} Manipulations of this sort are designated as "heterogeneous immunoassays".

The original intent of this work was to design the appropriate instrumentation and chemistry necessary for continuous in-situ monitoring of biochemically significant haptens and antigens. In the course of evaluating numerous devices, it became apparent that the kinetic limitations of immunochemical complex formation (i.e. large association and small dissociation rate constants) must be overcome in order to produce continuous, reversible, antibody-based FOCSs.⁷⁸ Interestingly, factors which may be detrimental to reversibility can become valuable assets when "single-shot" probes are constructed.

Single-shot probes supply data for only one measurement. Multiple determinations are performed with identically prepared probes. They respond quickly (at a mass transport-limited rate) and irreversibly, and can be used to concentrate, resolve, and separate analyte from dilute solutions and complex matrices. In contrast, continuous sensors based on chemical equilibria must

utilize low-affinity antibodies in conjunction with the observation of optical changes (e.g. fluorescence energy transfer, quenching, lifetime changes) upon binding of antigen or hapten to antibody. Since low-affinity antibodies are less selective than conventional antibodies and binding-induced optical changes may be difficult to follow, the analytical tradeoff for continuous detection is diminished selectivity and sensitivity.

In consideration of this apparant analytical dilemma, the following work is focused on the development of highly sensitive, rapid, irreversible devices. To date, there have been no literature-reported antibody-based extrinsic FOCSs which rely upon distal-face excitation to detect large molecules on a continuous basis. The ensuing discussion, however, describes the design, construction and application of the first optical immunosensor (the FIS) capable of performing pseudo-continuous and heterogeneous measurements of large proteins and small haptens at femtomole levels of sensitivity.⁷³⁻⁷⁶

CHAPTER 1

FIBER OPTIC HARDWARE

A. Principles of Fiber Optics

Technological advances in the fabrication of optical fibers, fiber connectors, sources, and detectors have been propelled, in large part, by the communications industry. The rapid growth of fiber optic sensing has paralleled the commercial availability of reproducible low-loss optical components. The association between the appearance of a new field of measurement science and the accessibility of novel materials underscores the importance of understanding the principles of fiber optics. In this manner, improvements in the performance of optical sensors can continue to mirror innovations in materials and instrumentation.

Light Transmission in Optical Fibers

An optical fiber is a transparent dielectric waveguide designed to transmit light over long distances. As illustrated in Figure 1.1, it consists of an inner cylinder of glass or plastic (the core) surrounded by a shell of glass or plastic (the cladding). An additional coating of plastic (the jacket) usually surrounds the cladding in order to provide mechanical and environmental protection. Figure 1.2 is an illustration of the geometrical optics

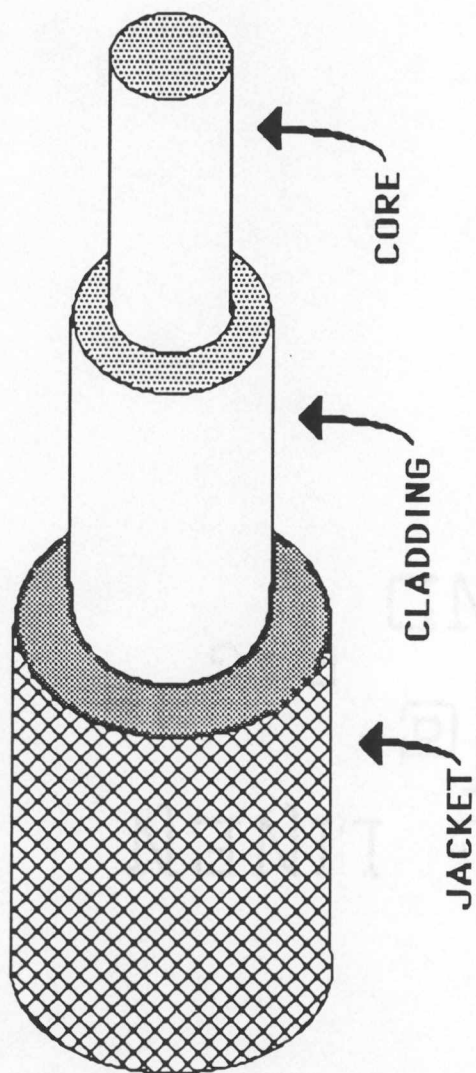


Figure 1.1. Optical fiber cross section

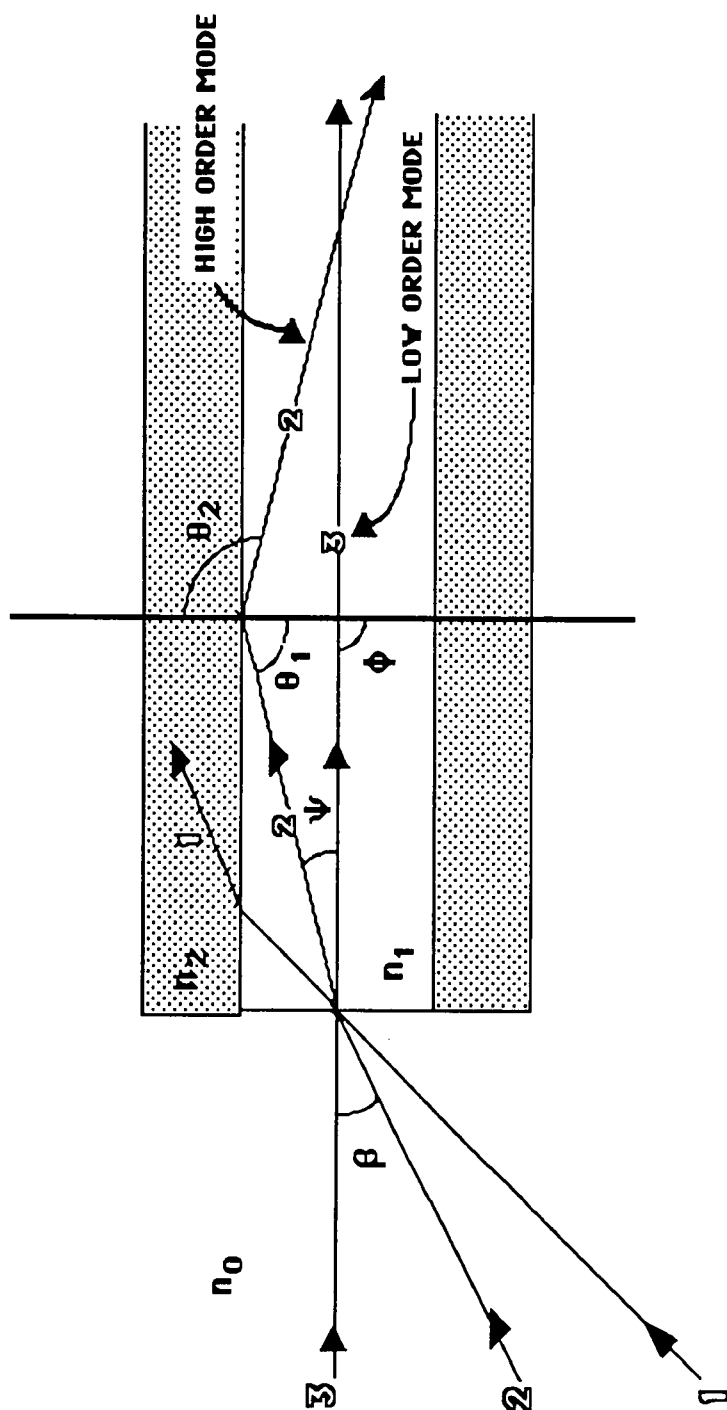


Figure 1.2. Geometrical optics model of light propagation along multi-mode step-index fiber.

model of light propagation along a fiber. The core refractive index, n_1 , is greater than the cladding refractive index, n_2 . In order for transmission to occur, θ_2 must be greater than or equal to 90° . Substituting $\theta_2 = 90^\circ$ into Snell's law ($n_1 \sin \theta_1 = n_2 \sin \theta_2$) yields $\theta_1 = n_2/n_1$. If a light ray enters the fiber at an angle, θ_1 , that is greater than or equal to the critical angle, θ_c , it will be totally internally reflected at the core/cladding interface. Accordingly, the maximum acceptance angle, 2β , is determined by n_2/n_1 and the refractive index of the medium n_0 . Light entering the maximum acceptance angle is transmitted along the length of the fiber. Light rays 2 and 3 (Figure 1.2) satisfy this requirement and are referred to as "bound rays" since their paths are confined within the core. Light ray 1 is known as a "skewed" or "refracted" ray since it is refracted at the interface. Skewed rays are also partially reflected but they are quickly attenuated due to absorption and scattering in the cladding. These relationships can be used to derive the fiber's numerical aperture, NA, and f/number:⁸⁴⁻⁸⁸

$$NA = n_0 \sin \beta = (n_1^2 - n_2^2)^{1/2} \quad (1.1)$$

$$f/\text{number} = 1/(2 \tan \beta) \quad (1.2)$$

Table 1.1 is a compilation of numerical apertures, acceptance angles, and f/numbers for various values of n_2/n_1 .

Fiber Modes

A thorough understanding of light transmission in optical fibers requires the application of electromagnetic field theory to wave propagation in dielectric media. In this approach, equations which relate field vectors at arbitrary points in space and time (Maxwell's equations) are solved within the cylindrical boundary conditions imposed by the fiber. In contrast to the geometrical optics treatment, physical optics analysis concludes that optical power is carried only through discrete sets of guided modes (electromagnetic field distributions) which correspond to discrete angles of incidence. Light rays cannot interfere with one another since each allowed mode is separated in angle of propagation by λ/d radians, where λ is the wavelength in the waveguide and d is the core diameter.⁸⁴ Complete physical optics analyses have appeared in several articles and texts.⁸⁶⁻⁸⁹

Fiber Types

The two general classes of fiber are: 1) step index and 2) gradient index. Step index fibers (Figures 1.2 and 1.3) are characterized by an abrupt refractive index change between core, n_1 , and cladding, n_2 . According to equation (1.3), the total number of modes that a step-index fiber can carry, M , depends upon fiber

TABLE 1.1. Basic Fiber Parameters

NA	$2\theta^a$	f/number	n_2	n_2/n_1^b
0.1	11.5	5.0	1.497	0.9978
0.2	23.1	2.4	1.487	0.9911
0.3	34.9	1.6	1.470	0.9798
0.4	47.2	1.1	1.446	0.9638
0.5	60.0	0.9	1.414	0.9428
0.6	73.7	0.7	1.375	0.9165

^a Total acceptance angle in degrees.

^b Core refractive index, $n_1 = 1.500$.

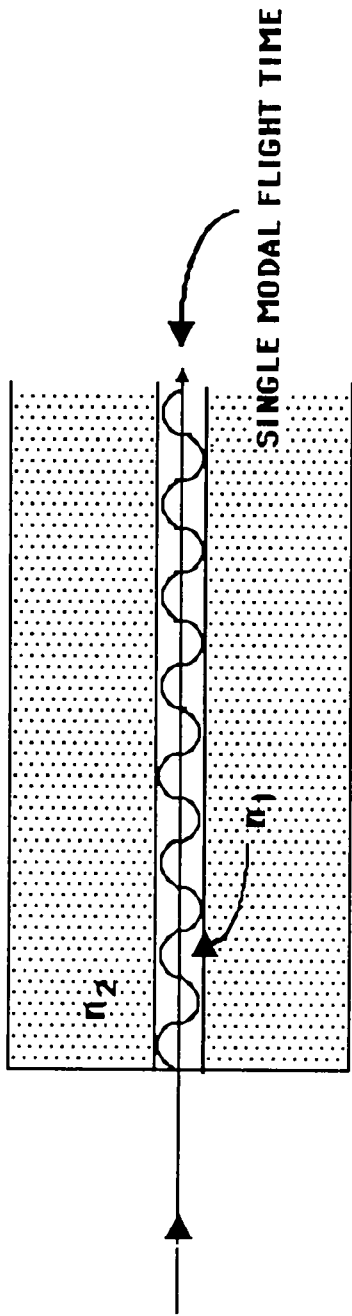


Figure 1.3. Single mode step-index fiber.

diameter, d ; wavelength of light, λ ; and numerical aperture, NA.^{85,90}

$$M \approx (2)[(\pi \times d \times \text{NA}) / \lambda]^2 \quad (1.3)$$

The amount of optical power that can be coupled to a fiber is proportional to the number of modes it can support. Multimode waveguides with large diameters and numerical apertures have the highest power throughputs. Minimizing fiber diameter and NA and increasing wavelength will result in the propagation of fewer modes. Single-mode step-index fibers have low power throughputs and core diameters which range between 1 and 10 μm . As illustrated in Figure 1.3, they are capable of transmitting a single mode of light.

Figure 1.2 is a geometrical ray optics representation of the differences between modes in a multimode step index fiber (typically 50-1000 μm in core diameter). The lowest order mode is symbolized by ray 3 which travels along a path that is coincident with the central axis. Meridional rays corresponding to higher order modes are propagated along zig-zag paths, repeatedly crossing the fiber's central axis and turning at the core/cladding interface. Since the time for energy to propagate to the end of the waveguide is path-length dependent, ray 2 will arrive at the output later than ray 3. The difference in arrival time is designated as "modal dispersion". It can be calculated using

equation 1.4:⁸⁴⁻⁸⁶

$$\begin{aligned}\Delta t &= t_{\text{highest}} - t_{\text{lowest}} = [L(\sec \psi) / n_1 c] - [L / n_1 c] \\ &\approx (Ln_1 / c)\Delta\end{aligned}\tag{1.4}$$

Energy launched along the axial path of a 1-km waveguide with $n_1 = 1.53$, $n_2 = 1.50$, $\psi = 15^\circ$, $\Delta = (n_1 - n_2) / n_2$, and $c = 3 \times 10^8$ m/s will arrive at the output in 5.1 μs . Light propagating along the path corresponding to the highest order mode will arrive in 5.28 μs . If the input energy is provided to an "ideal fiber" in the form of a pulse, it will experience a 0.18 μs pulse broadening due to modal delay distortion. In a real fiber, however, there are probably a number of bends and/or fluctuations in diameter or refractive index. This will allow the transfer of energy between modes (mode coupling) until a steady state power distribution is obtained. At this point, the different modes do not carry equal amounts of energy (as assumed in this calculation) and the observed broadening is slightly less than indicated.^{85,91}

The effects of modal dispersion are inconsequential for most extrinsic sensors. The primary requirements for extrinsic devices include: low cost, high optical throughput, and ease of handling. These needs can be met by utilizing relatively short, large-diameter, multimode fibers. Long-distance communication and intrinsic sensing applications, however, require

low-dispersion fibers. The use of small-diameter (1-10 μm), low NA, single-mode fibers eliminates modal dispersion. Unfortunately, transmission in these fibers is highly wavelength dependent,⁸⁵ and, due to their small size, light/fiber and fiber/fiber coupling can be extremely difficult.

Multimode gradient-index fibers can also reduce the effects of pulse spreading. As shown in Figure 1.4, this type of fiber has a core refractive index which is highest along the fiber's axis and tapers off roughly parabolically toward the core/cladding interface. The gradient structure allows skewed rays to travel in a helical pattern without crossing the central axis. Modes corresponding to meridional rays are propagated along sinusoidal paths with turning points that are dependent upon β and the index profile. Despite their long paths, modes launched at large angles of β experience transit times comparable to axially propagating modes. This is due to the fact that meridional rays spend a greater amount of time in low-optical density regions of the core. Pulse spreading in step-index fibers is typically reduced by a factor of 20-100 for comparably-sized gradient-index waveguides.^{84,85,92}

Attenuation Mechanisms

The rate of optical power loss along a fiber is described as attenuation. It is defined by the following equations:^{93,94}

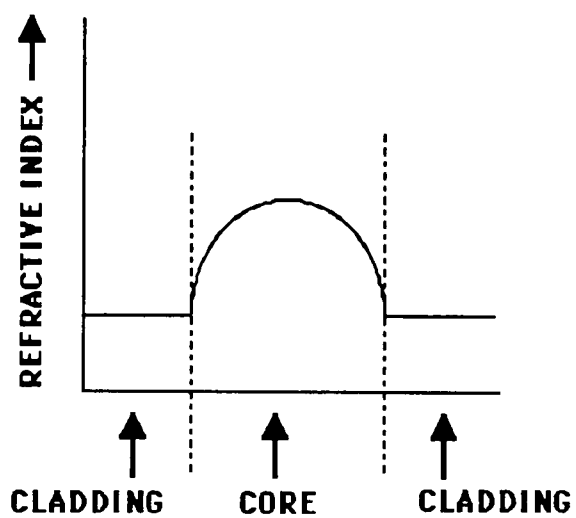
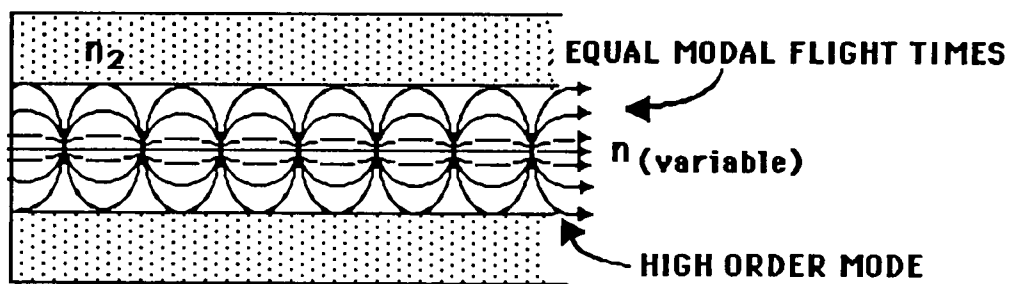


Figure 1.4. Multi-mode gradient index fiber.

$$P(z) = P(0) 10^{-(\alpha z/10)} \quad (1.5)$$

$$\alpha(z) = -10 \log [P(z) / P(0)] \quad (1.6)$$

where $P(z)$ is the power at distance z , $P(0)$ is the power at $z = 0$, and $\alpha(z)$ is the attenuation coefficient in decibels per unit length (dB/z). If, at a given wavelength, 100 mW of optical power were launched into a fiber with an attenuation coefficient $\alpha(z) = 10$ dB/km, $P(z)$ for a 1-meter length would be 99.77 mW and $P(z)$ for a 1-km length would be 10 mW. Clearly, long distance remote sensing applications may suffer from diminished sensitivity due to significant power loss. Common laboratory devices use short sections of fiber and are often unaffected by $\alpha(z)$ values less than 50 dB/km.

There are a number of factors which influence optical attenuation in dielectric waveguides. In general, these losses are determined by the waveguide's composition, method of preparation, and dimension. Composition and preparation losses can be attributed to intrinsic and extrinsic mechanisms, each of which can be further sub-divided into absorptive and radiative (scattering) components. Figure 1.5 is an illustration of the relationship between transmission losses and wavelength for a typical fused silica waveguide.⁹⁵

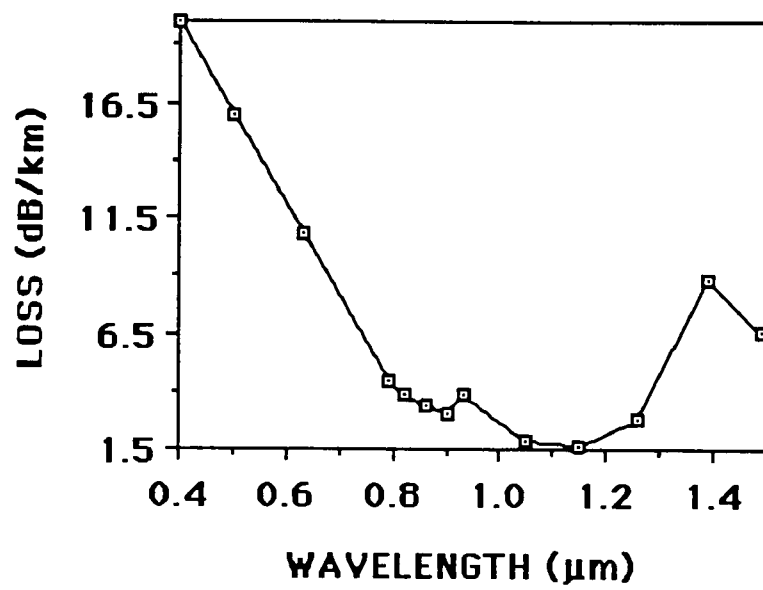


Figure 1.5. Transmission loss versus wavelength for fused silica fiber.

Intrinsic attenuation. The total intrinsic attenuation, α_i , can be expressed as:⁹³

$$\alpha_i = A/\lambda^4 + [B_1 \exp(B_2)]/\lambda + [C_1 \exp(-C_2)]/\lambda \quad (1.7)$$

where the coefficients A , B_1 , B_2 , C_1 , and C_2 are constants. The first term in equation (1.7) refers to Rayleigh scatter loss. It represents the lower attenuation limit of optical fibers and is caused primarily by variations in atomic density of the basic material (e.g. fused silica for communication-grade fibers) and intentionally-introduced dopants (B_2O_3 , GeO_2 , P_2O_5). These small (with respect to wavelength) inhomogeneities are responsible for microscopic waveguide refractive index fluctuations. They produce scattering intensities that are inversely proportional to the fourth power of the wavelength. Rayleigh scatter is the most significant attenuation factor for the transmission of visible and ultraviolet light.⁸⁴⁻⁸⁸ For example, at $\lambda = 400$ nm Rayleigh scatter may account for all of the observed 15 - 20 dB/km loss, but at $\lambda = 1.05$ μm it contributes only about 0.95 dB/km.

Raman scattering can also contribute to intrinsic losses. Under normal conditions in large-diameter multimode fibers it is insignificant. Due to its inverse dependence on core cross-sectional area, however, it has been observed at high light power levels in small-diameter (usually single mode) fibers.⁸⁵

The second and third terms in equation (1.7) describe intrinsic absorption losses due to electronic transitions in the primary fiber material (second term) and multiphonon (lattice vibration) processes. The first and second terms decrease while the third term increases with increasing wavelength.⁹³

Extrinsic attenuation. Extrinsic losses are caused primarily by absorption (and some scattering) of light by unintentionally-introduced impurities. These effects are most pronounced in the near infrared region. In glass fibers, they are attributed mainly to the presence of transition metal ions and dissolved water.

Fe and Cu ions are the largest contributors to metal absorption. The magnitude and range of their influence is dependent upon concentration and oxidation state. Attenuation occurs without characteristic maxima throughout the visible-near IR region.

Attenuation due to the fundamental vibration caused by OH⁻ ion absorption (from water) appears at 2.73 μm . First, second, and third harmonics appear independently at 1.37, 0.95, and 0.74 μm . These overtones also combine with fundamental silicon-oxygen bond vibrations, producing a series of combination frequencies at various wavelengths in the 0.80 - 1.05 μm region.

An additional extrinsic attenuation mechanism may arise from non-ideal manufacturing procedures. Large inhomogeneities such as bubbles, cracks, and unreacted materials can cause significant

scattering losses. As the size of the defect approaches the transmission wavelength, Mie scattering becomes particularly significant.

Leaky ray attenuation. Intrinsic and extrinsic loss mechanisms do not account for all of the observed power attenuation in fibers. A significant portion of light coupled to a waveguide resides in the cladding. These rays are designated as "leaky rays" since they contribute to power loss in a radial direction. The total allowable power, P , can be expressed as:⁸⁵

$$P = P_{\text{core}} + P_{\text{cladding}} \quad (1.8)$$

$$P_{\text{core}} / P_{\text{cladding}} = 3/4 (M^{1/2} - 1) \quad (1.9)$$

where M , the total number of modes, is described by equation (1.3).

Cylindrical waveguides can support two types of leaky rays. They are "tunneling" and "refracting" rays. When skewed light is coupled to a fiber, refracting rays will bifurcate at the core/cladding interface. The reflected component will be transmitted in the core for a short distance. The primary component of the skewed ray is refracted into the cladding where it is lost. Virtually all refracting rays dissipate rapidly over short lengths of fiber and are insignificant contributors to total optical power.⁹⁶

Tunneling rays (Figure 1.6) originate in the core from totally

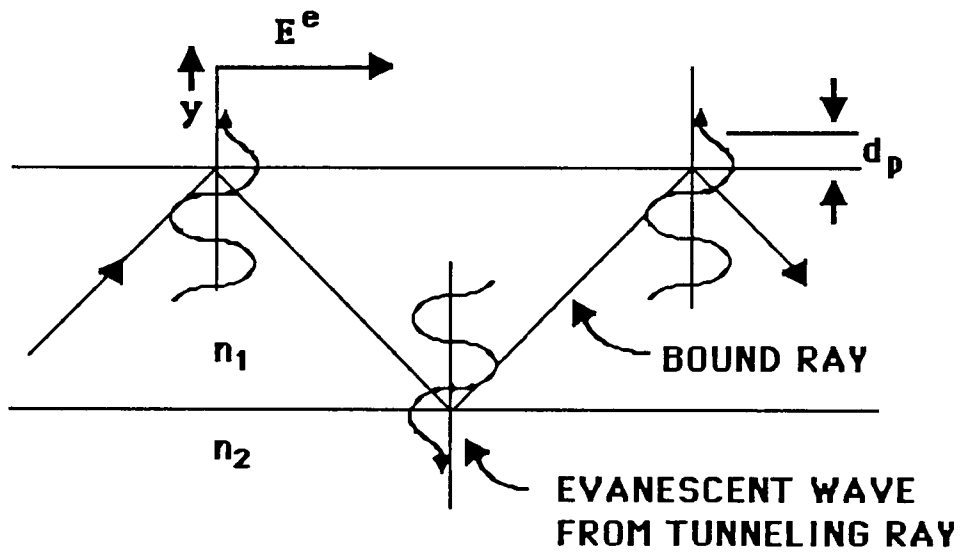


Figure 1.6. Evanescent wave propagation.

internally reflected bound rays. As each bound ray is reflected at the core/cladding turning point, optical power "tunnels" indefinitely in a radial direction from core to cladding. The field associated with the superposition of incident and reflected wave is termed "evanescent". The evanescent wave is propagated in an axial direction along the interface. Its electric field amplitude, E^e , in the core is equal to its amplitude right at the interface in the cladding. E^e decays with increasing distance (y) normal to the transmission axis according to the following equations:⁸²

$$\begin{aligned} E^e(y, \text{perp}) &= [E_{(2,0,\text{perp})} / E_{(1,0,\text{perp})}] \exp (-y/d_p) \\ &= (2 \cos \theta_1) / \{ [1 - (n_2/n_1)^2]^{1/2} \} \exp (-y/d_p) \end{aligned} \quad (1.10)$$

$$d_p = \lambda_0 / \{ 2\pi [2n_1^2 \sin^2 \theta_1 - n_2^2]^{1/2} \} \quad (1.11)$$

where $E^e(y, \text{perp})$ is the perpendicularly polarized evanescent electric field amplitude at distance (y) from the interface, $E_{(2,0,\text{perp})}$ and $E_{(1,0,\text{perp})}$ are the perpendicularly polarized electric field amplitudes at the interface in the cladding (or less dense medium) and core, respectively, λ_0 is the wavelength of light in a vacuum and d_p , the evanescent wave penetration depth, is the distance from the interface where the electric field amplitude has decayed to $1/e$ of its initial value (typically on the order of λ_0).

Since a fraction of bound-ray power is transmitted across the core/cladding interface creating an evanescent field, the loss can be expressed by a power transmission coefficient, T :⁹⁶

$$T = 1 - (\text{power in reflected ray} / \text{power in incident ray}) \quad (1.12)$$

Equation (1.12) can be related to a power attenuation coefficient due to tunneling:⁹⁶

$$\alpha_{\text{tunneling}} = T / z_p = N(T) \quad (1.13)$$

where z_p is the ray half-period between successive reflections and N is the number of reflections per unit length of fiber. By substituting $\alpha_{\text{tunneling}}$ into equation (1.5), $P(z)$, the attenuation of $P(0)$ at an axial distance (z) can be determined. The difference between $P(z)$ and $P(0)$ corresponds to the total evanescent field intensity absorbed by the cladding at distance (z).

In order to compare the efficiency of TIRF sensors with distal-face designs, it is necessary to evaluate the power associated with the evanescent field. From equations (1.10-1.13) it can be seen that the evanescent field intensity and penetration depth vary strongly with θ_1 . This can be explained, in a qualitative sense, by considering the total number of reflections, N , at the interface. As θ_1 changes, N increases or decreases

causing a concomitant fluctuation in T (and $\alpha_{\text{tunneling}}$). Changes in T alter the number and intensity of radially propagated tunneling rays. These variations, in turn, influence the magnitude of the evanescent field. The relationships between $E^e_{(0,\text{perp})}$, d_p , and θ_1 are illustrated in Figures 1.7 and 1.8.

The prediction that evanescent field intensity and penetration depth are functions of incidence angle has significant practical implications. In order to optimize detectability, a properly designed TIRF device must be capable of varying the coupling angle between light source and fiber. Further complications arise from the fact that each bound mode enters the waveguide with a unique θ_1 and contributes unequally to the depth and magnitude of the evanescent field. Large surface areas must therefore be probed in order to average out the effects of local field inhomogeneities.

Since the total number of modes, M , that can be propagated by a typical 200- μm diameter multimode sensing fiber (operating at 500 nm with a $\text{NA} = 0.22$) is roughly 10^5 , there are a considerable number of meridional rays corresponding to allowed modes which can contribute to average evanescent power. Substitution of M into equation (1.9) yields $P_{\text{core}}/P_{\text{cladding}} \approx 240$. The primary contributors to P_{cladding} are modes which correspond to leaky tunneling rays (refracting rays are dissipated close to the source.) According to equation (1.14):⁹⁷

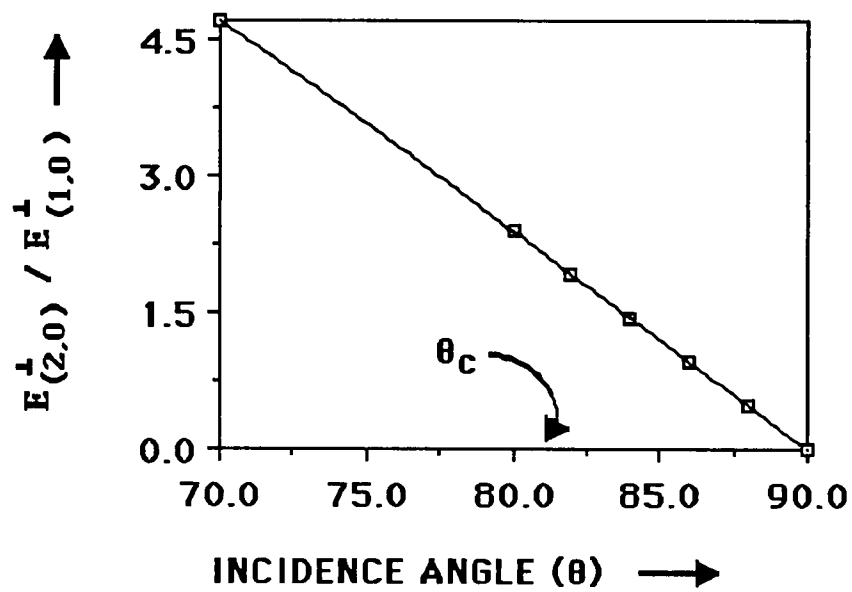


Figure 1.7. Magnitude of evanescent field vs. incidence angle.

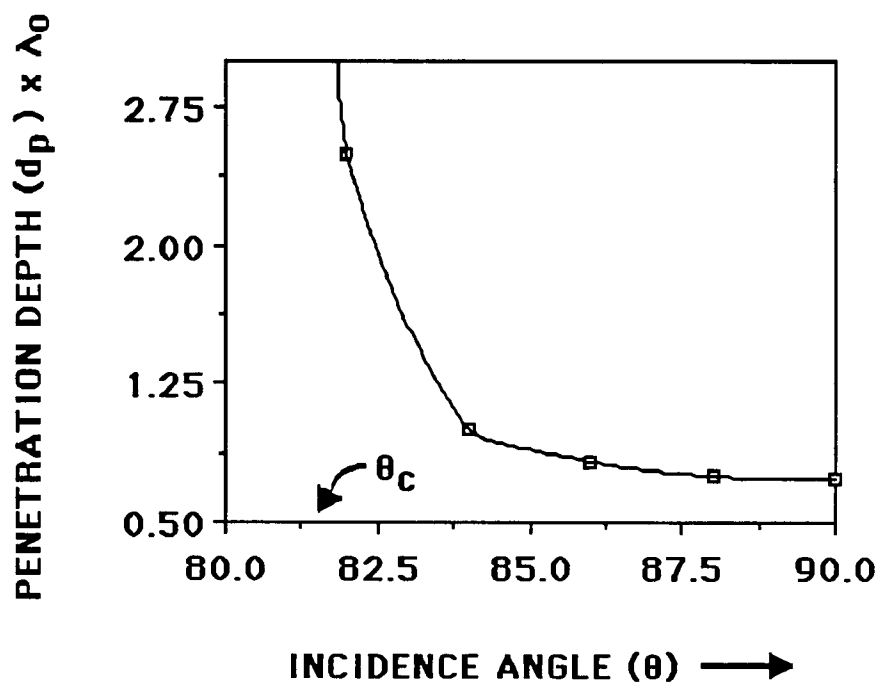


Figure 1.8. Penetration depth vs. incidence angle.

$$z_0 \approx \{d / [4 \cos^{-1}(n_2 / n_1)]\} \exp [(2M)^{1/2} / 2] \quad (1.14)$$

where z_0 is the distance at which tunneling ray power has decreased by 90%, attenuation of tunneling rays over large distances is insignificant for fibers with large M values. The intensity of the evanescent wave, however, is not the same at the end of a long fiber as it is on a short segment. Distance-dependent evanescent field variations are primarily due to the gradual loss of higher-order modes via mode coupling and attenuation. Higher-order modes are the most pronounced contributors to large evanescent field intensities and penetration depths since they correspond to rays with large values of θ_1 . Fluctuations in the frequency and range of θ_1 values will therefore cause variability in the evanescent wave intensity and penetration depth as a function of axial distance (z). These assumptions lead to the conclusion that relatively short fibers should be used in order to generate large homogeneous fields. Literature-reported TIRF devices are typically 10 cm or less,^{70,72} though a 1-meter long probe has been reported.⁹⁸ TIRF sensors usually possess larger exposed surface areas than distal face devices. Unfortunately, for in-situ applications, short fibers with large surface areas are less versatile than small-diameter distal face sensors.

Under optimum conditions, coupling of a 100 mW source onto the previously described sensing fiber will generate the equivalent

of $100(1/240) \approx 0.4$ mW of P_{cladding} in the form of evanescent-wave power. Assuming transmission losses due to other mechanisms amount to 50 dB/km, power at the distal face of a 1-meter long fiber will measure roughly 98.18 mW. This calculation is an approximation since the reagent phase of an extrinsic TIRF sensor would define n_2 and the resulting core/reagent-phase power distribution. It is useful, however, in demonstrating that TIRF sensors must employ larger surface areas than distal-face devices in order to produce comparable signals. A further limitation to TIRF sensors is the poorer collection efficiency of evanescently-excited fluorophores. Assuming $\theta_c \approx 80^\circ$, it can be shown that the emission from an evanescently-excited fluorophore has an allowable re-entry angle, $\theta \approx 10^\circ$. In contrast, the emission from a fluorophore positioned at the fiber's distal face is, on average, 1.5 times as likely to be collected owing to its 15° average allowable re-entry angle. From this information alone it can be seen that in order to observe comparable emission intensities, TIRF sensors require at least $(1.5 \times 240) = 360$ times the surface area of distal-face probes. Evanescent field variability may further increase this factor.

Bending losses. When a fiber is bent, bound rays become leaky rays and power is lost by tunneling and/or refraction. Heavy losses will occur if the bend's radius of curvature, R , is less than

the critical radius of curvature, r_c :⁸⁵

$$r_c = (3/4\pi) \lambda n_1^2 / NA^3 \quad (1.15)$$

or if R is less than 10^3 the fiber's radius. The critical radius of curvature can be minimized by decreasing the wavelength and increasing the difference between core and cladding refractive indices. For a typical PCS sensing fiber where $NA = 0.22$, $n = 1.5$, and $\lambda = 500$ nm, the minimum allowable r_c is 25 μm . In most instances the actual bend radius is enormous compared with r_c and the core dimensions. Bending losses are therefore negligible in practical multimode waveguides. Intrinsic microbend sensors, however, utilize mechanical microbend transducers to detect the presence of thermal, acoustic, and magnetic fields by the observation of deformation-induced losses.⁸¹

Fiber Materials

The primary physical characteristics required of optical fiber materials include transparency, strength, flexibility, and core/cladding compatibility. Most waveguides are fabricated from glass since it can be manufactured to satisfy all these requirements. Plastic fibers are less commonly used due to their high attenuation coefficients ($\alpha(z) = 100\text{-}1000$ dB/km at 0.85

μm); though their low cost, high flexibility (particularly at low temperatures), and large NAs (0.5-0.6) may make them attractive for some applications.

Glass waveguides have losses which typically range from 2-60 dB/km (at 0.85 μm). They are composed either of silica or silicates. Silica systems are also referred to as "fused-silica" and "quartz". They contain SiO_2 intentionally doped with GeO_2 , P_2O_5 , or B_2O_3 . Fabrication is accomplished using the vapor deposition technique.^{93,99} Additions of GeO_2 and P_2O_5 increase the 1.46 refractive index of pure silica, while B_2O_3 has the opposite effect. Pure SiO_2 is insoluble in water and has a low coefficient of thermal expansion. Most additions to SiO_2 cause increases in thermal expansion coefficients. When silica is used as the cladding material (glass/glass fibers), differences in viscosity and thermal expansion between the core and cladding must be minimized in order to inhibit material stresses. Because of this restriction, Δn and NA values are generally small (NA = 0.1 - 0.2).

Glass/glass fibers have core and cladding diameters which range from 3 - 400 μm and 50 - 500 μm , respectively. Due to the excellent transmission characteristics of fused silica, typical losses for doped-silica fibers (at 0.85 μm) range from 2-10

dB/km. It should be noted, however, that the total optical power throughput for a given sensor is highly dependent on wavelength and fiber length.

Plastic-clad silica (PCS) fibers are coated with a transparent plastic cladding material (usually silicone resin). They are most commonly found in 50 - 1000 μm core and 125 - 1200 μm cladding diameters. These fibers generally have larger numerical apertures ($\text{NA} = 0.2 - 0.4$) and larger core diameters than glass/glass communication-grade fibers. Their low attenuation coefficients (10-50 dB/km at 0.85 μm), UV transparency, and large light gathering capabilities make PCS fibers ideally suited for extrinsic sensing applications. Another useful feature is the ease with which the plastic cladding can be removed from the core. This facilitates direct attachment of reagent phase to the waveguide.

Silicate fiber systems consist of silicon, oxygen, and one or more metal(s). Examples include soda-lime-silicate, sodium-borosilicate, and alkali-lead-silicate. Due to their lower melting points and solubility in water they are easier to handle than SiO_2 glass and can be fabricated into fibers with large numerical apertures ($\text{NA} = 0.10 - 0.55$) and 50 - 600 μm core diameters. The primary drawback to silicate fibers is their poor (vs. SiO_2) transmission characteristics. Typical $\alpha(z)$ values range from 20 - 60 dB/km (at 0.85 μm), with particularly large

attenuations in the UV region. Ironically, the abundance of impurities and functional groups responsible for light attenuation in silicate fibers may actually contribute to their versatility as chemical sensors. These active sites (particularly surface Si-OH groups) can be chemically derivatized and used for reagent-phase immobilization. Silicates can also be etched more easily and generally have larger surface areas than fused-silica.¹⁰⁰

Chromatic Dispersion

The primary type of chromatic dispersion encountered in fibers is known as material dispersion. It is the fundamental limit to pulse broadening in waveguides and can be expressed by:⁹⁰

$$M(\lambda) = [(\Delta\lambda/\lambda) L/c] \lambda^2 (d^2n/d\lambda^2) \quad (1.16)$$

where $(d^2n/d\lambda^2)$ is the wavelength dependence of the core refractive index, $\Delta\lambda$ is the source spectral bandwidth, and L is the fiber length. Figure 1.9 illustrates the relationship between $\lambda^2(d^2n/d\lambda^2)$ and λ . It indicates that at about 1.3 μm , $M(\lambda) \approx 0$ for fused silica. Optical attenuation in this region is also low (≈ 5 dB/km) and, for these reasons, fiber communication systems generally operate at 1.3 μm . Further reductions in dispersion can be achieved by minimizing $\Delta\lambda$ through the use of coherent sources, e.g. lasers.

A practical consequence of chromatic dispersion is its effect on NA as a function of λ . Table 1.2 indicates that, for a given length of fiber, the NA and acceptance cone (2β) decrease with decreasing wavelength. These parameters also decrease with decreasing L, though this effect is primarily attributable to the distance-dependent loss of higher order bound and skewed modes. The magnitude of material dispersion in optical waveguides is demonstrated by the fiber optic spectrometer, a device which was designed to exploit the chromatic dependence of refractive index by temporally resolving multi-wavelength information.^{101,102}

Light Coupling

Coupling efficiency compares the quantity of optical power transmitted by bound rays in the fiber, P_{br} , to the total source optical power, P_s . It is an important parameter in sensor instrumentation because it defines the system's throughput, which in turn, is a major determinant of sensitivity and detection limit. Coupling efficiency, CE, can be expressed as:^{103,104}

$$CE = \epsilon \times S_f = (P_{br} / P_{face}) \times (P_{face} / P_s) \quad (1.17)$$

where $\epsilon = (P_{br} / P_{face})$ is the fraction of optical power imaged on

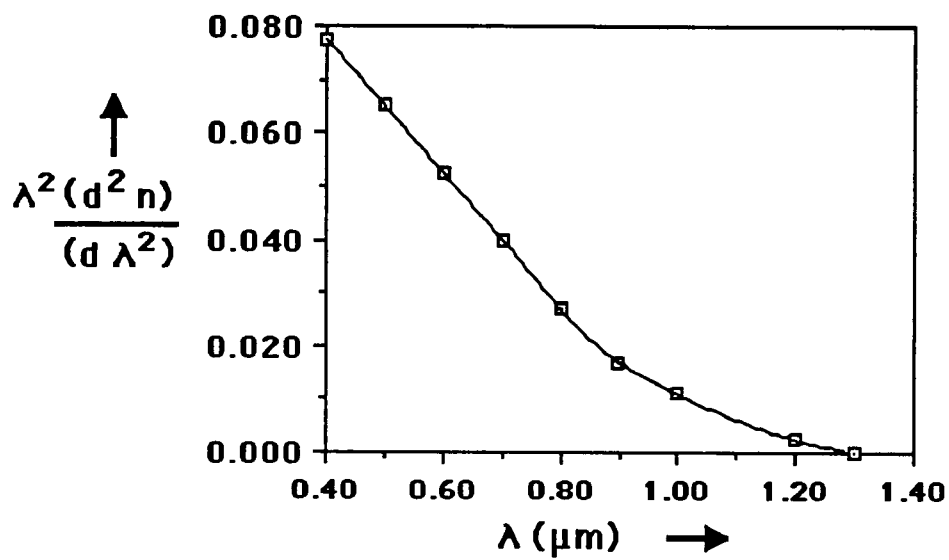


Figure 1.9. Chromatic dispersion vs. wavelength for fused silica fiber (Ref. 90).

TABLE 1.2. Effect of Chromatic Dispersion and Length on Numerical Aperture^a

Length (mm)	Numerical Aperture	
	245 nm	546 nm
150	0.31	0.52
250	0.28	0.46
500	0.23	0.36
1000	0.19	0.30
1800	0.15	0.26

^a For fused silica fiber bundle. (Ref. 105).

the fiber's input face which is transmitted as bound rays and $S_f = (P_{\text{face}} / P_s)$ is the fraction of the source reaching the input face of the fiber. P_{face} is the amount of optical power imaged on the input face of the fiber, P_{br} is the amount of bound-ray power, and P_s is the source power. For diffuse sources which emit over 4π steradians, S_f is roughly $0.5 (A_{\text{face}} / A_s)$ when the fiber is in contact with the source. A_{face} is the surface area of the fiber's input face and A_s is the area over which source power is distributed.

Diffuse sources. It can be shown that the bound ray power fraction, ϵ , for diffuse sources is:¹⁰³

$$\epsilon = 2 (n_2 / n_0)^2 [(n_1^2 - n_2^2) / 2n_1^2] \approx (n_2 / n_0)^2 (\theta_c)^2 \quad (1.18)$$

where n_0 is the refractive index of the coupling medium. If a 200- μm diameter multimode fiber with $\text{NA} = 0.22$, $n_1 = 1.5$, and $n_2 = 1.484$ is placed against a 4.5- mm^2 Xe-arc source, $S_f = 0.5 (\pi \times 0.1^2) / 4.5 \text{ mm}^2 = 3.5 \times 10^{-3}$, $\epsilon = 4.7 \times 10^{-3}$, and $\text{CE} = 1.6 \times 10^{-4}$. Assuming uniform source illumination and neglecting small

reflectance losses at fiber/air interfaces, P_{br} at 550 nm for a 1000-watt lamp (which delivers roughly 260 watts at 550-nm)¹⁰⁵ is 13.7 mW. Unfortunately, a fiber in contact with a polychromatic source is incapable of transmitting monochromatic radiation necessary for most sensors. By positioning the fiber 10 mm away from the source, a bandpass filter can be inserted for wavelength selection. In this case, consider, for simplicity, the 4.5 mm² source to be a "point". Power will be distributed over a spherical surface with a 10-mm radius. The fraction of source power observed by the fiber is : $S_f = (3.14 \times 10^{-2} \text{ mm}^2) / [4 \times \pi \times (10)^2]$ and $CE = 0.047 \times S_f = 1.2 \times 10^{-6}$. With the use of a 50 %T filter, approximately 160 μ W of 550-nm light will be transmitted down the fiber.

The introduction of focusing optics can result in coupling efficiency improvements for fibers separated from the source. For example, consider a 4.5 mm²-diameter source which is located 10 mm from a 10-mm diameter f/1 collimating lens. Approximately 6.3% of the source power will be collected by the lens. If a matching lens is used for focusing, roughly $(A_{face} / A_s) = 0.7\%$ of the source image will be directed on the fiber. The resulting coupling efficiency is $(0.047 \times 0.063 \times 0.007) = 2.1 \times 10^{-5}$. This corresponds to the transmission of nearly twenty times more light ($\approx 3\text{mW}$) using a two-lens configuration. In the case of diffuse

sources, however, the use of lenses cannot improve coupling efficiencies beyond those values attainable when the fiber is in direct contact with the source. Further improvements in optical power transmission for both focused and unfocused systems can only be achieved by increasing fiber diameter.

Collimated sources. Assuming the divergence of a collimated laser beam, θ , is less than the fiber's acceptance half-angle, β , the bound ray power fraction, ϵ , for collimated laser sources is 1. Coupling of a beam which has a larger diameter than the fiber is simply a function of S_f . Approximately 4%, 36%, and 100% of the power carried in a 1-mm diameter beam will be coupled to 200- μm , 600- μm , and 1000- μm diameter fibers, respectively. Laser/fiber coupling efficiencies can be improved by a factor of $(r_L/r_F)^2$, where r_L is the laser beam radius and r_F is the fiber radius, provided the following two conditions are met:¹⁰³ 1) F , the lens focal length, $\geq \psi$, where $\psi = (r_L) \times \{[(n_0/n_1)^2 - \sin^2 \kappa]^{1/2} / \sin \kappa\}$, and $\kappa = \cos^{-1} (n_2/n_1)$ and 2) diameter of the focused beam, $2\omega_0 = (4\lambda/\pi)[F/(2 r_L)] \leq 2 r_F$. If an $f/2$ lens with $F = 20$ mm is used to focus a 1-mm diameter 488-nm laser beam onto the previously described fiber, $\psi = 2.2$ mm and $2\omega_0 = 31 \mu\text{m}$. Coupling efficiencies will be improved for

the 200- μm and the 600- μm fibers by 25% and 2.8%, resulting in transmission of 100% of the incident optical power.

A summary of the coupling efficiencies for collimated and diffuse-source illumination of 200- μm , 600- μm , and 1000- μm diameter fibers is presented in Table 1.3. CE values are calculated for both focused and unfocused configurations. From this information it is clear that for applications requiring monochromatic light, sensor illumination with laser light (if available) is highly preferable to diffuse source coupling. It should be noted that CE values are given without considering the deleterious effects of interfacial reflection on throughput. As much as 4% of incident light can be reflected at each glass/air interface. For a more detailed discussion of the influence of reflected light on throughput and optical background see Chapter 1, Optical Background.

B. FIBER OPTIC INSTRUMENTATION

Intrinsic Sensors

Phase-modulated intrinsic devices are, perhaps, the most widely publicized type of fiber optic sensor. Instrumentation requirements generally include a coherent laser source and two polarization preserving single-mode fibers. Light is coupled onto each fiber and environmental effects may perturb one or both

TABLE 1.3. Source/Fiber Coupling Efficiency^a

Source	Conditions	Coupling Efficiency		
		Diam. = 200 μ m	600 μ m	1000 μ m
Collimated ^b	unfocused	0.04	0.36	1
	focused ^c	1	1	1
Diffuse ^d	0 mm from source	1.6×10^{-4}	1.5×10^{-3}	4.1×10^{-3}
	10 mm from source	1.2×10^{-6}	1.0×10^{-5}	2.9×10^{-5}
	10 mm with focusing ^e	2.1×10^{-5}	1.9×10^{-4}	5.0×10^{-4}

^a neglecting reflections at interfaces, ^b 1-mm beam diameter, ^c single-lens focusing, ^d 4.5 mm² source area, ^e two-lens focusing optics, see text.

fibers differently. If this is the case, a phase shift will occur and interferometric detection can be utilized in order to quantify the magnitude of the external effect. The four most commonly used instrumental configurations are the Mach-Zehnder, the Michelson, the Fabry-Perot, and the Sagnac interferometers.^{12,81,106} Of these, the Mach-Zehnder interferometer is the most frequently encountered configuration and will be discussed in some detail.

Mach-Zehnder interferometer. Figure 1.10 is an illustration of a typical Mach-Zehnder fiber interferometer. Laser light is focused onto a single-mode, polarization-preserving, sensing fiber designed to respond to the phenomenon of interest. A passive coupler splits 50% of the injected beam into a second single-mode polarization-preserving fiber which serves as the interferometer's reference arm. The coupler is a commercially available device which utilizes evanescent energy transfer between fibers in order to divide the beam. It is designated as a "3dB" coupler because, as described in equation (1.6), a 3dB loss results in a 50% power split. The two light beams are recombined by a second 3dB coupler which converts the phase-modulation to intensity-modulation. The optical signal is processed and correlated to a phase shift in the sensing fiber.

If the pathlengths (L) and the core refractive indices (n_1) of the sensing and reference fibers are the same, the light in both arms

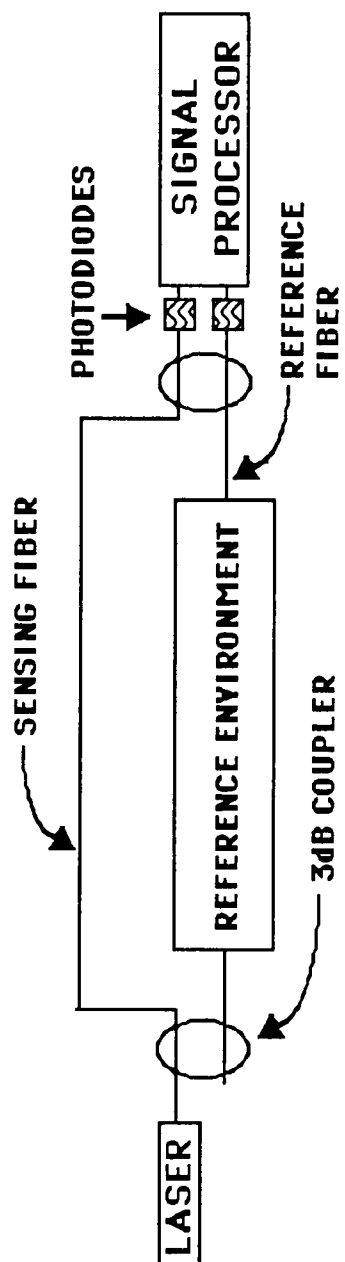


Figure 1.10. Mach-Zehnder fiber interferometer.

is in phase. Under these conditions, the recombined beam intensity is at its maximum. Output intensity is at its minimum when the two beams are $\lambda/2$ out of phase. The relationship between phase angle, ϕ , core refractive index, n_1 , and fiber length, L , for light travelling in a fiber is:^{81,106}

$$\phi = 2\pi n_1 L / \lambda_0 \quad (1.19)$$

where λ_0 is the wavelength of light in a vacuum and ϕ is the phase angle in radians. Changes in length and/or refractive index will cause phase changes according to:

$$\phi + \Delta = (n_1 L + n_1 \Delta L + L \Delta n_1) \quad (1.20)$$

As illustrated in Figure 1.11, the interferometer's output is a function of relative phase shift. When the phase shifts an integral number of wavelengths ($\Delta L = \lambda, 2\lambda, 3\lambda$, etc.) ϕ is zero or an even integral multiple of π , ($0, 2\pi, 4\pi$, etc.). Combination of the interferometer's arms results in constructive interference and maximum output intensity. When ΔL is an odd integral number of half wavelengths ($\lambda/2, 3\lambda/2, 5\lambda/2$, etc.), ϕ is an odd integral multiple of π , ($\pi, 3\pi, 5\pi$, etc.) and the two light beams destructively interfere, resulting in minimum output intensity.

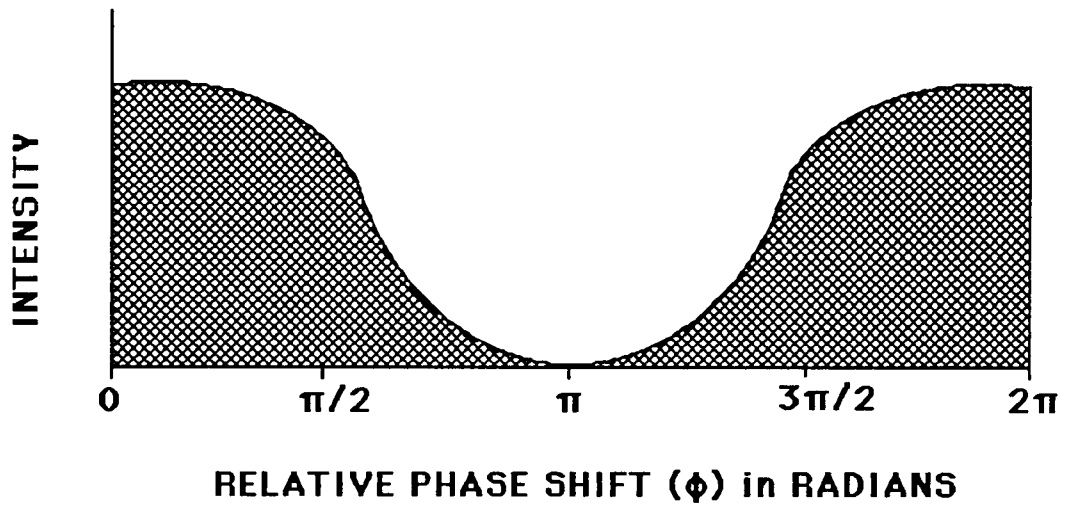


Figure 1.11. Interferometer output.

The minimum detectable phase shift for most interferometers is about 1 μ -radian.⁸¹ This corresponds to ΔL values of approximately 10^{-13} meters. Sensitivity is primarily a function of the type and amount of coating material on the sensing arm. Acoustic sensors may use several meters of sensing fiber coated with a compliant elastomer. Temperature sensors, on the other hand, may require less than a meter of fiber coated with a thermally sensitive material. Unfortunately, many of these devices suffer from background fluctuations and drift. This is due, in large part, to the lack of selectivity of the sensing element and to the extreme sensitivity of both arms to external fields (e.g. temperature and pressure).^{12,81} An interesting chemical sensor has been reported which attempts to overcome the selectivity limitation by employing an enzyme-coated sensor arm. Generation of heat during the enzyme/substrate reaction causes changes in sensor length.¹⁰⁷ This principle has also been applied to a hydrogen sensor which uses palladium as the immobilized reagent.⁶³ The application of selective chemical coatings which can reversibly expand, contract, or generate heat upon binding to ligand is a potentially fertile and largely unexplored area of chemical analysis.

Extrinsic Sensors

Extrinsic fiber optic sensors are generally more versatile, less expensive, and more frequently encountered than intrinsic devices. They are broadly classified as either "evanescent" or "distal face" sensors according to the type of reagent-phase excitation employed. Instrumentation is fashioned in a manner which best exploits their amplitude-modulated response. Specific design features including light source, fiber type, and reagent phase are determined by the requirements of the analytical system. The availability and variety of each of these components has engendered the creation of a host of devices.

Distal face sensors. The most frequently encountered extrinsic sensor design utilizes distal-face excitation of a mechanical transducer or an immobilized reagent phase. Both single-fiber and fiber-bundle configurations are employed. Single-fiber designs are generally capable of greater collection efficiencies than either dual-fiber or multiple-fiber bundles. Figure 1.12 is an illustration of a typical single-fiber sensing configuration. Light is directed onto the fiber through a beamsplitter. Dichroic filters have been used as beamsplitters for fluorescence measurements. Absorbance and reflectance detection, however, require wavelength-independent splitters such

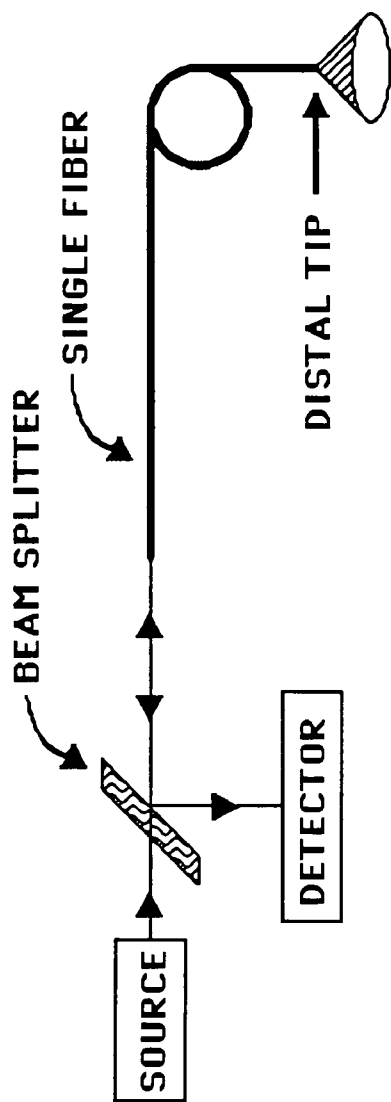


Figure 1.12. Single-fiber, distal-face sensing configuration.

as prisms and 3dB fiber couplers. If a laser source is employed, a beam-size mirror or, conversely, a large mirror with a beam-size hole can be used.

Light travels the length of the fiber and encounters the chemical or mechanical transducer at the sensing tip. Interaction between the transducer and the environment will produce changes in the optical signal. A portion of the modified signal is collected by the fiber and transmitted to the detector. The fraction of light collected by the fiber, Ω , is designated as its "collection efficiency". The distance a fiber can "see" beyond its distal face, Y , is roughly twice the fiber's core diameter.^{108,109} The observed optical signal diminishes with distance in spite of the greater number of interrogated species. This is due to the fact that, as shown in Figure 1.13, average re-entry angle (θ_{ave}) and collection efficiency decrease with increasing distance from the fiber. Both Ω and Y depend upon fiber diameter and numerical aperture. For a 200- μm diameter fiber with $NA = 0.22$, $\theta_c \approx 80^\circ$, and $2\beta \approx 25^\circ$: $\Omega(ave) \approx (\text{ave. re-entry angle} / 360^\circ) \times (4\pi \text{ steradians}) \approx 15^\circ / 360^\circ (4\pi \text{ sr}) \approx 0.5 \text{ sr}$, and $Y \approx 2(D_f) \approx 0.4 \text{ mm}$, where D_f = fiber diameter. The estimated $\Omega(ave) \approx 0.5 \text{ sr}$ is calculated for species on the face of the fiber and is comparable to $\Omega \approx 1\text{sr}$ observed for conventional backscattering

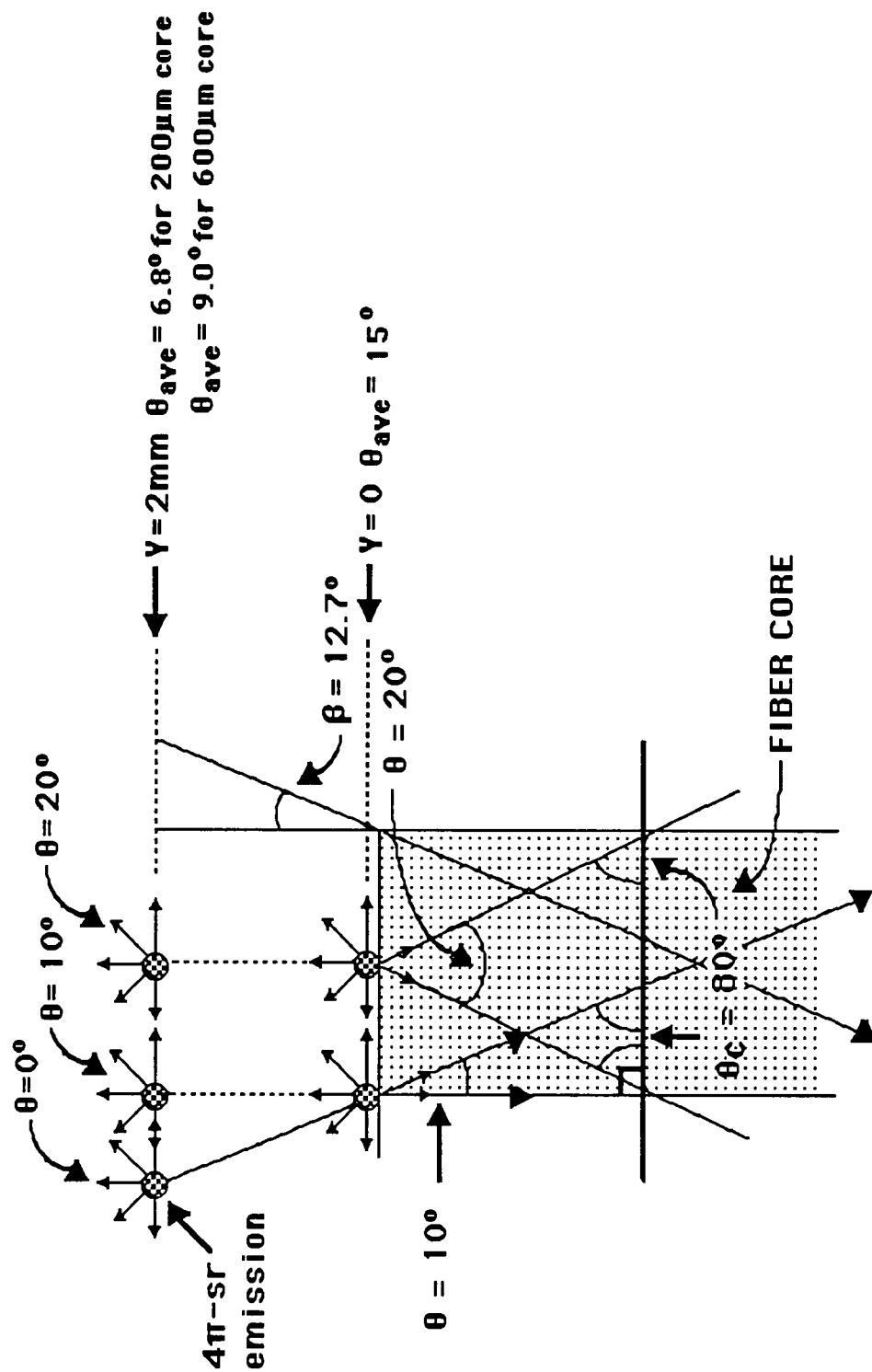


Figure 1.13. Fiber viewing region.

optics.^{110,111} Collection efficiencies can be improved by increasing fiber diameter and NA, and by placing micro-lenses at the sensing tip.

Placement of scattering or reflective materials within reasonable proximity of the distal face will disrupt the viewing volume. The response of absorbance and reflectance sensors is often dependent on the collection efficiency of surface-reflected light.³⁶ The viewing region and collection efficiency of these devices is a function of the fiber size, NA, and the orientation and distance of the reflective material from the fiber.¹¹²

A fiber bundle sensor configuration is illustrated in Figure 1.14. Bundles are used, in many cases, because of their convenience. Source/fiber coupling efficiencies are potentially superior to single fiber arrangements, particularly when large diameter bundles are used with diffuse sources. Viewing volumes and collection efficiencies, however, are generally poorer. Despite the fact that a number of fibers may be positioned around a central input fiber, the overlap between the collection cones and the source cannot exceed the viewing overlap experienced by a single fiber device. Furthermore, overlap regions diminish with increasing proximity to the fiber face, and the region of greatest source intensity ($Y = 0$) is not viewed at all. As previously stated, more analyte is interrogated at greater distances from the face; however, collection efficiencies are lower at larger distances. An additional drawback to bundles is the fact that they can not be

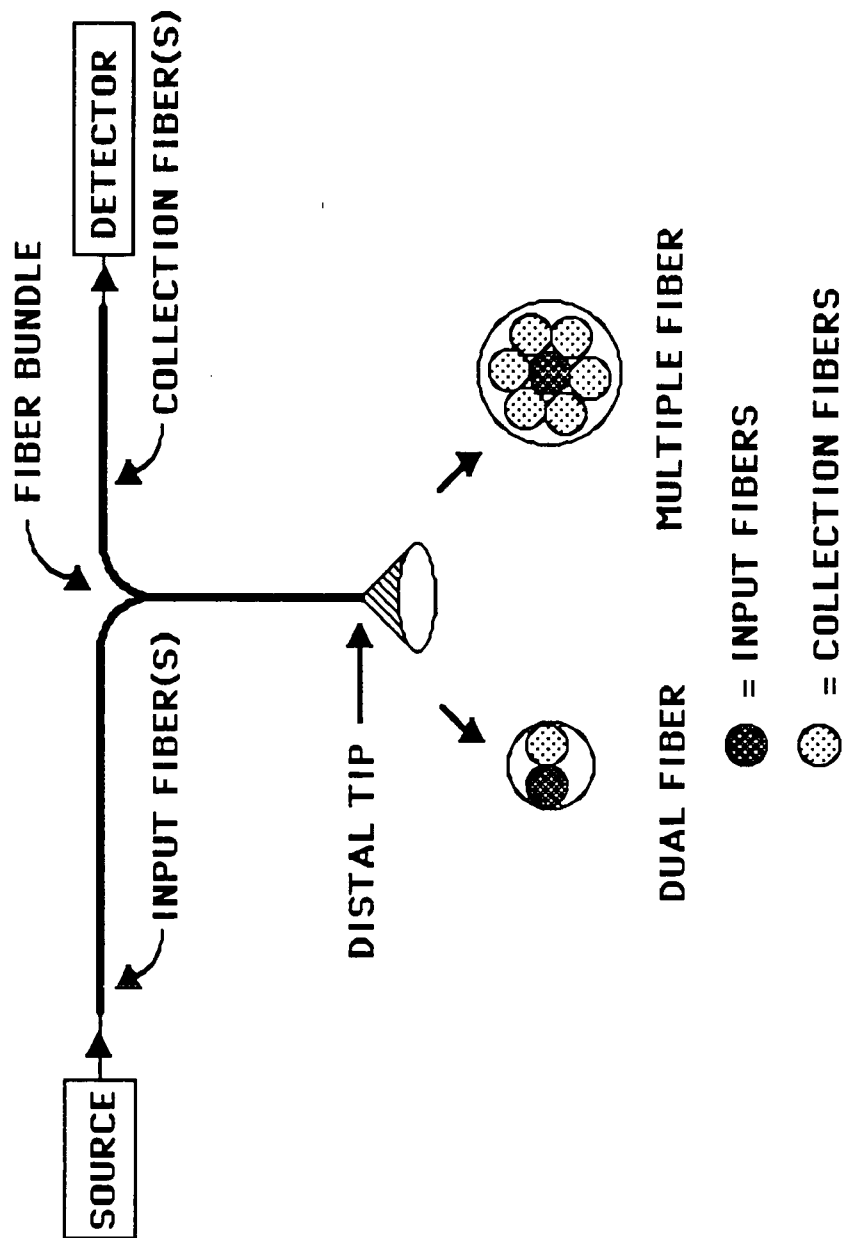


Figure 1.14. Fiber bundle sensing configuration

miniaturized beyond single fiber dimensions.

Bundles may be superior to single fibers when absorbance or scattering is the measured optical property. Since most of the optical power emerging from a multimode input fiber is in the form of divergent meridional rays, collection fibers will preferentially observe reflected illumination. Single fiber devices, on the other hand, may exhibit poorer collection efficiencies since divergent reflected light is excluded from their field of view.

TIRF sensors. TIRF sensors are generally fabricated by removing 2-10 cm of the fiber's jacket and cladding^{70,72,98} and coating the core with a reagent phase. Plastic-clad silica fibers can be stripped mechanically or chemically; however, glass claddings must be removed chemically, usually with hydrofluoric acid (HF). HF etching efficiently reduces the mass of fused silica while leaving the surface smooth and featureless.¹⁰⁰ Moderate increases in surface silanol groups (Si-OH) have also been observed with HF. This may be beneficial when OH-dependent immobilization strategies are employed. Source and detector may be separated by a beamsplitter and positioned at the fiber's input end (Figure 1.13). In this arrangement, the fiber's distal face is usually silvered or coated with blackened epoxy in order to minimize bulk solution viewing.⁹⁸ Alternatively, the detector may be placed opposite the source at the fiber's distal end.⁷⁰

Optical background levels are generally much higher in the latter configuration. Drawbacks to TIRF devices include their previously described evanescent wave heterogeneity and their lack of in-situ versatility. Additional sensitivity difficulties may arise when the source is opposite the detector or silvered fibers are used since these designs require high-rejection-ratio, low-throughput emission selection optics.

Optical background. High levels of optical background are encountered in all types of intensity-modulated extrinsic sensor instrumentation. The source of stray light is principally reflections from surfaces of optical components, particularly fiber faces. For materials of different refractive indices, n_a and n_b , the amount of interfacially reflected light, ρ , is:¹¹³

$$\rho = (n_a - n_b)^2 / (n_a + n_b)^2 \quad (1.22)$$

If focusing and beamsplitting components are coated with anti-reflection materials, their contribution to total backscattered light (ρ_{total}) may be as little as 0.4% per interface. Assuming $\rho = 4\%$ and 0.4% for air/fiber and fiber/water interfaces respectively, total backscattered light may be as high as 6% of the incident power (ρ_{total} may be as high as 12 - 13% without anti-reflection coated optics). If fluorescence is monitored,

emission selection optics may have roughly a 3% throughput and a 10^4 rejection ratio. For fluorophores immobilized at the distal face of the fiber, $\Omega(\text{ave}) \approx 0.5 \text{ sr} \approx 4\%$ collection efficiency. Assuming the fluorescence quantum efficiency is unity, the equivalent of $(0.03 \times 0.04 \times 0.96) \approx 0.1\%$ of the incident power will be seen as signal. If the optical background (from optical components) is $(0.06/10^4) \approx 6 \times 10^{-4} \%$ of incident power, un-rejected backscattered light will constitute roughly 0.6% of the observed optical signal. If absorbance or reflectance detection is employed, stray light (unwanted backscatter) is not spectrally rejected and optical signal is usually collected from a reflector placed a few millimeters beyond the distal face. In this case, detectable optical signal, generated by the fraction of light returned to the fiber, must exceed the 6% backscattered light contribution by a factor of two. Clearly, reductions in backscattered radiation can result in significant sensitivity improvements in absorbance and, to a lesser extent, fluorescence detection.

Several techniques have been suggested to minimize the impact of backscattered light. These include employing high-rejection-ratio double monochromators¹¹⁴ and bevelling the fiber's incident and distal faces at angles which exclude the reflected light from the detector's view.¹¹⁵ Limitations to these techniques include the low throughputs of double monochromators, and the cumbersome, irreproducible nature of angling small quartz

surfaces. Time-resolved techniques have also been utilized. These include the temporal discrimination between long-lived reagent-phase fluorophors and the instantaneous backscatter,⁷⁷ and the utilization of high-frequency phase resolution to temporally discriminate between the optical signal emanating from the end of a long-path-length sensing fiber and the short-path-length optical background which occurs in the vicinity of the input face.¹¹² In the latter experiment, 30 meters of fiber were required to achieve the 90° phase shift necessary for complete background cancellation at a modulation frequency of 844 kHz.

Sensing termini. A number of sensing termini have been described which utilize either surface reflectance or backscattering collection geometries. As previously described, absorbance probes generally employ a reflective surface positioned at some fixed distance, b , from the distal face. Both single-fiber and fiber-bundle arrangements are common. Light encounters the measurement solution with a path length of $2b$, and absorbance can be correlated to reductions in reflected power. A close relative to this type of device is the mechanical reflectance transducer. Since reflected light throughput is a function of mirror position, the mirror is connected to a membrane or polymer which is sensitive to the measured field, e.g. temperature, pressure, etc. Changes in field intensity produce optical signal

variations based on mirror position.¹¹⁴

In contrast to surface reflectance configurations, backscattering collection geometries are generally utilized in conjunction with fluorescence detection. Both reflectance and backscattering collection, however, are widely used in the design of fiber optic chemical sensors (FOCSs). FOCSs are characterized by their chemically selective reagent phase at the fiber's sampling terminus. Their purpose is to provide simple, rapid, in-situ analyses of trace chemicals. Figures 1.15 (a-f) illustrate the most common types of FOCS termini.

The reagent phase should, ideally, be a highly specific, rapidly reacting, reversible binder for the ligand of interest. Many devices are actually single-shot disposable probes since reagent phase kinetics hinder reversibility.⁷³⁻⁷⁷ These reagent phases generally have large formation constants (K_a) and are capable of excellent speed, sensitivity, and selectivity. Probes with these features can function, at best, in a pseudo-continuous fashion only if excess binder is used. At the conclusion of each measurement the probe is "reset" so that the signal from the recently formed product becomes the baseline for the next analysis. Alternatively, the reagent phase can be reset by exposing it to "chaotropic" conditions where the ligand/binder reaction is reversed.¹¹⁶ Critical response parameters for these devices include the amount of binder and the rate of analyte mass transport to and in the reagent phase. Ideally a thin layer of reagent is covalently

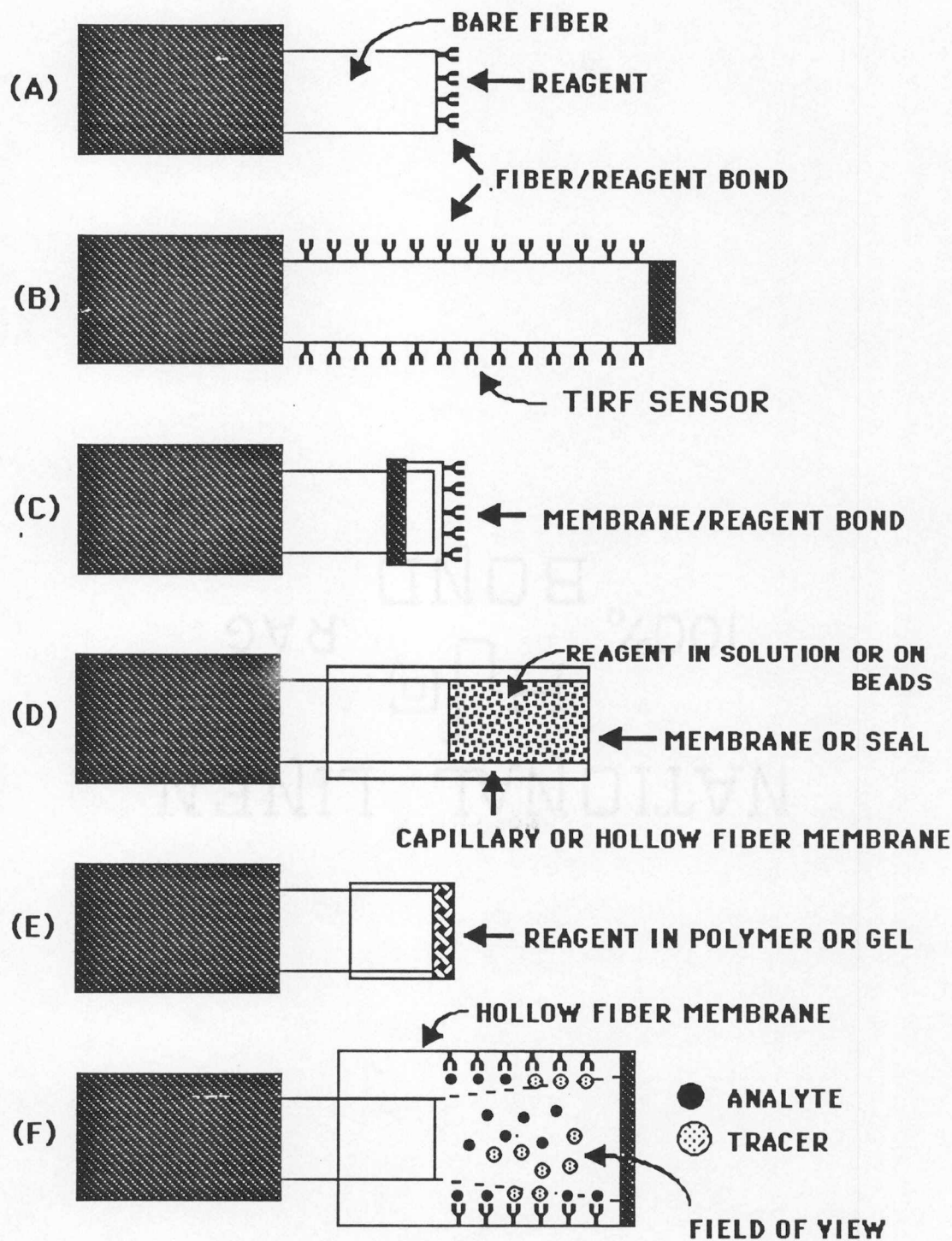


Figure 1.15. Possible FOCS termini.

attached to the surface of the fiber (Figures 1.15-a and 1.15-b) or to an optically transparent substrate (Figure 1.15-c). If there is not enough detectable material, higher loadings of reagent can be achieved by immobilizing it behind a membrane either in solution or on beads (Figure 1.15-d), or entrapping it in a gel or polymeric matrix (Figure 1.15-e). Due to scattering, swelling, and settling effects, however, the latter three immobilization strategies may only be useful in specialized applications. Response times for sensors whose reagent phases are thicker than a monolayer or entail transport through a membrane may be slower than those which employ a monolayer of binder. Furthermore, mass transport conditions must be scrupulously controlled since time-dependent non-equilibrium measurements may be performed with these devices. Provided there is sufficient binder, long incubations can pre-concentrate and even separate analyte from complex matrices. Examples of this type of reagent phase include biochemically significant ligand/binder combinations such as antibody/antigen, enzyme/substrate, and lectin/carbohydrate reactions.¹¹⁷

A second type of reagent phase utilizes the rapid reversibility and color change associated with indicator reactions.³⁹⁻⁴³ The extent of spectral shift is proportional to the magnitude of the measured phenomenon. Because of their low association constants, these reagents are significantly less sensitive and selective than the previously described ligand/binder reactions. Their equilibrium-based response, however, is fast, continuous,

and reversible, and the amount of indicator is sufficiently small that analyte concentration is not perturbed. The majority of extrinsic chemical sensors utilize either acid/base,^{44-46,48,54-58} complexation,⁴⁹ ligand exchange,⁶¹ or luminescence quenching^{46,52,59,60,62} indicator reactions. Immobilization geometries are identical to those employed in irreversible ligand/binder sensors.

A third type of reagent phase is based on competitive equilibria between a binder and two ligands. One of the ligands has a unique optical property, e.g. fluorescence, and is designated as the tracer. The other ligand (analyte) competes with tracer for reagent phase binding sites. Various detection schemes can be employed in order to perform analyses. One of the most successful configurations is the "field of view" sensor illustrated in Figure 1.15-f.³³ Binder is immobilized on the inner surface of a hollow-fiber membrane and the sensor is loaded with tracer--out of the fiber's field of view. The end of the membrane is sealed in order to maintain its molecular-weight cutoff characteristics and to prevent the fiber from viewing the bulk solution. Tracer must be large enough to remain inside the membrane. This can be accomplished by crosslinking or labeling with a large fluorophore, e.g. phycobiliproteins.¹¹⁷ Analyte diffuses into the sensor and competes with tracer for a limited number of binding sites. Displaced tracer migrates into the field of view causing an increase in fluorescence signal which is directly proportional to

analyte concentration. As previously described, the more sensitive and selective the binder, the poorer the reversibility. Response times are primarily dependent upon the rate of analyte transport across the membrane material and, under certain circumstances, the ligand/binder association and dissociation rate constants. Detection can be accomplished by utilizing spectral shifts, temporal discrimination, or field of view in order to distinguish between bound and free tracer.

CHAPTER 2

PRINCIPLES OF FLUOROIMMUNOASSAY

Immunological methods are among the most sensitive, selective, and widely applicable techniques available for bio-analysis. They are used primarily to detect low concentrations of drugs, toxins, proteins, hormones, and microorganisms in complex biological matrices. Radioimmunoassay is the first and the most frequently used immunological method. Despite its excellent sensitivity and precision, hazards and inconveniences associated with the handling of radiolabeled materials have stimulated the pursuit of alternative immunochemical techniques.

Recent developments in fluorescence labeling and detection strategies have significantly expanded the range of immunoassay applicability. Along with advances in fiber optic instrumentation, these innovations have been the driving force behind antibody-based fiber optic sensor development. In order to intelligently design and apply devices which perform immunochemical measurements, antibody structure and function must be understood. Accordingly, the ensuing discussion is devoted to these topics.

Antibody Structure

Normal human plasma protein levels are about 70 g/L.¹¹⁹ The globular protein, albumin, constitutes about half of the total plasma protein concentration. Immunoglobins are the second most abundant human plasma protein, accounting for roughly 11 % - 26 % of the total protein concentration¹¹⁹. Approximately 80 % of immunoglobulins belong to the "G" class.¹¹⁹ These immunoglobulin G (IgG) molecules are the primary contributors to the immune response and are typically referred to as "antibodies".

Figure 2.1 is an illustration of an IgG antibody. It is composed of two identical "light" (L) protein chains and two identical "heavy" (H) protein chains. Each H and L chain is joined by a disulfide bond. The H chains are attached to one another via a disulfide linkage in the flexible hinge region of the molecule. The entire structure is Y-shaped and has a molecular weight of approximately 160,000.

The amino half of light chains and amino quarter of heavy chains have regions where there is amino acid sequence heterogeneity. These variable regions are the ligand binding sites. They vary between antibodies and their exact sequence determines which molecule(s) will combine with the IgG. Each H-L pair therefore contributes one binding site and the entire 4-chain molecule is bivalent.¹²⁰

At neutral pH, the enzyme papain cleaves the IgG molecule and produces three large fragments. Papain acts by selectively

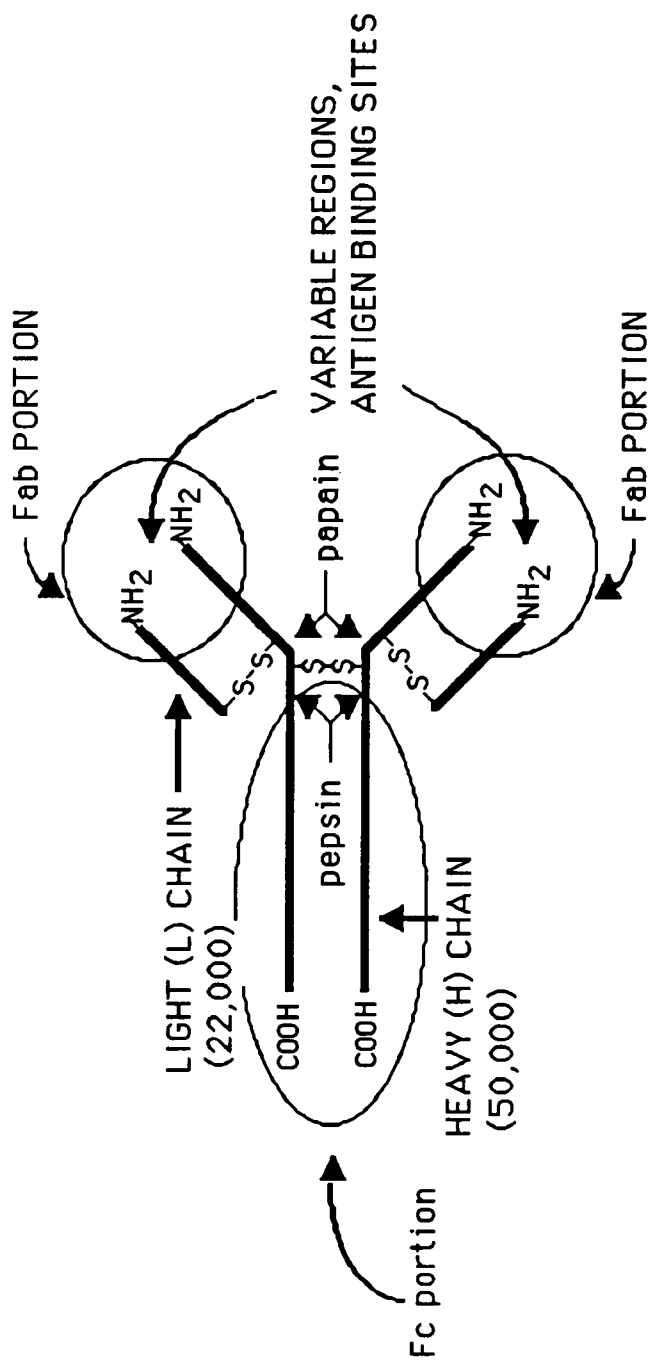


Figure 2.1. IgG antibody structure.

attacking each H chain at a site just N-terminal to the interheavy chain disulfide bonds. Two of the resulting fragments are identical (MW $\approx$ 45,000) and have a single binding site. They are designated as Fab fragments--ab standing for "antigen binding". The third fragment, Fc, has a molecular weight of roughly 50,000. The "c" stands for crystallizable, since, in a cold neutral buffer, this portion of the IgG digest will spontaneously crystallize. The amino acid sequence in the Fc portion does not vary between molecules.¹²¹

The enzyme pepsin attacks the C-terminal side of the interheavy chain disulfide bonds. A single large bivalent fragment designated as $F(ab')_2$ is produced. The interheavy chain disulfide bonds can be reduced to yield two univalent Fab' fragments. Each Fab' fragment is terminated with a sulfhydryl (-SH) moiety and is therefore amenable to different immobilization strategies than the Fab fragments. Understanding the relationship between structure and the availability of derivatizable functional groups is a critical component in the design of immunosensors based on immobilized immunochemical reagent phases.¹²²

Antibody Function

The reversible non-covalent combination between antibody and its specific antigen or hapten occurs in the variable Fab portion of the molecule. Antigens are molecules which can elicit an immune response in an animal. In order to successfully induce antibody

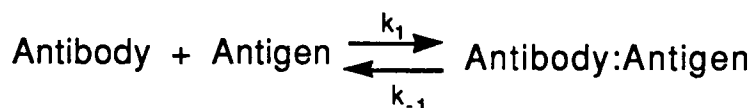
production, antigens usually have molecular weights greater than 5000 - 10,000.¹¹⁹ Haptens, on the other hand, are small molecules which do not stimulate antibody production. Both antigens and haptens have structural features known as epitopes or antigenic determinants. The Fab portion of the IgG molecule is produced to recognize these antigenic determinants.

Antibodies can be broadly classified as either polyclonal or monoclonal. Polyclonal antibodies are raised in hyperimmunized animals. They are formed in response to a single antigen and possess several different binding sites which are capable of combining with each of the antigen's multiple epitopes. The association strength between an antibody and a single epitope is termed affinity. This interaction energy varies with the antigenic determinant and, for polyclonal antibodies, the average interaction strength for heterogeneous antibodies is referred to as avidity.¹²³

Monoclonal antibodies (MoAb) are raised from single antibody-producing cells fused to a myeloma cell, the latter providing immortality and selective phenotype for isolation and single antibody production. The hybrid cells (hybridomas) produce molecules capable of recognizing a single antigenic determinant. MoAb are characterized by a single association constant which defines their affinity for a particular antigen or hapten. They are particularly valuable in immunosensors since their binding site homogeneity produces a uniform response.^{120,123}

The affinity constant (K_a) for immunochemical complex

formation is an indicator of polyclonal avidity and monoclonal affinity. It is described by:



$$K_a = k_1/k_{-1}$$

Typical K_a values range from $10^6 - 10^{12} \text{ M}^{-1}$ for antibody/hapten complexes.¹¹⁹

Forward reaction rates are generally less variable than reverse reaction rates.^{119,121} Values for k_{-1} and k_1 range from $10^2 - 10^{-4} \text{ s}^{-1}$ and $10^7 - 10^9 \text{ M}^{-1}\text{s}^{-1}$ respectively.¹²⁴ Due to these large k_1 values, the rate of antibody binding is primarily mass-transport controlled.^{119,125,126} Both k_1 and k_{-1} must be considered in determining the response times of immunosensors which rely upon dynamic equilibria to operate on a continuous basis. At the expense of sensitivity and selectivity, rapid equilibrium-based sensors can be constructed with low affinity antibodies;⁷⁸ however, response times will never approach those attainable with indicator-based reagent phases (Chapter 1). In order to maximize the benefits of immunochemical reagent phases, the speed, specificity, and concentrating capability of high affinity antibodies should be exploited. This can be accomplished by using large excesses of homogeneous antibodies in

non-equilibrium assays or by regenerating binder following equilibrium measurements.

Binder regeneration can be achieved by inducing immune complex dissociation. This involves disrupting the non-covalent interactions between antigenic determinant and antibody combining site. Since optimal antibody binding generally occurs in the pH 6 - 8 range, chaotropic reagents which alter the pH or ionic strength of the medium will "unzip" the associated determinant. Some effective chaotropic reagents are: 6 M guanidine hydrochloride, 3 M potassium thiocyanate, 8 M urea, and 1 M propionic acid.¹¹⁶ After exposure to the appropriate reagent, the protein can be returned to its original conformation by renaturing at neutral pH. A significant amount of ligand binding capacity may be lost with each dissociation/renaturing cycle, though a procedure for near-perfect regeneration has been reported.¹²⁷ Success appears to be dependent upon the type of antibody, the chaotropic reagent, and the immobilization procedure.

Antibody Measurements

Fluoroimmunoassays (FIAs) are typically classified according to whether a separation step is necessary in order to distinguish antibody-bound analyte from free analyte. In homogeneous FIAs, antibodies are added to a sample and measurements are performed without separating antibody-bound material from the reaction mixture. Analyte concentration is usually determined by

measuring binding-dependent changes in the fluorescence properties of antigen or antibody. Among the most commonly detected phenomena are binding-mediated variations in lifetime, polarization, quenching, energy transfer, and spectra.^{119,128-131} Because homogeneous measurements do not require separation of antibody-bound analyte from the sample, they are generally faster and more straightforward than heterogeneous techniques. Unfortunately, the simplicity of homogeneous measurements is often offset by their poor sensitivity. Sensitivity-limiting factors include: the presence of interferences, high levels of optical background, and the fact that small changes in fluorescence properties are measured. Because of these drawbacks, most applications of homogeneous FIA involve the detection of analytes which occur in high concentrations, e.g., drugs.¹²⁹

In heterogeneous FIAs antibody-bound analyte is separated from free analyte prior to measurement. Separation is typically accomplished by precipitation, dialysis, or by using solid-phase-bound antigens or antibodies.^{119,128,129,132} Of these, solid-phase separation on easily washable surfaces which display low fluorescence backgrounds is most common. Since analyte is removed from the sample matrix and can be rinsed, interference and optical background effects are minimized. Furthermore, fluorescence intensity is measured rather than changes in fluorescence properties. As a result, heterogeneous

FIAs are more versatile, sensitive, and usually have lower detection limits than homogeneous techniques.

Measurement strategies for both heterogeneous and homogeneous FIAs can be grouped into three broad categories. They are: 1) competitive binding assays, 2) direct assays of natural fluorophors and 3) double-antibody (sandwich) assays. Figure 2.2 illustrates the principles of each of these methods. In competitive binding measurements (Figure 2.2-a) fluorophor-labeled analyte (tracer) competes with unlabeled analyte for a limited number of antibody binding sites. Provided there is sufficient material for detectability, sensitivity is inversely proportional to the amount of antibody.¹³³ An inverse relationship between fluorescence intensity (or fluorescence property) and the concentration of unlabeled analyte is also observed. Linear dynamic ranges are typically small, extending over approximately one decade of concentration.⁷³ Among the most frequently-used fluorescent labels are: fluorescein isothiocyanate (FITC), rhodamines, phycobiliproteins, and metal chelates.¹²⁹ In general, labels should be stable, have a high fluorescence quantum efficiency, should be distinguishable from optical background, and should not change characteristics upon coupling to analyte (or antibody). Properties of some common fluorescent labels are listed in Table 2.1.

Direct assays (Figure 2.2-b) are performed by incubating analyte with excess antibody. Since analyte is a natural

Figure 2.2. Fluoroimmunoassays: **(A)** Competitive Binding, **(B)** Direct, **(C)** Double Antibody (Sandwich Assay).

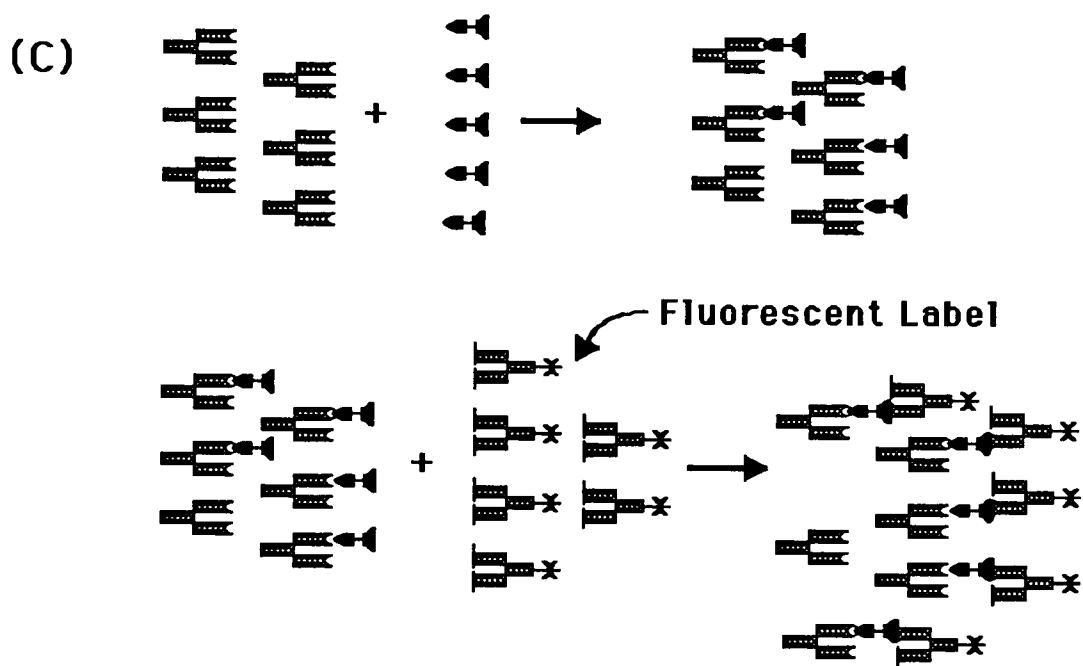
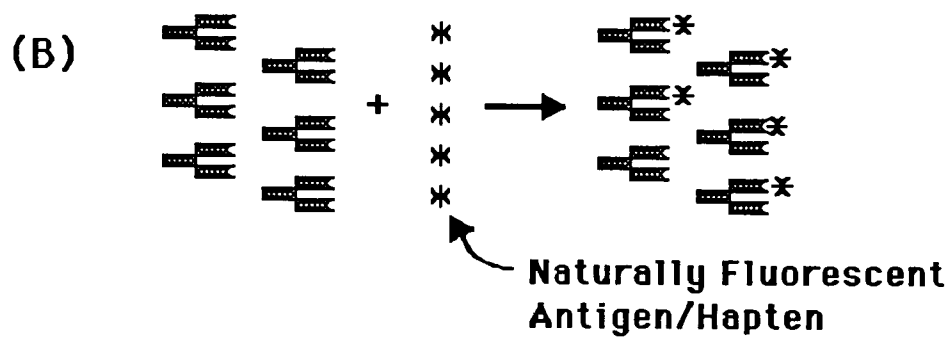
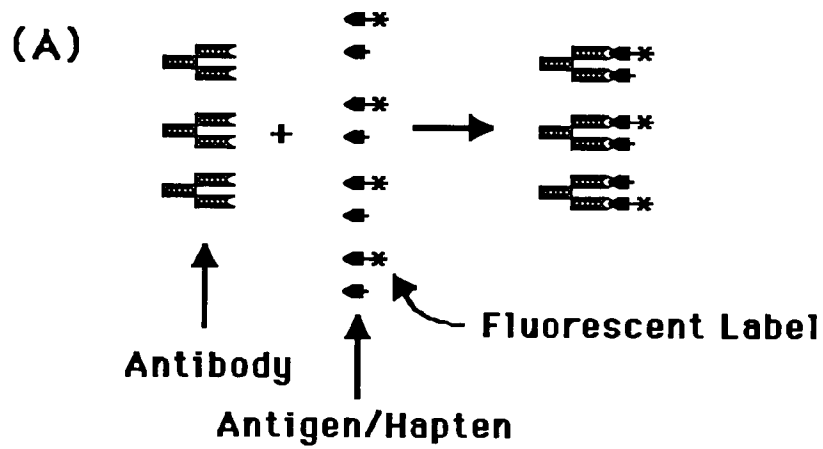


Table 2.1. Properties of Common Fluorescent Labels^a

Label ^b	λ_{ex} (nm)	λ_{em} (nm)	ϵ (L/mol·cm)	ϕ_{fl}	τ (ns)
FITC	490	520	7×10^4	0.85	4.5
RBITC	550	585	1.2×10^4	0.70	3.0
DANS	340	480-520	3.4×10^3	0.30	14
Phycobili- proteins	550-620	580-660	7×10^5 - 2.4×10^6	0.50-0.98	
Eu-(β -NTA) ₃	340	590,613	3×10^4	5.0×10^5	

^a From Ref. 128, 129.

^b FITC, fluorescein isothiocyanate; RBITC, rhodamine B isothiocyanate; DANS, dansyl chloride; NTA, naphthoyltrifluoroacetone

fluorophor, response (fluorescence intensity or fluorescence property) is proportional to analyte concentration and sensitivity is proportional to the amount of antibody. Measurements can be conducted either under equilibrium or non-equilibrium conditions and linear dynamic ranges are potentially large, extending over more than one decade of analyte concentration.⁷⁶ Double-antibody or "sandwich" assays (Figure 2.2-c) are conducted by incubating unlabeled, non-fluorescent analyte with an excess of "primary" antibody. The antibody-antigen complex is then incubated with a high concentration of fluorophore-labeled "second" antibody. The second antibody is selected to recognize antigenic determinants of the primary antibody, thus forming an antibody "sandwich" with analyte occupying the center. Sensitivity is proportional to the amount of primary antibody and response is proportional to analyte concentration. Linear dynamic ranges can extend over several decades of concentration. Commonly-used fluorescent labels include those listed in Table 2.1.

Limits of detection for these immunoassays are a function of incubation time, the K_a , and the detectability of the measured optical parameter.⁷⁶ Instrumentation which utilizes laser-based fiber optic fluorescence detection is generally sensitive enough to allow each of these techniques to be adapted to a FOCS design. The remaining considerations in attempting to construct successful sensors are primarily associated with the nature of the reagent

phase. In order to illustrate the interplay between instrumental and chemical requirements, the ensuing chapters will focus on the design, development, and application of two types of antibody-based FOCS. The first fluoroimmunosensor (FIS), described in Chapter 3, utilizes the principles of competitive binding fluoroimmunoassay in order to detect rabbit antibodies. The second FIS design, described in Chapter 4, incorporates direct fluoroimmunoassays for the detection of fluorescent metabolites of the carcinogenic hapten, benzo(a)pyrene.

CHAPTER 3

FIBER OPTIC CHEMICAL SENSORS BASED ON COMPETITIVE BINDING FLUOROIMMUNOASSAY

This chapter describes the development of a fiber optic chemical sensor based on the principle of competitive binding fluorescence immunoassay. Rabbit Immunoglobulin G (IgG) is covalently immobilized on the distal sensing tip of a quartz optical fiber. The sensor is exposed to fluorescein isothiocyanate (FITC)-labeled and unlabeled anti-rabbit IgG. The 488-nm line of an argon-ion laser provides excitation of sensor-bound analyte. This results in fluorescence emission at the optical fiber's sensing tip. Sensor response is inversely proportional to the amount of unlabeled anti-IgG in the sample.

Limits of detection (LOD) vary with incubation time, sample size, and measurement conditions. For 10- μ L samples, typical LOD are 25 femtomoles of unlabeled antibody in a 20-minute period. These results indicate that each fiber optic fluoroimmunosensor (FIS) can be constructed to perform a single sensitive, rapid, low-volume immunoassay, in in-situ or benchtop applications.

Introduction

In order to detect trace amounts of chemicals with high sensitivity and specificity, the principles of solid-phase

immunoassay can be applied to FOCS design. This involves the covalent immobilization of receptor molecules (antibody or antigen) to the distal face of a single-strand quartz optical fiber. The fiber and immobilized receptor molecules form a stable, selective FIS. Careful selection of the proper immobilization procedure enhances FIS stability by minimizing receptor leakage. Provided there is sufficient immobilized reagent for reasonable sensitivity, this direct attachment of antibody or antigen via organosilanating reagents is preferable to those techniques which utilize membrane or gel-entrapped reagent phases. This is due to the fact that sensor response times are limited by mass transport to the fiber and immunochemical kinetics. Despite their potential for higher loadings, these processes may be slower in membrane or gel systems.^{40,44}

As described in Chapter 2, several types of immunoassays can be performed. The appropriate FIS immunoassay method is determined by the chemical characteristics of the antigen/antibody system of interest. For example, naturally fluorescent antigens and haptens should be analyzed by direct measurement. Non-fluorescent haptens should be detected via competitive binding or sandwich techniques, though the scarcity of hapten epitopes (antibody recognition sites) may diminish the sensitivity of sandwich analyses. An alternate means of gaining sensitivity in direct and sandwich assays entails increasing the absolute amount of immobilized receptor by increasing fiber

surface area. This can be accomplished with small-diameter fibers by utilizing evanescent-wave excitation of biomolecules bound to the circumference of the sensing tip. As described in Chapter 1, these TIRF sensors are characterized by large exposed surface areas and low evanescent wave penetration depths. Their limitations include: fluctuating evanescent-wave intensities and penetration depths, and the fact that evanescently-excited fluorescence is coupled less efficiently into the optical fiber than distal-face excitation.

In the studies described in this chapter, the FIS is exposed to fluorophor-labeled and unlabeled ligand. This results in competition for FIS binding sites. Signal intensity using this competitive binding immunoassay scheme is inversely proportional to analyte (unlabeled ligand) concentration. Though the fiber's diminutive surface area restricts the absolute amount of bound analyte, excellent sensitivity is achieved since an efficient distal face fluorescence collection geometry is used. In addition, the small amount of immobilized receptor serves as the limiting reagent in the competitive assay. As a result, rapid, low-volume analyses can be performed. Moreover, factors which alter fluorescence signals, such as quenching, matrix interferences, and self-absorption are relatively insignificant for the FIS. This is partly due to the fact that the sensing tip is thoroughly washed following analyte incubation and all measurements are made in a controlled matrix.

Experimental Section

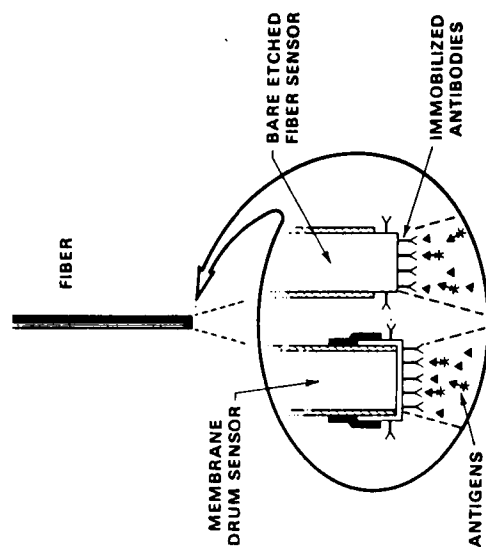
Materials. Rabbit immunoglobulin G (IgG), polyclonal anti-rabbit IgG, polyclonal fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG, bovine serum albumin (BSA), and ovalbumin were purchased from Cooper Biomedical, Inc., Malvern, PA. Spectrapor cellulose dialysis membrane (MW cutoff = 12,000) was acquired from Thomas Scientific, Philadelphia, PA. 3-Glycidoxypyrrolyltrimethoxysilane (GOPS) was obtained from Aldrich Chemical Co., Milwaukee, WI. Phosphate-buffered saline (PBS), pH 7.4, and all other reagents were supplied by Sigma Chemical Co., St. Louis, MO.

Multi-mode, fused-silica, 600- μm diameter optical fibers (numerical aperture = 0.22) were supplied by Math Associates, Westbury, NY. Fluoroimmunosensor (FIS) fibers were cut into 0.6-m lengths and terminated with SMA connectors (Math Associates). Both ends were hand-polished with 15- μm , 3- μm , and 0.3- μm lapping film.

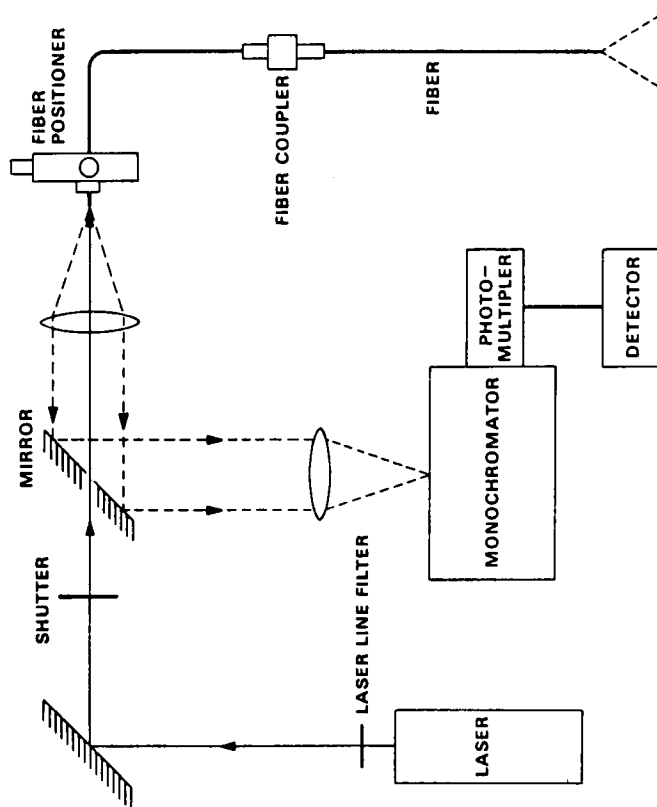
Apparatus. A Perkin-Elmer 650-40 spectrofluorimeter was used to evaluate the immobilization procedures for protein binding capacity and stability. All FIS measurements were performed with the instrument shown in Figure 3.1-a. Light from a Spectra-Physics Model 162-A forced-air-cooled argon-ion laser (approximately 10 mW at 488 nm) is directed through a 488-nm

Figure 3.1. (a) Instrumental apparatus and (b) magnified view of possible sensing termini.

(b)



(a)



laser line filter (Corion Corp., Holliston, MA) and a Uniblitz variable shutter (Vincent Associates, Rochester, NY). Since the shutter permits the delivery of controlled-duration excitation pulses (a 1-second aperture is used), FITC photodegradation is minimized and fiber-to-fiber reproducibility is enhanced. Excitation light passes through a mirror with a small hole in the center and is focused by a 25-mm diameter f/2 lens onto the incident end of an f/2.2 optical fiber. A portion of the red-shifted FIS fluorescence emission is collected at the distal face. This signal returns to the incident end and diverges at a half angle of ≈ 12.5 degrees. The 25-mm diameter f/2.0 lens collimates the FIS signal. The mirror directs the collimated emission through a 40-mm diameter f/3.6 focusing lens, a 550-nm bandpass filter (Corion Corp., FWHM = 50 nm), and into an Instruments SA H-10 (f/3.5) monochromator. Photocurrent generated by an RCA 1P28 photomultiplier tube operated at -700V dc is detected by a Keithley Model 485 picoammeter. Data is simultaneously collected and stored in the memory of the picoammeter and on a strip chart recorder.

Procedures. *Surface silanization with 3-glycidoxypropyltrimethoxysilane (GOPS).* Fibers were stripped of approximately 3 mm of cladding. The quartz cores were washed in hot 1 M HNO_3 and rinsed in water. A 10% aqueous solution of GOPS was heated to 90 °C and maintained at pH 3 with 1 M HCl.¹³⁴ Fibers were

submerged, allowed to react for 2 hours, then dried overnight at 105 °C.

Membrane drum preparation. Wet cellulose membranes were stretched over the ends of 16-gauge stainless steel tubes and affixed with heat-shrink tubing (Figure 3.1-b). Membranes were treated in 1 mM EDTA and distilled, deionized water prior to activation. Fibers were placed inside the drum assembly.

Surface activation and protein attachment. GOPS-derivatized fibers and membrane assemblies were oxidized in 0.1 M periodic acid for 1 hour.^{134,135} The resulting aldehydic surfaces were washed in water and phosphate-buffered saline (PBS) pH 7.4. Fibers and membranes were then suspended in polypropylene vials containing 1 mL of 3-6 mg/mL protein and allowed to react for 24 hours at 4 °C. During this time, glass- and membrane-bound aldehydes formed Schiff bases with protein amino groups. Gentle reduction of the Schiff bases was accomplished by the addition of NaBH₄ in PBS for three consecutive 15-minute intervals.^{134,135} Fibers and membranes were rinsed in PBS and stored in 1% ovalbumin/PBS in order to minimize the effects of non-specific binding.¹³⁶ Sensors prepared in this manner were stable for several weeks.

Measurements. *Determination of optimum substrate for immobilization.* In order to determine the extent of protein surface coverage and degree of non-specific binding, fibers and

membranes were prepared with rabbit IgG and BSA reagent phases. Sensors were incubated with 0.1 mg/mL anti-rabbit IgG-FITC for 60 minutes at room temperature. Following incubation, sensors were rinsed well with phosphate-buffered saline (PBS) then reacted with 3 M potassium thiocyanate (KSCN) chaotropic reagent. The amount of specifically bound protein was determined by measuring FITC fluorescence in the chaotropic wash at $\lambda_{ex} = 488$ nm, $\lambda_{em} = 520$ nm with a Perkin Elmer 650-40 spectrofluorimeter.

The rate of protein loss following covalent attachment (leakage) was determined by immobilizing anti-rabbit IgG-FITC on 5 identically prepared membranes and fibers. Each sensor was suspended in a polypropylene vial containing 1 mL of PBS. Samples were shaken at room temperature for approximately 2.5 hours. During this time, FITC fluorescence from successive vials was measured at regular intervals.

Competitive binding assays. All measurements were performed with GOPS-derivatized fibers. FIS reagent phases consisted of rabbit IgG. Sample solutions contained varying amounts of anti-rabbit IgG. Two types of competitive binding assays were investigated.

The first method involved simultaneous incubation of the FIS in a mixture of labeled and unlabeled analyte. Dose-response curves were obtained for 1-mL stirred, 1-mL unstirred, and 10- μ L solutions. Each solution in these three categories contained different amounts of anti-rabbit IgG along with 31 picomoles

(1-mL solutions) or 15.5 femtomoles (10- μ L solutions) of anti-rabbit IgG-FITC. Blank solutions consisted of pure FITC labeled antibody in PBS.

A separate FIS was used for each sample. Data points on each dose-response curve were obtained using identically prepared fibers. All fibers were rinsed well in 20 mL of PBS after the appropriate incubation period. Measurements were performed following rinsing in 1 mL of fresh PBS. Excitation was provided by the 488-nm laser line. Fluorescence signals were detected at 550 nm with an 8-nm bandwidth. Background signals were obtained for each fiber prior to incubation in 1 mL of fresh PBS. All signals were collected during the one-second period the shutter was open. Peak intensities (I) were recorded in the picoammeter memory (data read rate was 3 points per second) and on the strip chart recorder. Instrumental noise (typically 0.3 nA) was determined by measuring the recorder signals for unincubated sensors in PBS. The percentage of bound labeled analyte (%B) was determined using equation (3.1):¹³⁷

$$\%B = [(I_{\text{sample}} - I_{\text{background}})/(I_{\text{blank}} - I_{\text{background}})] \times 100 \quad (3.1)$$

A second competitive binding method involved a two-minute pre-incubation of the FIS in varying concentrations of 1-mL stirred, unlabeled analyte. Fibers were then rinsed and incubated for 2.0 minutes in 1-mL stirred anti-rabbit IgG-FITC (0.5 mg/mL).

Measurements were performed following rinsing in 1 mL of fresh PBS. Signal detection procedures for this pre-incubation assay were identical to those described for the simultaneous-incubation technique.

Results and Discussion

FOCS used in fluoroimmunoassays are based on highly specific antibody-antigen interactions. Just as in other ligand-binder competitive assays, FIS analyses require competition between labeled and unlabeled analyte for a limited number of receptor binding sites. All measurements involve separation of free labeled ligand from a receptor-bound fraction. The FIS competitive assay can therefore be classified as a heterogeneous solid-phase fluoroimmunoassay.

Optimal measurement conditions for heterogeneous solid-phase competitive assays are usually found when matrix interferences are virtually eliminated and receptor binding sites nearly saturated by labeled ligand.¹³⁷ This requires that the solid surface be stable, easily washable, and that the amount of immobilized receptor be maintained as low as reasonably possible for adequate detectability. Both the membrane-drum and GOPS-derivatized fibers were evaluated with regard to these criteria.

Figure 3.2 indicates that, following immobilization, the amount of protein lost due to rinsing was slightly higher for the

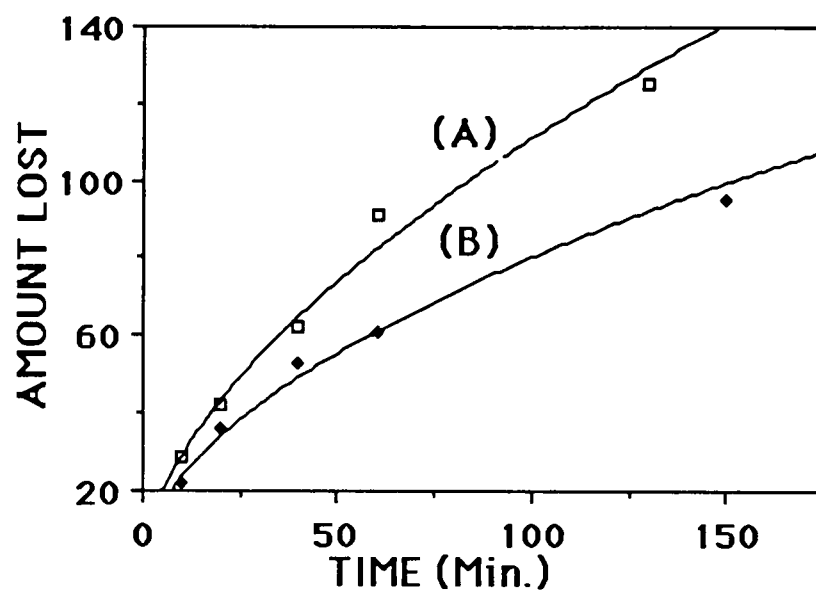


Figure 3.2. Amount of protein lost (ng/cm^2) vs. time: (A) membrane-drum and (B) GOPS-derivatized fiber sensors.

membrane-drum FIS. In order to determine the extent of non-specific binding to the FIS two types of proteins, rabbit IgG and bovine serum albumin (BSA), were immobilized on membranes and fibers using the previously-described procedures. After incubating membranes and fibers in anti-IgG-FITC and washing in phosphate-buffered saline (PBS), sensors were reacted with 3 M KSCN in order to disrupt non-covalent antigen-antibody bonds. Table 3.1 shows that BSA membranes and fibers interacted relatively poorly with anti-IgG-FITC. This result was expected since anti-IgG should not specifically recognize BSA. The extent of non-specific interaction, however, was greater for fiber surfaces ($\approx 13\%$) than membrane surfaces ($\approx 1\%$). This may have been due, in part, to slightly more effective PBS rinsing of the membranes.

By assuming the anti-rabbit IgG-FITC to be approximately univalently bound to immobilized rabbit IgG and the 3 M KSCN chaotropic rinse to be reasonably effective, GOPS derivatization yields protein loadings $\approx 3 \mu\text{g}/\text{cm}^2$. This corresponds to 8×10^{-9} grams or 50 femtomoles immobilized rabbit IgG ($\text{MW} \approx 1.6 \times 10^5$) on the distal end of a 600- μm diameter FIS. This procedure was repeated several times and FIS loadings were consistently 18-50 femtomoles ($1\text{-}3 \mu\text{g}/\text{cm}^2$). These values agree well with previously reported $1 \mu\text{g}/\text{cm}^2$ antibody loadings on quartz surfaces using aminopropyltriethoxysilane derivatization.⁷⁰ Since the membrane-drum sensors were slightly less stable, more

Table 3.1. Optimum Substrate For Immobilization

Anti-IgG-FITC lost from surface ($\mu\text{g}/\text{cm}^2$) following wash in 3M KSCN		
<u>Type of Surface</u>	<u>Immobilized Reagent</u>	
	<u>IgG</u>	<u>BSA</u>
cellulose membrane	0.77	0.66
quartz optical fiber	2.9	0.43

cumbersome to construct, and yielded lower protein loadings, GOPS-derivatized fibers were used in the FIS competitive binding experiments.

In order to determine whether a single FIS or multiple sensors were necessary for data collection, FIS signal reproducibility was investigated. Five identically prepared fibers with rabbit IgG reagent phases were incubated for 5 minutes in 1-mL stirred solutions of 3.1×10^{-9} M anti-rabbit IgG-FITC. The relative standard deviation for measured signals was 10.2%. Repeated exposure of similarly prepared sensors to 3 M KSCN chaotropic reagent followed by rinsing in PBS and re-incubation in 3.1×10^{-9} M anti-rabbit IgG-FITC failed to restore signals to their original levels. In fact, after one chaotropic wash, FIS response was typically 30-40% poorer. After 3 washes with 3 M KSCN, most fibers failed to bind specific antibody. Because of these results, each data point on the competitive binding dose-response curves was obtained using a separate, identically prepared FIS. In spite of the reasonably reproducible between-fiber response, efforts are in progress to identify effective, non-destructive chaotropic reagents for convenient, single-fiber use.

Simultaneous-incubation dose-response curves are illustrated in Figures 3.3 and 3.4. General curve shapes concur with expected FIS competitive binding behavior. Samples containing greater amounts of unlabeled analyte exhibit lower signals due to the fact

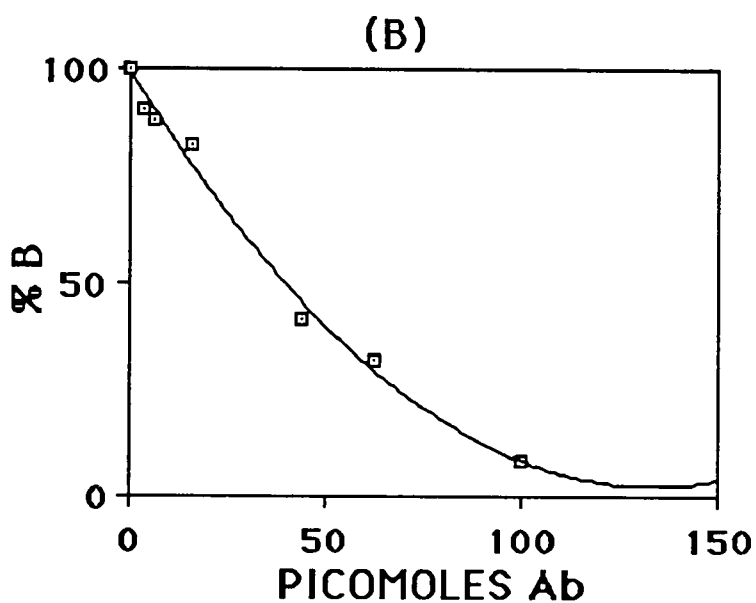
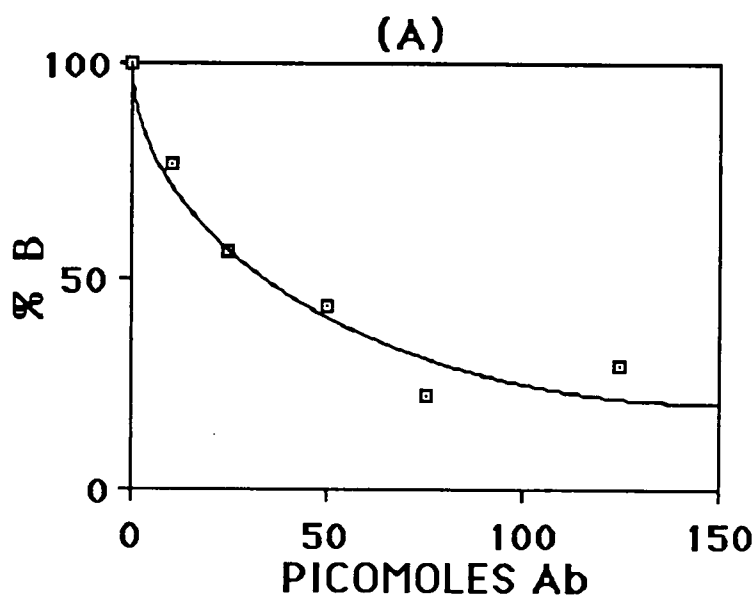


Figure 3.3. Simultaneous-incubation FIS dose-response: Percent bound (%B) labeled anti-IgG vs. amount of unlabeled anti-IgG (Ab) for (A) 1.0-mL unstirred solutions, twenty-minute incubations; (B) 1.0-mL stirred solutions, five-minute incubations.

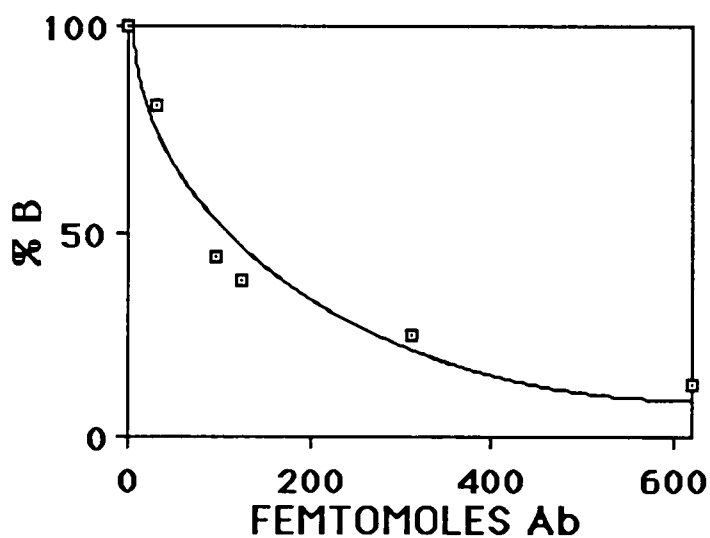


Figure 3.4. Simultaneous-incubation FIS dose-response for 10- μ L drops: Percent bound (%B) labeled anti-IgG vs. amount of unlabeled anti-IgG (Ab), twenty minute incubations.

that proportionally more labeled analyte is displaced from the limited number of FIS binding sites. The 10- μ L (Figure 3.4) and 1-mL unstirred samples exhibit significant curvature. This is primarily attributable to three factors: (1) the heterogeneous range of avidities of the polyclonal anti-rabbit IgG antibodies, (2) possible differences in the avidities of the FITC-labeled and unlabeled antibodies (due to labeling) and (3) the immobilized rabbit IgG binding sites were randomly oriented.¹³⁸ Regions of optimal detectability are on the steepest portions of the curves. As with most competitive assays, this covers a narrow interval of analyte concentrations and results in linear dynamic ranges (LDR) of approximately one order of magnitude, at best. Limits of detection (LOD) were obtained by determining the amount of unlabeled analyte at two R.S.D. units below $I = I_B$ (i.e., %B $\approx$ 80). This corresponds to 8 picomoles, 13 picomoles, and 25 femtomoles for the 1-mL unstirred, 1-mL stirred, and 10- μ L drop samples. It should be emphasized that these LOD are strongly dependent on a number of factors including incubation time, extent and reproducibility of fiber surface coverage, instrumentation, and immunochemical response. As a result, day to day LOD may fluctuate significantly.

The effect of sample stirring on the FIS was also investigated. Signal levels attained with five-minute, stirred 1-mL sample incubations (Figure 3.3-b) were approximately the same as those produced with 20-minute, unstirred, 1-mL samples. This is a good

indication that, for the high avidity antibodies which have relative rapid reaction kinetics, the FIS response rate is limited by the rate of antibody transport to the fiber surface. Recently reported experimental evidence supports this notion.^{125,126}

An additional feature of the stirred 1-mL sample dose-response data is that it displays less curvature and greater LDR than data from the unstirred solutions. Since an absence of curvature in the competitive binding dose response usually indicates a relatively homogeneous antibody population, the rapid rate of supplying the FIS with antibody during forced convection (stirring) may increase the proportion high avidity antibodies which react with the IgG on the fiber surface.

The second competitive binding approach, pre-incubation, yielded a dose-response which is illustrated in Figure 3.5. The detection limit using this measurement technique was approximately 100 picomoles for 1-mL solutions. This value is strongly influenced by incubation times and concentration of labeled antibody. Selection of the appropriate incubation time in 0.5 mg/mL labeled antibody was accomplished by recording the sensor's time-dependent response in a 1.0-mL stirred solution. These results, illustrated in Figure 3.6, demonstrate that saturation of FIS binding sites is nearly complete after only two minutes.

The most important feature of the pre-incubation assay is that it can be used in-situ. For example, sensors could be placed in-vivo for a specified period of time, removed, and "developed" in

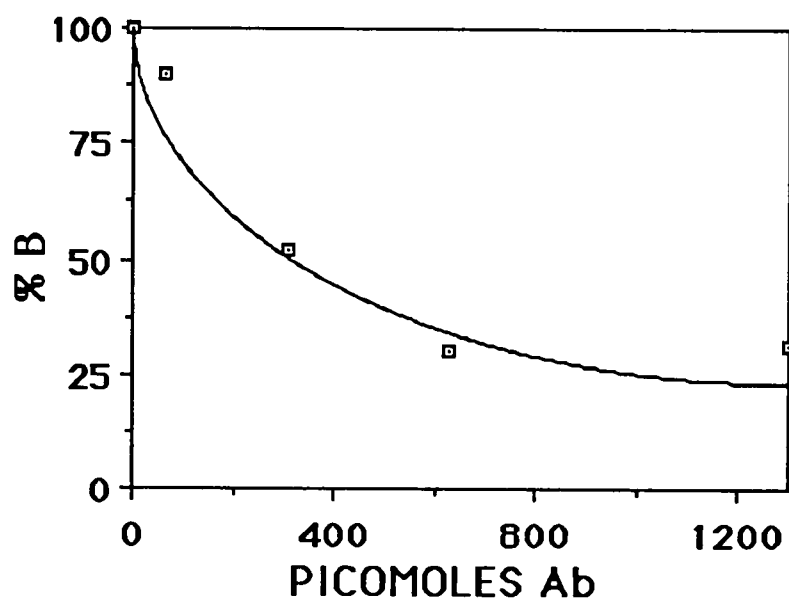


Figure 3.5. Pre-incubation FIS dose-response: Percent bound (%B) labeled anti-IgG vs. amount of unlabeled anti-IgG (Ab) for 1.0-mL stirred solutions, two-minute incubations.

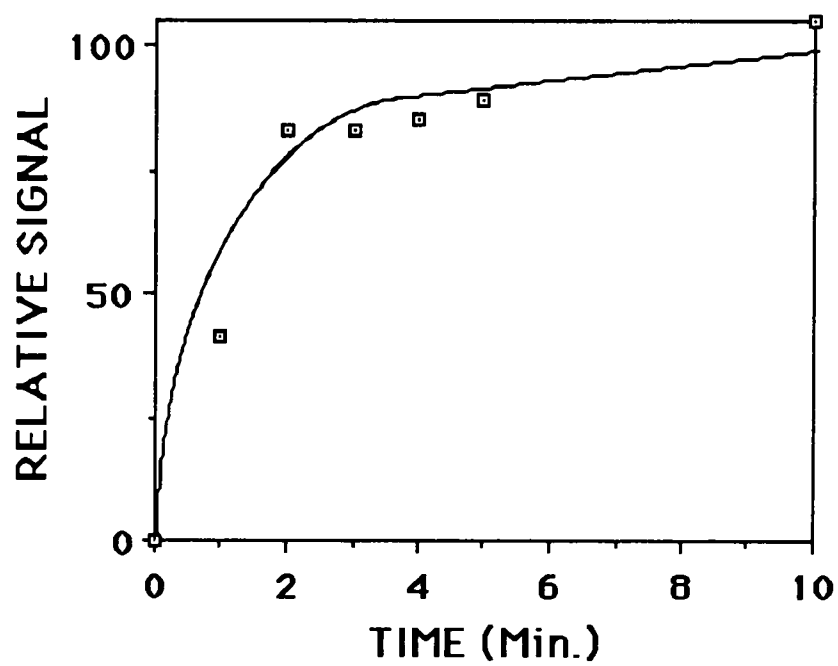


Figure 3.6. Relative signal vs. time (min.): FIS in 0.5 mg/mL stirred anti-IgG-FITC.

a high concentration of labeled analyte. Standardization would be difficult since pre-incubation conditions for calibration solutions would have to be identical to samples. This would entail simulating in-vivo matrices and mass transport conditions.

Simultaneous incubation, on the other hand, could not be used for in-vivo measurements. Due to its extremely low sample volume requirements, rapid response times, and single sample manipulation step, this type of assay may be useful only for benchtop analyses.

A major drawback to both types of assays is the fact that multiple fibers must be used. As a result, measurement error can often be attributed to imprecision in fiber derivatization and biomolecule immobilization. At present, this work indicates that fiber-to-fiber relative standard deviation is about 10%. It is hoped that continued attention to the variables which control the extent of fiber surface coverage will minimize the effects of this source of measurement imprecision. Additional factors which influence FIS reproducibility and detectability include the cumbersome manual SMA coupling procedure, instrumental noise, and optical background. The use of high speed fiber optic switches is currently being investigated in order to minimize these effects and further facilitate rapid, simultaneous, multi-site analyses.

In conclusion, the results presented in this chapter demonstrate that antibody-based fiber optic chemical sensors can be developed to perform simple, rapid immunoassays in microliter

volumes of analyte. Assay sensitivity varies in a manner which is consistent with competitive binding measurements. Though linear dynamic ranges (LDR) are poor, excellent sensitivity is achievable over a narrow interval of analyte concentrations. As described in the preceding chapter, FIS applications can be extended by applying the principles of antibody sandwich and direct assay to sensor design. Manipulation of these techniques, along with the pre-incubation competitive assay, will allow full exploitation of the in-situ measurement possibilities offered by fiber optic sensor instrumentation.

CHAPTER 4

DEVELOPMENT OF ANTIBODY-BASED FOCS FOR DETECTION OF BENZO[a]PYRENE METABOLITES

This chapter describes the development of an antibody-based fiber-optic sensor designed to detect benzo(a)pyrene (BaP) metabolites, in this case r-7,t-8,9,c-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (benzopyrene-tetraol or BPT), a naturally fluorescent hydrolysis product of DNA adducts which form during BP metabolism. The sensor's principle of operation is based on diffusion of BPT into the fiber's reagent phase where it complexes with monoclonal anti-BPT antibody. Reagent phase is immobilized inside a reusable sensing tip and consists of either membrane-entrapped liquid antibody or antibody immobilized on 7- μ m silica beads. The antibody binds and concentrates BPT in the fiber's viewing region until equilibrium with the sample is achieved. Typical measurements are performed in 15 minutes under non-equilibrium conditions. The 325-nm line of a He:Cd laser provides excitation of the immune complex. The intensity of the resulting fluorescence emission is directly proportional to the amount of BPT in the sample.

Sensor response rate varies with BPT concentration in the sample and, under certain circumstances, with antibody concentration in the sensor. At high antibody concentrations,

sensor response rate is linear for about two hours. For 15-minute incubations, typical LOD are 5×10^{-10} M. Significant chemical selectivity is also demonstrated despite the presence of structurally and spectrally similar polynuclear aromatic (PNA) interferants. These results indicate that the fluoroimmunosensor (FIS) is capable of performing highly sensitive and selective immunoassays and could be useful as a screening instrument for BP metabolites in biological samples. Furthermore, the information provided by this work should be generally applicable to the development of devices for chemically similar systems.

Introduction

Among the primary considerations involved in the design and development of fiber optic chemical sensors (FOCSs) are the nature of the reagent-phase/analyte interaction and the detectability of the measured optical property. In view of these circumstances, it is not surprising that a large number of FOCSs have been described which utilize the excellent sensitivity of fluorescence detection in conjunction with the chemical specificity of ligand/binder reactions.³⁹⁻⁴³ As previously discussed, the majority of these devices have been used detect ions and small molecules. The FOCS measurement of large molecules ($MW \geq 200$) has been accomplished primarily by incorporating biologically significant ligand/binder reactions into the sensor's reagent phase. Successful examples of these devices include those which exploit enzyme/substrate,⁶⁴⁻⁶⁷

lectin/carbohydrate³³ antibody/antigen (Ab:An),^{68-73,76,77} and antibody/hapten (Ab:Hn)^{74,75,78} interactions. The versatility of these fiber-optic bio-sensors is generally a function of the commercial availability (or ease of manufacture) of the required biochemicals and their capacity to be adapted to an appropriate sensor design.

FOCSs which employ immunochemical reagent phases are particularly promising since they have the potential to perform highly sensitive and selective in-situ measurements of a broad variety of compounds. Antibodies to an extensive range of materials are commercially available or can be produced. Because they rely upon the outstanding specificity of antibody/antigen recognition, antibody-based FOCSs can be fabricated to detect trace amounts of materials in complex matrices. By coupling immunoassays with small-diameter fiber-optic instrumentation, site-specific analytical measurements can be conducted which probe nanoliter to low microliter sample volumes.

The overall sensitivity of FOCSs with immunochemical reagent phases is a dual function of the Ab:An affinity constant (K_a) and the sensitivity of the measured optical parameter. Using high-affinity antibody reagent phases and laser-excited fluorescence detection, we have developed fluoroimmunosensors (FISs) which are capable of femtomole limits of detection.^{73,74,77} These FIS reagent phases, described in Chapter 3, have consisted of roughly a monolayer of immunochemical covalently bound to the fiber's

sampling terminus. This approach is useful for competitive binding measurements since, provided there is sufficient material for detectability, assay sensitivity is inversely proportional to the amount of immobilized limiting reagent.¹³³ In the case of direct immunoassays of natural fluorophores and antibody "sandwich" assays, however, sensitivity and linearity are directly proportional to the amount and availability of immobilized binder.¹³³ Optimum sensor performance can therefore be achieved for these measurements by maximizing reagent phase loadings and binding site orientation.

In this work, an FIS design was required which could perform direct immunoassays of the naturally fluorescent, polynuclear aromatic (PNA) compound *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (benzopyrene-tetraol or BPT). BPT's significance is derived from the fact that it is a bio-indicator of an individual's exposure to the carcinogenic PNA benzo(a)pyrene (BaP). BaP is metabolized in-vivo to a specific diol-epoxide derivative of BaP; *r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)-pyrene (BPDE) which covalently binds to DNA.^{139,140} The resulting BPDE-DNA adducts are eliminated through urine and feces.¹⁴¹ In order to determine the extent of BPDE-DNA adduct formation, weakly fluorescent BPDE-DNA can be converted (via acid hydrolysis) to the highly fluorescent BPT.¹⁴² Analysis of BPT in hydrolyzed biological samples is, therefore, evidential of an individual's exposure to benzo(a)pyrene.¹⁴³

In the work described in this chapter, BPT FISs were constructed using purified monoclonal antibodies immobilized at the end of 200- μm and 600- μm core-diameter optical fibers. Because of previously encountered difficulties associated with preparing, handling, and regenerating sensors with reagent phases covalently-bound to fibers,⁷³ reusable sensing tips capped with highly permeable dialysis membranes were fabricated for this work. Measurements were performed with two types of reagent phases. The first consisted of a high concentration of liquid-phase antibody that was entrapped within a sensing tip. The second was composed of solid-phase antibody immobilized on 7- μm diameter Protein-A-derivatized silica particles suspended in a sensing tip.

Sensor response was dependent upon analyte (i.e., BPT) diffusion across the 7- μm thick membrane. Once inside the sensor, BPT was concentrated within the fiber's observation zone. Due to a high affinity constant for Ab:BPT complex formation, the amount of antibody-bound BPT continued to increase for several hours until equilibrium was achieved. During routine FIS assays, incubation times were much less than those required for sample equilibration to occur. These non-equilibrium measurements provided roughly a factor of two enhancement of BPT fluorescence intensity (vs. bare fiber measurements) within 15 minutes. This concentrating capability permitted the sensor to function in a pseudo-continuous fashion. Under these conditions unbound BPT did not noticeably contribute to the observed signal. Thus, measurements could be

performed without separation from the sample matrix (homogenously) until binder saturation (equilibrium) was achieved. Additional measurements were conducted in complex matrices containing serum proteins and structurally similar spectral interferants. These analyses were implemented by separating the FIS from the sample after an appropriate incubation period (heterogeneous immunoassays). Each FIS was then rinsed in "blank" solutions to remove unbound interferants.

Data from these investigations are organized in a manner which permits evaluation of the sensor's analytical figures of merit, including: linearity, precision, limits of detection, selectivity, and rate of response. FIS performance is further discussed with regard to the practical utility of the device and the influence of the analyte's characteristics on sensor design.

Experimental Section

Materials. Due to the fact that PNAs are haptens, i.e., they are too small to elicit an immune response, anti-PNA antibodies must be raised to protein-conjugated PNAs. Accordingly, monoclonal antibody 8E11 was developed from the spleen cells of a mouse immunized with BPDE-guanosine covalently coupled to the protein bovine serum albumin (BSA). In order to isolate the immunoglobulin G (IgG) antibody fraction, crude mouse ascites fluid was purified by precipitation with 30% ammonium sulfate. The precipitate was

redissolved, dialyzed against phosphate buffered saline (PBS), pH 7.4, and diluted to 1.5 mg IgG/mL.

Crossreactivity studies were performed using competitive enzyme-linked immunosorbent assay (ELISA). These results have shown that antibody 8E11 recognizes DNA adducts and free BPT with approximately the same high affinity.¹⁴⁴ In addition, the antibody recognizes BP-7,8-diol and BP-9,10-diol, but not BP itself. A thorough description of antibody production, sensitivity, and specificity has been reported.¹⁴⁵ The value of K_a for Ab:BPT complex formation in PBS was determined by FIS measurements to be approximately 10^8 M^{-1} .

All PNA haptens were obtained either from the Midwest Research Institute (MRI), Kansas City, MO, or from the National Bureau of Standards (NBS), Gaithersburg, MD. Figure 4.1 is an illustration of BPT and two structurally similar PNAs used in a series of interference studies. Stock solutions (0.05 mg/mL) of 3,4-dihydroxy-1,2-epoxy-1,2,3,4-tetrahydrobenz(a)anthracene (anthracene-diol-epoxide or ADE, Fig. 4.1a), BPT (Fig. 4.1b), and 7,8,-dihydroxy-7,8,dihydrobenzo(a)pyrene (BP-diol or BPD, Fig. 4.1c) were prepared in ethanol. Working solutions ranging from 10^{-6} - 10^{-10} M were made by diluting stock solutions in PBS. Additional interference studies were performed using a solution that was 8.9×10^{-8} M BPT in fetal calf serum (FCS) spiked with 16

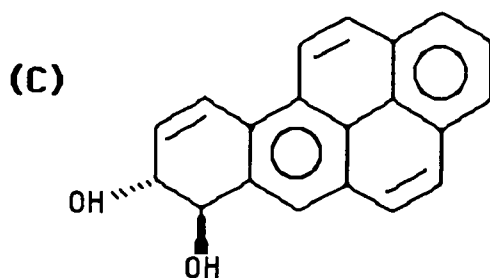
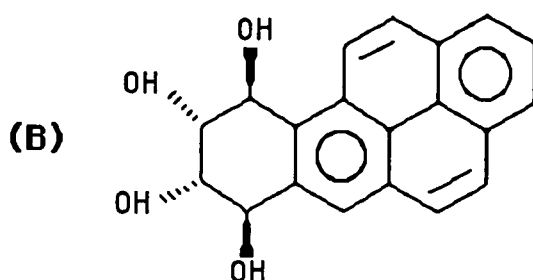
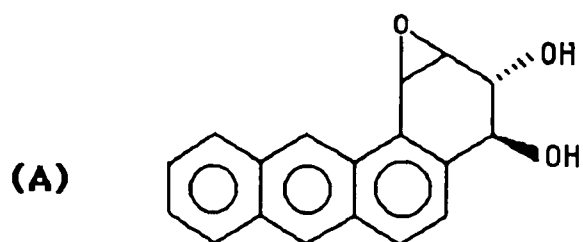


Figure 4.1. Structures of PNA metabolites:
(A) 3,4-dihydroxy-1,2-epoxy1,2,3,4-tetrahydrobenz-(a)anthracene (benzoanthracene-diol-epoxide or ADE),
(B) r-7,t-8,9,c-10-tetrahydroxy 7,8,9,10-tetrahydrobenzo(a)pyrene (benzopyrene-tetraol or BPT),
(C) 7,8,-dihydroxy 7,8, dihydrobenzo(a)pyrene (benzopyrene-diol or BPD).

PNA_s from an NBS standard reference material (SRM 1647). The final concentrations of the 16 PNA interferants in the FCS sample are listed in Table 4.1.

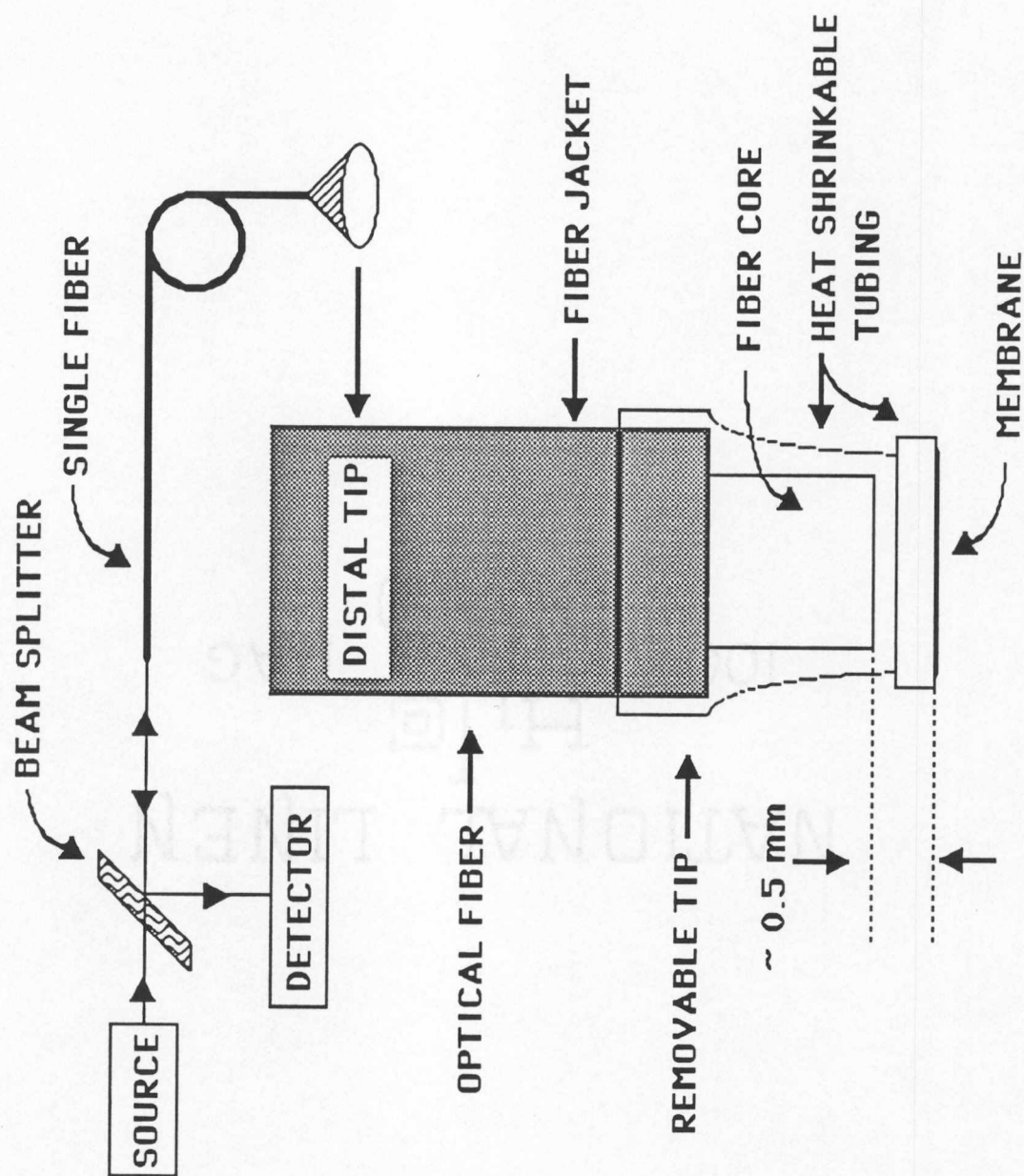
Apparatus. A detailed description of the FIS apparatus is provided in Chapter 3. The primary components of the instrument used in this study are schematically illustrated in Figure 4.2. The excitation source consists of an 8-mW model 4230 Liconix He:Cd laser (Liconix Inc., Sunnyvale, CA). The 325-nm line of the laser is focused onto the fiber after passing through a 20 %T laser line filter (Corion Corp., Holliston, MA), and a beamsplitter (a 25-mm diameter mirror with a 1.5-mm diameter hole in the center). Selection of the PNA fluorescence emission is accomplished using a 40 %T 40-nm FWHM band-pass filter centered at 400 nm (Corion Corp.). The fluorescence emission is detected by a Hamamatsu model R760 photomultiplier tube operated at -1000V dc. The output of a Keithley model 485 picoammeter (Keithley Instruments Inc., Cleveland, Ohio) used to process the photocurrent is displayed on a strip chart recorder. A Uniblitz model SD-10 shutter (Vincent Associates, Rochester, New York) is programmed to open for one second every 90 seconds in order to provide semi-continuous, controlled-duration laser excitation. This allows observation of immune-complex formation in near-real time and helps minimize photothermal/photochemical effects.

Table 4.1. Composition of NBS Spike (SRM 1647)

Constituent	Amount (ng/mL)
1) Anthracene	32.9
2) Benz [a] anthracene	50.3
3) Benzo [a] pyrene	53.0
4) Fluoranthene	101.0
5) Pyrene	98.4
6) Naphthalene	225.0
7) Acenaphthylene	191.0
8) Acenaphthene	210.0
9) Fluorene	49.2
10) Phenanthrene	50.6
11) Chrysene	46.8
12) Benzo [b] fluoranthene	51.1
13) Benzo [k] fluoranthene	50.2
14) Benzo [ghi] perylene	40.1
15) Dibenzo [a,h] anthracene	36.8
16) Indeno [1,2,3-cd] pyrene	40.6

Spiked sample consisted of 8.9×10^{-8} M BPT in Fetal Calf Serum with the above concentrations of PNAs.

Figure 4.2. Schematic diagram of instrumental apparatus and magnified view of sensing tip.



Sensor Fabrication. Sensors were constructed either from 200- μm or 600- μm core-diameter plastic-clad, multimode, fused silica fibers (General Fiber Optics, Cedar Grove, N.J.). The FIS designs reported in Chapter 3 utilized separate reagent-bonded fibers for each measurement. In this study, a single 1-meter-long fiber remained dedicated to the instrument and each analysis was performed with a reusable sensing tip (illustrated in Figure 4.2). Tips were fabricated by tapering heat-shrink tubing around the fiber's jacket and core. Very high permeability cellulose dialysis membrane (Ref. no. 10.17, Diachema AG, Zurich, Switzerland, molecular weight cutoff = 10,000) was stretched across the face of the tip and positioned with another piece of heat-shrink tubing roughly 0.5 mm from the fiber's face. When assembled, a seal was formed between the fiber jacket and the tubing. Each tip could easily be slipped off the fiber to be re-filled with fresh reagent phase.

Two types of reagent phases were employed. The first consisted of 10^{-6} M - 10^{-8} M liquid-phase antibody (MW $\approx$ 160,000) solutions entrapped behind the sensing tip's 10,000 molecular-weight-cutoff membrane. All liquid-phase sensors were constructed with 200- μm diameter fibers. Total sensor volume for these devices was approximately 20-40 nL, resulting in roughly 3-4 ng of immobilized antibody.

The second type of reagent phase was composed of antibody immobilized on silica beads suspended in a sensing tip.

"Durasphere" silica particles (7- μm diameter, 500-Å pore size) were purchased from the manufacturer (Alltech Inc., Deerfield, IL.) with covalently-bound Protein A. Reagent phase was prepared by incubating, overnight, 100 μL of 1.5 mg antibody/mL (in PBS, pH 7.4) with 12 mg (dry weight) of beads. The non-covalent antibody/Protein A linkage was selected due to its excellent stability (K_a for Ab:Protein A complex formation $\approx 10^6$), simplicity, and binding site orientation. Protein A is known to specifically bind the Fc or "tail" portion of the antibody molecule, thus leaving the antibody's binding sites available to interact with antigen.¹⁴⁶ The bead binding capacity was determined to be roughly 1.9 mg antibody/gram. Sensor tips containing solid-phase antibodies were assembled on 600- μm diameter fibers. The bead density was adjusted to 16.6 mg/mL in order to minimize optical problems associated with settling and scattering. Tip volume was approximately 140 nL, resulting in a total amount of immobilized antibody (4.2 ng) which was comparable with liquid-phase sensors.

Measurements. All FIS assays were performed either in a sequential, stepwise, or pseudo-continuous fashion. Sequential analyses were implemented by filling the sensing tip with a constant concentration of fresh antibody prior to each measurement. Measurements were completed by incubating sensors for a specified interval (typically 15 minutes for liquid-phase and 45 minutes for solid-phase tips) in 1-mL stirred

solutions. During this period, materials which were permeable to the membrane diffused into the reagent phase and became conjugated with their specific antibody. Following incubation, the sensor was rinsed in PBS for at least 4.5 minutes. During rinsing, unbound interferants were dialyzed out of the sensing tip leaving only antibody-bound material confined to the fiber's viewing region. Signal levels were obtained from the difference between pre- and post-incubation signal-to-noise ratios (S/N) acquired while the sensor was in PBS.

Stepwise data collection procedures took advantage of the large amount of unbound antibody which remained in the sensor following each short incubation. Measurements were performed by loading the tip with excess antibody and incubating the FIS for a specified interval (typically 15 minutes) in several samples. The FIS was re-zeroed prior to each analysis by allowing the PBS rinse signal from the previous measurement to serve as the baseline for the next determination. As with sequential measurements, difference information was used to record signal levels.

Pseudo-continuous operation was achieved by observing concentration-dependent changes in S/N without rinsing in PBS. Because of antibody binding, the sensor's response to BPT continued to increase until equilibrium was achieved (approximately 12 hours). S/N values were calculated for stepwise, sequential, and pseudo-continuous analyses by using

peak-to-peak noise levels obtained during measurements of blank PBS solutions.

In order to confirm the concentrating effect of immune-complex formation, blank measurements were conducted which utilized non-specific protein reagents. Liquid-phase blank sensors were loaded with 1.5 mg rabbit IgG/mL PBS (Cooper Biomedical Inc., Malvern, PA). Solid-phase devices contained 16.6 mg Protein A beads/mL PBS. Sensors were incubated in several different concentrations of BPT using stepwise and sequential procedures. FIS response increased until signal levels reached those obtained during bare-fiber measurements (about 15 minutes), after which no further elevations in signal were observed. PBS rinses of blank sensors caused the FIS signal to return to baseline within 3 minutes, indicating negligible non-specific binding effects.

Results and Discussion

The FISs described in this chapter were designed to perform sensitive, low-volume assays of naturally fluorescent bio-indicators of benzo(a)pyrene-DNA adducts. Although applications of this device are limited to in-vitro analyses of BPT in hydrolyzed biological samples, FIS performance characteristics indicate that it is capable of functioning in a variety of matrices with comparable or superior speed, sensitivity, and/or selectivity to established techniques.^{143,147-152} Of equal importance is the

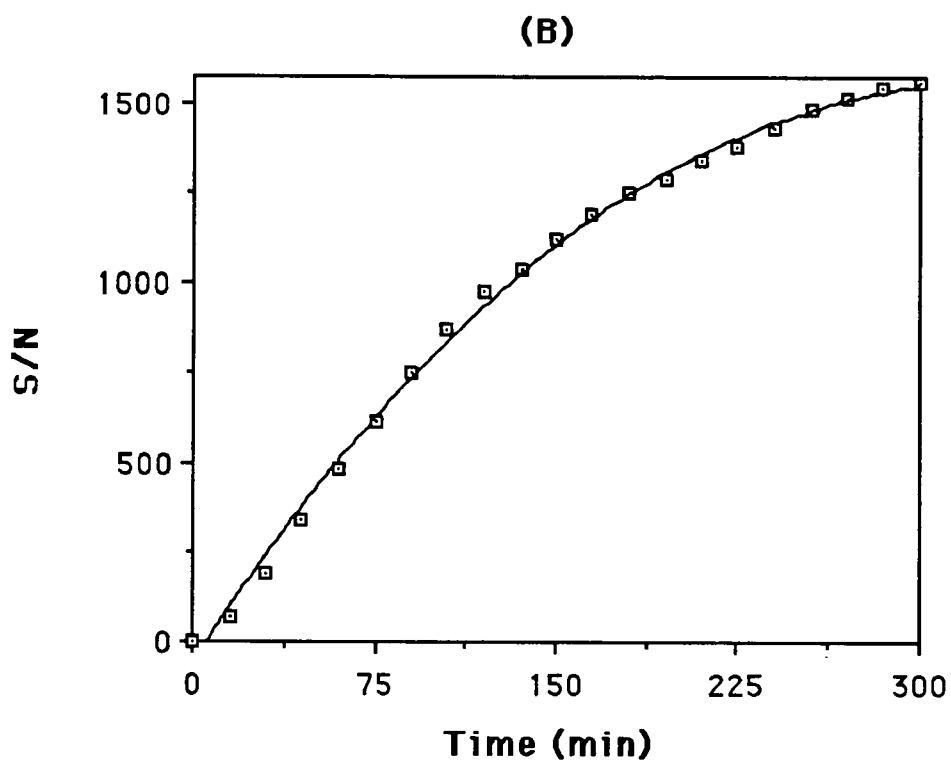
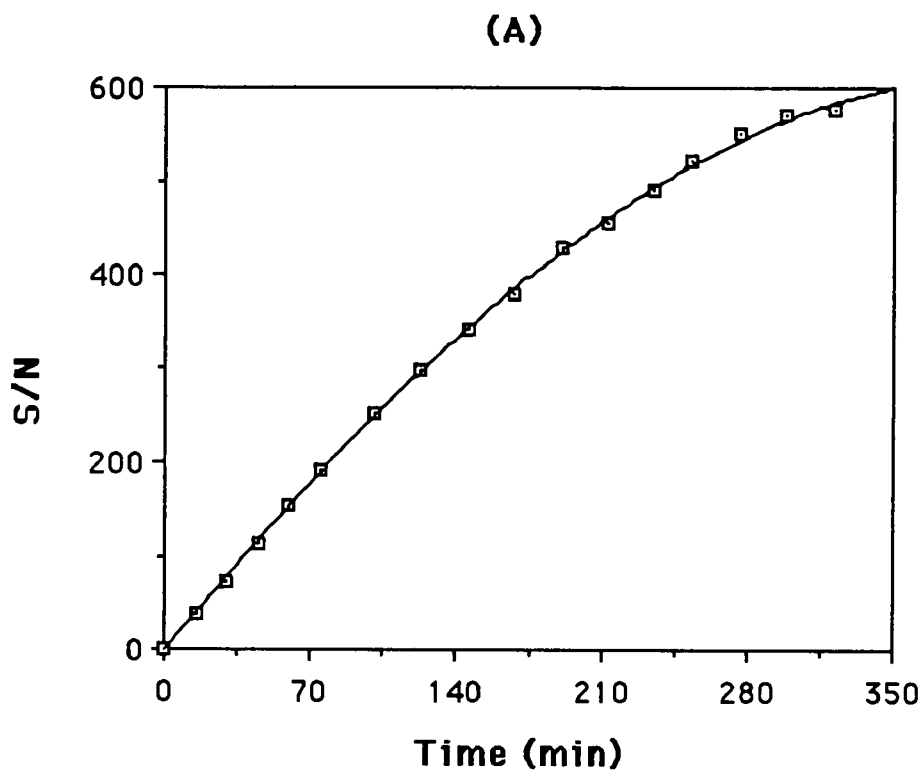
fact that this study validates the sensor's principles of operation. By defining the FIS's fundamental response features, similarly constructed devices could be used for in-vivo analyses of chemically analogous systems.

In order to determine which factors influence FIS performance, dose-response data were obtained for BPT solutions under a variety of conditions. Among the parameters examined were the sensor's time-dependent concentrating effect, its ability to discriminate between analyte and interferences, and the consequence of variations in BPT and antibody concentration on FIS response. Results from these investigations are used to evaluate the sensor's analytical figures of merit. Accordingly, the ensuing discussion focuses on FIS linearity, limits of detection, precision, response kinetics, and selectivity.

Linearity. The sensor's temporal behavior was examined for approximately 12 hours in order to determine the period of time over which the FIS was capable of a linear response. Conditions for the time-dependent study, illustrated in Figure 4.3, consisted of either 1.9×10^{-6} M liquid-phase or 0.03 mg/mL solid-phase antibody loaded in the sensing tip. The liquid reagent-phase sensor was incubated in 1.6×10^{-8} M BPT and the solid-phase device was placed in 1.6×10^{-7} M solutions.

In contrast to blank sensors, antibody-filled FIS signals continued to rise over the entire measurement period. S/N levels

Figure 4.3. FIS temporal response: S/N vs. Time for (A) liquid antibody reagent-phase sensor in 1.6×10^{-8} M BPT and (B) bead-immobilized antibody reagent-phase sensor in 1.6×10^{-7} M BPT.



were greater for the solid-phase assay (Figure 4.3-b) because the solid-phase device utilized a larger diameter fiber and probed a more concentrated solution. The linear response period was about the same for both sensors, extending for roughly 2 hours.

The "concentrating factor" for each sensor was determined by comparing antibody-filled FIS signals with those obtained from blank sensors incubated in the same BPT solutions. These results, listed in Table 4.2, indicate that the liquid-phase device was capable of nearly a factor of two greater concentrating effect. At 300 minutes the solid-phase S/N was 20 times the blank level and the sensor had appeared to reach equilibrium. In contrast, the 300-minute liquid-phase S/N was roughly 40 times greater than the blank's and the response continued to increase for nearly six more hours.

The time (t) for each sensor to reach equilibrium was determined by performing a least-squares, third-order polynomial regression fit on the temporal response data. The equations for the liquid and bead sensors are, respectively:

$$S/N = 11.70 + 2.705t - 0.002t^2 \quad (4.1)$$

$$S/N = -67.72 + 10.81t - 0.022t^2 + 1.17 \times 10^{-5} t^3 \quad (4.2)$$

Setting $d(S/N)/dt = 0$ in each case yields $t_{(\text{equilibrium-liquid})} = 11$ hrs. and $t_{(\text{equilibrium-bead})} = 5$ hrs. These results indicate that the FIS is capable of responding linearly (and non-linearly)

Table 4.2. Time-Dependent Concentrating Factors

Time (Min.)	Concentrating Factor	
	<u>liquid reagent phase</u>	<u>bead reagent phase</u>
15	2.6	0.9
30	5.0	2.5
45	7.8	4.4
75	13.2	8.1
120	20.5	12.7
165	25.9	15.5
255	35.7	19.3
300	39.1	20.3

for long periods of time. The actual working range is probably a function of the relative concentrations of antibody and hapten, the K_a , and the occurrence of fluorescence inner-filter effects. Inequalities in one or more of these factors may, therefore, have accounted for the bead sensor's reduced dynamic range.

Temporal response data can also be used to provide fundamental information including the K_a and the concentration of antibody binder in the sensing tip. Substituting $t_{\text{(equilibrium)}} \approx 11$ hrs. into equation (4.1) yields $S/N_{\text{(equilibrium)}} = 930$. Because of the fact that free [BPT] $\ll$ [Ab:BPT], virtually all of the observed equilibrium signal was contributed by Ab:BPT. As a result, using independently obtained calibration data, $S/N = 930$ corresponded to [Ab:BPT] $\approx 1 \times 10^{-6}$ M. Since the initial free [Ab] was 1.9×10^{-6} M, the final free equilibrium [Ab] was roughly 9×10^{-7} M. Furthermore, neglecting membrane adsorption and polarization effects (unequal distribution of BPT across the membrane), the free equilibrium [BPT] inside the sensor was approximately equal to the [BPT] in the sample. Substituting these values into the equilibrium expression for Ab:BPT formation:

$$K_a = [\text{Ab:BPT}] / ([\text{Ab}] [\text{BPT}]) \quad (4.3)$$

yields $K_a \approx 8 \times 10^7 \text{ M}^{-1}$. During long incubations, our experiments indicate that membrane adsorption and polarization may actually

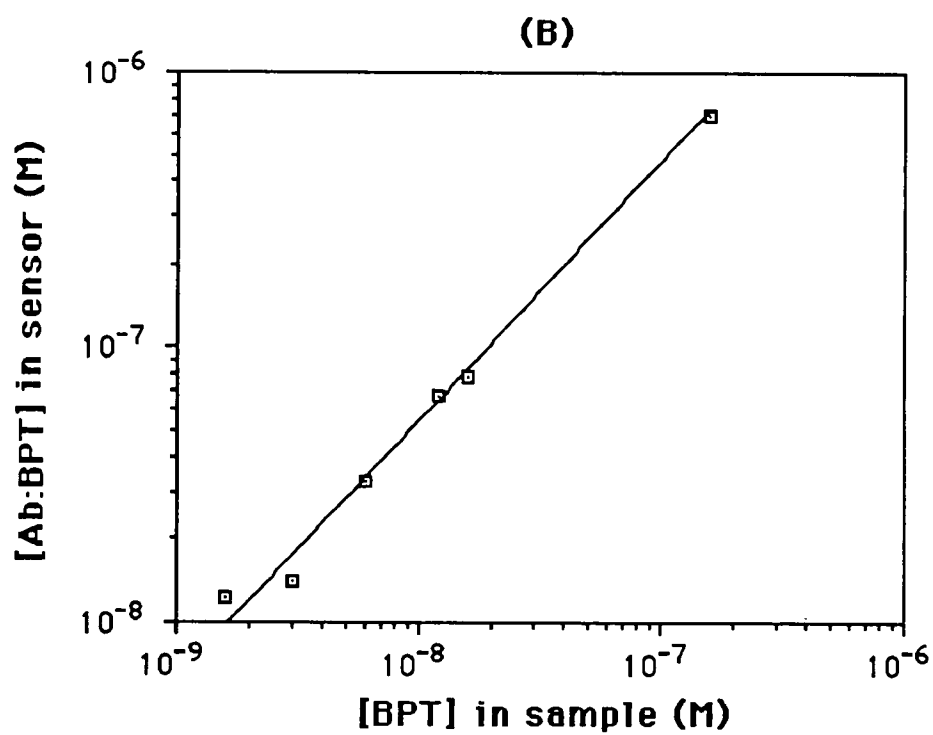
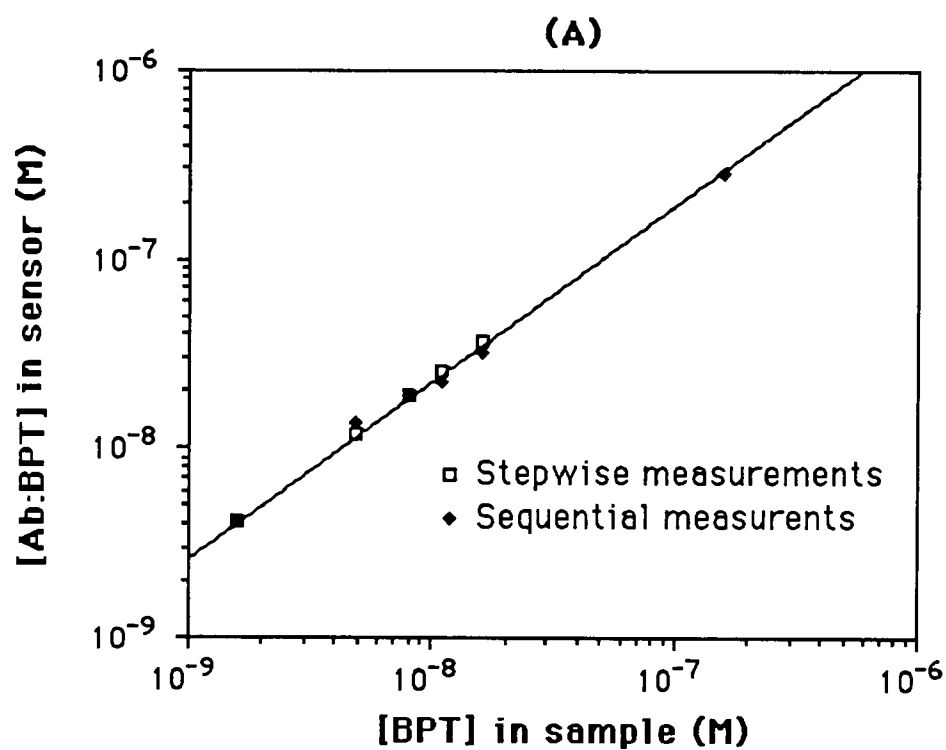
have occurred. Accordingly, the exact value for free [BPT] inside the sensor was probably less than 1.6×10^{-8} M and the K_a may have been slightly larger than estimated. In principle, this FIS-derived determination of the formation constant is equivalent to the well-established equilibrium dialysis technique.¹⁵³

Alternately, when the K_a is known through independent experiments, the concentration of free antibody in the sensing tip can be derived from temporal response data and equation (4.3). If the initial free [Ab] is unknown it can then be deduced from the sum of bound and free components.

Concentration-dependent linearity was evaluated by obtaining the dose-response data illustrated in Figure 4.4. Sensors were loaded with a constant amount of antibody and incubated for either 15 minutes (liquid tips) or 45 minutes (bead tips) in varying concentrations of BPT. These incubation times were selected because temporal response data indicated they were of sufficient duration to observe approximately a factor of two concentrating effect. The [Ab:BPT] values appearing in Figure 4.4 were obtained by correlating sensor S/N levels to those obtained from bare-fiber Ab:BPT calibration plots.

Liquid-phase sensors were used to perform stepwise and sequential measurements (Figure 4.4-a). The average absolute difference between data obtained with both procedures was $10.5\% \pm 8.9\%$. A linear response was observed for up to 2 hours without changing reagent phases during stepwise measurements. This

Figure 4.4. FIS dose response: $[Ab:BPT]$ vs. $[BPT]$ for (A) liquid antibody reagent-phase sensors, 15-minute incubations and (B) bead-immobilized antibody reagent-phase sensors, 45-minute incubations.



relatively long stepwise sensor lifetime was consistent with the FIS's previously observed time-dependent linearity. Bead-sensor assays were conducted sequentially (Figure 4.4-b). As illustrated in Figures 4.4-a and 4.4-b, the linear response region for sensors using either type of reagent phase extends over approximately 2 decades. The slope, $d[\text{Ab:BPT}]/d[\text{BPT}]$, is the antibody concentrating effect observed for each assay. Values of 1.8 and 4.3 were derived from the slopes of liquid and bead sensor calibration plots, respectively. These concentrating factors agree reasonably well with those reported at the 15 and 45 minute marks of the liquid and bead temporal studies (Table 4.2). This agreement is particularly encouraging since calibration and temporal studies were performed on different days under slightly different instrumental and experimental conditions.

Dynamic range can be extended, at low and high BPT concentrations, by manipulating incubation times. At high [BPT], further extensions in dynamic range should occur by increasing [Ab]. In fact, antibody immobilization on beads was pursued in an effort to elevate its effective concentration beyond liquid-phase levels. Unfortunately, the Protein A beads used in this study did not produce the desired increase in [Ab].

Limits of detection. FIS limits of detection (LOD) are dependent upon assay incubation times, the antibody-hapten association constant (K_a), and the sensitivity of the measured

optical parameter. In this work, K_a was determined to be $8 \times 10^7 \text{ M}^{-1}$, the minimum detectable [Ab:BPT] for fiber measurements was approximately $1 \times 10^{-9} \text{ M}$, and [Ab] for typical liquid-phase assays was $2 \times 10^{-6} \text{ M}$. Substitution of these values into equation (4.3) yields $[\text{BPT}] = 6 \times 10^{-12} \text{ M}$. This figure represents the minimum concentration of unbound BPT inside the sensor that will yield a detectable equilibrium concentration of Ab:BPT. When the FIS is at equilibrium with regard to the sample, $[\text{BPT}]_{(\text{inside})} = [\text{BPT}]_{(\text{outside})}$, and $6 \times 10^{-12} \text{ M}$ also represents the lowest concentration of BPT which can be detected in the sample.

Estimation of sample detection limits can be further understood by considering the effect of dropping sample BPT concentration below $6 \times 10^{-12} \text{ M}$. Under these conditions, $[\text{BPT}]_{(\text{inside})}$ values are reduced. Substituting smaller $[\text{BPT}]_{(\text{inside})}$ levels into equation (4.3) yields Ab:BPT concentrations which are below the fiber's LOD. Therefore, under the above equilibrium conditions, $[\text{BPT}]_{\text{LOD}} = 6 \times 10^{-12} \text{ M}$. Lower detection limits can be achieved by improving instrumental sensitivity, increasing [Ab], or by using higher affinity antibodies. These extremely low detection limits are not, however, encountered in practical FIS applications due to the prohibitively long incubation times required to reach equilibrium. In addition, at long incubation times, the appearance of membrane adsorption

and polarization effects would cause $[BPT]_{(inside)} < [BPT]_{(outside)}$.

As a result, the observed BPT detection limits would be slightly higher than 6×10^{-12} M since greater $[BPT]_{(outside)}$ levels would be required in order to maintain sufficient $[BPT]_{(inside)}$ for Ab:BPT detectability.

In the case of non-equilibrium FIS measurements, LOD can be derived from the sensor's time-dependent concentrating effect and the bare-fiber detectability of Ab:BPT. For 15-minute liquid-phase sensor measurements, a concentrating factor of 2 was observed. Consequently, $[BPT]_{(bulk)}$ levels as low as 5×10^{-10} M can be probed in order to satisfy the 1×10^{-9} M minimum [Ab:BPT] detectability requirement. LOD for 45-minute bead sensor incubations (based on a factor of 4 concentrating effect) were 2.5×10^{-10} M. Further reductions in FIS detection limits can be achieved by increasing incubation times, though the merits of this approach must be evaluated by weighing the benefits of lower LOD against the disadvantages of longer analysis times. Additional LOD modifications can be affected by altering the sensor's fundamental response parameters.

An experiment designed to illustrate the FIS's excellent low-volume sensitivity was conducted in a 5- μ L droplet of 1.6×10^{-9} M BPT. Results indicate that BPT was easily detected (S/N = 39) after a 30-minute incubation. In contrast, bare-fiber FIS measurements yielded a S/N that was close to the detection limit.

The large response observed during the 30-minute incubation was partly due to solvent evaporation which increased the BPT concentration and the rate of diffusion into the sensor. Nevertheless, the absolute amount of BPT in the sample was 8 femtomoles; indicating that the absolute LOD for BPT is in the sub-femtomole region even when relatively short incubations are employed.

Precision. FIS precision was evaluated by performing multiple assays of identical BPT solutions. In a series of five, 45-minute long, bead-sensor sequential measurements of 3×10^{-9} M BPT, the average S/N was 13.85 ± 0.86 . The 6.2% relative standard deviation (RSD) displayed by these sequential bead-sensor assays was approximately the same as the RSD encountered during sequential liquid-phase measurements. Stepwise analyses, however, were nearly 2.5 times less reproducible, exhibiting a 15% RSD. Membrane memory effects may have been responsible for this discrepancy since the sensing tips were thoroughly washed (inside and out) between sequential assays while stepwise rinses consisted of a brief exposure to PBS dialysis solutions.

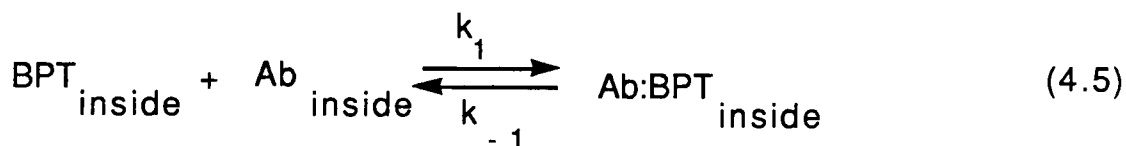
Additional factors which may contribute to imprecision include variations in instrumental response and irreproducible measurement conditions. Fluctuations in the latter, particularly rate of analyte mass transport, can be extremely deleterious when

highly sensitive, non-equilibrium assays are performed. Reproducing in-situ conditions is relatively straightforward for benchtop measurements. It is, however, a critically important challenge which must be addressed before the FIS can provide accurate information from in-vivo samples.

Response kinetics. FIS response can be described by a two-step mechanism involving 1) transport of BPT across the membrane into the reagent phase and 2) reaction of BPT with antibody to form Ab:BPT. Step 1 is represented by:



The rate constants k_d and k_{-d} for this relatively slow process are a function of BPT's diffusion through the membrane material. In step 2:



the rate constants k_1 (typically 10^7 - $10^9 \text{ M}^{-1}\text{s}^{-1}$) and k_{-1} (typically 10^2 - 10^{-4} s^{-1}) are responsible for the rapid rate of

immune complex formation inside the reagent phase.^{121,124} At high $[Ab]_{inside}$, the rate of BPT diffusion in the reagent phase does not significantly influence liquid and bead-sensor response, though it may be important in determining the response rate for gel or polymer-based devices.

Since BPT_{inside} is a rapidly reacting intermediate whose time-dependent concentration, $d[BPT]_i/dt$, is approximately zero, the entire kinetic scheme can be simplified by applying the steady state approximation to equations (4.4) and (4.5). By assuming that k_1 is much greater than k_{-1} , the rate of Ab:BPT formation becomes:

$$d[Ab:BPT] / dt \approx k_1 k_d [Ab]_i [BPT]_o / (k_{-d} + k_1 [Ab]_i) \quad (4.6)$$

where the subscripts "i" and "o" represent "inside" and "outside" the sensing tip. When $[Ab]_i$ is large, equation (4.6) reduces to:

$$d[Ab:BPT] / dt \approx k_d [BPT]_o \quad (4.7)$$

and sensor response rate is simply a function of the external analyte concentration and the rate constant for its diffusion across the membrane.

Figure 4.5 summarizes sensor behavior by illustrating the

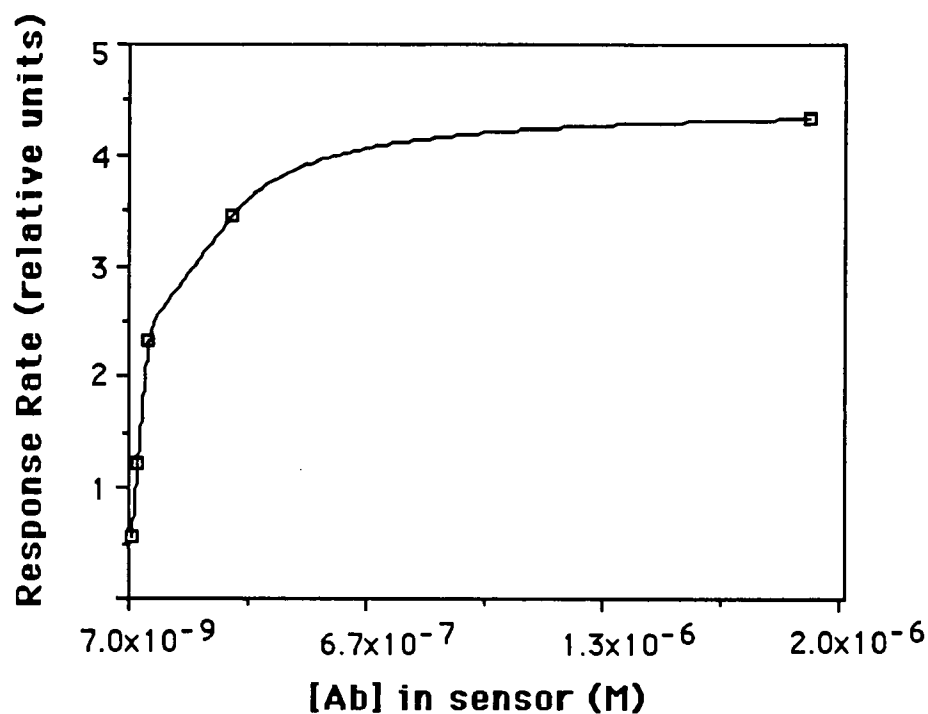


Figure 4.5. FIS response rate (change in S/N during 15-minute incubations) as a function of sensor [Ab] for liquid antibody reagent-phase sensors.

influence of $[Ab]_i$ (at constant $[BPT]_0$) on response rate. At high $[Ab]_i$, sensor response rate is nearly independent of antibody concentration. In this region, antibody binder is in excess. Sensor response kinetics can be described by equation (4.7), and small perturbations in $[Ab]_i$ have little or no influence on response rate.

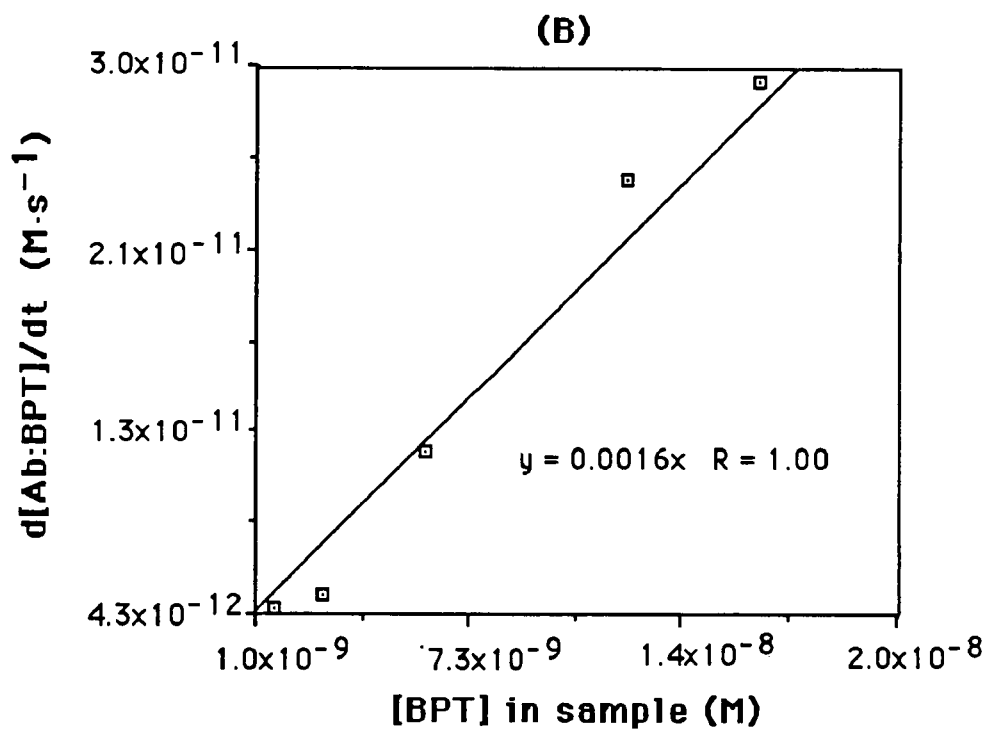
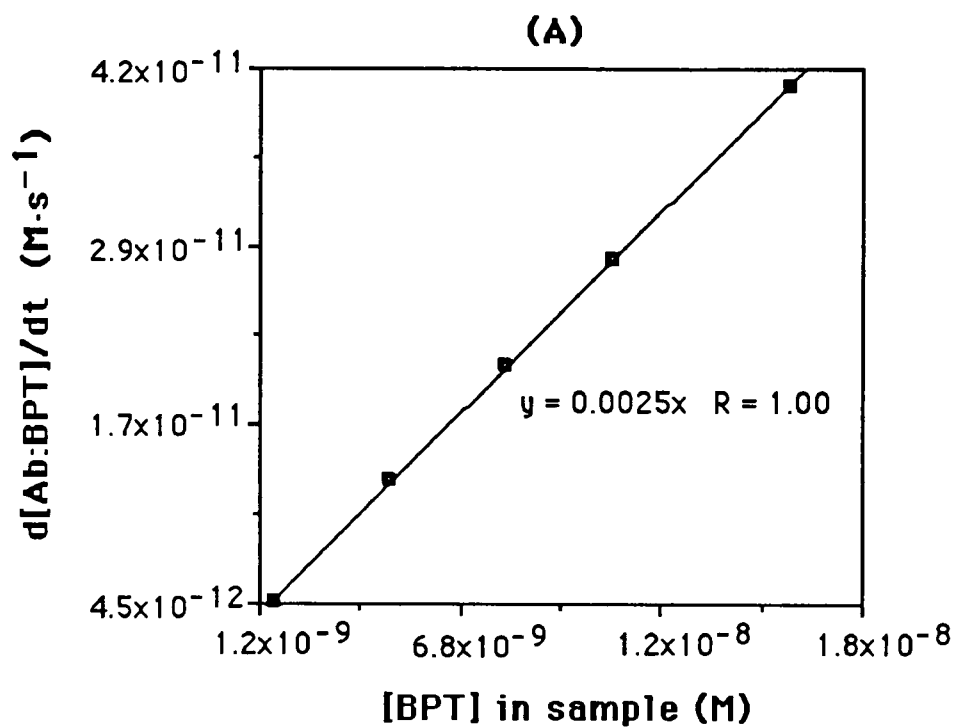
In contrast, at sufficiently low antibody concentrations $k_d \gg k_1[Ab]_i$ and equation (4.6) reduces to:

$$d[Ab:BPT] / dt \approx k_1 k_d / k_{-d} [Ab]_i [BPT]_0 \quad (4.8)$$

In this case, sensor response kinetics are probably second order. These conditions are represented by the steep portion of Figure 4.5. In this low $[Ab]_i$ region, small changes in $[Ab]_i$ produce large variations in response rate. Since it is not desirable to operate the FIS under these rapidly changing conditions, a high concentration of Ab should be maintained inside the sensor for most applications.

When $[Ab]_i$ is large, a plot of the change in response over a 15-minute interval ($d[Ab:BPT]/dt$) vs. $[BPT]_0$ should yield a straight line whose slope, k_d , is the rate constant described in equation (4.7). Calibration data is re-displayed in Figures 4.6-a and 4.6-b to illustrate this relationship. For liquid reagent phases

Figure 4.6. FIS response rate as a function of sample [BPT]: (A) liquid antibody reagent-phase sensors, 15-minute incubations and (B) bead-immobilized antibody reagent-phase sensors, 45-minute incubations.



(Figure 4.6-a) $k_d = 2.5 \times 10^{-3} \text{ s}^{-1}$. When bead reagent phases are employed, $k_d = 1.6 \times 10^{-3} \text{ s}^{-1}$ (Figure 4.6-b). The agreement between liquid and bead rate constants is reasonable, though the slightly slower bead kinetics may be attributable to the presence of solid particles interfering with membrane transport. It should be noted that rinsing times were short (4.5 minutes) relative to the time required to achieve a concentrating factor of one, indicating that $k_{-d} > k_d$; perhaps due to a slight pressure differential between the "closed" 20-nL sensor and the open 1-mL sample.

The FIS-derived response constant can also be used to calculate sensor detection limits for any incubation time, "t". Integration of equation (4.7) as a function of time yields, for sequential measurements:

$$[\text{Ab:BPT}]_i = k_d t [\text{BPT}]_o \quad (4.9)$$

If k_d and the minimum detectable $[\text{Ab:BPT}]_i$ are known, equation (4.9) can be used as a general expression to provide time-dependent $[\text{BPT}]_{\text{LOD}}$. Detection limits calculated in this manner are only valid when $[\text{Ab}]$ is in excess.

Selectivity. Figure 4.7 summarizes the results of an interference study, performed in PBS, using the PNA interferants

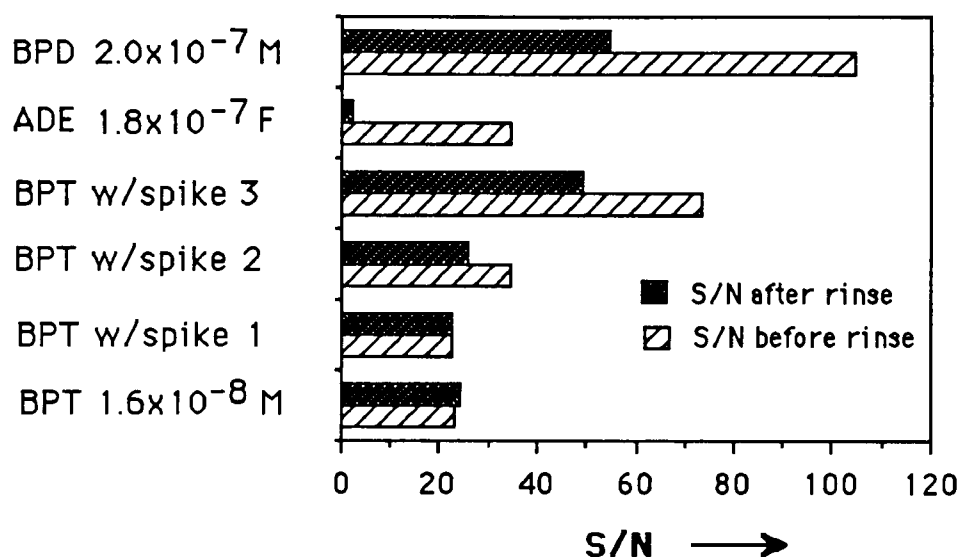


Figure 4.7. FIS interference study in PBS. Liquid antibody reagent-phase sensors, 15-minute incubations, 4.5 min. rinses. All samples contained 1.6×10^{-8} M BPT. Spike contents: (1) 1.8×10^{-8} F ADE. (2) 1.9×10^{-6} F ADE. (3) 1.9×10^{-6} F ADE and 4.0×10^{-8} M BPD.

illustrated in Figure 4.1. Analyses were conducted by incubating liquid reagent-phase sensors for 15-minutes in BPT and BPT plus PNA solutions. Signal levels observed prior to rinsing were generally greater than those observed following a 4.5 minute rinse. These elevated pre-rinse signals were indicative of the extent of spectral interference provided by the ADE and BPD interferants. In the case of two-component BPT/ADE mixtures (spikes 1 and 2), post-rinse FIS signals returned to those obtained for pure BPT solutions, despite the two-order-of magnitude ADE excess in spike 2. These results were not surprising since, as the data in Figure 4.7 illustrate, pure 1.8×10^{-7} F ADE was poorly retained by antibody. The formal analytical concentration is reported since, with time, aqueous ADE hydrolyzes to anthracene-tetraol. The exact molar concentration of ADE was therefore not known.

In the case of the three-component BPT/BPD/ADE mixture, post-rinse signals dropped precipitously from pre-rinse levels. The observed signal reduction was probably due primarily to the loss of unbound ADE from the sensor, though the disappearance of unbound BPD may also have contributed to this effect. It is likely that the latter phenomenon occurred to a lesser extent since, as indicated by the pure BPD measurement, significant antibody:BPD binding was observed.

Additional interference studies were carried out in a biological matrix, fetal calf serum (FCS). As illustrated in Figure 4.8, measurements were performed in spiked and unspiked 8.9×10^{-8}

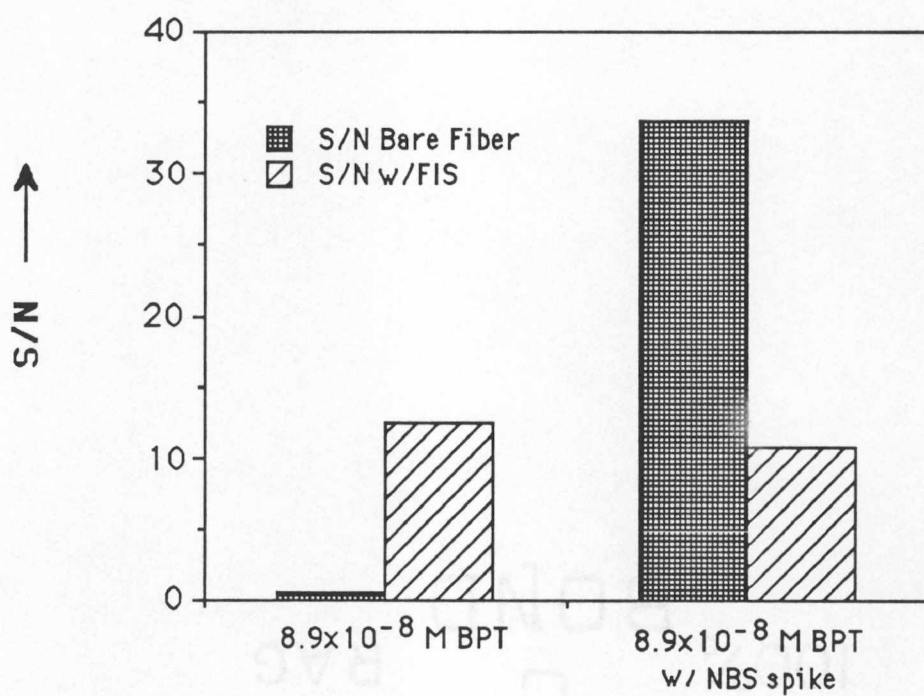


Figure 4.8. FIS interference study in fetal calf serum (FCS). Liquid antibody reagent-phase sensor, 15-minute incubation, 4.5 min. FCS rinse.

BPT/FCS. The spike was composed of 16 PNA interferants from NBS SRM 1647. Final spike concentrations are listed in Table 4.1. Both bare-fiber and liquid-phase FIS analyses were conducted. FIS rinses were performed in pure FCS. The bare-fiber measurement revealed that, at the excitation and emission wavelengths used in this work, SRM 1647 was responsible for significant spectral interference. The post-rinse FIS signals for spiked and unspiked solutions, however, were roughly the same, indicating that the FIS was capable of excellent chemical selectivity. The sensor was also observed to display slightly diminished sensitivity, probably due to FCS-related elevations in optical background.

The specificity of antibody/hapten interactions is not the sole parameter which determines the sensor's ability to discriminate between structural interferants. Additional selectivity factors include the membrane's molecular-weight cutoff, thickness, and analyte compatibility, as well as the solubility of the analyte in the sensor's reagent phase. In the case of SRM 1647 measurements, a number of hydrophobic components may not have crossed the hydrophilic membrane material. Overall performance was, therefore, probably attributable to the simultaneous action of more than one selectivity determinant.

In conclusion, these results demonstrate that small, hydrophilic, fluorescent haptens can be reliably detected using membrane-entrapped antibody reagents. Rapid, non-equilibrium measurements can be conducted for long periods of time, provided

there is sufficient excess antibody in the sensing tip. An additional unique feature of the FIS is its ability to perform measurements in complex biological matrices.

Data obtained during FIS analyses can be used to characterize sensor response as well as provide fundamental immunochemical information. Improvements in sensor performance can be achieved by optimizing parameters which influence detectability. Among the potential drawbacks to these devices are membrane-related difficulties including memory effects and response-limiting trans-membrane diffusion. Memory effects can be addressed by improved rinsing; however, additional impediments to sensor precision may arise if mass transport conditions and incubation times are not precisely reproduced. Despite these obstacles, it is expected that the FIS's outstanding sensitivity, selectivity, and simplicity will encourage further applications and design refinements so that increasingly versatile sensors can be constructed.

CHAPTER 5

CONCLUSIONS

Selection of the proper FIS design is dependent upon the analytical characteristics of the system of interest. Large non-fluorescent molecules are best detected using a fluorescent tracer in a competitive binding assay. Sensitivity is inversely proportional to the amount of binder since it is the limiting reagent. Monolayer fiber reagent-phases are therefore well suited to these measurements, provided there is sufficient immobilized binder for detectability and the immunochemicals are not significantly denatured during immobilization. Higher loadings can be achieved by immobilizing binder on solid particles and confining them behind membranes or frits.

Solid reagent phases can also be appropriate for sandwich assays and direct assays of natural fluorophores. Sensitivity for these measurements is directly proportional to the amount of binder. In order to optimize sensor response, reagent-coated beads and binder-entrapped gels can be used. Analyte diffusion and solubility in the reagent phase must also be considered. When the analyte is incompatible with membrane and gel materials, monolayer fiber reagent phases should be used at the expense of lower reagent phase loadings. The rapid diffusion of materials in

monolayer reagent phases can significantly decrease sensor response times.

Small, hydrophilic, fluorescent haptens can be conveniently analyzed using membrane-entrapped liquid reagent phases. The use of membrane tips eliminates the inconvenience and potential irreproducibility of solid-phase immobilization on multiple fibers. A single fiber can be used for long periods of time provided there is excess antibody in the sensing tip. Potential drawbacks to these devices include membrane memory effects, slow (sensor response limiting) diffusion across the membrane, and the fact that non-equilibrium assays are performed. These factors can increase the potential for error between samples and standards, particularly if mass transport conditions and incubation times are not precisely reproduced.

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APPENDIX

APPENDIX

GLOSSARY OF ACRONYMS AND ABBREVIATIONS

Ab	Antibody.
ADE	Benzanthracene diol epoxide.
An	Antigen.
BaP	Benzo(a)pyrene.
BPD	Benzopyrene diol.
BPDE	Benzopyrene diol epoxide.
BPDE-DNA	Benzopyrene diol epoxide-DNA adduct.
BPT	Benzopyrene tetraol.
BSA	Bovine serum albumin.
FCS	Fetal calf serum.
FIS	Fluoroimmunosensor.
FITC	Fluorescein isothiocyanate.
FOCS	Fiber optic chemical sensor.
GOPS	3-Glycidoxypropyltrimethoxysilane.
Hn	Hapten.
IgG	Immunoglobulin G.
KSCN	Potassium thiocyanate.
LDR	Linear dynamic range.
LOD	Limits of detection.
MoAb	Monoclonal antibody.
NBS	National Bureau of Standards.

PBS	Phosphate-buffered saline.
PCS	Plastic-clad silica.
PNA	Polynuclear aromatic compound.
RSD	Relative standard deviation.
SMA	SMA-type fiber optic coupler.
S/N	Signal-to-noise ratio.
SRM	Standard reference material.

VITA

Bruce Jason Tromberg was born in Cincinnati, Ohio on November 25, 1957. He grew up and attended schools in Washington, D. C., graduating from Woodrow Wilson High School in that city in 1974. In May 1979 he received a Bachelor of Arts degree in Chemistry and Psychology from Vanderbilt University in Nashville, Tennessee. After working for one year in a pathology research laboratory at Vanderbilt University Medical Center, he began study toward a Master's degree in Chemistry at The University of Tennessee, Knoxville. This degree was awarded in June 1983.

Following graduation from The University of Tennessee, he worked for one year as a research chemist at Oak Ridge National Laboratory. In September 1984, he re-entered The Graduate School of The University of Tennessee, Knoxville. In August 1986, he received a U. S. Department of Energy/Oak Ridge Associated Universities pre-doctoral fellowship to pursue his dissertation research. In March 1988 he was awarded the Doctor of Philosophy degree in Chemistry.

The author is a member of several scientific and professional societies including The American Chemical Society, The American Association for the Advancement of Science, and The Society of Photo-Optical Instrumentation Engineers. He has been recognized by the editors of Research and Development magazine as co-creator of one of the top 100 technical innovations in 1987.

After graduation, Dr. Tromberg will be employed as a post-doctoral research fellow at the Beckman Laser Institute and Medical Clinic, University of California, Irvine.