Development of antibody-based fiber-optic sensors

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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 (fluoroimmuno-sensors) capable of in-situ detection of drugs, toxins, and naturally occurring biochemicals.

Fluoroimmuno-sensors (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, 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 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.

1. INTRODUCTION

Immunological methods 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.[1] Along with advances in fiber optic instrumentation, these innovations have been the driving force behind antibody-based fiber optic sensor development.

2. FIBER OPTIC CHEMICAL SENSORS

Extrinsic fiber optic 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 the observation of analyte-induced changes in the optical properties of the sensor's reagent phase.[2] Because the reagent phase (or external

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transducer) recognizes the analyte, the specificity and sensitivity of the device is regulated by the transducer's fundamental characteristics. For antibody-based FOCS, these critical features may include: antibody availability, abundance, association constant (K_a), and other factors relevant to the particular immunoassay.

2.1. Antibody-Based FOCS

In this work, a sensor design was required which could perform simple, rapid, in-situ analyses of large molecules (M.W.> 200) in complex matrices. These stipulations demanded the adoption of an extrinsic sensing configuration 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.[3-12]

Many of these sensors have been used to detect small molecules,e. g.CO_2[5,6], NH_3[11],metal ions[9,10,12], and pH.[3,5,7,8] In order to perform highly sensitive and selective detection of trace amounts of large molecules, the specificity of biologically significant ligand/binder reactions can successfully be applied to FOCS design. These reactions can include enzyme/substrate[13], antibody/antigen (Ab:An)[14], and antibody/hapten (Ab:Hn)[15] interactions. Kronick and Little first described this approach in their report of a total internal reflection fluorescence (TIRF) immunoassay of dye-labeled antibodies bound to an antigen-coated planar waveguide.[16] Angel[17], Sutherland and Dähne, et al.[18], and Andrade et. al[19], 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 varies considerably with the light source's incidence angle and waveguide's structure (i.e., planar or cylindrical).[20] These restrictions demand the use of relatively short (5-10 cm) reagent-coated fibers. This design maintains adequate interfacial excitation intensity, however, the use of short fibers with large exposed surface areas limits the in-situ capability of these devices.

2.1.2. Distal face devices. In an attempt to circumvent the limitations of TRIF designs, a fluoroimmunosensor (FIS) was fabricated which utilizes an immobilized immunochemical reagent phase located at the distal face of a single sensing fiber. By locating the reagent phase in this position, the sensing element experiences maximum excitation light intensity and emmission collection efficiency. Either fiber bundles or single fibers can be used. Advantages to single-fiber designs include their superior fluorescence emission collection efficiencies 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.[6,7,1,15] These features typically result in single-fiber sensors capable of femtomole limits of detection.[14,15]

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 can be addressed by recording binding-dependent reagent phase changes. These changes may include variations in luminescence energy, intensity, and lifetime[21], and "field of view" detection of unbound indicator molecules.[22]

Extrinsic devices which employ immunochemical reagent phases and distal-face excitation have the potential to enhance selectivity, sensitivity and versatility over TIRF immunosensors and FOCSs 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 allow an immunosensor to concentrate analyte within the sensor's observation zone. Depending upon the type of immunoassay, this may permit the sensor to function without separation from the analyte (homogeneously), since the only observable contributor to the optical signal may be reagent-bound analyte. If this is not the case, unbound interferants can be rinsed away in a buffer solution by removing the fiber from the measurement matrix after a pre-determined incubation period. Manipulations of this sort are designated as "heterogeneous immunoassays."

3. ANTIBODY FUNCTION AND CLASSIFICATION

The reaction between antibody and its specific antigen or hapten is reversible and non-covalent. Antigens are molecules which can elicit an immune response in an animal. In order to successfully induce antibody production, antigens must have molecular weights greater than 5000 - 10,000. 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 variable regions in the Fab portion of the IgG antibody molecule, illustrated in Figure 1, are produced to recognize these antigenic determinants.[23]

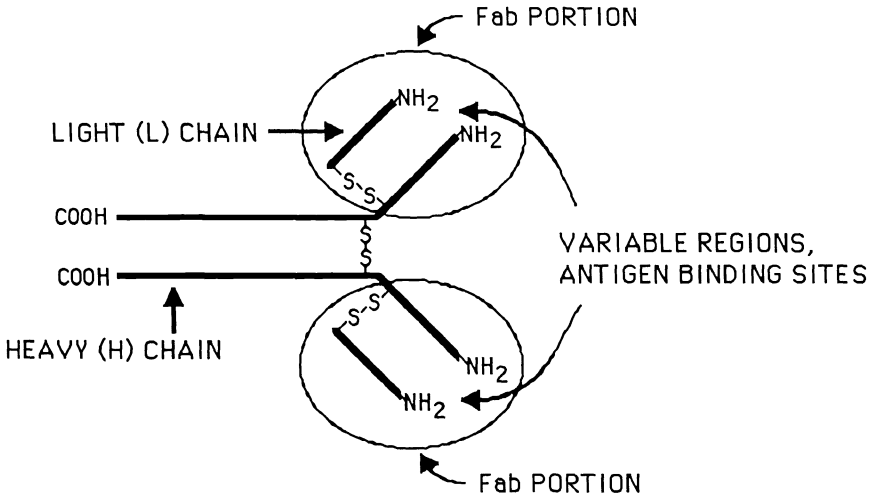

Figure 1: Structure of antibody of IgG class, MW ~ 160,000.

Antibodies can be broadly classified as either polyclonal or monoclonal. Polyclonal antibodies are raised in hyperimmunized animal sera. 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.[24]

Monoclonal antibodies are raised from fused cells (hybridomas) which produce molecules capable of recognizing a single antigenic determinant. They are characterized by a single association constant which defines their affinity for a particular antigen or hapten. Monoclonals are particularly valuable in immunosensors since their binding site homogeneity produces a uniform response.[23,24]

3.1. Reactions of Antibodies

The association constant (K_a) for immunochemical complex formation is an indicator of polyclonal avidity and monoclonal affinity. It is described by:

$$\text{Antibody} + \text{Antigen} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Antibody:Antigen}$$

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

Typical K_a values range from 10^6 - 10^{12} M^{-1}. Forward reaction rate constants are generally less variable than reverse rate constants.[25] Values for k_{-1} and k_1 range from 10^2 - 10^{-4} s^{-1} and 10^7 - 10^9 $M^{-1}s^{-1}$ respectively.[26] Due to these large k_1 values, the rate of antibody binding is primarily mass-transport controlled. 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 or replacing binder following equilibrium measurements.

Binder regeneration can be accomplished by inducing immune complex dissociation. 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 variable Fab regions. Some effective chaotropic reagents are: guanidine hydrochloride, potassium thiocyanate, urea, and propionic acid.[27] After exposure to the appropriate reagent, the protein can often 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.[28] Success appears to be dependent upon the type of antibody, the chaotropic reagent, and the immobilization procedure.

4. SINGLE-MEASUREMENT PROBES

The kinetic limitations of immunochemical complex formation (i.e., large association and small dissociation rate constants) must be overcome in order to produce continuous, rapidly responding, reversible, antibody-based FOCSs.[19,29] 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. Their primary limitations are relatively poor reproducibility and inconvenience. In contrast, continuous antibody-based sensors are less sensitive and selective. These devices require the use of low affinity antibodies as well as the application of homogeneous detection strategies which may be difficult to follow.[1] The resulting analytical tradeoff for continuous detection is diminished selectivity and sensitivity.

In consideration of this apparent analytical dilemma, much of our work has focused on the development of highly sensitive, rapid, irreversible devices. We anticipate that a thorough understanding of the capabilities and limitations of single-shot probes will engender the successful construction of antibody-based continuous FOCSs.

5. INSTRUMENTATION

Figure 2 illustrates the general features of most single-fiber sensors. Specific components, including light source, beam splitter, fiber type, and reagent phase are determined by the requirements of the analytical system. Both single-fiber and fiber bundle designs are employed. Single-fibers were utilized in this work because they are generally smaller and are capable of greater fluorescence collection efficiencies. Bundles are used, in many cases, because of their simplicity. In addition, the coupling of diffuse light to large-diameter bundles can be more efficient than to a single fiber. Fluorescence collection efficiencies, however, are generally poorer since overlap in the collection regions diminishes with increasing proximity to the area of greatest source intensity: the excitation fiber's face. An additional minor drawback to bundles is the fact that they can not be miniaturized to single fiber dimensions.

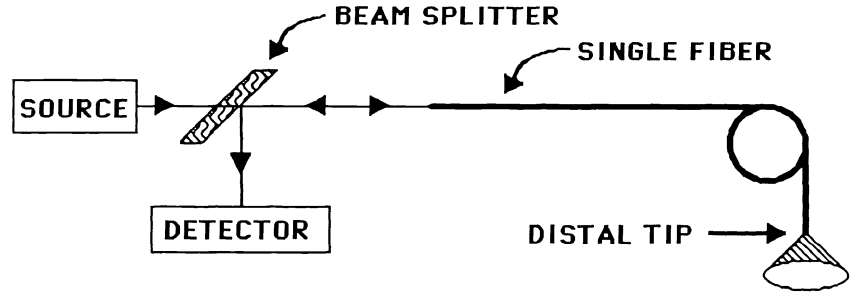

Figure 2: Extrinsic single-fiber sensor instrumentation.

As depicted in Figure 2, light is directed onto the fiber through a beamsplitter. Dichroic filters are commonly used as beamsplitters in fluorescence measurements to separate excitation and emission radiation. Wavelength-independent splitters such as prisms and fiber couplers have also been employed. If the light source is a laser, a beam-sized mirror or, conversely, a large mirror with a beam-sized hole can be used. Lasers are primarily used because of their high intensities and superior source/fiber coupling efficiencies. Since it is relatively easy to focus laser beams to low-μm spot diameters, nearly 100% of a focused beam can be coupled to a typical 200-μm diameter plastic-clad silica fiber. Laser/fiber coupling efficiency is generally limited only by reflections at the fiber's incident end. In contrast, using a conventional two-lens collimating/focusing system, the same fiber will accept orders of magnitude less light from a diffuse source.[30]

Light travels the length of the fiber and encounters the chemical 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." Collection efficiencies can be improved by increasing fiber diameter and numerical aperture (NA), and by placing micro-lenses at the sensing tip.

5.1. Sensing Termini

A number of sensing termini have been explored in our work. Each is characterized by a chemically selective reagent phase at the fiber's sampling terminus. Figures 3(a-e) illustrate the most common types of FOCS termini.

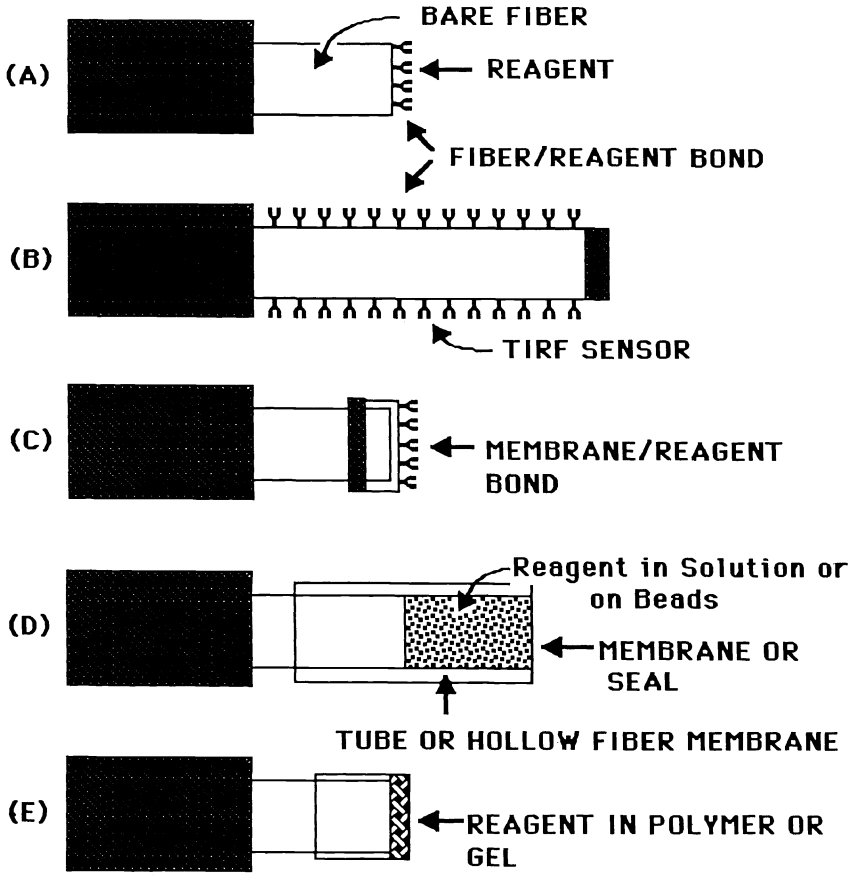

Figure 3: Possible sensing termini.

The reagent phase should, ideally, be a highly specific, rapidly reacting binder for the ligand of interest. When excess binder is used, a single probe can function in a pseudo-continuous fashion. In this type of measurement, the probe is simply re-zeroed at the conclusion of each measurement so that the signal from the recently formed product becomes the baseline for the next analysis. 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 attached to the surface of the fiber (Figures 3a and 3b) or to an optically transparent substrate (Figure 3c). 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 3d), or by entrapping it in a gel or polymeric matrix (Figure 3e). 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 antibody binder, long incubations can pre-concentrate and even separate analyte from complex matrices.

6. FIS MEASUREMENTS

6.1. Competitive Binding Assays

Several 0.6-m long, 600-μm diameter fluoroimmunosensors were prepared for single-shot competitive binding measurements of anti-rabbit IgG. The general FIS apparatus is depicted in Figure 2. The 488-nm line of a Spectra-Physics forced-air-cooled argon-ion laser was used to provide excitation. Additional instrumentation details appear elsewhere.[14]

Sensing termini resembling Figure 3a were prepared by covalently attaching rabbit IgG to the quartz surfaces via an organosilane derivatizing agent, (3-Glycidoxyproyl)trimethoxysilane (GOPS).[14] Fiber protein loadings were determined to be 1-3 $\mu g/cm^2$. Each sensor was incubated for five minutes in 1-mL stirred PBS solutions containing varying amounts of unlabeled analyte (anti-rabbit IgG) along with 31 picomoles of fluoresceinisothiocyanate-labeled analyte (anti-IgG-FITC). Following incubation, each fiber was removed from the sample and rinsed in PBS. Measurements were performed after rinsing in fresh 1-mL PBS solutions. The dose-response curve illustrated in Figure 4 summarizes these results.

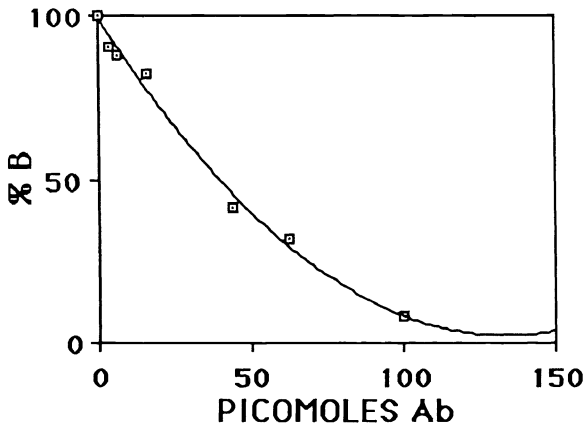

Figure 4: Competitive binding dose-response.

Figure 4 compares the percent of fiber-bound tracer (anti-IgG-FITC) with the amount of unlabeled analyte in each sample. At 100% bound (%B), optical signal was derived solely from a solution which contained only FITC-labeled tracer. As the concentration of unlabeled analyte increased, %B and optical signal decreased since more unlabeled analyte competed with tracer for a limited number of fiber binding sites. The assay limit of detection (LOD) was obtained by determining the amount of unlabeled analyte at two relative standard deviation (RSD) units below the 100 %B response. Since the between-fiber RSD in this work was roughly 10%, the LOD at approximately 80 %B corresponded to 13 picomoles of anti-rabbit IgG. The linear dynamic range (LDR) was, for competitive assays, characteristically narrow, covering roughly 1 order of magnitude of analyte concentration.

6.2. Direct Assays of Natural Fluorophores

A sensor for the carcinogenic fluorescent hapten r-7,t-8,9,c-10-Tetrahydroxy-7,8,9,10-Tetrahydrobenzo(a)pyrene (BPT) was constructed in a manner similar to that illustrated in Figure 3d. BPT is the acid hydrolysis product of benzo(a)pyrene/DNA adducts. Since the sensitivity for immunoassays of natural fluorophors is directly proportional to the amount of immobilized binder, a high concentration (typically 0.5 mg/mL in PBS) of liquid-phase monoclonal anti-BPT antibody (produced by Professor Regina Santella of Columbia University, N.Y., NY) was entrapped in a 40-nL removable tip constructed from small-diameter shrinkable tubing. The tip was capped by a highly permeable, 7-μm thick, cellulose dialysis membrane (Diachema AG, Zurich Switzerland, molecular-weight cutoff = 10,000) and slipped over the distal end of a 0.6-m long 200-μm diameter sensing fiber. The 325-nm line of an 8-mW Liconix model 4230 He:Cd laser (Liconix Corp., Sunnyvale, CA) was used for excitation of the antibody:hapten (Ab:Hn) complex. Emission intensity was monitored at 400 nm.

The antibody-filled sensor was incubated for 15 minutes in varying concentrations of 1-mL stirred BPT/PBS solutions. During this time, hapten diffused across the 10,000 molecular-weight cutoff membrane and became conjugated with antibody. Following each incubation, the sensor was rinsed for 4.5 minutes in PBS. Blank sensors loaded with rabbit IgG showed no retention of BPT during PBS rinses. In contrast, optical signals obtained during PBS rinses of the anti-BPT-filled FIS were typically twice as high as the blanks. Data was collected in a sequential and a stepwise manner. Sequential measurements were performed by re-loading the sensor tip with the same concentration of antibody prior to

each BPT incubation. Stepwise determinations involved loading the tip with antibody a single time and re-zeroing each analysis by subtracting the previous incubation's signal. Stepwise measurements of different BPT concentrations were performed in a variety of sequences without significant effect on sensor response. A working curve illustrating S/N vs. [BPT] for stepwise 15-minute incubations and 4.5 minute rinses is shown in Figure 5. The average difference between stepwise and sequentially obtained data was 10.5% ± 8.9%.

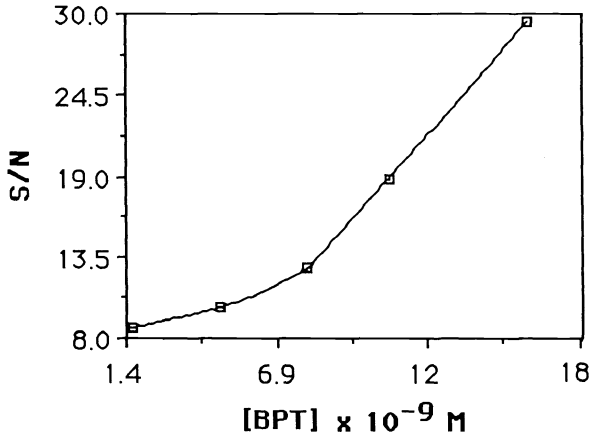

Figure 5: S/N vs. [BPT] for stepwise 15-minute incubations

A linear response was obtained using the stepwise sensor for up to 2 hours. Equilibrium was achieved in about 12 hours for measurements in 16 nM BPT. These results indicate that, provided there is sufficient excess antibody, this type of non-equilibrium device could be used in a pseudo-continuous fashion. The sensor's ability to perform in this manner would depend on its ability to concentrate hapten to the point where analyte would be the primary contributor to optical signal. Longer incubation times produce a greater concentrating effect and higher optical signals. After one hour in a 16-nM BPT solution, the FIS signal was roughly ten times that observed by a bare fiber. Based on these results (and the fact that the LOD for the bare fiber observation of BPT = 10^{-9} M), LODs were 5 x 10^{-10} M for 1 x 10^{-10} M for 15 and 60 minute incubations.

7. 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 fluorphors. 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.

8. ACKNOWLEDGEMENTS

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