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Fluorescent Optical Sensors

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ABSTRACT

Optical sensors are prepared by immobilizing an indicating layer on the distal end of a fiber optic cable. Dyes, enzymes, and antibodies can all be incorporated into the layer using a variety of immobilization techniques. Much of the present work is devoted to developing novel indicating schemes by combining appropriate recognition schemes into polymeric matrices.

Index Entries: Optical sensors; fluorescent indicators; fiber optics; immunosensors; controlled release polymers.

INTRODUCTION

Interest in continuous monitoring has blossomed during the last decade (*1*). Progress in microfabrication and materials science, and advances in electronic devices, such as detectors, optoelectronic components, and light sources, have focused attention on developing self-contained, inexpensive, low-power monitoring devices called sensors. One category of sensors is based on fiber optics. Optical sensors have significant advantages over their electrochemical counterparts, most notably, freedom from electromagnetic interference, their extremely small size, and the capability for internal calibration (*2,3*). In addition, conventional indicator chemistry with over a century's worth of development can be used to prepare these sensors. An optical sensor consists of a fiber-optic strand with appropriate indicating chemistry disposed at the far or distal tip of

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the fiber. Light is introduced into the proximal end, where it travels to the distal tip via total internal reflection. The indicating layer interacts with the analyte of interest and alters the light in proportion to the analyte concentration. Changes in absorbance, luminescence, polarization, and refractive index can all be used as optical transduction mechanisms. The returning light can be carried either through the same fiber or a separate detection fiber. Light is then processed through the appropriate detection system and correlated with the analyte concentration.

The key feature of any optical sensor is the selective indicating layer. In its simplest form, a colorimetric indicator is immobilized on the tip of the fiber and reacts reversibly through an equilibrium reaction with the analyte of interest (*4*). In more complex systems, the indicator is coupled with an enzyme (*5,6*) or with other indicators to furnish a layer with the requisite selectivity and sensitivity. The most desirable indicator scheme is a reversible system in which either an equilibrium or steady-state reaction is established as a function of analyte concentration. Reversibility is not an absolute criterion, however. Irreversible indicating schemes, such as binding of a metal ion with an indicator to form an essentially irreversibly bound complex or reaction of an antibody with an antigen to form an extremely tightly bound complex are effectively irreversible systems. These materials can be used, however, by measuring the rate of binding or by exposing subsaturating concentrations of the analyte to the sensor. Under these conditions, the sensor is capable of measuring analyte concentrations until it becomes saturated with analyte. At saturation, the indicator layer must either be recharged, or must undergo treatment to destroy or remove the bound analyte. Another potential class of indicating schemes is the use of reagents that react with analytes to provide an optical signal, such as absorbance, fluorescence, or chemiluminescence. A wide variety of these reagents have been developed for clinical and environmental analyses. These reagents must be used on a one-time basis only and cannot be used to prepare continuous sensors.

IMMUNOSENSORS

One important class of selective binding species is antibodies. Antibody-antigen reactions are essentially irreversible because of their large association constants (10^5–$10^9 M^{-1}$) (*7*). These association constants are comprised of large forward and small reverse rate constants ranging from 10^7 to $10^9 M^{-1}s^{-1}$ and 10^2 to $10^{-4}s^{-1}$, respectively. These kinetic properties make antibodies highly selective for a particular antigen. The slow off rates relegate them to one-time use or to a limited number of uses by exposing the sensor to subsaturating solutions of antigen (*8*). An alternative approach is to regenerate the antibody by exposure to chaotropic agents (*9*). One salient exception is the preparation of an optical sensor using a rapid off-rate antibody (*10*).

In order to obviate this limitation, we have developed immunosensors that are based on continuous release of fresh immunoreactants (*11*). These sensors utilize the same controlled release polymers that have been used conventionally as drug delivery systems (*12*). A salient feature of these polymers is their ability to sustain a continuous release of material for extremely long time periods. This feature, coupled to the entirely passive nature of release, provides a simple strategy for developing long-lasting optical immunosensors. In previous work, we showed that controlled-release polymers could be used to release pH indicators continuously for time periods of nearly 6 mo (*13,14*). These indicators possessed either a dual-wavelength pH sensitivity enabling an internal ratiometric measurement or were coupled with a surrogate indicator to provide an independent measurement of the release rate. Using either of these strategies, the precision of the pH measurements over 6 mo ranged between 0.05 pH units for the former internal ratioing strategy to 0.1 pH units for the two dye indicator system.

The first design parameter for developing optical immunosensors is the selection of an optical transduction mechanism. It is necessary to couple the antibody-antigen reaction to an optical signal that correlates the degree of this binding interaction. Many commercially available immunoassay systems employ an enzyme to amplify the antigen-binding event. In these systems, after the immunoreaction, the bound species are exposed to an enzyme substrate analog that generates color in proportion to the amount of bound antigen. Although this strategy enables extremely sensitive measurements to be made, it suffers from the need to conduct the assay under heterogeneous conditions with one or more washing steps. This complexity precludes its use in a sensor mode. The more attractive type of immunoassay is a homogeneous one in which all the reagents are in solution. Several possible optical transduction systems can be employed with homogeneous systems. All of them involve a fluorescent labeled antibody (*15*). In a direct assay, antigen binding to the fluorescent labeled antibody either enhances or quenches the fluorescent signal. Although simple, this approach is uncommon in that very few analytes are capable of interacting with the fluorescent label to provide sufficient signals for sensitive detection. We have employed a fluorescence energy-transfer mechanism. This type of assay is general and can be applied to virtually any antibody-antigen system. In this scheme, a competition reaction is set up between the solution antigen and a labeled antigen present in the assay medium (as shown in Eqs. *1* and *2*).

$$*\text{Ab} + \text{Ag} \leftrightharpoons *\text{Ab} \sim \text{Ag} \quad (1)$$

$$*\text{Ab} + \text{Ag}^* \leftrightharpoons *\text{Ab} \sim \text{Ag}^* \quad (2)$$

* = Label.

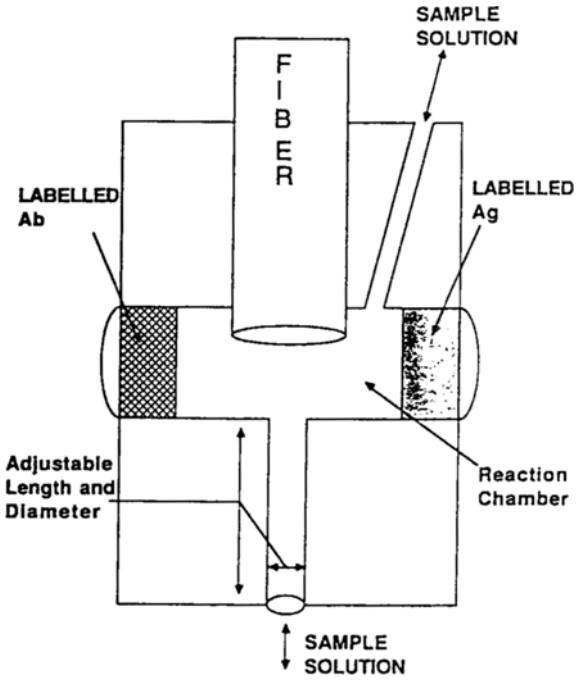

Fig. 1. Controlled-release sensor operates by reagent release and analyte diffusion to reaction chamber.

In this system, fluorescent labeled antigen competes with the analyte antigen for a limited number of antibody-binding sites. When the fluorescent labeled antigen binds to fluorescent labeled antibody, a transfer of energy occurs through a Förster mechanism from the labeled antibody to the labeled antigen. This energy transfer manifests itself as a change in the relative emission intensity of the two fluorescent labels.

The first controlled-release immunosensor prepared was a model system that used IgG as the antigen (*11*). In this system, fluorescein labeled antiIgG was used in conjunction with Texas Red labeled IgG. Each of these immunoreactants was incorporated separately into an ethylene vinyl acetate (EVAc) copolymer. EVAc is dissolved in methylene chloride, and then the protein immunoreactant is added to provide a suspension that is then cast and allowed to cure by solvent evaporation. The resulting polymer contains small particles of protein that are released slowly when contacted by water. The polymers are placed in a reaction chamber as shown in Fig. 1.

When the sensor is immersed in the sample medium, analyte diffuses through the access ports and mixes with the Texas Red labeled IgG in a reaction chamber, where it encounters the fluorescein labeled antibody. The resulting competition reaction for the limited amount of antibody provides fluorescence emission spectra that change as the proportion of unlabeled to labeled antigen changes.

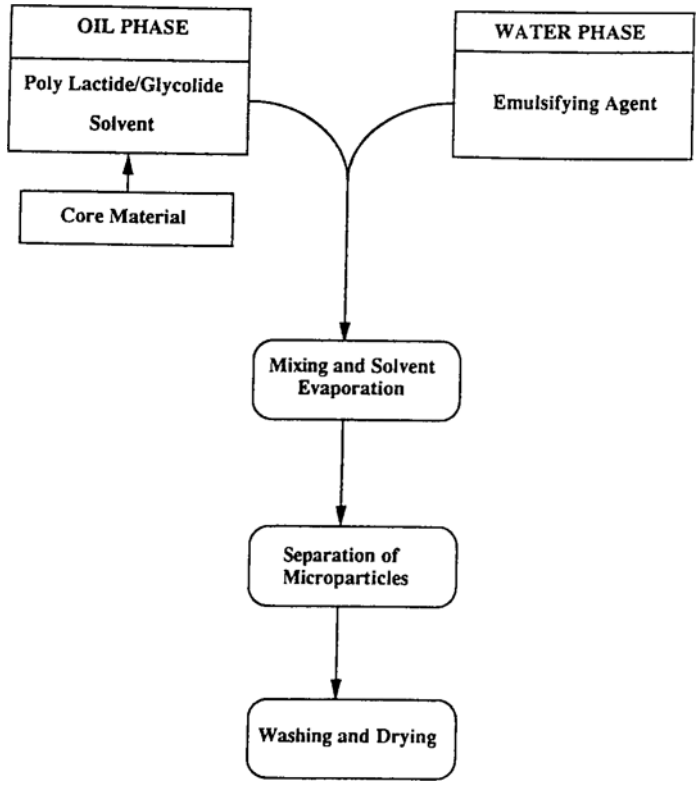

Fig. 2. Protocol for microparticle preparation.

With a minor amount of mathematical manipulation, one can use these optical signals to account not only for changing IgG concentrations, but also for the changing release rates of the fluorescent labeled species. This feature allows us to offset the release rate variability that is inevitable with these types of polymers. The reader is encouraged to consult the references for details of the analysis. Using this approach, the sensor lasted for nearly 30 d, and was able to distinguish between the presence and absence of nanomolar IgG concentrations.

Despite the initial success with this sensor, it exhibited several design flaws. First, excursions in release rate caused the ratiometric measurement to exceed its calibration boundaries and thereby lose its quantitative measurement capabilities. Second, the response time was several hours owing to the macroscopic nature of the sensor tip as well as the long diffusion times required for the macromolecules involved in the immunoassay. Consequently, we have developed a miniaturized version of our sensor system. In this system, we employ microparticles prepared through a solvent evaporation emulsion procedure (*16*) (Fig. 2). A micrograph of the microparticles is shown in Fig. 3.

These microparticles can be prepared in a variety of sizes, depending on the emulsification procedure. For our initial studies using this ap-

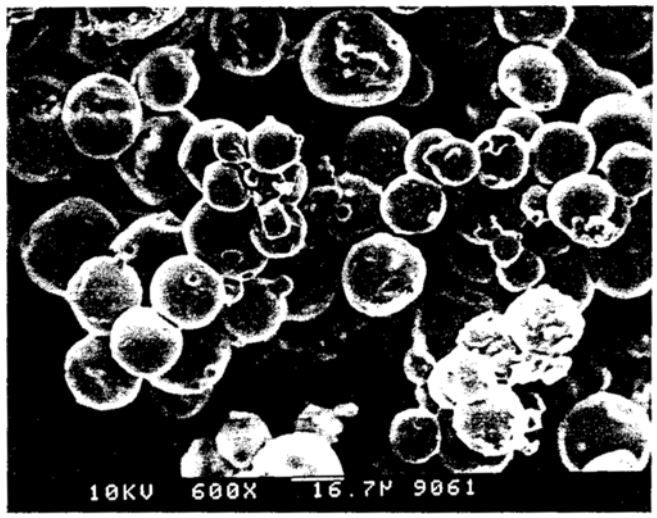

Fig. 3. Scanning electron micrograph of lactide-glycolide microspheres containing fluorescein diacetate.

proach, we incorporated several pH indicators into the microparticles. On exposure to buffer, indicators were released in a sustained fashion over many hundreds of hours. As can be seen in Fig. 4, a sustained release of reagent occurs for nearly 1000 h. This time frame is significantly less than for reagent release using bulk polymer incorporation, however, it provides sufficient longevity for a wide variety of monitoring applications. After microparticle preparation, the sensor is fabricated by suspending the microparticles in a polymerization medium containing an appropriate hydrogel, such as polyacrylamide or polyhydroxyethylmethacrylate (Fig. 5). These hydrogels provide a support matrix for the microparticles as well as a diffusion barrier allowing sufficient reagent to build up after reaction with analyte for a measurement to be made. Because the polymer layer is very thin, the sensor responds in several minutes. Microparticles have been fabricated out of both EVAc copolymer and lactide-glycolide copolymers. The latter operate via surface hydrolysis of their ester bonds on contact with water.

The other key components of sensor performance are the labeled immunoreagents. In our previous work, where fluorescein labeled antiIgG and Texas Red labeled IgG were employed, the amount of energy transfer was restricted owing to the macroscopic nature of the macromolecules. This size limitation prevented very efficient energy transfer from occurring because of the large intramolecular distances between dye molecules after binding. Our recent efforts have been aimed at increasing the efficiency of energy transfer between the labeled antibody-antigen complex. We have found that by using low-molecular-weight antigens, the efficiency of energy transfer can be increased approximately fourfold. This increased efficiency presumably stems from the shorter transfer distances

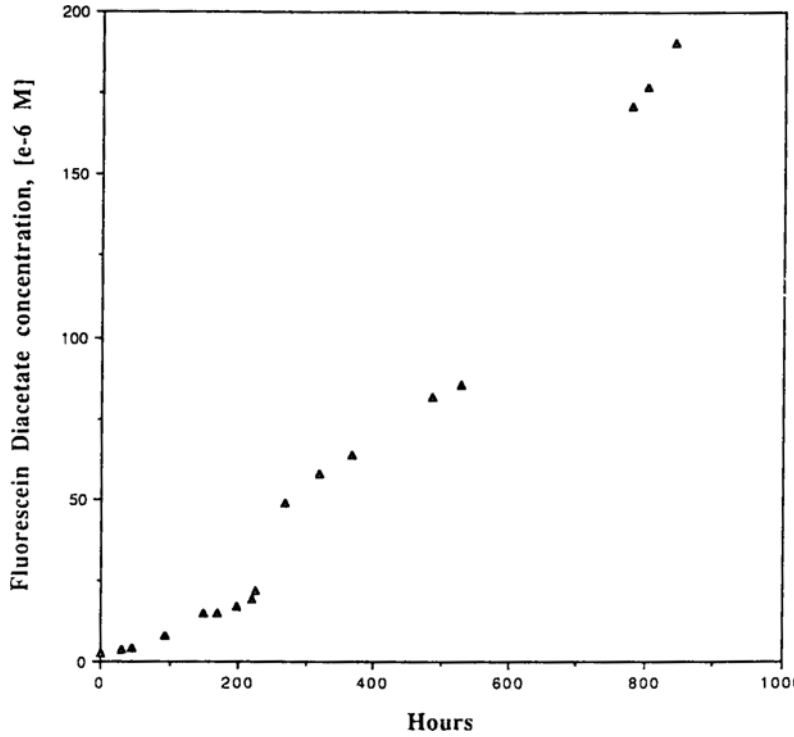

Fig. 4. Release rate of fluorescein diacetate from microparticles is sustained for hundreds of hours.

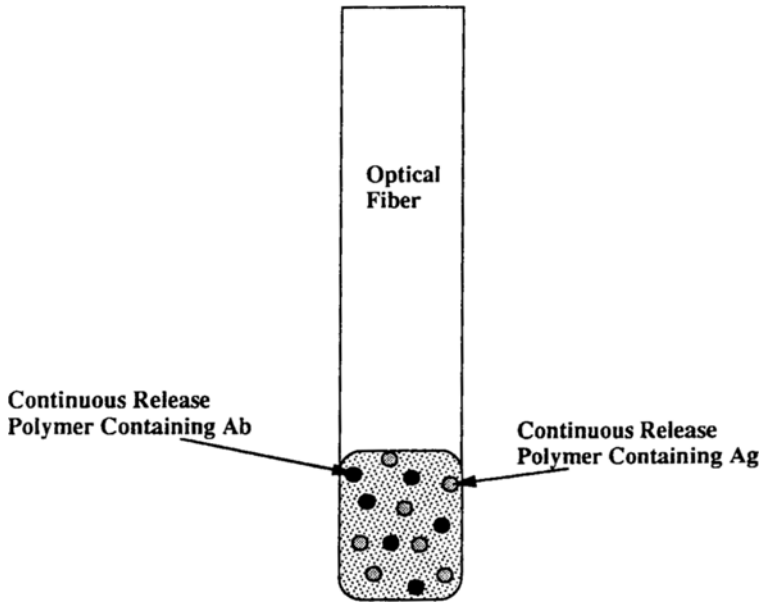

Fig. 5. Configuration of immunosensor based on microparticles containing antibody and antigen.

in these smaller antibody-antigen complexes. This aspect of the work is perhaps the most critical as well as most technically demanding. Conjugation of a dye label to an antigen requires a great deal of synthetic selectivity in order to avoid destroying the binding properties of both the antigen and antibody through the conjugation procedure. Each antibody-antigen pair must be tailored to avoid loss of binding capability.

After labeling, the antibody and antigen will be separately incorporated into microparticles. These microparticles will then be dip-coated and incorporated into a hydrogel as shown in Fig. 5. The hydrogel will then serve as a reaction chamber in which labeled antigen and labeled antibody will be released as solution analyte antigen enters the hydrogel and reacts. The amount of labeled antibody microparticles will be designed to provide limiting antibody-binding sites at all times to facilitate the competition reaction between labeled and unlabeled antigen. In this way, we hope to speed the reaction time and create sensitive quantitative immunosensors.

OPTICAL SENSOR ARRAYS

In a preliminary communication, we have reported the use of imaging fibers containing thousands of individual pixels coupled with a CCD video camera to provide spatial resolution of discrete indicating regions on the distal tip of a fiber (*17*). The imaging fibers are comprised of several thousand individual fibers melted and drawn together to produce a solid, single-fiber element having thousands of individual pixels capable of transmitting images through the fiber. These regions of indicator are deposited selectively by sequential excitation through the fiber with the fiber's tip residing in a photopolymerization solution. Light is introduced at the proximal end of a fiber through a pinhole-sized spot and travels with little dispersion through the fiber. Thus, polymer forms only where light emanates from the fiber. By selectively depositing polymers containing different analyte-sensitive indicators on the fiber tip, one can prepare a sensor array. The key to the successful implementation of this strategy is a precise polymerization scheme, as well as selecting the appropriate indicator chemistries. As shown in Fig. 6, we can microposition and deposit upwards of nine discrete sensing spots on a single 350-μm fiber. This ability contrasts sharply with the conventional "one sensor one fiber" concept.

The ultimate power of this technique will depend on our ability to incorporate a multiplicity of selective indicating chemistries into these polymers. For example, one can imagine employing dyes either singly or in combination, as well as coupled to enzyme layers and antibody-containing polymer layers to provide an array with a wide variety of specific indicating chemistries. This type of sensor can be used for rapid screening in low-volume samples (e.g., blood) or can be used as a field

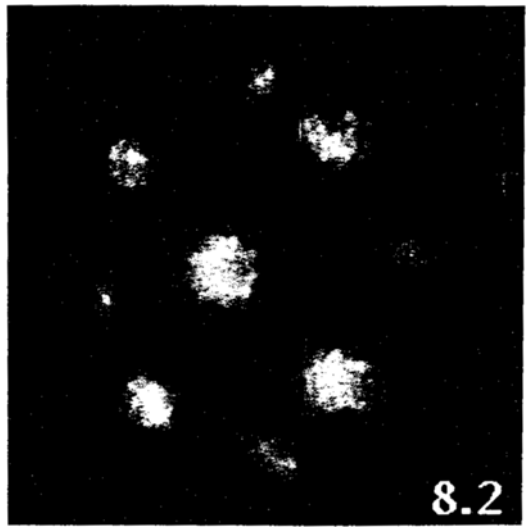

Fig. 6. Fluorescence image of a single 350-μm imaging fiber containing nine pH sensitive cones.

screening technique for environmental samples. Both reversible and irreversible chemistries can be applied to this type of array depending on the desired longevity.

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