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# Applications of Fiber-Optic Evanescent Wave Spectroscopy

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## ABSTRACT

*The evanescent wave (EW) component of light propagated via fiber-optic waveguides can be used to both sense and transmit information regarding the immediate environment of the fiber's surface. In this article, an outline of the theoretical and practical aspects of this emerging methodology is given, as well as a discussion of the advantages, disadvantages, and limitations of the technique. Examples are given of how EW spectroscopy may be used in the analysis of pharmaceutical systems. Evaluation of attributes of components of EW spectroscopy allows prediction of the future for this rapidly evolving area of photonics.*

## INTRODUCTION

Although still in its infancy, the biosensor industry holds great promise for addressing the need for simple, fast, and continuous in situ monitoring techniques. The potential lies in the ability to measure the interaction of analytes with biological systems through a biomolecular recognition capability. Modern optical biosensors have evolved from revolutionary progress in the communications industry (the minuscule optical sources and sensors), information technology (exemplified by microprocessors and optical fibers), and molecular biology (biomolecules that can recognize a target substance). A multitude of optical measuring techniques exist for optical sensing of real-time fluoroimmunoassays by miniaturized instrumentation using new fluorescent labels. Optical sensors utilize fiber-optic circuits in combination with fluorescent indicator molecules, which are essentially an adaptation of benchtop spectroscopy (1). This older technology required extensive, and therefore expensive, optical processing, either by a grating monochromator or by a xenon lamp combined with mechanical filter wheel and photomultipliers, to detect the signal. Advances of the previous decade in photonics, laser, and fiber-optic technology have revolutionized the communications industry and have, for the most part, replaced electrical wires with fiber optics for telephone transmission. One tangible result is the reduced complexity of optical components, the

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benefits of which are reshaping old approaches to chemical analysis.

Biosensors based on piezoelectric crystals, field effect transistors, and thermistors find a role in clinical biochemistry in low-volume testing, patient self-testing, and in vivo monitoring (2). The International Union of Pure and Applied Chemistry (IUPAC) defines *biosensors* as a subgroup of chemical sensors in which a biologically based mechanism is used for analyte detection. One characteristic of biosensors that distinguishes them from other bioanalytical methods, such as immunoassays and enzyme assays, is that the analyte tracers or catalytic products can be directly and instantaneously measured. For antibody-based biosensors, analyte tracers or unlabeled antibodies are directly detected in a single step, whereas for most immunoassays, an enzyme is attached to the analyte of interest, and measurement of the binding of the antibody to the antigen is a multistep process. Another advantage that biosensors have over bioanalytical assays is that they can regenerate and reuse the immobilized biological recognition element. For enzyme-based biosensors, an immobilized enzyme can be used for repeated assays rather than being discarded after each measurement; this feature allows these devices to be used for continuous or multiple assays. For antibody-based biosensors, chemical immobilization of the antibody to the signal transducer can be beneficial. In some cases, after the analyte has been measured (i.e., as a result of the antibody-analyte binding), the analyte can be stripped from the immobilized antibody and another assay can be performed. In other cases, antibody-based biosensors have been shown to respond reversibly to chemical compounds within seconds or minutes. By contrast, immunoassays, including the enzyme-linked immunosorbent assay (ELISA), are typically based on irreversible binding and are thus used only once and discarded.

Fiber-optic fluorimeters are used to monitor the binding of antigen to antibody immobilized near the distal end of a long optical fiber, improving the speed, sensitivity, and utility of immunoassays. Low-loss optical fibers permit remote continuous monitoring at locations separate from the optoelectronic instrumentation. In one scheme, excitation light passes from the source through one leg of a bifurcated fiber bundle to a sensing element comprised of a microcuvette or lumen of a semipermeable membrane. This sensing element contains the fluorescent indicator molecules in a matrix. A portion of the fluorescent light emitted by the indicator is captured within the second leg of the fiber bundle and delivered to a detector. In this scheme, the optical waveguide is used to transmit information about changes in the optical characteristics of the environment of the sensor tip.

An alternate and more innovative scheme employs the evanescent wave (EW) component of a completely internally reflected light beam both to sense and to transmit optical changes on the fiber surface. This review focuses on a specific class of fiber-optical sensing element for evanescent wave spectroscopy (EWS) and its related phenomenon, surface plasmon resonance (SPR) (3). Pertinent optical measurement techniques and instrumentation have been described for EWS (4) only. The interested reader is directed to the more detailed treatment of optical techniques (5), fluorescence spectroscopy (6), and general chemical sensing with fiber optics (7) readily available in the literature. The EWS sensor attributes are discussed and compared to more conventional techniques. Finally, potential applications and the current state of the art are reviewed with an eye toward what may be in store for the future for this rapidly developing area of photonics.

## THEORY OF EVANESCENT WAVE SPECTROSCOPY

Optical biosensors, based on the EW, are analytical devices that measure the interactions between biomolecules in real time without the need for any labels. EWS and SPR are highly promising techniques for optical sensing induced by molecules on the surface of the waveguide (e.g., for measurement of a chemical reaction), which can be in the form of a slab guide, a planar integrated optic, or an optical fiber. EWS, also known as attenuated total reflection (ATR) spectroscopy, is based on light inside the core having an angle of incidence relative to the cladding less than the critical angle; hence, it is totally reflected. Figure 1 shows how light traveling in a waveguide is confined to the core in accordance with the law attributed to Snell. The light waves are propagated along the fiber by total internal reflection (TIR). TIR depends on the angle of incidence and the refractive indices of the media.

$$\sin \alpha / \sin\theta = n_2/n_1 \ (\textit{Snell's Law})$$

When $\theta = \pi/2$ and $\alpha = \alpha_c$, the $\alpha_c = n_2/n_1$, where $\alpha_c$ = critical angle for TIR. Thus, if $\sin \alpha < n_2/n_1$, we have refraction and reflection. When the angle of reflection $\theta$ is such that $\sin \theta_c = n_2/ n_1$ and $n_1 > n_2$, infrared (TIR) predominates.

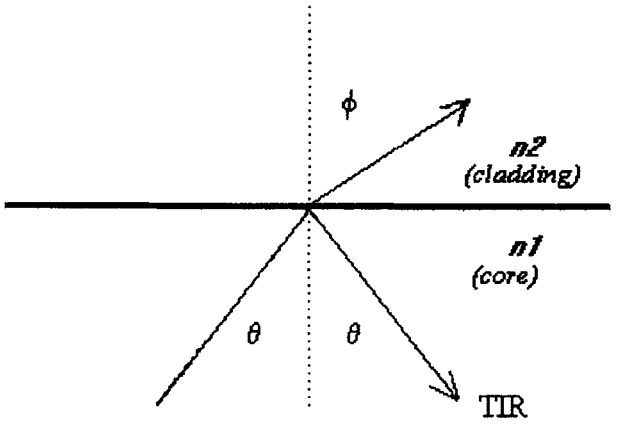


**Figure 1.** Principle of total internal reflection.

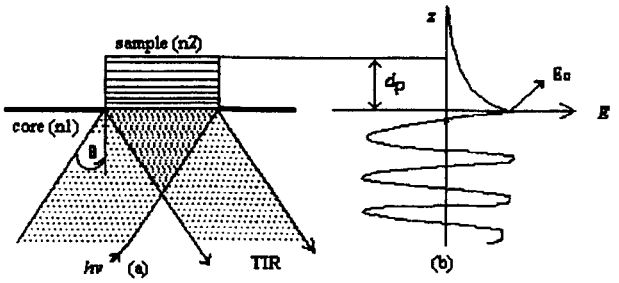


**Figure 2.** Principle of evanescent wave spectroscopy: (a) light guided inside the waveguide measures analyte properties on the surface by the evanescent wave; (b) the electric field intensity of the EW falls off exponentially outside the waveguide. (From Ref. 4.)

Actually, a small portion of the light enters the cladding (or surrounding medium) far enough (less than a wavelength) to recognize the different refractive index. An EW sensor exploits the fact that some of the energy passing through an optical fiber penetrates the light-guiding cladding. When totally internally reflected in a transparent solid at its interface liquid, the excitation light beam penetrates only a short distance into the liquid. This surface electromagnetic field, called the *evanescent wave*, can selectively excite fluorescent molecules in the liquid near the interface. The operating principle behind either EWS or SPR is derived from this small portion of internally reflected light energy associated with the guided modes that penetrate into the surrounding lower refractive index medium. Such information may be observed by determining the degree of light scattering induced by analyte on the surface of the probe by absorption spectroscopy or by fluorescent changes. Figure 2 shows how the light trapped within the guide can be used to sense chemical changes in the environment immediately surrounding the probe.

If $\theta > \theta_c$, there is an EW refracted through the interface in the $Z$ direction that penetrates the $n_2$ medium a distance $d_p$, which is of the order of a wavelength. The electric field amplitude $E$ decays with distance $z$ into the rarer medium:

$$E = E_0 \exp^{(-z/d_p)}$$

where $E_0$ is the electric field at the surface of the guide. The depth of penetration $d_p$ is defined as the distance for the electric field amplitude to fall to $1/e$ of this value at the surface.

The depth of penetration $d_p$ can be related to other factors by the equation (8)

$$d_p = \frac{\lambda/n_1}{2\pi\,[\sin^2\theta - (n_2/n_1)^2]^{1/2}}$$

where $n_1$ and $n_2$ are the indexes of the guide and the surrounding medium, respectively, and $\theta$ is the angle of propagation in the guide. One can see from Eq. 3 that, qualitatively, $d_p$ decreases with increasing $\theta$ and increases as $n_2/n_1$ tends to unity. One can thus select a value of $d_p$ by an appropriate choice of values of $\theta$, $n_1$, and $\lambda$. For example, with a quartz waveguide, $n_1 = 1.46$ and, if the sample is in water, $n_2 = 1.34$. This gives a value for $\theta_c$ of 66°. If $\theta$ is set to 70° and $\lambda$ to 500 nm, the value of $d_p$ is 270 nm, which will easily contain a monolayer of an immunological component with a diameter of about 25 nm. Penetration depths are typically 50–1000 nm for visible light ($d_p < \lambda$), which implies that the EW interacts with many monolayers at the surface of the probe (9).

However, $d_p$ is just one of the four factors that determine the attenuation of reflection. The others are the polarization-dependent electric field intensity of the reflecting surface, the sampling area, and the matching of the two refractive indices $n_1$ and $n_2$. An effective thickness $d_e$ takes account of all these factors. It represents the thickness of film required to produce the same absorption in a transmission experiment. In order to enhance the sensitivity, multiple reflection may be used. If the number of reflections $N$ is a function of length $L$ and thickness $T$ of the waveguide and the angle of incidence $\theta$ so that

$$N = (L/T)\cot\theta$$

then the longer and thinner the waveguide, the larger is $N$ and the more frequently the EW interacts with the surface layer of analyte. If $R$ is the reflectivity,

$$R = 1 - \alpha d_e$$

where $\alpha$ is the absorption coefficient. Then, for $N$ reflections,

$$R^N = 1 - N\alpha d_e$$

The wave in a single-mode fiber is carried coherently by constructive interference; thus, a continuous evanescent field is present at the interface. An optimum geometry exists following removal of the cladding that permits the evanescent field present at the interface to be available at the point of reflection. Lew and Depeursinge suggested, in a theoretical evaluation, that the sensitivity of such a device depends on the path length of the exposed core and the amount of the total field present at the interface (10). The configuration of the probe tip was shown to be a critical limiting factor in signal acquisition (11). Probes with a radius unaltered from that of the original core inefficiently returned the signal produced on binding the fluorescent-labeled antigen. Conditions that conserve excitation power, such that power in the EW is optimized, must be met to obtain a maximal signal. The threshold sensitivity for the optimal step-etched fiber probe was improved by over 20-fold compared with a sensor region of uniform radius. Tapering the radius from the fiber core radius (100 μm) to an end radius of 29 μm was found to produce a maximal signal (12).

Early reviews (13,14) of TIR described its use to examine the cell/substrate contact regions and to measure the chemical kinetic binding rates and surface diffusion constants. An EW optrode normally substitutes the sample for the cladding and uses the optical evanescent field of guided modes in a fiber (or planar waveguide) to probe the vicinity of the interface between the decladded core and the environment (15). Antibodies or other biomolecules attached to the surface of a decladded fiber can bind the compound to be sensed, which then absorbs part of the EW and thereby produces a signal. The waveguide may be coated with a reagent or fluorescent indicator, depending on the type of sensor. This is a preferred configuration as fluorescent sensors are generally more selective than absorption-based sensors because the sensing is performed at a different wavelength from the excitation, and it is unlikely that other fluorescent species will be present at the same absorption/emission wavelengths. In practice, a large Stoke's shift is desirable to allow separation of the excitation from the fluorescence at the sensor output.

A fluorescent indicator attached as a marker to detect an antigen/antibody reaction may be used to increase the selectivity of the probe. Fluorescence created in solution radiates isotropically, but fluorescence from molecules close to the surface is preferentially coupled into the waveguide by tunneling (16), effectively boosts fluorescent intensity by a factor of 5 to 10, and simultaneously filters the analyte signal from the background fluorescence and other sources of stray light in the surrounding environment.

Initial work combined EWS with immunometric procedures for monitoring of antibody-antigen reactions at a solid-liquid interface (17). If analyte changes the optical properties of an absorbance- or fluorescence-based indicator, these changes can be measured by the evanescent field. The light absorbed can be analyzed by evanescence spectroscopy, or the evanescent field can provide a means for coupling florescent light into the fiber. Figure 3 shows a typical component setup for a simple fiber-optic sensor based on intensity measurement for oxygen determination with a sol-gel coating on the optical fiber (18). Light from a bulk optics source or a solid-state source (e.g., laser diode or light-emitting diode [LED]) is launched into a fiber with suitable optics that collimate, focus, and, optionally, also selectively filter, modulate, or polarize the light beam. In this case, the sample forms the cladding. Light transmitted to the sensor tip by TIR interacts with the environment, and then it is directed back to a

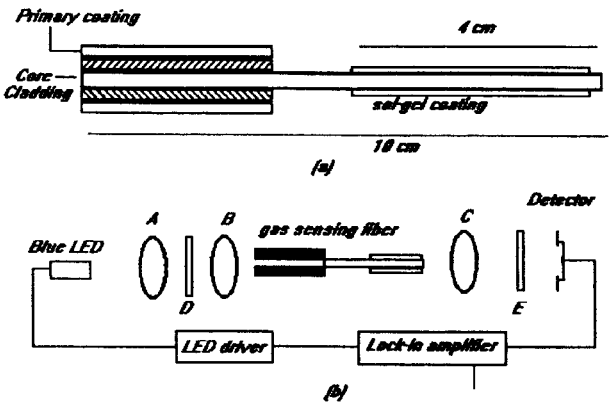


**Figure 3.** Evanescent wave sensor with an optical fiber for oxygen determination: (a) sensing fiber; (b) instrumentation. Light from a blue light-emitting diode (LED) is injected by lenses (A, B) and a short-wavelength pass filter (D) into the fiber. Fluorescent light from the fiber is collected by a lens (C) and passed through a long-wavelength pass filter (E) at the top of the detector. (From Ref. 15.)

suitable detector (e.g., pin photodiode, charge-coupled device array, or photomultiplier tube) for readout. Noise reduction and minimization of ambient illumination is effected by additional coupling elements that divide the light to provide a reference signal, employing a pulsed light source, or signal condition and detection in a phase-sensitive manner (i.e., lock-in amplifier). Unlike electrochemical transducers, a reference sensor is optional. Instead, the ratio of the signal can be determined at an analytical and reference wavelength to eliminate scatter and source fluctuations.

Ideally, the excitation wavelength should be in the visible or near-IR (NIR) region of the spectrum because LED/laser sources are inexpensive and readily available for these regions. Specific ligands are immobilized to a sensor surface, and a solution of receptor or antibody is injected over the top. Binding is measured by recording changes in the refractive index caused by the molecules interacting near the sensor surface within the evanescent field. The many possible combinations of biological recognition elements and transducers targeted to specific compounds render the detection of small molecules by EW fiber-optic technology promising. The optical signal can be processed by alternative forms of modulation (wavelength, amplitude, time, phase, or polarization). Choice of light sources range from simple lamps, LEDs, and gas, dye, or solid-state lasers.

Surface plasmon resonance occurs at a dielectric-metal layer interface when light that is totally reflected with an underlying dielectric induces a collective oscillation in the free-electron plasma at the metal film boundary. Energy carried by photons of light can be ''coupled'' or transferred to electrons in a metal. The wavelength of light at which coupling (i.e., energy transfer) occurs is characteristic of the particular metal and the environment of the metal surface that is illuminated. The conditions for this to occur are that the momentum of the photons in the plane of the film should match that of the surface plasmons on the opposite surface of the metal film (19). This occurs at a defined critical incident angle of the light. When there is a match or resonance between the energy of the light photons and the electrons at the metal surface, a transfer of energy occurs. The resulting effect is to produce a large change in the reflection coefficient at this resonance angle.

A typical sensor employs a silver film of thickness of 40 to 50 nm, which is evaporated onto a glass plate or prism. The sample is placed on the silver layer. The intensity of the electric field of the EW within the sample layer is enhanced by two orders of magnitude compared with the interface without the metal film. The use of an SPR optical biosensor requires that one of the biomolecules be immobilized to the sensor surface; when another molecule binds, it results in a change in the angle at which the EW is generated. The sample properties shift the resonance angle, providing a highly sensitive means of monitoring small changes due to the surface reaction.

The development of ''direct'' sensors, such as those using SPR, has been hampered by problems of nonspecific binding and poor sensitivity to small molecules. ''Indirect'' sensors (for example, those employing a fluorescent-labeled reagent) overcome many of the problems of direct sensors, but require more-sophisticated instrumentation because of the low light levels detected. Enhanced sensitivity over conventional total internal reflection fluorescence (TIRF) and SPR techniques was achieved by a combination of the best features of the two techniques: the surface field intensity enhancement boosted the emission from a fluorescent-labeled immunoassay complex at a metal surface for assaying for human chorionic gonadotrophin in serum (20).

## ATTRIBUTES OF EVANESCENT WAVE SPECTROSCOPY COMPONENTS

A fluorescence-based EW biosensor combines the selectivity of molecular recognition of bioreceptors (e.g., antibody, enzyme, nucleic acid probes) and the exceptional sensitivity of spectrochemical detection technologies. The simplicity, selectivity, and sensitivity of the method make the use of fluorescent techniques particularly attractive. In most applications, fluorogenic labeling is used to increase the sensitivity of other methods and to determine protein characteristics due to environmental changes of the sample matrix. In fluorogenic labeling, a fluorescent molecule is normally coupled either covalently or noncovalently to a nonfluorescent molecule, and the fluorescence emitted is detected (21).

The practical applicability of any fluorescent labeling method depends both on its intrinsic sensitivity (primarily determined by the quantum yield of the label) and on the presence of interfering fluorophores. Detection sensitivity has been improved recently by high excitation intensities common to laser light sources. Fluorescent labeling may be the best approach even if the analyte itself is fluorescent, but has excitation and emission maxima that are in a spectral region for which detection is difficult. The major requirements for fluorescence labels are high quantum yield, large Stokes shift, excitation maxima accessible to inexpensive light sources, chemical and photochemical stability, low susceptibility to fluores-

cence quenchers (22), and reactive functional groups in the label molecule (23).

Fluorescence measurement involves the determination of light intensity above a zero background, and minimization of background fluorescence is an important goal. Sometimes, the wavelength of the emitted photon is in a region in which reabsorption by the matrix molecules is likely (short-wavelength ultraviolet [UV]) or the matrix itself is actively fluorescent in this region (long-wavelength UV and visible region). In those cases when the analyte molecule of interest fluoresces only in the spectral regions of high interference or is not fluorescent at all, we can introduce fluorescence tracer molecules that will absorb and fluoresce in the region removed from that of the sample matrix so that most of the interference can be eliminated. A multitude of labels are available for use in the visible spectral region that have most of the desired characteristics. However, their relatively short wave absorption and fluorescence maxima ensure that the background fluorescence of the sample matrix is substantial. Significant numbers of dyes are available commercially for use as labels; however, the absorption maxima for most of the labels are below 600 nm (24), which coincides with the spectral region in which many biomolecules autofluoresce. Therefore, the detection of the label is usually limited by the background fluorescence. This has prompted research to find fluorogenic labels that do not have this disadvantage.

Davidson and Hilchenbach (25) summarized the use of fluorescent probes in immunochemistry, the problems associated with fluorometric analysis, and the reactions used to conjugate dye to proteins. Perhaps the most widely used commercial labels are the highly reactive isocyanate and isothiocyanate fluorescein derivatives. The isothiocyanate group of the dye reacts with primary amine or hydroxyl groups of the analyte to form a stable, covalent linkage with the analyte. Although fluorescein has a high quantum yield, its absorption and emission maxima are in the spectral region for which interference is likely, although less so than in the UV or the short-wavelength visible region. Fluorescein isothiocyanate is sensitive to pH, conjugates with gamma-amino groups of lysine, and possesses a quantum yield that is much reduced on conjugation. It possesses only a small Stokes shift and undergoes photofading on irradiation.

Rhodamine isothiocyanate gives a conjugate that is far more stable than fluorescein. Its use has been limited by its emission spectrum, which lies in a region for which the human eye is not particularly sensitive. Lucifer yellow (LY) conjugates very efficiently under mild conditions. Unfortunately, the Stokes shift is not particularly large, and transmission properties of the dichroic filters in fluorescence microscopes, which are attuned to fluorescein, are not ideal for LY. Dansyl chloride becomes highly fluorescent on conjugation, but its excitation wavelength (320 nm) also is increased by the level of background autofluorescence. Phycoerythrins are unconjugated molecules that show little fluorescence, high extinction coefficients, and high quantum yields. They are normally used as streptavidin conjugates and are linked to the desired protein via biotinylation of the protein, which produces a very large protein-biotin-streptavidin and phycoerythrin complex that is useful in dual-labeling methodologies. An optical-fiber biosensor, based on a ruthenium(II) complex with a luminescence that is sensitive to oxygen changes, for free cholesterol monitoring in serum samples was used as an optical transducer of the oxidation of cholesterol by cholesterol oxidase (26). Ruthenium complexes have also been employed in a miniaturized, rapid (2 sec) glucose sensor at least 25 times faster and with an absolute sensitivity 5–6 orders of magnitude higher than that of current glucose optodes (27).

The far-visible and the NIR spectral region (600–1000 nm) are areas of low interference in which only a few classes of molecules exhibit significant fluorescence (28). Fluorophores with longer absorption and emission wavelengths, especially NIR chromophores, are superior with regard to interference because only a few families of compounds are able to absorb and fluoresce in this spectral region. Background light absorption in this spectral region is not negligible in most cases because of overtone vibrational bands of solvents containing hydrogen atoms. Although overtones of different vibrational bands (such as hydroxyl) are present, they do not contribute to the fluorescence signal. Careful choice of the excitation and emission wavelength and appropriate interference filters permit the selective detection of the fluorescent probe's emission wavelength. These features of the NIR spectral region make it ideal for using fluorescence labels.

## ADVANTAGES AND DISADVANTAGES OF THE TECHNIQUE

In spite of their numerous advantages, NIR fluorogenic labels are still on the "ground floor" of their development. Until recently, there were no commercially available isothiocyanate-derivatized cyanine dyes for the NIR spectrum (29). Using NIR dyes as covalently bound fluorescence labels widens their applicability to biomolecules. In theory, the binding of these dyes to proteins can be achieved in a straightforward synthetic chemistry,

and specific dye-to-molecule ratios can be obtained. The NIR fluorophore label can be excited using several types of laser devices, further improving the sensitivity of their detection. For the covalent labeling of biomolecules and analytes containing primary amino functional groups, isothiocyanate derivatives for fluorophores are most suitable labels because they form stable thioureas. Amino derivatives, which are precursors to isothiocyanate-substituted dyes, can also be used to conjugate the fluorogenic label to thiol groups on proteins. A classic reactant that uses this chemistry is *N*-succinimidyl-3-(2-pyridyldithio)propionate, which has been widely used in immunochemistry (30). Using appropriate synthetic steps, however, one can introduce various functional groups (31). The synthesis of labels containing the aryl N-chlorosuccinimide moiety (the "sticky" portion of the molecule, i.e., point of attachment/conjugation) is rather straightforward because the method has successfully been applied to the synthesis of visible fluorescein and rhodamine isothiocyanate fluorogenic labels.

There are shortcomings and potential pitfalls associated with the application of NIR fluorogenic labels. Fluorescence in the NIR region requires extensive conjugation in the molecule, which increases instability and photobleaching and shortens the shelf life of the label. The labels are necessarily large, hydrophobic molecules, often with molecular weights of more than 1000. Binding of NIR fluorogenic labels to large molecules (e.g., albumin or globulins) can induce pronounced spectral changes (32). The lack of commercial availability of NIR fluorogenic labels has led several research groups to employ noncovalently bound labels such as the dye indocyanine green (IG), which is approved by the Food and Drug Administration (FDA) (33–36). Polymethine cyanine dyes absorb and fluoresce in the NIR region (600–1200 nm) and have large molar absorptivities and good fluorescent properties. A major drawback of noncovalent polymethine cyanine labels has been damage to size-exclusion columns caused by free unassociated dye (37). Semiconductor laser absorbance and semiconductor laser fluorimetry have been used to detect polymethine dyes at the ultratrace level (38).

Until recently, the NIR spectral region has been less accessible to inexpensive instrumentation. The sensitivity of the most widely used detector, the photomultiplier tube, significantly decreases in this region. This situation has changed rapidly since the introduction of semiconductor-based detectors (e.g., photodiodes and the recently available NIR semiconductor lasers). Many important advances have been made in the past few years, primarily as the result of improvements in instrumental techniques, such as the proliferation of photodiode and semiconductor laser diode excitation sources. Laser diodes in the wavelength range of 660–680 nm are commercially available (39). Unfortunately, diode laser emission wavelengths are restricted to the NIR region, at which many compounds do not absorb and most cannot be induced to fluoresce.

Progress in fiber-optic photonics is very much a function of component technology. In this section, limitations and alternative strategies are considered. Many biosensors are ready to progress from the research bench to the field study stage (40). However, as evidenced by the paucity of commercially available biosensors, overcoming the technical, regulatory, and market obstacles is not a trivial matter. These devices must compete with other, fairly well established field analytical methods such as chemical sensors, immunoassays, and chemical test kits. In addition to the expected technical challenges (producing and packaging a durable device for field use, developing training programs, establishing regulatory acceptance procedures, adhering to quality assurance protocols), biosensors must offer new capabilities or significant improvements over existing methods to be successful in an increasingly competitive marketplace.

Properties of fiber-optic biosensors can be controlled by shrouding the tip of the sensor in a membrane that permits the analyte to pass while restricting enzyme activity to the probe's surface. Polymers used as membranes have been reviewed in the sensor literature (41,42). The particular properties of the membranes of relevance for this application are the ease of refractive index modulation and the potential of the hydrogel to act as a permselective barrier in which a colorimetric agent may be immobilized.

The analytical features of this type of biosensor depend on enzyme loading, thickness of the various reagent layers, and intrinsic chemical and physical properties of the reagents involved. As for all biosensors, enzyme loading controls sensitivity, stability, and, to some extent, selectivity. The goal is to load sufficient enzyme at the sensing tip so that the rate-limiting step is molecular diffusion and not reaction kinetics (43). In doing so, the maximum sensitivity is maintained with longer operational lifetimes and less sensitivity to components of the sample matrix, which can either inhibit or inactivate the enzymatic activity. Thicknesses of the enzyme and dye layers are other critical parameters that affect response times and sensitivity. Thicker layers require more time for the steady-state condition to be established, which lengthens response times. In addition, the density of the enzyme layer will affect sensor response times be-

cause the reaction products must diffuse through this region before entering the chemical mediation layer for detection. Slower mass transport through the enzyme layer lengthens the time for material to reach the detection layer, thereby increasing response times. Thicker layers should increase sensitivity. Ultimately, sensor selectivity is governed by the selectivity of the biocatalyzed reaction and the extent to which the fluorescence measurement is influenced by components in the sample. Certainly, other substrates of the enzyme would constitute positive interferences, and other quenching agents would constitute negative interferences. Finally, sensor lifetime is controlled by the stabilities of the enzyme and the dye. Photodegradation and thermal degradation pathways must be considered for these key reagents.

A disadvantage of an antibody-based biosensor is the long equilibration time (generally 30 min, even with small quantities of antibody). Another practical disadvantage is the inability of the sensor to respond reversibly, which renders sensor calibration extremely difficult. A number of approaches to the problem of maintaining sensitivity of the biosensor are under consideration. One is to place two biosensors into the system, with one of the biosensors in an off-line regeneration mode and the other switched to the on-line sensing mode (44). A more elegant approach is to employ pulsed DC currents across the active surface of the biosensor. Such pulses would be designed to accelerate the dissociation of the antigen: antibody complex and thus maintain the ability of the biosensor to report the concentration of antigen promptly (45). A rapid (3 sec) automated optical biosensor system based on fluorescence excitation and detection in the evanescent field of a quartz fiber was used to detect oligonucleotides. Thermal regeneration allowed hundreds of assay cycles to be performed with the same fiber with a signal variation of only 2.4% (46). Another oligonucleotide sensor has been observed to be regenerable (minimum of five cycles) and to sustain full activity after prolonged storage times (1 year), harsh washing conditions (sonication), and sterilization (autoclaving) (47).

The problem associated with fiber-to-fiber variability in measured signals has been addressed by labeling a portion of the immobilized capture antibody with the fluorescent cyanine dye Cy5.5 (emission lambda maximum = 696 nm) (48). The antigen was then labeled with fluorescent Cy5 (emission lambda maximum = 668 nm). Both fluorophores were excited by 635-nm light, and their emission was collected using both a fiber-optic spectrometer and a biosensor optimized to collect fluorescence at two wavelengths. The fluorescence from the Cy5.5-labeled capture antibody served as a calibration signal for each fiber and corrected for differences in optics, fiber defects, and varying amounts of capture antibody present on the fiber. Normalizing the signal measured from Cy5-labeled antigen binding to the Cy5.5 signal provides a standardization process for greatly reducing signal variance among individual fibers.

At present, the spectroscopic application of fiber optics is in its infancy; this is reflected in a wide choice of fibers, none of which possesses ideal properties for biosensor applications. The silicone fibers are mechanically stable and have a high throughput. The simplest and cheapest is the plastic-clad-silica (PCS) multimode fiber. The plastic cladding can be removed easily over a suitable fiber length, exposing the core and allowing access to the evanescent field. The exposed region, typically several centimeters in length, is usually coated with a polymer of sol-gel film containing the indicator dye. However, the disadvantage of the multimode fiber is that the sensitivity is dependent on the mode distribution and hence on launching conditions and external disturbances.

Waveguides for different wavelengths of light are made from different materials: $> 450$ nm, plastic (polyacrylamides); $> 350$ nm, glass; $< 350$ nm, fused silica; $> 1000$ nm, germanium crystal. The quality of NIR fibers has improved markedly over the last 7 years, but this is more a result of the demands of the communications industry rather than the need for improved biosensors. Moreover, the NIR region offers an excellent "fingerprint" region for identification (49). The bands are weaker than those in the mid-infrared (MIR) fingerprint region, but similar identification characteristics are retained. The major attraction of step index multimode fiber is the ease with which they can collect light. Their core diameters start at 100 μm, and some all-plastic cores have cores larger than a millimeter. Typical numerical apertures (NAs) are 0.2–0.4 for silica fibers and 0.4–0.75 for plastic fibers. This combination of large core and large NA allows the use of inexpensive large-area light sources and avoids the need for extremely precise connectors. Indeed, tolerances are so large for 1-mm core all-plastic fibers that signals can be transmitted even when the fibers are partly out of the connector. However, these advantages come at the cost of limited transmission bandwidth and higher losses—much higher for plastic fibers.

The main use of multimode step-index fibers has been in short data links at low speeds as they accept light from inexpensive LEDs and allow the use of inexpensive connectors with large tolerances. The larger cores offer larger collecting areas and permit the fibers to handle higher laser powers, but make the fibers stiffer and more costly. Fibers made entirely of polymethylmetha-

crylate surrounded by lower index materials, such as fluorine-containing polymers, lead to large core-cladding index differences and a full acceptance angle of 60°. All-plastic fibers have some important attractions (low cost, better flexibility, and ease of handling), and they have been used for many years in applications such as light outage indicators in cars and many types of fiber-optic bundles. Flexibility and low cost are important in large-core fibers because silica types tend to be stiff and expensive. All-plastic fibers have much higher attenuation and less resistance to high temperatures than glass types. Losses are lower at visible wavelengths than in the NIR region, in which glass fibers have the lowest attenuation. Low glass transition temperatures limit most plastics to temperatures below 125°C. Most uses of plastic are likely to remain in light piping and image transmission through bundles, for which the goal is to carry light no more than a few feet, and bandwidth (in the communications sense) is meaningless.

The availability of excellent light sources is a spinoff of the relentless quest on the part of laser disk manufacturers to maximize recording density of optical storage. This has pushed the development of semiconductor lasers that oscillate at shorter wavelengths to permit tighter focus of the laser beam on a smaller area. Since the need to compress more information in less space is not likely to diminish, inexpensive laser diodes in the red, or even blue, region of the visible spectrum are to be expected. Along these lines, the advent of the fiber laser has demanded the greatest change in our perception of the laser since the invention of semiconductor devices. Fiber lasers are compact, will permit further miniaturization and cost savings, and, in some cases, may be spliced directly onto conventional optical fibers with minimum insertion loss. When such devices become commercially available, conventional immunofluorescent dyes with greater stability (such as rhodamine isothiocyanate) will become practical. Commercially available solid-state light sources will unleash a torrent of progress in semiconductor laser fluorometry.

## EXAMPLES OF THE USE OF EVANESCENT WAVE SPECTROSCOPY

New methods being developed show promise for continuous, in situ monitoring of compounds in various biological matrices by EWS (50,51). The analytical community continues to search for portable analytical techniques that can give reliable, on-site results for a variety of matrices and a host of analytes. Biosensors are beginning to move from the proof-of-concept stage to commercialization. Biosensors can operate in harsh temperature and pressure extremes, environments of extreme electromagnetic interference, and volatile conditions of high concentrations of organic solvents (e.g., methanol and acetonitrile) and can be used for in situ monitoring of process streams. They can be constructed from a wide array of immunochemicals and even genetically engineered microorganisms, and they can be configured to be reversible.

The first biosensors were reported in the early 1960s and comprised enzymes immobilized to oxygen electrodes (52). Continued development of this kind of biosensor led to the commercialization of various devices for such applications as the measurement of glucose in blood and the detection of glutamate, aspartame, sulfite, lactose, and ethanol in food products. Advances in biochemistry, molecular biology, and immunochemistry have expanded the range of biological recognition elements, and developments in fiber optics and microelectronics have expanded the capabilities of signal transducers. Evanescent-wave-based biosensors are being used to study an increasing number of applications in the life sciences, including the binding and dissociation kinetics of antibodies and receptor-ligand pairs, protein-DNA and DNA-DNA interactions, epitope mapping, phage display libraries, and whole cell- and virus-protein interactions (53). The durability, sensitivity, and low cost of signal transducers and the growing availability of enzymes, antibodies, and genetically engineered microorganisms have contributed to the recent interest in applying biosensors to the pharmaceutical sciences.

A decided advantage of EWS is the sensor's ability to measure molecules localized on or near the outer surface of the fiber, even in the presence of turbid or highly absorbing solutions. Like other solid-phase immunoassays, the antigen-specific antibodies bound to the fiber tend to concentrate the analyte, making a stronger signal. Novel methods for dissolution testing that circumvent manual or flow-through sampling have been applied to tablets; these methods involve spectrophotometric measurements by means of fiber optics, with the measurements made in turbid solution without previous filtering of the sample. Recently developed biosensor technology allows near-real-time in situ measurement of gas tension ($pCO_2$ and $pO_2$) and of pH on arterial blood, cerebrospinal fluid, and brain parenchyma. Comparison of simultaneous biosensor measurements and discrete arterial blood sampling for traditional blood gas analyses indicated a high level of correlation for $pCO_2$, $pO_2$, and pH (54). The advantage of the EW is that it has a limited penetration

depth, which restricts detection of the fluorescent complexes bound to the fiber's surface. Since measurements occur at the fiber's surface in the EW, the sensor only detects bound fluorophores.

Applications of biosensors range in their development stages from proof of concept to commercial availability. There are currently four commercially available EW biosensors on the market and two commercially available instruments, the IAsys (from Affinity Sensors) and the BIAcore (from Pharmacia Biosensor), that use an EW detection system to provide a relatively facile way to assess the affinity of one biomolecule for another. Because biosensors are relatively small, they can be used separately or as modular detectors in larger systems. The Ocean Optic miniature fiber-optic spectrophotometer is a low-cost, high-sensitivity spectrometer configured especially for measuring fluorescence via its fiber-optic oxygen sensor (55). For example, these devices can be used in flow injection analysis formats, as detectors for liquid chromatographic systems, or as stand-alone sensors at the end of an optical fiber.

Many biosensors are on the brink of commercialization, such as the U.S. Navy's continuous-flow immunosensor, which is expected to be licensed later this year. The Naval Research Laboratory has developed a fiber-optic biosensor and a continuous-flow immunosensor that can be used to measure explosives in discrete samples or to monitor process streams (56,57). The fiber-optic system is based on a competitive immunoassay performed on the fiber core of a long optical fiber. The flow system is a displacement immunoassay with response measured by changes in the fluorescent signal in several minutes. Immunosensors such as these combine the advantages of conventional immunoassay methods with the option of obtaining real-time monitoring measurements with data integration capabilities.

Biosensors are currently available for rapidly monitoring biochemical oxygen demand (BOD) and are in use at water treatment facilities in Europe and Japan (58). The short response time and high sensitivity of these microorganism-based sensors make them desirable for water monitoring. Japanese studies indicate that a biosensor using immobilized *Trichosporon cutaneum* in combination with a dissolved oxygen electrode can be used to measure BOD values in industrial wastewater in as little as 15 min (traditional BOD measurements take 5 days).

Conceivably, multiple tests can be miniaturized on the tip of a evanescent waveguide. An optical penicillin biosensor was fabricated by selective photodeposition of penicillin-sensitive matrices immobilized as micrometer-size particles in a polymer hydrogel with a covalently bound pH indicator (59). This array of penicillin-sensitive and pH-sensitive matrices allows for the simultaneous, independent measurement of pH and penicillin concentration produced during a *Penicillium chrysogenum* fermentation. Thus, the foreseeable applications to fulfill the demands for real-time analyses of biotechnology and pharmaceutical processes are readily apparent.

Fiber-optic evanescent fluoroimmunosensors have been developed to detect and quantitate cocaine rapidly (60,61). Monoclonal antibody (mAb) immobilized covalently on quartz fibers has been used as the biological sensing element in the portable fluorometer. Comparison of different methodologies showed that biosensor values for coca alkaloid content were not significantly different from gas chromatography values. The biosensor assay was rapid, did not require cleanup of the crude leaf extracts, and fibers (mAb coated) stored at 37°C gave stable responses for 14 days (62).

A new technique for viewing a sample and measuring surface chemical concentrations is based on the deposition of a thin, analyte-sensitive polymer layer on the distal surface of a 350-μm-diameter imaging fiber. The pH sensor array and an acetylcholine biosensor array contain approximately 6000 optical sensors and can display visual information about a remote sample with 4-μm spatial resolution, allowing alternating acquisition of chemical analysis and visual histology (63).

The accuracy and rapid response time of EW sensors show great potential for development as a clinical diagnostic tool. Fibrin degradation products present in patients with sepsis and thrombotic disorders were assayed by a sandwich fluoroimmunoassay immobilized in the sensing region of an EW biosensor. Physiological concentrations could be determined in plasma samples on calibrated fibers in 11 min, and the concentrations determined by fiber-optic assay were strongly correlated with those determined by ELISA (64). Evanescent wave technology is rapidly developing as an alternative, in-process method for detecting microbial contaminates. Lipopolysaccharide (LPS) endotoxin, the causative agent in the clinical syndrome known as sepsis, is responsible for more than 100,000 deaths annually, in large part due to the lack of a rapid, reliable, and sensitive diagnostic technique. An LPS assay using the fiber-optic biosensor applicable to both clinical and environmental testing was recently demonstrated (65). The detection of LPS from *Escherichia coli* at very low concentrations in 30 sec was performed using an EW fiber-optic biosensor with polymyxin B covalently immobilized onto the surface of the fiber-optic probe to selectively bind fluorescently labeled LPS. A portable fiber-optic biosensor for easy monitoring

of clinical samples or on-site analysis of suspect food samples for staphylococcal enterotoxin B (a causative agent of food poisoning) quantitates toxin in human serum, urine, and aqueous extract of ham (66). A fiber-optic biosensor was used to detect the fraction 1 antigen from *Yersinia pestis*, the etiologic agent of plague. The instrument employs an argon ion laser (514 nm) to launch light into a long-clad fiber and measures the fluorescence produced by an immunofluorescent complex formed in the EW region (67). A sandwich immunoassay scheme was used to detect ricin in the picograms-per-milliliter range by an antiricin immunoglobulin G (IgG) immobilized directly to the silanized fiber using a cross-linker and to avidin-coated fibers incubated with biotinylated antiricin IgG to immobilize the antibody using an avidin-biotin bridge (68). The assay using the avidin-biotin-linked antibody demonstrated higher sensitivity and wider linear dynamic range than the assay using antibody directly conjugated to the surface. Sensitive fiber-optic immunosensors that produce EWs have been described for the rapid and specific detection of *Staphylococcus aureus* in clinical specimens and foods (69), antibodies specific for the parasite *L. donovani* in human serum (70), and Botulinum toxin (71,72).

Evanescent wave biosensors capable of measuring prognostic and diagnostic infection indicators (including interleukin-6 [IL-6], tumor necrosis factor alpha, IL-12, C-reactive protein, and thrombin) are in various stages of development. A minimally invasive fiber-optic EW Fourier transform infrared spectroscopy method using ultrasensitive fiber probes with low losses in the MIR region of the spectrum (850–1800 $cm^{-1}$) has been used for noninvasive and rapid (seconds) direct measurements of the spectra of normal and pathological breast tissue (about 1 mm) in vitro, ex vivo, and in vivo to perform accurate testing of tumor tissues at the early stages of their development.

Biosensors bring the actual testing directly to the site rather than transporting the sample to the laboratory. One of the prime objectives of medical technology is to develop methodology and instrumentation that is simple to use and technically insensitive and provides rapid test results of medical value. By having diagnostic test results in time frames approaching "real time," clinicians are in a position to institute acute interventional therapy based on more-complete findings of clinical and biochemical data, to monitor and adjust dosages of drugs with very restricted therapeutic and toxic windows, and to reduce a patient's hospitalization and the psychological trauma of waiting for a diagnostic test result. The single attribute of the ability to permit the instrument to be brought to the sample rather than vice versa explains their widespread use.

Near-patient (NP) testing represents the fastest-growing segment of the $16 billion worldwide medical diagnostic testing market. Quantech Limited has submitted to the FDA its 510(k) premarket approval for its first cardiac marker Myoglobin and is developing other tests, including the cardiac markers CK-MB and troponin and the pregnancy marker hCG. Quantech's critical care system, based on its patented SPR technology, will have the ability to perform a complete range of clinically related, quantitative whole blood tests on a single instrument near the patient and still provide results at least twice as fast as those from the central laboratory. This time reduction in receiving test results will allow for more rapid and specific patient treatment and significant cost savings. Further tests are expected to be developed to provide a complete range of clinically related, quantitative whole blood tests using a single instrument near the patient.

## CONCLUSIONS

The above examples are representative of the type of problems that have been investigated using EWS. Our preliminary efforts have been directed toward the application of EW biosensors to determine the activity of proteolytic enzymes and their impact on delivery of peptide and protein drugs in the nasal mucosa. The inadequate bioavailability of peptides and proteins, even in the presence of absorption enhancers, suggests that there is another barrier, an enzymatic barrier, that limits absorption. The mucosal membranes of the nasal cavity are known to have various types of peptidase and protease activities, with activities comparable to those in the ileum (73). The significance of these enzymes is shrouded since the structural organization of these peptidases can be destroyed on homogenation. It is envisaged that, as the knowledge base concerning EWS widens, the use of this technique will permit us to elucidate the pharmacokinetics of drugs by this route. In any event, the photonics revolution of the last decade will permit all aspects of pharmaceutical sciences and medical care to rethink a profound question in chemical analysis: do we take the sample to the instrument or vice versa?

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