Design and Development of a Fiber-Optic Immunosensor Utilizing Near-Infrared Fluorophores

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The design and application of a fluorescent fiber-optic immunosensor (FFOI) are reported. The FFOI is utilized for the detection of antibody/antigen binding within the near-infrared (NIR) spectral region. The technique is developed through the combined use of fiber-optic, semiconductor laser-excitation, fluorescence detection, NIR dye, and immunochemical techniques. The antibody is immobilized on the FFOI and utilized as a recognition component for trace amounts of specific antigen. The FFOI is constructed to utilize an antibody sandwich technique. The assay involves the immobilization of the capture antibody on the sensing tip of the FFOI followed by the exposure of the immobilized sensing tip to the antigen. The antigen-coated FFOI is then introduced to a second antibody previously labeled with the NIR dye. Typical measurements are performed in about 15 min. A semiconductor laser provides the excitation (780 nm) of the immune complex. The resulting emission is detected by a silicon photodiode detector (820 nm). The intensity of the resulting fluorescence is directly proportional to the concentration of the antigen. The sensitivity of the analysis reaches 10 ng/ml and the response time is 10–15 min.

INTRODUCTION

Conventional immunoassays are considered to be powerful approaches for analyzing minor components in complex media. However, they generally require much skill with automation in order to obtain a reliable analytical result. The development of immunosensors offers the opportunity to reduce the skill required in immunoassays. Immunosensors are a subclass of biosensors which utilize the binding between an antigen or hapten and its corresponding antibody. Most immunosensors are based on electrical or optical detection techniques.[1] Of these, optical immunosensors coupled to fiber optic have received considerable attention.[1]

The key features of any immunosensor are the selectivity and sensitivity, which characterize antibody–antigen recognition.[2,3] The exploitation of fiber-optic technology in immunosensing offers a number of advantages; among these are freedom from electrical interference, miniaturization, remote and on-line capability, and low cost.[1]

Different types of fiber-optic immunosensors can be developed based on the type of assay and the choice of detection technique.[2] The immunoassay format appropriate for a fiber-optic immunosensor depends on the chemical characteristics of the antigen–antibody system under investigation.[2] Nonfluorescent analyte systems may be detected via direct, antibody sandwich, or competitive binding immunoassay techniques.[4,5]

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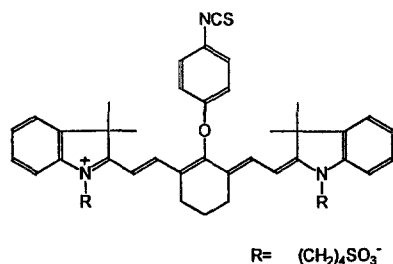[4] To whom correspondence should be addressed.
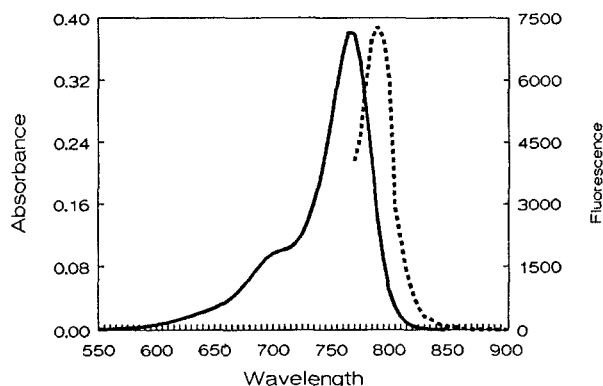
Fig. 1. Structure of Dye I.

Fig. 2. Absorption and emission spectra of Dye I.

A number of fiber-optic immunosensors have been demonstrated for the detection of serum analytes, environmental pollutants, and a host of other compounds.[6-14] The advantages of these fiber-optic immunosensors are simplicity and sensitivity; however, their application has been limited to the detection of fluorescence in the visible region. Unfortunately, the visible detection range is a high interference area where a large number of compounds are known to exhibit absorption and emission maxima, making it difficult to detect extrinsic fluorescence.[15-17]

In an attempt to overcome the limitation of visible fluorophores, we began evaluating the use of NIR fluorophores as alternative labels for use in fluorescently labeled fiber-optic immunosensors (FFOI).[16,17] NIR spectral regions extend from 700 to 3000 nm. This region is an area of low biological interference, where only a few compounds exhibit significant absorption or fluorescence, making it ideal for detection of extrinsic fluorescence.[15-17]

In addition, the spectral characteristics of NIR dyes make them favorable to use with semiconductor lasers and photodiode detectors. Application of semiconductor lasers offers a number of advantages including an excitation wavelength in the NIR spectral region; monochro-

matic, narrow, intense, coherent, and collimated (with correcting optics) beam; long lifetime; low power consumption; high efficiency; and small size. Similarly, photodiode detectors are favored because of their high sensitivity and responsivity, high signal-to-noise ratio (S/N), fast response, and low cost. Excitation of the NIR fluorophores with semiconductor lasers and detection of the generated fluorescence with photodiodes will create highly sensitive and yet affordable FFOIs.

This report describes the design and development of a simple, practical, sensitive, and selective NIR-FFOI. The FFOI is constructed to utilize an antibody sandwich technique. The assay involves the immobilization of the capture antibody on the sensing tip of the FFOI followed by the exposure of the immobilized sensing tip to the antigen. The antigen-coated FFOI is then introduced to a second antibody previously labeled with the NIR dye. Typical measurements are performed in about 15 min. A semiconductor laser provides the excitation (780 nm) of the immune complex. The resulting emission is detected by a silicon photodiode detector (820 nm). The intensity of the resulting fluorescence is directly proportional to the concentration of the antigen. The sensitivity of the analysis reaches 10 ng/ml and the response time is 10–15 min.

EXPERIMENTAL

Materials

The poly(methyl methacrylate) (PMMA) optical fiber (core diameter = 1000 μm and numerical appreture = 0.52) was purchased from Mitsubishi Cable America, Inc. (New York). *N,N*-Dimethylformamide (DMF), methanol (MeOH), and dichloromethane (CH₂Cl₂) were purchased from Fisher Scientific Company (Fair Lawn, NJ). Hydrochloric acid (HCl) was obtained from J. T. Baker, Inc. (Pillipsburg, NJ). The remaining high-purity immunochemicals, chemicals, and reagents used in this study were purchased from Sigma Chemical Company (St. Louis, MO).

A noncommercial fluorescent NIR dye (Fig. 1), Dye I, was synthesized in our laboratory as described previously.[18] The labeling involves a reaction of the isothiocyanate group of the dye with an amino group of the antibody. The excitation and emission spectra of Dye I (Fig. 2) exhibit main absorption and emission peaks at 770 and 793 nm, respectively, with a Stokes's shift of 23 nm. The absorbance maximum of Dye I is a good match for the output wavelength of the 780-nm semiconductor laser, and ultrasensitive NIR detection is pos-
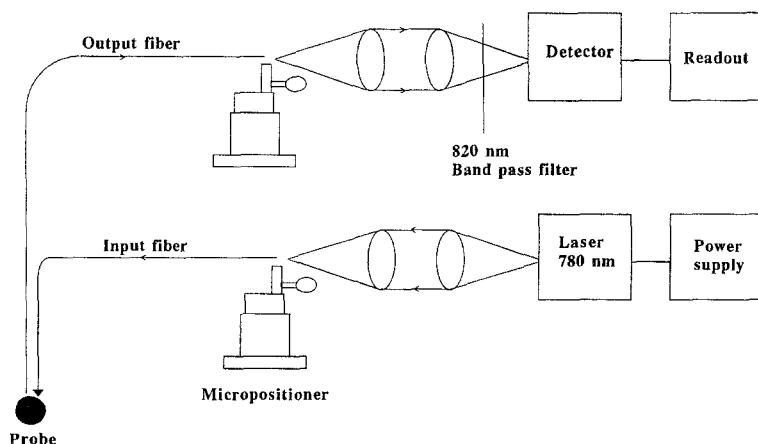
Fig. 3. Diagram of the NIR-FFOI instrumentation.

sible with this dye.[19] The emission maximum of Dye I makes it feasible to collect fluorescent signals away from scattered light. The molar absorptivity of Dye I is 1.4×10^5 L cm^{-1} mol^{-1}. The fluorescence quantum yield of Dye I was estimated to be 40% in MeOH. The NIR dye used as the standard for the quantum yield determination was IR-125 (Eastman Kodak, Rochester, NY) with a quantum yield of 13% in MeOH. Dye I data was well fit to a monoexponential decay of 0.815 nsec and χ^2 of 9.43. Dye I was found to be stable when its fluorescence intensity was monitored over 24 h in the dark, and photostable to sun light over the same period of time.

Instrumentation

The fluorescence unit (Fig. 3), excluding the optical probe, was designed and generously donated to our laboratory by LI-COR (Lincoln, NE). The FFOI consisted of two strands of plastic optical fibers connected at the common distal ends. The fluorescence unit (Fig. 3) was made by focusing a 30-mW semiconductor laser, with a 780-nm excitation wavelength and a spectral width of 2 nm, on one of the free optical-fiber ends. The fiber ends were held in microposition holders for maximum fluorescence signal. As the light traveled from the proximal end to the distal end of the FFOI, it induced fluorescence of the dye. The fluorescence signal was collected by the second optical fiber, which was routed to the opposite end of the FFOI. The distal end was positioned in front of a microscope objective lens which focused the radiation onto a silicon photodiode detector. Because the fluorescence band was broad and extended beyond 800 nm, it was feasible to collect the fluorescence signal without interference from scattered excitation. Therefore, the detector system was equipped with a 820-nm filter (10-

nm BW, OD > 5). By using the 820-nm filter, $<3\%$ of scattered light from the laser's excitation wavelength reached the detector. However, $>20\%$ of the fluorescence signal was measured at 820 nm. The system background was measured by blocking the excitation beam and monitoring the detector response. Therefore, the signal was found to be eight times stronger than the noise.

A Perkin–Elmer Lambda 2 UV/vis/near-IR spectrophotometer (Norwalk, CT) was used for the absorption measurements. The spectrometer was interfaced to a Zenith 286 computer equipped with a PECSS program used to store data and control the spectrophotometer. The excitation wavelength was determined from the absorption spectrum using the maximum absorbance wavelength. Fluorescence spectra, stability, and lifetime studies of Dye I were performed with an ISS K2 multifrequency phase fluorometer (Champaign, IL). The spectrofluorometer was equipped with a laser diode excitation source (Laser Max, Rochester, NY) which emitted up to 50 mW at 780 nm. The spectrofluorometer was interfaced to a Gateway-486 computer that contained ISS K2 operating software. Spectras were obtained using a 2-mm slit width.

Methods

A stock solution of Dye I (1 mg/ml) was prepared in 20% (v/v) DMF/water. Stock solutions (1 mg/ml) of each of the antigens (human IgG and IgM) and goat anti-human IgG antibodies (F_{ab} and γ chain specific) were prepared individually in phosphate-buffered saline (PBS; pH 7.4), and dilutions were made as required. The labeling of the antibody (goat anti-human IgG antibodies) was adapted from the antibody labeling procedure of Riggs et al. and used without further modification.[20] A
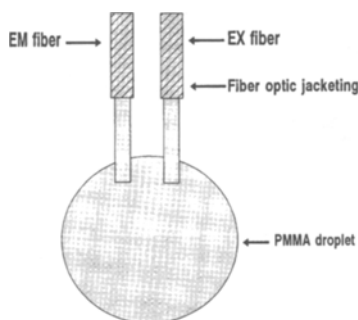
Fig. 4. Schematic of the FFOI optical probe.

γ chain-specific labeled antibody was used in all experiments, except in the selectivity study, which utilized an F_ab-specific conjugate.

The design of the NIR-FFOI is based on a double-stranded optical fiber[16,17] as depicted in Figs. 4 and 5. Two strands of PMMA plastic fiber were stripped of their jackets, then the cores were polished and covered with a PMMA solution (3 g/10 ml CH_2Cl_2) to form a droplet holding the two strands together (Fig. 4). The polymer was dried overnight at room temperature. The dried polymer droplet was then incubated in HCl (6 M) at room temperature for 18 h, which caused hydrolysis of the methoxycarbonyl groups on the surface of the polymer to carboxylic acid groups. The carboxylic acid groups were utilized for immobilization without further activation. Further, the FFOI was examined under an electron microscope to ensure the smoothness of the reaction site. The FFOI was then rinsed with distilled water and incubated in the anti-human IgG antibody (0.1 μg/ml) for 45 h at 4°C (Fig. 5). Following the antibody immobilization, the FFOI was immersed in a solution of the antigen (0.1 μg/ml) and incubated for 30 min at 4°C (Fig. 5). The antigen-coated FFOI was immersed in PBS to measure the reference signal level and then in the NIR-labeled antibody (0.1 μg/ml) to monitor the response of the fluorescent complex. The reference and the fluorescent signals were each measured 10–15 min after incubation of the FFOI in the corresponding solution. Once all measurements were completed, the fluorescence/reference ratios (relative fluorescence) were calculated and plotted versus the antigen concentrations as depicted in Figs. 6–8.

RESULTS AND DISCUSSION

Immobilization

An important step in the development of the FFOI is the generation of a large number of activated polymer

binding sites. This determines the antibody loading, which in turn affects the sensitivity. The FFOI (originally containing methyl ester groups, PMMA-COOCH_3) was incubated in HCl to generate surface carboxylic groups (PMMA–COOH). The time required for maximum surface activation of the polymer binding sites was determined by comparing the fluorescence intensity of a series of FFOIs activated for different time increments ranging from 1 to 24 h. Following the formation of the fluorescent complex, the fluorescence response of each FFOI was monitored. The fluorescence intensity increased with increasing activation period up to 18 h, and a gradual decrease in the response was observed once the activation time exceeded 18 h. This decrease in the signal may be due to several possibilities. (a) The overgeneration of the number of binding sites causing the overimmobilization of the antibody; therefore, it is likely that each antibody is bound at too many sites to the polymer and is thus sterically hindered from binding to antigen. (b) The overgeneration of the number of binding sites might alternatively cause the excessive formation of the fluorescent complex; therefore, it is likely that excited molecules are self quenching and thus resulting in quenched fluorescence. (c) Alternatively, extended exposure of PMMA to HCl might degrade the polymer. On the basis of these findings the FFOIs used in this work were activated for 18 h.

Another important step involved in the development of the technique was the determination of the incubation period required for obtaining optimum antibody immobilization. Several activated FFOIs were prepared and incubated with the antibody. The incubation period ranged from 24 to 50 h. The fluorescence intensity of each FFOI was measured by immersing the antibody-coated FFOI in the antigen solution for 30 min and then in the labeled antibody solution. The intensity increased with increasing incubation period; however, the increasing signal reached a steady state at about 45 h. Assuming that the experiment optimizes the amount of antibody immobilized, then at 45 h one of the two possibilities has been reached. (a) The maximum amount of antibody has been attached to the PMMA, or (b) the amount of antibody on the PMMA is high enough that the concentration of either the antigen or the labeled antibody is the limiting factor in the formation of the fluorescent complexes at the surface. Hence, in order to optimize the antibody immobilization, successive activated FFOIs were incubated in the antibody for 45 h.

Sensitivity

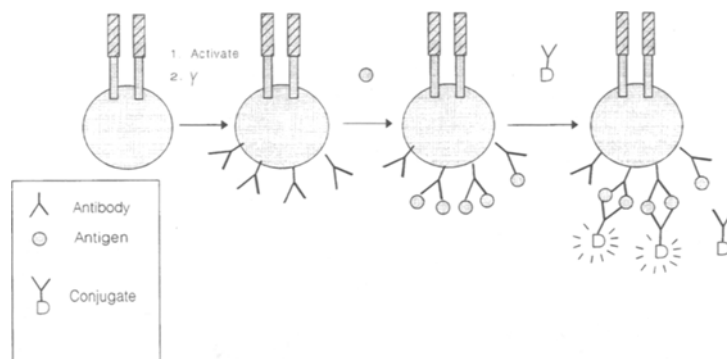Eight individual FFOIs were activated and immobilized with anti-human IgG antibody as described. Dif-

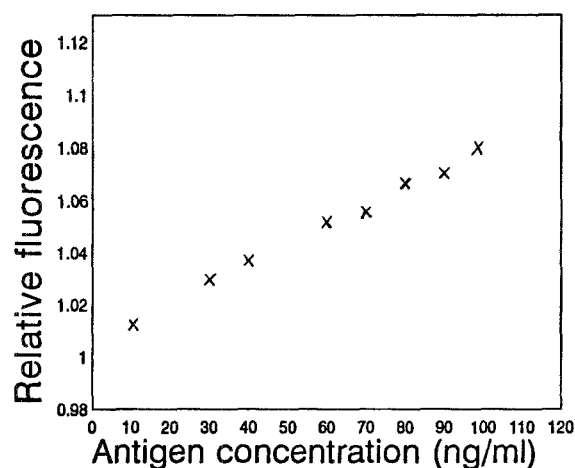**Fig. 5.** Formation of fluorescent complex on the FFOI.

Fig. 6. The plot of relative fluorescence versus antigen concentration. Different antigen concentrations correspond to the IgG concentrations used to coat the different antibody-immobilized FFOIs. The reference and fluorescence signals were measured as described under Methods, and relative fluorescence was then calculated and plotted versus antigen concentration. For each IgG concentration, three identical FFOIs were tested with SD = ±0.003.

Fig. 7. The plot of relative fluorescence (in FFOI immobilized with F_{ab}-specific antibody) versus antigen concentration. Various concentrations of the antigens (IgG and IgM) were used to coat the different antibody-immobilized FFOIs. The reference and fluorescence responses were measured as described under Methods, and relative fluorescence was then calculated and plotted versus antigen concentration. For each antigen concentration, three identical FFOIs were tested with SD = ±0.003.

ferent concentrations of human IgG, ranging from 10 to 100 ng/ml, were used to coat the eight FFOIs. The reference signal was measured as described under Methods. The fluorescence intensity of each FFOI was then measured by immersing the IgG-coated FFOI in a vial containing the labeled antibody. The plot of relative fluorescence versus antigen concentration is shown in Fig. 6. For each concentration plotted in Fig. 6, three identical FFOIs were tested, which exhibited similar results (SD = ±0.003). The response of the FFOI was found to be linear, indicating that the FFOI has a dynamic range from 10 to 100 ng/ml of IgG (Fig. 6). The result of this experiment suggests that an increasing concentration of IgG enhances the binding of a larger num-
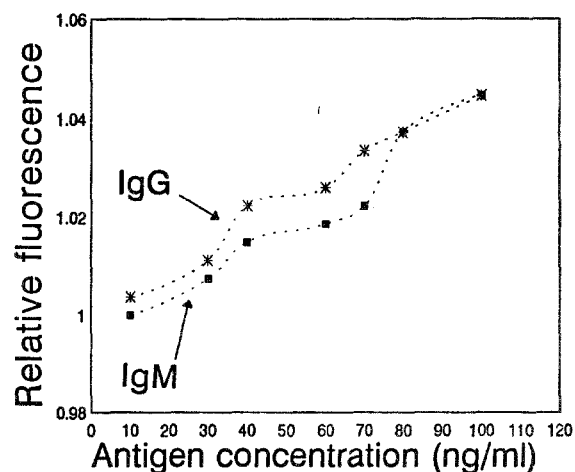
ber of NIR-labeled antibodies, resulting in greater fluorescence response. It may be possible to reduce the detection limit further by optimizing the instrument (i.e., designing a steady arrangement at the fiber-to-laser and the fiber-to-detector connections) and enhancing the conjugation procedure.

Selectivity

To test the selectivity of the FFOI, two sets of experiments (A and B) were designed.
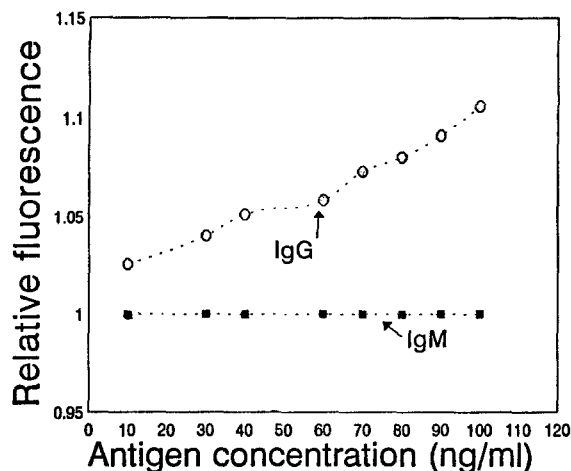
Fig. 8. The plot of relative fluorescence (in FFOI immobilized with γ chain-specific antibody) versus antigen concentration. Various concentrations of the antigens (IgG and IgM) were used to coat the different antibody-immobilized FFOIs. The reference and fluorescence responses were measured as described under Methods, and relative fluorescence was then calculated and plotted versus antigen concentration. For each antigen concentration, three identical FFOIs were tested with SD = ±0.003.

Experiment A

Two FFOIs were prepared as before using anti-human IgG antibody (F_{ab} specific) for immobilization. Further, one of the FFOIs was coated with IgG and the other with IgM. Following the reference signal measurement, the fluorescence intensity of each FFOI was monitored by immersing the FFOI in a vial containing the labeled antibody. The plot of relative fluorescence versus antigen concentration is shown in Fig. 7. For each concentration plotted in Fig. 7, three identical FFOIs were tested, which exhibited similar results (SD = ±0.003). Figure 7 shows that the fluorescence response increases with increasing concentration of either antigen, IgG or IgM. This result is apparently due to the fact that F_{ab}-specific anti-IgG antibody contains both heavy and light chains of the antibody molecule which can bind the F_{ab} fragment of IgG or IgM. In conclusion, the FFOI prepared using F_{ab}-specific anti-IgG antibody for immobilization is capable of detecting both IgG and IgM and is not selective for the detection of IgG or IgM individually.

Experiment B

An experiment parallel to experiment A was carried out by immobilizing γ chain-specific anti-human IgG antibody. Figure 8 shows the plot of relative fluorescence versus antigen concentration. Triplicate FFOIs were tested at each antigen concentration (Fig. 8) and each exhibited similar results (SD = ±0.003). As can be seen, for the IgG-coated FFOI, the fluorescence response increases with increasing IgG concentration. In contrast, in the second FFOI there is no signal change with an increasing concentration of IgM. These findings may be due to the fact that since γ chain-specific anti-human IgG antibody can bind only the γ chain of IgG, it cannot bind IgM because IgM lacks the γ chain. Hence, upon the introduction of the FFOI to the labeled antibody, a fluorescent complex forms in the case of IgG, but this is not the case with IgM. In summary, an FFOI immobilized with γ chain-specific anti-human IgG antibody is selective for the detection of IgG alone.

CONCLUSION

The proposed FFOI provides a promising new type of immunosensor which utilizes an NIR fluorophor for the assay of IgG and possibly a variety of other immunocompounds. The described immunosensor results in reduced analytical expenditure and a short response time. The chemistry and instrumentation of the technique have been developed but are still amenable to optimization and miniaturization.

Several parameters must be investigated and optimized further before such a device can be utilized at its full potential. The NIR-FFOI reported requires a high concentration of immobilized antibody. Antibody is the limiting factor at a high antigen concentration. Improving the immobilization technique may help reduce this problem and increase the sensitivity and selectivity by allowing the maximum amount of antibody to be loaded onto the FFOI with preserving the antibody activity on bonding to the FFOI. In addition, protein labeling ideally calls for a water-soluble dye, whereas Dye I is partially soluble in water. This issue may be addressed by an appropriate dye design. Cyanine dyes (such as Dye I; Fig. 1) have an *N*-alkyl substituent in the parent nucleus, and the solubility of such a dye can be controlled by variation of the *N*-alkyl substituent.

The mentioned areas are currently under investigation, and we believe that despite these obstacles, it is expected that the FFOI's sensitivity, selectivity, and simplicity will encourage further application and design refinement so that increasingly versatile immunosensors can be constructed.

ACKNOWLEDGMENTS

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