AC RESEARCH

# Dynamically Modified, Biospecific Optical Fiber Sensor for Riboflavin Binding Protein Based on Hydrophobically Associated 3-Octylriboflavin

**Faye K. Ogasawara,† Yunlong Wang, and Donald R. Bobbitt***

*Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701*

**A new approach to the production of optical fiber sensors is described which is based on a dynamic modification procedure. In this approach, the optical fiber surface is rendered hydrophobic through the covalent attachment of a $C_{18}$ moiety. Specific sensing ligands are then associated with this surface through either their inherent or designed hydrophobicity. To investigate the feasibility of the approach, an optical fiber sensor has been constructed for riboflavin binding protein in which 3-octylriboflavin is associated with the fiber surface. Fluorescence quenching occurs upon binding of the protein to the immobilized riboflavin derivative. The sensor possesses a minimum measurable quantity of 7.3 pmol of binding protein in a probe volume which is less than 10 μL. With this approach, the sensing surface was repetitively regenerated 15 times over a 1-h period with less than a 5% variation in sensor-to-sensor performance. The approach is general, and with minor variations it can be used in a variety of sensing situations.**

## INTRODUCTION

Recent developments in the field of chemical analysis have been directed toward the miniaturization of chemical sensors. The impetus for this effort lies in the desire to produce in vivo sensors capable of detecting a variety of chemical and biochemical materials indicative of the diseased state. To probe these systems adequately without perturbing the system under study is one of the great challenges in the field of chemical analysis today. One exciting development has been the use of optical fibers to deliver and collect light from specific regions where the light interactions at the distal end of the fiber can be used to gain knowledge about the chemical events occurring at the fiber tip.[1] Optical fibers, because of their small diameters, can be used to produce extremely small optical probes (optrodes), and because light can be propagated in optical fibers over long distances without appreciable loss, they can be used effectively in remote monitoring situations. As an example, an optical fiber sensor has been described which is suitable for monitoring glucose levels via the effect of the solution refractive index on the transmission properties of the optical fiber.[2] Optical fiber sensors have also been developed which can respond to pH changes (using an immobilized indicator),[3-5] temperature,[6] oxygen levels,[7] and for sensing a variety of dissolved gases.[8-10]

A number of chemical assays have been developed which utilize an optical fiber in the measurement process, and these sensors have been shown to be both sensitive and small enough for in vivo applications. However, in measurements involving biochemical materials which are important from a physiological perspective, biological complexity demands that other methods be coupled to the light measurement to improve the selectivity of the analysis. Primarily, this is a consequence of the fact that in complicated matrices many materials will possess similar spectral characteristics and spectral discrimination alone cannot differentiate amongst all of the many possibilities. One approach to add selectivity to the light measurement process is to couple a biospecific reagent to the optical system through either direct attachment to the optical fiber via a chemical bond or to a host material which is then mechanically fastened to the fiber. This ligand enhances the ability of the sensor to discriminate between analyte and interferent by virtue of the inherent selectivity of most biological reactions. For example, several recent reports have demonstrated the feasibility of using an optical fiber probe to kinetically measure enzyme activities.[11,12] In such a probe, substrate material is immobilized at the end of the optical fiber and the changes in the spectral properties of the substrate or product[11] are used to assess the extent of the reaction. Extremely selective optical fiber sensors have also been reported which are based on the covalent immobilization of

* To whom correspondence should be addressed.
† Present address: Department of Chemistry, Michigan State University, 320 Chemistry Building, E. Lansing, MI 48824.

(1) Peterson, J. I.; Vurek, G. G. *Science* **1984**, *224*, 123-127.
(2) Ross, I. N.; Mbanu, A. *Opt. Laser Technol.* **1985**, *17*, 31-35.
(3) Moreno, M. C.; Martinez, A.; Milan, P.; Camara, C. *J. Mol. Struct.* **1986**, *143*, 553-556.
(4) Jordan, D. M.; Walt, D. R.; Milanovich, F. P. *Anal. Chem.* **1987**, *59*, 437-439.
(5) Peterson, J. I.; Goldstein, S. R.; Fitzgerald, R. V.; Buckhold, D. K. *Anal. Chem.* **1980**, *52*, 864-869.
(6) Grattan, K. T. V.; Palmer, A. W. *Proc. SPIE—Int. Soc. Opt. Eng.* **1986**, *630*, 256-265.
(7) Peterson, J. I.; Fitzgerald, R. V.; Buckhold, D. K. *Anal. Chem.* **1984**, *56*, 62-67.
(8) Arnold, M. A.; Ostler, T. J. *Anal. Chem.* **1986**, *58*, 1137-1140.
(9) Lubbers, D. W.; Gehrich, J.; Opitz, N. *Life Support Syst.* **1986**, *4*, 94-108.
(10) Wolfbeis, O. S.; Posch, H. E.; Kroneis, H. W. *Anal. Chem.* **1985**, *57*, 2556-2561.
(11) Arnold, M. A. *Anal. Chem.* **1985**, *57*, 565-566.
(12) Wolfbeis, O. S. *Anal. Chem.* **1986**, *58*, 2874-2876.

0003-2700/92/0364-1637$03.00/0 

antibodies at the fiber tip. These optrode systems have been used in both competitive and noncompetitive assays.[13,14]

None of the methods reported to date for associating specific ligands with optical fiber surfaces is general enough to allow application to a wide range of systems, and they have been plagued by a loss of activity and operating lifetime after modest use due to their fragile nature. Further, chemically bonded substrates have only limited use in nonreversible systems or in kinetic assays due to the finite amount of reagent which can be bound to the fiber or substrate surface.

One approach to circumvent the limitations cited above would be to associate ligands with the optical fiber through a mechanism which is less severe and which lends itself to regeneration. If such a mechanism could be identified, it would assure the biological integrity of the ligand and allow measurements on nonreversible systems. Recently, it has been demonstrated that ion-exchange phases can be created from reversed-phase HPLC packings through a dynamic modification interaction.[15] In this procedure, the hydrophobic portion of octadecanesulfonic acid is associated with the hydrophobic packing material thereby permitting the sulfonic acid moiety to be accessible to the bulk solution. This functionality then serves as an ion-exchange site for cationic species.

A similar mechanism is proposed as a general method for the production of optical fiber biosensors which would maintain a greater degree of activity for fragile ligands. In this procedure, the surface of the optical fiber is rendered hydrophobic through the covalent attachment of an octadecyl moiety. The desired ligand is then associated with this surface through either its inherent or designed hydrophobicity. The advantages of this approach lie in the mild conditions used to achieve coverage of the optical fiber with the biochemical species, thereby assuring biological activity, and in the fact that ligand molecules can be renewed or changed by simply washing off the existing molecule and then immersing the fiber in an appropriate solution of the desired ligand species.

One area in which this technology could make a significant impact is in the study of flavoproteins. Flavoproteins comprise an important class of oxidative–reductive enzymes ubiquitous in living systems. Riboflavin binding protein (RBP) is known to serve as a storage function for the coenzyme (riboflavin), and it has been extensively studied in order to understand the site and mechanism of apoprotein attachment to the flavin moiety. An optical fiber sensor for riboflavin binding protein would extend the range of systems which could be studied under physiological conditions thereby allowing flavoprotein interactions to be more fully characterized. In this paper, a new and general approach for the production of optical fiber biosensors is described which is based on a dynamic modification procedure. Through this procedure, a riboflavin binding protein sensor was developed which utilizes riboflavin as the sensing ligand. The hydrophobicity of riboflavin was enhanced by the addition of a $C_8$ group to the N-3 position. The modified riboflavin was then dynamically associated with the hydrophobic surface of a $C_{18}$-modified optical fiber. When apo-RBP is present, it binds to the riboflavin at the fiber surface and this association results in efficient quenching of riboflavin fluorescence. Optimized synthetic procedures are described for the modification of the fiber surface and for the attachment of an octyl moiety to riboflavin to increase its hydrophobicity. The sensor will be characterized in terms of its analytical capabilities, and the generality of the approach will be explored.

(13) Vo-Dinh, T.; Tromberg, B. J.; Griffin, G. D.; Ambrose, K. R.; Sepaniak, M. J.; Gardenhire, E. M. *Appl. Spectrosc.* **1987**, *41*, 735–738.
(14) Tromberg, B. J.; Sepaniak, M. J.; Vo-Dinh, T.; Griffin, G. D. *Anal. Chem.* **1987**, *59*, 1226–1230.
(15) Cassidy, R. M.; Elchuk, S. *Anal. Chem.* **1982**, *54*, 1558–1563.

## EXPERIMENTAL SECTION

**Materials.** The silica optical fiber used for these studies had a 1000-$\mu$m core diameter and a protective jacket composed of Nylon (Fiberguide Industries, Stirling, NJ, Type Superguide UV). For fiber modification, dichlorodimethylsilane was obtained from Aldrich (Milwaukee, WI) and distilled over calcium hydride prior to use. Chlorodimethyloctadecylsilane was also obtained from Aldrich and used without further purification. Hydrochloric acid, methylene chloride, methanol, and hexane were obtained from Fisher Scientific (Houston, TX) and used as received. Toluene was obtained from Aldrich and further purified by distillation over calcium hydride. For the modification of riboflavin, both riboflavin and 1-iodooctane were obtained from Aldrich and used as received. Acetic anhydride, glacial acetic acid, perchloric acid, potassium carbonate, sodium hydroxide, and potassium phosphate monobasic were obtained from Fisher Scientific and used without additional purification. Riboflavin binding protein was obtained in the apo-form from Sigma Immunochemicals (St. Louis, MO). Deionized water was purified through a Fisher Scientific Ultrapure Cartidge prior to use. The nitrogen used as a blanket for the synthetic procedures was oxygen free and further purified by passage through anhydrous calcium sulfate prior to use.

Synthesis of 3-octylriboflavin was followed by thin-layer chromatography using plastic-backed silica gel sheets containing a fluorescent indicator (Eastman Kodak Co., Rochester, NY type 13181). Spot visualization was achieved using illumination from a UV source at 365 nm. The solvent system was a 18:1:1 mixture of chloroform:methanol:glacial acetic acid.

**Synthetic Procedures. Preparation of $C_{18}$-Modified Optical Fiber.** The optical fiber surface was prepared by successive polishing with 60-, 9-, 1-, and 0.3-$\mu$m lapping film. Five polished fibers were placed in a 1000-mL round-bottom flask containing 500 mL of 2 N HCl. The fiber surface was activated by reflux in the acid for 6 h, and the fibers were then dried at 100 °C for 4 days. The activated fibers were placed in a 500-mL round-bottom flask containing 150 mL of toluene and 100 mL of dichlorodimethylsilane. The mixture was stirred and refluxed for 4 h under nitrogen. The deactivated fibers were then placed in a 250-mL round-bottom flask containing 150 mL of toluene and 1 g of chlorodimethyloctadecylsilane. The mixture was stirred and refluxed under nitrogen for 6 h. The fibers were then placed in a Soxhlet extractor for polymerization via water for 12 h, at which time the water was replaced, in turn by methanol, methylene chloride and hexane. Each solvent wash was 24 hours in duration.

**Preparation of 2′,3′,4′,5′-Tetraacetylriboflavin.** A 5.0-g sample of riboflavin was added to a 400-mL beaker containing 200 mL of a 1:1 (v:v) mixture of glacial acetic acid and acetic anhydride. After the dropwise addition of 1 mL of 70% perchloric acid, the mixture was stirred for 30 min at 40 °C. The mixture was cooled in an ice bath and diluted with an equal volume of water, and the solution was then extracted three times with 25 mL of chloroform. The combined chloroform extracts were washed four times with 25 mL of deionized water followed with a 25-mL extraction with a saturated solution of NaCl. The solution was evaporated to dryness and the product was then recrystallized from 95% ethanol producing a product with a melting point of 238–239 °C.

**Preparation of 2′,3′,4′,5′-Tetraacetyl-3-octylriboflavin.** In a septa-stoppered 125-mL Erlenmeyer flask, 1.7 g of 2′,3′,4′,5′-tetraacetylriboflavin was magnetically stirred with 0.5 g of potassium carbonate in 20 mL of dimethylformamide. A 1-mL aliquot of 1-iodooctane was added dropwise to the mixture. Stirring was continued for 8 h at room temperature. The suspension was filtered by suction and rinsed with 25 mL of chloroform. The filtrate was extracted with 1–10 and 3–5-mL portions of water, followed by a final wash with 10 mL of a saturated solution of NaCl. The organic layer was evaporated to a reduced volume and then extracted with 25 mL of isooctane. The isooctane was evaporated to dryness yielding product.

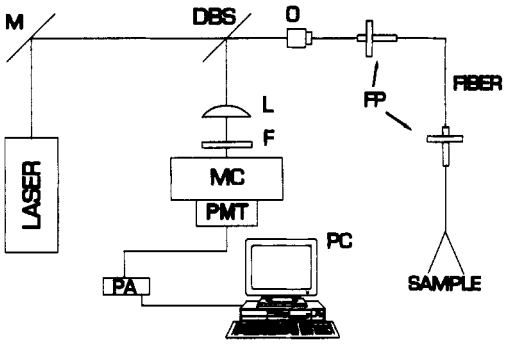


**Figure 1.** Optical configuration used for single fiber fluorescence measurements: laser, argon-ion laser; M, mirrors; DBS, dichroic beam splitter; O, microscope objective; FP, fiber positioner; L, focusing lense; F, Raman holographic filter; MC, monochromator; PMT, photomultiplier tube; PA, picoammeter; PC, personal computer.

**Preparation of 3-Octylriboflavin.** The product from the previous step was dissolved in methanol, and the solution was transferred to a 100-mL round-bottomed flask. After evaporation of the methanol, the compound was refluxed in 2 N HCl for 6 h. The acid solution was evaporated to dryness and the product rinsed twice with water. The product was then purified by recrystallizing it twice from 95% ethanol.

**Instrumentation.** Surface modification of the optical fiber was followed by diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy using a Nicolet Instruments FT-IR instrument (Madison, WI, Model 205 DX). Ten scans at a resolution of 8 $cm^{-1}$ were taken on 1-cm sections of both bare and hydrophobically modified fibers. The spectral range spanned 4500–400 $cm^{-1}$. The averaged scans obtained for the bare fiber were subtracted from data obtained with the modified fiber to produce a difference spectrum. HPLC data were obtained using a Shimadzu (Kyoto, Japan) LC-600 system which included a SPD-6AV UV/vis variable-wavelength detector and a CR601 data processing system. An Alltech (Deerfield, IL) Adsorbosphere 10-$\mu$m silica column (250 × 4.6 mm) was used to access the purity of the 3-octylriboflavin. The mobile phase was an 18:1:1 (v:v:v) mixture of chloroform:methanol:glacial acetic acid. Detection was at 475 nm. UV/vis spectra were taken on a Hewlett Packard diode array spectrophotometer (Palo Alto, CA, Model 8452). Fluorescence emission spectra of both native and modified riboflavin were obtained on a Perkin-Elmer Spectrofluorometer (Norwalk, CT, Model 650-40).

**Apparatus.** The optical configuration for fluorescence measurements using dynamically modified optical fibers is schematically represented in Figure 1. A single fiber was used to both deliver light to the interaction region and collect the resulting fluorescence. The apparatus was constructed on a 6-ft × 2-ft × 2.3-in. optical breadboard (Newport Corp., Fountain Valley, CA, Model XSN26). Light from an argon ion laser (Ion Laser Technology, Salt Lake City, UT, Model 490 ASL) operating at 488 nm was directed through a dichroic beam splitter (CVI Laser Corp., Albuquerque, NM) and into a microscope objective (Newport, Model M-10X, FL = 14.8 mm, NA = 0.25). Excitation power was adjusted using a neutral-density filter placed in the optical path of the excitation laser. The objective was rigidly held by an objective fiber coupler (Newport, Model F-91-C1) which focused the excitation light into the optical fiber and collimated the emission light. The fluorescence was reflected at the dichroic mirror and collected by a 75-mm focal length, 50-mm-diameter plano convex lense. The collimated light was passed through a holographic filter (Physical Optics Corp., Torrance, CA, Model RHE λ 488) and into a scanning monochromator (CVI, Model DK 240-2). Light exiting the monochromator was detected with a photomultiplier tube (Thorn EMI Gencom, Inc., Fairfield, NJ, Model 9816B) which was powered by a constant-voltage power supply (Bertran Associates, Model 215). The PMT was cooled to approximately 5 °C by a refrigerated circulating bath (Neslab Instruments, Inc., Portsmouth, NH, Model RTE-8). The signal from the PMT was amplified by a picoammeter (Keithley Instruments, Inc., Cleveland, OH, Model 485), digitized, and then sent to a laboratory computer (80286 based) via an IEEE-488 interface (National Instruments, Inc., Austin, TX, Model PC-2A).

**Procedure.** A 0.1 M solution of 3-octylriboflavin in methanol was prepared and the hydrophobically modified end of the optical fiber immersed in this solution for 3 min. The fiber was removed from the modifying solution, rinsed with water, and then air-dried. The modified fiber was then placed in a pH 7.3 buffer solution and the fluorescence measured. The modified 3-octylriboflavin was removed from the fiber by immersion of the tip in methanol.

For the study of the interaction of the dynamically associated 3-octylriboflavin with riboflavin binding protein (RBP), the optical fiber was first modified as described above and then immersed in 10 $\mu$L of solution containing varying amounts of RBP. The fluorescent signal was monitored for 10 min, the surface was renewed (washed and remodified), and another measurement was begun. A blank measurement was obtained for the buffer solution without RBP. All concentrations of RBP tested were repeated a minimum of three times.

## RESULTS AND DISCUSSION

The association of riboflavin with riboflavin binding protein (RBP) has been extensively studied and the interior of RBP is known to be fairly hydrophobic. Spectroscopic studies of riboflavin bound to RBP have shown that the 7,8 methyl groups reside in a hydrophobic environment in the complex, while the N-3 position appears to be outside the protein cavity and sees primarily bulk solution.[16] This suggests that modification of riboflavin at the N-3 position would enhance its hydrophobicity while minimizing the effect of the modification on its association with RBP. However, standard synthetic procedures were found to add $C_8$ groups to the active ribityl side chain in addition to the N-3 position.

Riboflavin was successfully modified at the N-3 position with a $C_8$ moiety by first acetylating the reactive hydroxyl groups on the sugar.[17,18] The acetylated hydroxyl groups were then restored following alkylation by reflux in 2 N HCl. This procedure minimized the deterioration of riboflavin which is greatly accelerated in alkaline solutions.[19]

The synthesis of 3-octylriboflavin was monitored by $^1H$ NMR, $^{13}C$ NMR, FT-IR, UV/vis, and fluorescence spectroscopies. $^1H$ NMR analysis clearly showed that the acetylation, alkylation, and restoration steps were successful. The identity of the final product was further confirmed by $^{13}C$ NMR and IR analysis. Exact assignment of both NMR resonances and IR bands has been reported.[20] However, attempts to obtain molecular weight information on the 3-octylriboflavin from mass spectral data were unsuccessful. If one assumes that the molar absorptivity of 3-octylriboflavin is identical to that of riboflavin, UV/vis absorption spectroscopy can be used to estimate the molecular weight of the modified species. Riboflavin has a molar absorptivity ($\epsilon$) of 1215 $M^{-1}$ $cm^{-1}$ at 465 nm. A 0.0386 g/L solution of 3-octylriboflavin demonstrated an absorbance of 0.0956. With the assumption noted above, the molecular weight of the modified riboflavin ($C_{25}H_{36}N_4O_6$) is determined to be 491, which is, within experimental error, identical to the theoretical value of 488. TLC and HPLC analysis of the product showed the presence of a single peak, in support of the spectroscopic data.

The fluorescence of 3-octylriboflavin was evaluated over a range in concentration spanning 5 orders-of-magnitude from $1 \times 10^{-10}$ to $1 \times 10^{-5}$ M. Riboflavin was also evaluated over

(16) Choi, J.; McCormick, D. B. *Arch. Biochem. Biophys.* **1980**, *204*, 41–51.
(17) McCormick, D. B. *J. Heterocycl. Chem.* **1970**, *7*, 447–450.
(18) McCormick, D. B. *J. Heterocycl. Chem.* **1974**, *11*, 969–977.
(19) *Merck Index*, 10th ed.; Windholz, M., Ed.; Merck and Co., Inc.: Rahway, NJ, 1983; pp 1064–1065.
(20) Ogasawara, F. K. Ph.D. Dissertation, University of Arkansas, Fayetteville, 1991.

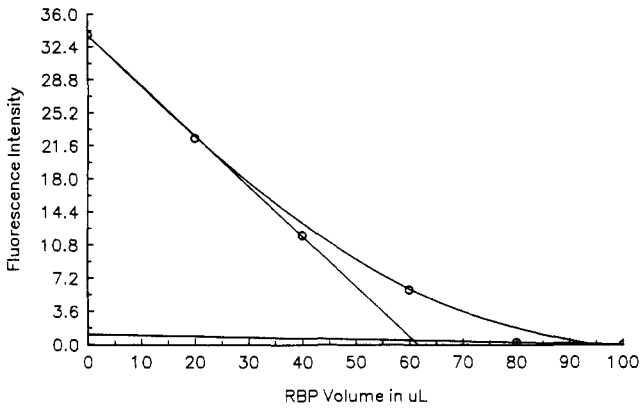


**Figure 2.** Titration of 3-octylriboflavin with RBP showing molar binding interaction. The concentration of 3-octylriboflavin was $8.8 \times 10^{-7}$ M, while the concentration of the binding protein was $1.8 \times 10^{-5}$ M. The open circles represent experimentally determined values. The region before and after the equivalence point has been emphasized by a best fit line to the data points. The intersection of the two regions represents the equivalence point for the binding of 3-octylriboflavin to RBP.

this span, and the modified and native riboflavin exhibited identical spectral characteristics. More important from the perspective of producing a sensor for RBP based on its association with 3-octylriboflavin is the ability of the modified riboflavin to complex with the binding protein. Solutions of riboflavin and 3-octylriboflavin were titrated with RBP using fluorometric detection. As discussed before, riboflavin fluorescence is effectively quenched upon binding to RBP. Figure 2 shows the fluorescence detected titration of RBP with 3-octylriboflavin. The open circles represent actual measured fluorescence intensities, while the curved line represents the best fit to the experimental data. To emphasize the equilvalence point, the best fit lines to the decending and postequilvalence point regions of the data are also shown. The intersection of the two lines gives a reasonable estimate of the equivalence point which is unbiased by equilibrium effects. From the experimental data depicted in Figure 2, it was determined that 3-octylriboflavin bound RBP in a molar ratio of 1.6:1, 3-octylriboflavin:protein. In contrast, riboflavin bound the protein in a molar ratio of 1:1. The decreased binding efficiency of the modified riboflavin may be due to hydrophobic repulsion between the modified hydrophobic riboflavin and the hydrophilic exterior of the protein. However, the reduction in binding efficiency is minimal and did not affect the use of 3-octylriboflavin as an associating ligand for RBP.

The procedure utilized to modify the optical fiber surface with an octadecyl moiety was chosen to maximize the coverage of the surface with the $C_{18}$ functionality.[21] Although the surface coverage of the optical fiber was not quantitatively determined here, previous studies utilizing high surface area silica has shown that this procedure will provide surface coverages on the order of 3 $\mu$mol/cm$^2$.[21] Endcapping was also considered unnecessary as recent reports have shown that the procedure utilized for the fiber modification provides effective insulation of unreacted silanol sites due to steric hindrance from the octadecyl moiety.[22] To evaluate the procedure, 1-cm section of optical fiber were stripped of their protective jacket and modified and diffuse reflectance infrared Fourier transform spectroscopy was used to follow the surface modification. Comparison of bare and modified fibers showed a series of three bands in the region between 3000 and 2800 cm$^{-1}$. A summary of these bands in given in Table I. The

**Table I. Summary of Hydrocarbon Bands Obtained with $C_{18}$-Modified Optical Fibers**

| experimental (cm$^{-1}$) | ref (cm$^{-1}$)[23] | description |
|---|---|---|
| 2958 | 2958 | $-CH_3$ |
| 2922 | 2915 | $-CH_2-$ |
| 2855 | 2848 | $-CH_2-$ |

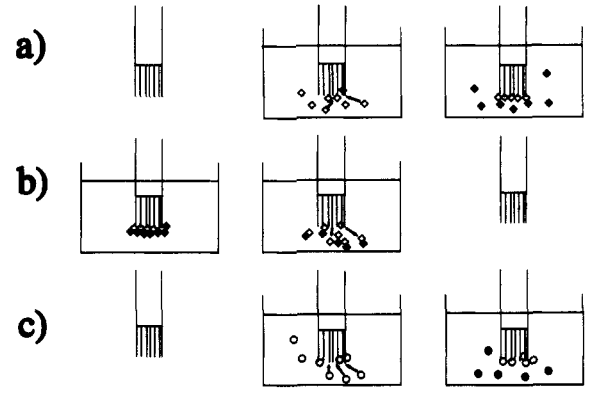


**Figure 3.** Development of fiberoptic biosensors by dynamic modification: (a) dynamic modification with diamond antigens for diamond antibody assay; (b) removal of dynamically modified reagent phase; (c) dynamic modification with circle antigens for circle antibody assay.

bands observed for the modified fiber were not present in the unmodified one. Thus the spectral evidence suggests that the fiber surface was modified with a hydrocarbon phase. Physical evidence for the modification was also evident as it was noted that the modified fibers were buoyant in water while the unmodified fibers did not float.

To further evaluate the potential of this synthetic route to provide a hydrophobic surface on the optical fiber for the purposes of dynamic modification, silica gel was modified through an identical procedure. Unmodified gel was then compared with the $C_{18}$-modified silica with respect to its ability to associate 3-octylriboflavin. The high surface area of the silica gel allowed the dynamic modification procedure to be followed visually. It was found that the unmodified silica did not adhere any 3-octylriboflavin after washing with water, while the $C_{18}$-modified silica strongly retained the fluorescent marker. This was evident from the bright yellow color of the silica after exposure to the 3-octylriboflavin. Washing with water did not diminish the intensity of the color. However, washing with methanol completely removed the modifying species leaving the silica gel white. This suggests that the dynamic association of 3-octylriboflavin with a hydrophobically modified optical fiber should be possible.

As discussed previously, the objective of these studies was to develop an optical fiber based assay for riboflavin binding protein using a dynamic modification procedure as a means to evaluate a new, potentially universally applicable method for the production of optical fiber biosensors. The dynamic modification approach requires that the fiber surface and the associating ligand possess similar properties which would drive the ligand to preferentially associate with the fiber surface rather than enter the surrounding solution. Hypothetically, a variety of different sensing systems could be produced from the same optical fiber. Since the associating surface is constant for a given fiber, measurement-to-measurement precision should be improved. Further, dynamic modification allows one to easily renew the sensing surface if the sensor performance degrades.

The dynamic modification procedure is schematically depicted in Figure 3. The permanent modification chemically transforms only the distal end of the optical fiber surface to provide a surface which dynamically attracts reagents that are similar to it. As depicted in Figure 3, ligand removal

(21) Aue, W. A.; Hastings, C. R. *J. Chromatogr.* **1969**, *42*, 319–327.
(22) Nawrocki, J.; Buszewski, B. *J. Chromatogr.* **1988**, *449*, 1–12.
(23) Anthoni, U.; Larsen, C.; Nielsen, P. H.; Christophersen, C. *Anal. Chem.* **1987**, *59*, 2435–2436.

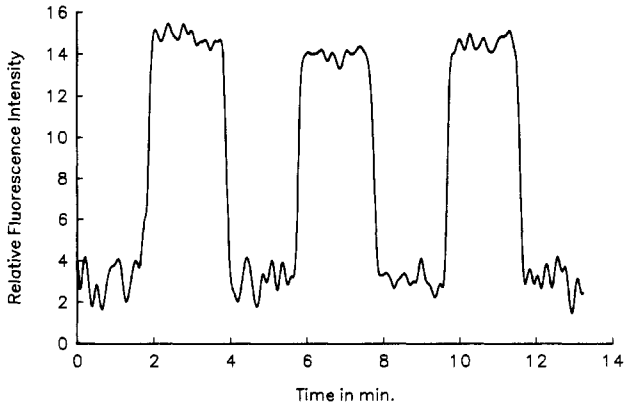


**Figure 4.** Regeneration and removal of 3-octylriboflavin from the hydrophobically modified optical fiber surface.

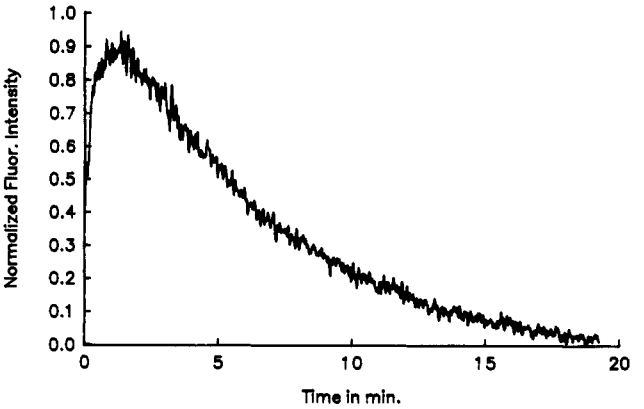


**Figure 5.** Observed fluorescence decay due to quenching of 3-octylriboflavin fluorescence upon binding to RBP. The test solution consisted of 10 μL of a 1 × $10^{-5}$ M solution of RBP.

requires only that the fiber be immersed in a solvent system in which the ligand is soluble. There are other interactions which could be exploited for dynamic modification which involve electrostatic attraction,[24,25] hydrogen bonding,[25] or hydrophilicity.[26] The approach thus can be made general by application of the appropriate interaction.

To demonstrate the regenerability and reproducibility of the dynamic modification approach, a hydrophobically modified optical fiber was repetitively modified with 3-octylriboflavin. Fluorescence originating at the fiber tip was monitored as the fluorescent ligand was alternately associated with and then removed from the fiber surface. Representative results from such a procedure are shown in Figure 4. From Figure 4, several points should be noted. First, it is clear that the hydrophobically modified optical fiber surface can associate 3-octylriboflavin in sufficient quantity such that the resulting fluorescence is easily measured. It has been determined that much of the experimental noise evident in the data of Figure 4 is a consequence of the way the data were sampled. Recently, substantial reduction in this noise has been observed using gated detection of the fluorescence signal. Second, the dynamic modification procedure does provide a regenerable and renewable sensing surface. One optical fiber was modified 15 times over a 1-h period. For these 15 experiments, the residual fluorescence of the fiber between modification steps, which is a measure of the effectiveness of removal of the modifying ligand, did not change from that observed with the fiber before any modification had been effected. In addition, by consideration of the magnitude of the fluorescence observed for a given modification event, over the 15 separate modifications the fluorescence intensity varied by less than 5%. Although this reproducibility only refers to regeneration of the optical fiber surface with the sensing ligand, this result is significant since it shows the potential for precise experiment-to-experiment correlation and it is due to the fact that the adhering surfaces are identical for all of the 15 sensors produced. In comparison with other biosensor systems, sensor-to-sensor reproducibilities on the order of 5–10% have been reported,[27-30] Thus, as demonstrated in these preliminary investigations, the dynamic modification procedure can provide experimental reproducibilities which are as good as or better than existing technologies. The 5% reproducibility reported here is actually an upper limit since the primary source of error with the current configuration can be traced to the inability to reproducibly position the optical fiber in the sample at the same distance from the bottom of the Teflon sample holder.

To evaluate the 3-octylriboflavin-modified optical fiber as a sensor for RBP, the fluorescence originating from the distal end of the fiber was monitored as the fiber was immersed in 10-μL samples containing varying amounts of the binding protein. One immediate concern was to determine whether the 3-octylriboflavin could leach from the hydrophobic surface into the buffer system. This would degrade the performance of the sensor and provide an anomalous signal which would be difficult to differentiate from quenching due to binding. Initial experiments showed that the fluorescence measured in the buffer solution decreased by approximately 10% over a 5-min period. Since the 3-octylriboflavin was dissolved in methanol for the dynamic modification procedure, it was proposed that the hydrophobic surface of the fiber could also associate methanol which would facilitate the leaching of the modification ligand into the buffer. To circumvent this limitation, the fiber was first immersed in the solution of 3-octylriboflavin, and the fiber tip was then dried by a gentle flow of air prior to immersion in the buffer solution. The drying step effectively eliminated the 3-octylriboflavin leaching and the measured fluorescence, within experimental error, was found to be constant over a 20-min period.

To evaluate the quantitative capabilities of the RBP sensor, a 3-octylriboflavin-modified optical fiber was immersed in buffered solutions containining varying amounts of the binding protein. Representative data from such an experiment are given in Figure 5. As predicted, the fluorescent intensity decreased with time as the 3-octylriboflavin complexed with RBP. Fluorescence intensity was monitored for at least 10 min although sufficient data were obtained in approximately 1 min to determine the rate of fluorescence quenching caused by the binding of RBP and 3-octylriboflavin. Large changes in the measured fluorescence were evident in the first 2–3 min following immersion; however, for the purposes of quantitation, data starting at 5 min were used. Although the rate of fluorescence quenching was decreased by this time, the data were found to be more reproducible. The initial large change may be indicative of RBP in the solution immediately adjacent to the modified surface binding to 3-octylriboflavin sites, while the longer time decay reflects diffusion of RBP to the surface from more remote locations. The initial response may eventually be sampled effectively; however, for the procedures used for these

(24) Werner, T. C.; Cummings, J. G.; Seitz, W. R. *Anal. Chem.* **1989**, *61*, 211–215.
(25) Shaksher, S. M.; Seitz, W. R. *Anal. Chem.* **1989**, *61*, 590–593.
(26) Snyder, L. R.; Kirkland, J. J. *Introduction to Modern Liquid Chromatography*, 2nd ed.; John Wiley and Sons: New York, 1979; Chapter 6.
(27) Alarie, J. P.; Sepaniak, M. J.; Vo-Dinh, T. *Anal. Chim. Acta* **1990**, *229*, 169–176.
(28) Alarie, J. P.; Bowyer, J. R.; Sepaniak, M. J.; Hoyt, A. M.; Vo-Dinh, T. *Anal. Chim. Acta* **1990**, *236*, 237–244.
(29) Bright, F. V.; Betts, T. A.; Litwiler, K. S. *Anal. Chem.* **1990**, *62*, 1065–1069.
(30) Choquette, S. J.; Locascio-Brown, L.; Durst, R. A. *Anal. Chem.* **1992**, *64*, 55–60.

studies in which the fiber must be dynamically modified, dried, and then immersed in the RBP solution, the data were very sensitive to the modification conditions, including the length of time the fiber was out of solution during drying. Future studies need to address this point so that data procurement can begin at times soon after immersion.

To determine that the sensors response was selective for RBP and not indicative of nonspecific adsorption of protein to the fiber surface, the dynamically modified optical fiber was immersed in a solution containing albumin in place of RBP. At concentrations of albumin 1 order-of-magnitude greater than the highest RBP concentration tested, the measured fluorescence remained constant over a 15-min period. When the albumin solution was removed and the fiber immediately immersed in a solution of RBP, the measured fluorescence began to decrease, indicative of RBP binding. Thus the dynamic modification procedure preserves the biospecific interactions between RBP and riboflavin, and over the time period required for measurement, it does not appear that nonspecific adsorption will significantly interfere with the measurement process.

Data for solutions spanning the range from $7.8 \times 10^{-11}$ to $2.1 \times 10^{-9}$ mol of RBP were tested with the dynamically modified sensor. The rate of fluorescence decay resulting from complex formation was measured over a fixed time period of 5 min. From a plot of the rate of fluorescence decay versus moles of RBP, a linear response was evident, providing a correlation coefficient of 0.998. The standard error of the slope of a buffer blank was determined to be $4 \times 10^{-4}$ V $s^{-1}$, while $7.8 \times 10^{-11}$ mol of RBP provided a signal of $8.5 \times 10^{-3}$ V $s^{-1}$. From this data, a minimum measurable quantity (MMQ) of $7.3 \times 10^{-12}$ mol of RBP in the 10-$\mu$L probed volume is estimated at a signal-to-noise ratio of 2.

## CONCLUSIONS

A new approach for the development of optical fiber sensors has been demonstrated which is based on a dynamic modification procedure. In measurement situations where nonreversibility is a concern or in situations where the reagent is consumed as a consequence of the measurement process, this approach can provide significant advantages. These advantages derive from the ease with which specific reagent phases can be removed and renewed on the optical fiber surface. A single optical fiber was used to produce 15 individual sensors which varied in performance by less than 5%. This level of sensor-to-sensor reproducibility is as good as the best reports to date using other methods which rely upon covalent attachment of the sensing ligand to the fiber surface. Using this technique, an optical fiber based sensor for RBP has been developed which utilizes hydrophobically associated 3-octylriboflavin. The dynamically modified optical fiber sensor can respond to RBP at the 0.7 $\mu$M level. Since the goal of these studies was to investigate the feasibility of the approach, minimal effort was expended to improve the quantitative capability of the sensing system although further improvements are expected using signal averaging and a more reproducible method to position the sensor in the sample holder. The time response of the sensor is longer than anticipated and may be a consequence of both the hydrophobic surface and the rate with which analyte can diffuse to the surface. Finally, a number of different interactions have been identified which could be applied to this dynamic modification approach thereby expanding the generality of the method.

## ACKNOWLEDGMENT

This research was supported by the National Institutes of Health through Grants 2 S07 RR07101-10 and 1 R03 RR04236-01, and by the Camille and Henry Dreyfus Foundation through a teacher-scholar fellowship (D.R.B.) We acknowledge the assistance of Mr. Marvin Leister in obtaining the NMR spectra.

RECEIVED for review January 23, 1992. Accepted April 28, 1992.