JOURNAL OF BIOMEDICAL OPTICS 4(4), 443–449 (OCTOBER 1999)

POLARIZATION-BASED SENSING OF GLUCOSE USING AN ORIENTED REFERENCE FILM

Joseph R. Lakowicz,[†] Ignacy Gryczynski,[†] Zygmunt Gryczynski,[†] Leah Tolosa,[†] Lisa Randers-Eichhorn,[‡] and Govind Rao[‡]

[†]University of Maryland School of Medicine, Department of Biochemistry and Molecular Biology, Center for Fluorescence Spectroscopy, 725 West Lombard Street, Baltimore, Maryland 21201; [‡]Medical Biotechnology Center and Department of Chemical and Biochemical Engineering, University of Maryland Biotechnology Institute, 725 West Lombard Street, Baltimore, Maryland 21201

(Paper JBO-227 received December 28, 1998; revised manuscript received August 12, 1999; accepted for publication August 16, 1999.)

ABSTRACT

We describe a new approach to glucose sensing using polarization measurements in the presence of a stretch-oriented reference film. The method relies on measurement of the polarized emission from the reference film and of a fluorophore which changes intensity in response to glucose. A glucose-sensitive fluorescent signal was provided by the glucose/galactose binding protein from *E. coli*. This protein was labeled with an environmentally sensitive fluorophore at a single genetically inserted cysteine residue, and displayed decreased fluorescence upon glucose binding. Using the protein and the reference film we observed glucose-sensitive polarization values for micromolar glucose concentrations. This method of polarization-based sensing is generic and can be used for any sensing fluorophore which displays a change in intensity. In principle, one can construct simple and economical devices for this type of glucose measurement. *© 1999 Society of Photo-Optical Instrumentation Engineers.* [S1083-3668(99)01204-6]

Keywords glucose; sensing; diabetes; transdermal sensing; fluorescence polarization; protein sensors.

1 INTRODUCTION

During the past several years we have seen the rapid introduction of new methods of fluorescence sensing.[1–3] These methods include the use of time-resolved fluorescence for sensing,[4–6] the development of red and near infrared probes,[7,8] and the introduction of long lifetime transition metal complexes.[9–11] Within the past year there have been several reports describing the use of reference fluorophores in sensor design. The basic idea is to use the reference to provide a constant internal standard, and to eliminate the dependence on the total intensity. In such sensors one measures the combined emission from the reference and of an analyte-sensitive fluorophore. This approach has been used with a long lifetime metal-ligand complex as the reference for sensing of pH and pCO_2.[12,13] We used a similar reference with low frequency modulation measurements to develop sensors for pH, calcium, and glucose.[14–16]

The approaches described above for combined sensors are based on phase-modulation fluorometry, and thus require an amplitude-modulated light source. To further simplify the design and construction of sensors we recently reported the development of anisotropy-based sensing.[17,18] Anisotropy measurements have previously been described for metal ion sensing.[19,20] In these reports[19,20] the sensing mechanism relied on the presence of a change in the anisotropy of the sample in response to the analyte. While this may seem to be a requirement for sensing, our approach to anisotropy-based sensing does not depend on anisotropy changes in the sample. Instead, we measure the combined emission from a polarized reference film and of the analyte-sensitive sample. The sample and optical conditions are such that the emission from the sample has an apparent polarization of 0 or −1, the latter value due to a horizontally oriented polarizer. A change in the intensity of the analyte-sensitive fluorophore results in a change in polarization of the combined emission. For clarity, we note the anisotropy and polarization are two measures of the same phenomena. In the present report we demonstrate the validity of this approach using samples of varying intensity, demonstrating the functional behavior of a polarization sensor. We then demonstrate glucose sensing using the glucose-galactose binding protein from *E. coli*, which when suitably labeled is known to display glucose-dependent changes in intensity.[14,21]

2 MATERIALS AND METHODS

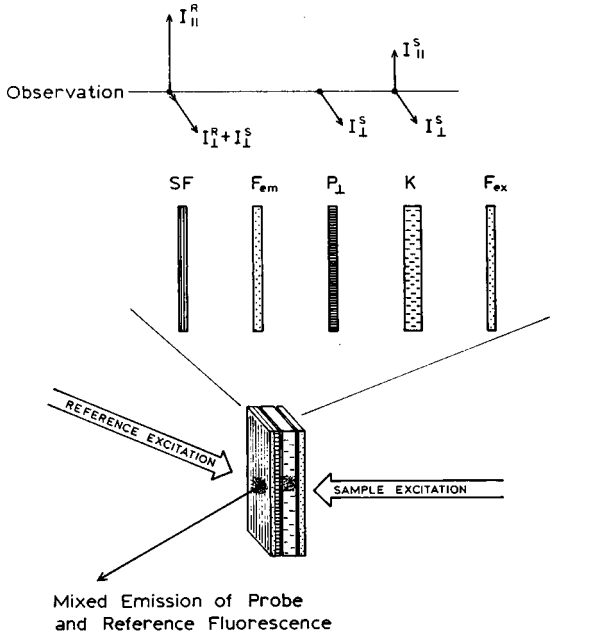
2.1 POLARIZATION SENSOR DESIGN

All experiments were performed using the apparatus shown in Scheme I. The sensor was contained within the T-format optical module of a SLM 8000

Address all correspondence to Joseph Lakowicz. Tel: 410-706-8409; Fax: 410-706-8408; E-mail: jf@cfs.umbi.umd.edu

1083-3668/99/$10.00 © 1999 SPIE

spectrofluorometer. Two light sources were used.



F_{ex} – Excitation Filter, SWP, $\lambda < 390$ nm, UV hand lamp
K – Cuvette with Probe
$P_{\perp}$ – Polarizer. Only the horizontal component of the probe fluorescence is transmitted.
F_{em} – Emission Filter, LWP, $\lambda > 410$ nm
SF – Stretched Film, Vertically Oriented, HOECHST 33342 Doped PVA

Scheme I. Experimental setup for anisotropy-based sensing with separate excitation of the sample and polarized reference. The reference excitation at 380 nm was isolated from a xenon arc lamp using a monochromator. The sample excitation centered at 390 nm was isolated from a UV hand lamp.

The sample was excited using a UV hand lamp with a filter to select wavelengths below 390 nm. The reference film was excited using the monochromator output from the SLM 8000 instrument, repositioned for front face excitation using mirrors. The combined emission of the sample and reference film was observed from the front surface of the sensor.

Several features of the sensor are worthy of detailed explanation. There is a polarizer ($P_{\perp}\rangle$ in the perpendicular orientation distal from the sample and sample excitation source. This polarization insures that the emission from the sample contributes only to the perpendicular total intensity (see Theory below). Because of this polarizer there is no need for the sensing fluorophore to display polarized emission, or a change in polarization in response to the analyte. A change in sample intensity results in a change in perpendicular sample intensity ($I_{\perp}^{S}$, top of Scheme I), and thus a change in the polarization measured for the combined emission. The use of this polarizer also allows for a wide range of polarization values (+1 to −1).

The reference signal is provided by a stretched film of Hoechst 33342 (HOE) in polyvinyl alcohol (PVA) film. When stretched the elongated HOE molecules are aligned along the stretching axis. Such film displays polarized emission even when excited with unpolarized light. Hence there is no need for a polarizer in front of the excitation source for the reference film.

While the presently used configuration may seem complex (Scheme I), it was ideally suited for these initial experiments to develop polarization-based sensing. The relative intensities of the reference film and of the sample could be adjusted by attenuating either of the excitation sources. This adjustability was important because the glucose-sensitive protein displays less than a twofold change in intensity in response to glucose.[14] Optimal changes in the polarization required adjustment of the relative sample and reference intensities. In future reports we will consider alternative optical configurations for polarization sensing. However, the use of two excitation sources does not seem limiting with modern solid state sources, such as light emitting diodes, laser diodes, or electroluminescent devices. In any event, it is clear that similar results could have been obtained with a single light source.

2.2 REFERENCE FILM

Hoechst 33342 was obtained from Molecular Probes, Inc. Polyvinyl alcohol films were prepared according to published procedures.[22,23] The process consists of dissolving the PVA in water and polymerizing at ~360 °K. HOE in methanol was added to a 10%–15% aqueous solution of polyvinyl alcohol. The HOE containing PVA solution was then cast on a plate, and dried over the period of several days in dust-free atmosphere. The PVA films were stretched at about 350 °K up to sixfold to orient the HOE molecules. The stretching ratio (R_S) is defined as the axial ratio a/b of an ellipse which is formed when stretching an imaginary circle in the unoriented film.[23] The volume of the circle or ellipse is assumed to be conserved. Under these conditions

$$R_S = N^{3/2}, \tag{1}$$

where N is the physical fold of the stretch.

2.3 SAMPLE PREPARATION

Human serum albumin (HSA) was obtained from Sigma, Inc., and used without further purification. The HSA concentration was 3.3 mg/ml or 5×10^{-5} M. Solutions of 8-aniline-4-naththalenesulfonic acid (ANS) was also obtained from Sigma, Inc., and its concentration determined using $\epsilon(372\,\text{nm}) = 7800\,\text{M}^{-1}\,\text{cm}^{-1}$. Solutions of HSA and ANS were in 0.05 M phosphate buffer, pH 7.

The glucose assay was accomplished using the glucose/galactose binding protein from *E. Coli*. We used a mutant which contained a single cysteine residue at position 26.[14] This position was labeled

with 2-(4′-iodoacetamidoanilino)naphthalene-6-sulfonic acid (I-ANS) from Molecular Probes, Inc. The labeled protein was purified as described previously,[14] and dissolved in 20 mM phosphate, pH 7.0.

3 THEORY

At first glance it may appear that the theory describing the polarization values from the apparatus in Scheme I would be complex. However, the range of polarization values, and factors which affect the polarization, can be readily explained by some simple considerations. The polarization of the combined emission from the sample and reference is given by

$$P = \frac{I_{\parallel}^{T} - I_{\perp}^{T}}{I_{\parallel}^{T} + I_{\perp}^{T}}, \tag{2}$$

where the superscript T indicates the sum of the intensities from the sample and reference. The subscripts indicate the parallel ($\parallel$) and perpendicular ($\perp$) components of the emission. The total parallel intensity is given by

$$I_{\parallel}^{T} = I_{\parallel}^{R} + I_{\parallel}^{S}, \tag{3}$$

where the superscripts R and S refer to the reference film and sample, respectively. Similarly

$$I_{\perp}^{T} = I_{\perp}^{R} + I_{\perp}^{S}. \tag{4}$$

Each intensity depends on a number of factors, including the excitation intensity, probe concentration, filter transmission, polarizer efficiency, quantum yield, observation wavelength, and the intensity of each light source. For simplicity we chose not to explicitly indicate these multiple factors.

The use of a polarizer ($P_{\perp}$) in front of the sample (K) results in elimination of the vertical component, so that $I_{\parallel}^{T} = I_{\parallel}^{R}$. Substitution of Eqs. (3) and (4) into the definition of polarization [Eq. (2)] yields

$$P = \frac{I_{\parallel}^{R} - I_{\perp}^{R} - I_{\perp}^{S}}{I_{\parallel}^{R} + I_{\perp}^{R} + I_{\perp}^{S}}. \tag{5}$$

The measured polarization depends on the relative intensity of the sample to that of the reference film. If the sample fluorescence is zero then the polarization is given by

$$P = \frac{I_{\parallel}^{R} - I_{\perp}^{R}}{I_{\parallel}^{R} + I_{\perp}^{R}} = P_R, \tag{6}$$

which is that found for the reference film (P_R). If the observed intensity is dominated by the sample emission, then the polarization approaches a value of −1

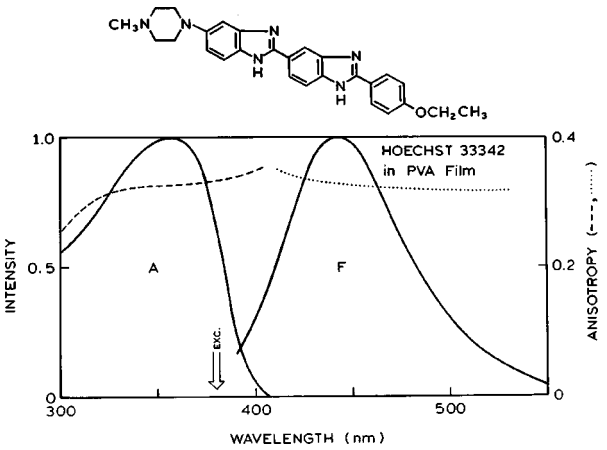


Fig. 1 Absorption, fluorescence, and polarization spectra of the reference fluorophore—Hoechst 33342 in an unoriented PVA film.

$$P = \frac{-I_{\perp}^{S}}{I_{\perp}^{S}} = -1. \tag{7}$$

The reference films typically display polarization values near 0.8. Hence one may expect a wide range of polarization values for this sensor configuration.

Using Eq. (5) one can readily imagine how the measured spectral properties of the reference and sample would affect the observed polarization. For instance, increasing the intensity of the reference film will shift the polarization value towards P_R. Similarly, increasing the intensity of the sample, or of the sample excitation intensity, will shift the polarization value towards −1. Selection of the observation conditions to select the sample emission with greater efficiency than the film emission would also shift the polarization towards −1. The configuration shown in Scheme I is highly versatile, allowing adjustment for the optical properties of the light sources, filters, samples, and reference films.

4 RESULTS

4.1 REFERENCE FILM

Prior to demonstrating the possibility of polarization sensing, it is valuable to understand the properties of the HOE/PVA film. Absorption and emission spectra of the stretched films are shown in Figure 1. Hoechst 33342 displays a good Stokes' shift, which results in minimal loss of polarization due to homo resonance energy transfer. Hence, the concentration of HOE in the film can be larger than practical for dyes like fluorescein which display a smaller Stokes' shift. The anisotropy is high in the PVA film because the HOE molecules are immobile during the excited state lifetime in this viscous media. The anisotropy is mostly constant across the long wavelength absorption band and the emission spectrum, providing a high anisotropy at all useful

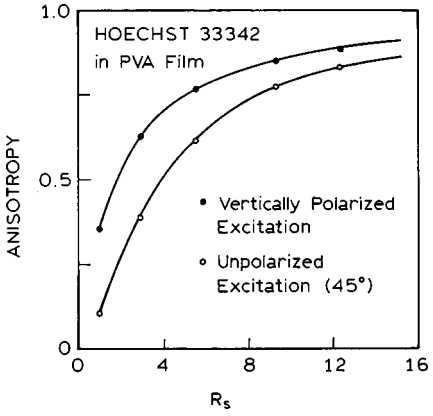


Fig. 2 Dependence of the fluorescence anisotropy of Hoechst 33342 in PVA on the stretching ratio $R_S = N^{3/2}$, where N is the physical fold of the stretch (see Ref. 23).

wavelengths. And finally, the excitation and emission spectra of the film are comparable to those needed for the ANS-labeled protein.

While Hoechst 33342 in the unoriented film could be used as the reference, we chose to obtain a higher anisotropy using a stretched film. The effect of stretching on the steady state anisotropy is shown in Figure 2. A stretching ratio of 12 results in an anisotropy near 0.85 when using vertically polarized excitation. An important property of the stretched films is that they provide highly polarized emission even when excited with unpolarized light. This is shown in Figure 2 for the excitation polarization oriented at 45°, which is equivalent to excitation with unpolarized light. Prior to stretching, the anisotropy values with 45° excitation are one-half of that found with vertically polarized light, which is the result expected from theory.[24] As the film is stretched the anisotropy with unpolarized excitation increases rapidly, and approaches the values found with polarized excitation. This effect occurs because the electronic transitions are oriented along the stretching axis. The orientation provides the same effect as the polarizer, to orient the emission dipoles vertically in the laboratory axis.

4.2 POLARIZATION SENSING OF ANS CONCENTRATIONS

We examined the properties of our polarization sensor (Scheme I) using solutions of ANS bound to human serum albumin. It is well known that the fluorescence intensity of ANS increases upon binding to proteins. The HSA concentration was maintained at a high constant concentration of 0.05 mM to insure that all the ANS remained bound to the protein.

Emission spectra of the HOE/PVA film alone, ANS-HSA alone, and of the combined sample are shown in Figure 3 (top). The emission maximum of ANS-HSA is at a longer wavelength than HOE/PVA. This results in a shift of the emission maxima

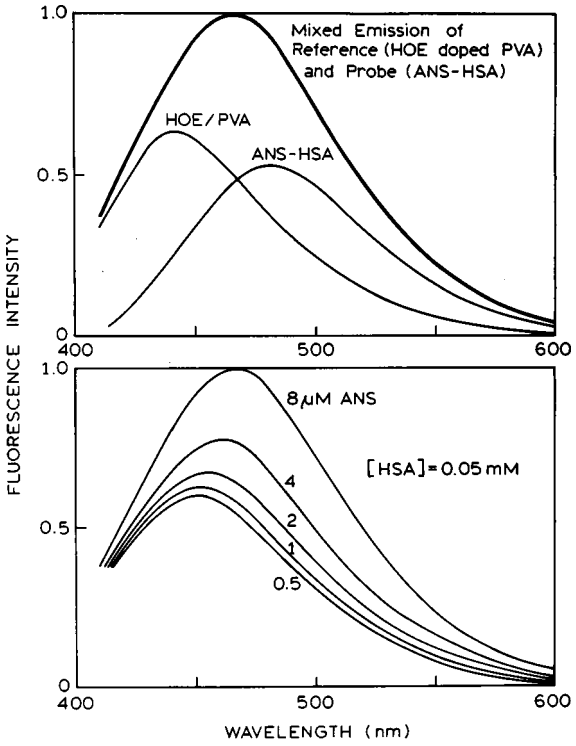


Fig. 3 Fluorescence spectra of the reference film HOE/PVA, the probe ANS-HSA, 8 μM ANS, and the total emission (top). The lower panel shows the combined emission spectra for HOE/PVA and various concentrations of ANS at a constant concentration of HSA.

for the combined emission as the ANS-HSA concentration increases (Figure 3, bottom).

We measured the emission wavelength-dependent polarization for the combined emission from the reference film and ANS-HSA (Figure 4). The polarization decreases for increasing concentrations of ANS-HSA. Also, the polarization decreases most dramatically on the long wavelength side of the emission, which is predominantly due to ANS-HSA. The polarization remains high at shorter

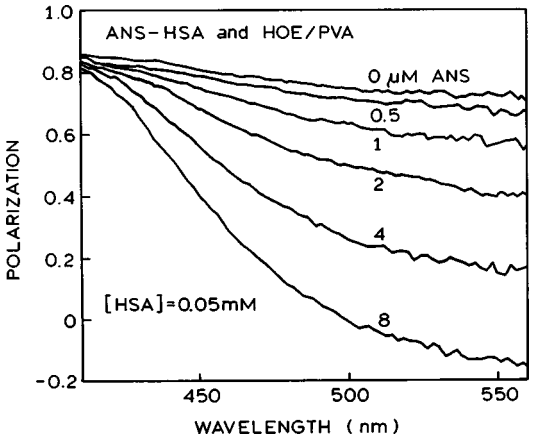


Fig. 4 Dependence of the polarization of the combined emission of HOE/PVA and ANS-HSA on observation wavelength.

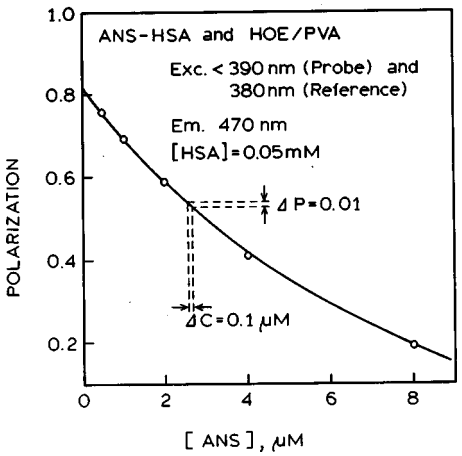


Fig. 5 Calibration curve for a polarization assay for the concentration of ANS-HSA.

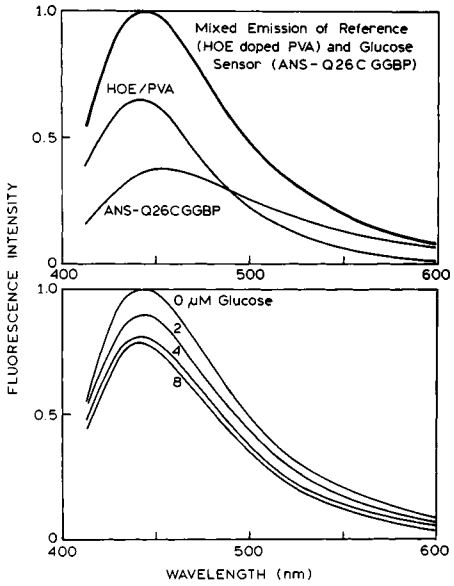


Fig. 6 Top: Fluorescence spectra of the reference (HOE/PVA), glucose sensor ANS-Q26C, and combined fluorescence of both (thicker line). Bottom: Glucose dependent total fluorescence of HOE/PVA and ANS-Q26 GGBP.

wavelengths because the emission at these wavelengths is dominantly due to the HOE/PVA film.

It is important to notice that the range of polarization values is very large, from 0.8 to −0.2. This large range is due to the high polarization of the reference film, and to the use of a perpendicular polarizer after the sample (Scheme I). This polarizer results in the sample contributing a negative polarization of −1 to the combined emission. Hence, one can expect the polarization to reach still lower values for higher concentrations of ANS.

We used the wavelength-dependent polarization values of 470 nm to develop a calibration curve for the ANS concentration (Figure 5). The polarization decreases as the concentration of ANS-HSA increases. Polarization values can easily be measured to ±0.01, which indicates the ANS concentration will be accurate to ±0.1 μM, or one part in 80. Hence, for sensors which display large changes in intensity due to the analyte, one can expect a high accuracy close to ±1%.

4.3 POLARIZATION SENSING OF GLUCOSE

Measurement of glucose is of widespread interest in the health care of diabetics. This is because there are long-term adverse health consequences of elevated blood glucose levels. A wide range of technologies have been used in attempts to develop more convenient and/or less invasive methods to measure blood glucose. The methods include near-infrared spectroscopy,[25–27] optical rotation,[28,29] colorimetric[30,31] and fluorescence detection.[32–34] In spite of these extensive efforts there remains the need for minimally invasive methods to measure blood glucose. Our own efforts are focused on methods to detect the low glucose concentrations which are known to be present in interstitial fluid.[35,36]

Our approach to glucose sensing is to use the glucose/galactose binding protein (GGBP) from *E. coli*. This protein binds glucose with a dissociation constant near 0.8 μM,[20,37] and has already been used to measure micromolar glucose concentrations.[14,21] This was accomplished by introducing a single cysteine residue into the sequence, and labeling this residue with an environmentally sensitive fluorophore.

In our use of GGBP the single cysteine residue was introduced at position 26, which was then labeled with I-ANS (ANS-Q26C GGBP). The emission intensity of the labeled protein is known to decrease by 50% upon binding glucose.[14] Addition of glucose results in a decreased intensity from the covalently bound ANS. This decrease has been interpreted as due to displacement of the ANS from a moderately hydrophobic site upon binding of glucose to GGBP. This results in a decrease in the intensity of ANS, which is known to be quenched in aqueous environments.

Because of this modest intensity change it is important to carefully balance the relative intensities from the labeled protein and the reference film in order to obtain the maximal possible change in polarization. This is an advantage of the configuration shown in Scheme I, the relative intensities can be adjusted without repeated changes of the Hoechst 33342 or protein concentration. The emission spectra shown in the top panel of Figure 6 illustrate that the intensities from the film and protein are of comparable intensity. These individual spectra were recorded by using just one of the excitation sources. When both sources are used, the emission maximum for the mixed emission is between the emission maxima of the film and protein, demonstrating that both contribute to the emission.

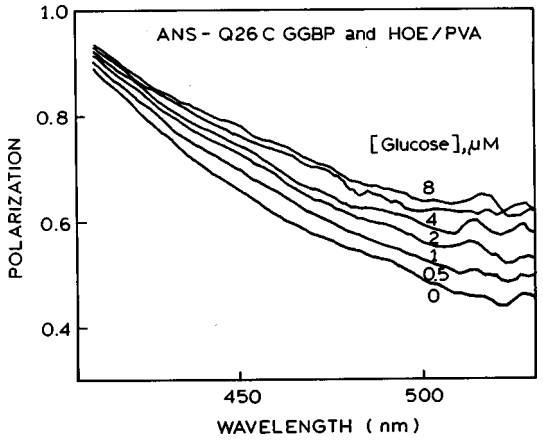


Fig. 7 Dependence of polarization of the glucose-dependent total emission on observation wavelength.

Emission spectra of the combined emission are shown in the lower panel of Figure 6. For the combined emission the glucose-dependent change is smaller, which is due to the constant intensity from the reference film. The change in emission maximum with changing glucose concentrations demonstrated that both the film and the protein contribute to the measured emission.

Since the reference film and labeled protein do not show the same emission maxima, the polarization should depend on the observation wavelength. Such a dependence was observed, with the polarization decreasing at longer wavelengths (Figure 7). We used these changes to develop a calibration curve for glucose (Figure 8). The polarization increases with increasing glucose concentration due to the decreasing intensity of ANS-Q26C GGBP with increasing amounts of glucose, and the increased relative contribution of the HOE/PVA film to the total emission. Based on polarization values accurate to ± 0.01, one can expect an accuracy of ± 0.2 μM in the glucose concentration. In the future we hope to obtain labeled derivatives of GGBP which display larger changes in intensity, which would provide higher accuracy in the glucose concentrations. In the present case the emission from the reference film was dominant, and became even more dominate as the glucose concentration increased. One could probably obtain a wider range of polarization values by selecting the initial intensities of the reference and sensing fluorophores to be optimal for a known intensity change due to the analyte.

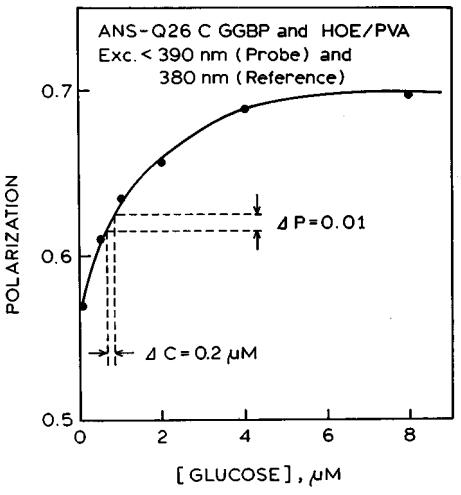


Fig. 8 Polarization based glucose sensing.

5 DISCUSSION

Chemical sensing based on polarization seems to have favorable properties for real-world applications. One advantage is that polarization measurements are intrinsically ratiometric. Also, only steady state measurements are needed. Another advantage is that a wide range of polarization values are possible, from 1.0 to −1.0 when using an oriented reference film and a perpendicular polarizer in front of the sensor.

Polarization sensing can be used with any sensing fluorophores which display a change in intensity, eliminating the need for ratiometric fluorophores. This can be especially advantageous for sensing of species such as chloride, oxygen, sodium, or potassium, for which the sensing fluorophores display little if any spectral shift.

With regards to glucose sensing, one can imagine a number of future possibilities. Changes in the amino acid sequence of GGBP can be used to modify the binding constant to the desired concentration range. If needed, more than one GGBP could be used to cover an extended range of glucose concentrations. In summary, our method of polarization sensing is rather generic, and can be used for a range of analytes.

While polarization sensing has many advantages, it is important to consider the potential problems. The analyte calibration curve depends on the relative intensities of the sample and reference fluorophores. Hence these concentrations must be carefully controlled in any device. The use of proximity focusing and a single light source should minimize these difficulties.

Another potential problem is using this method with turbid or absorbing solutions, which could change the measured intensity from the sensing fluorophore. In such cases it would be preferable to use a semipermeable membrane which allows the analyte to diffuse to the sensing fluorophore but which restricts the migration of interfering components in the sample. Nonetheless, it appears that polarization sensing of glucose can be accomplished with good reliability using the principles of polarization sensing.

Acknowledgments

This work was supported by a grant from the National Institutes of Health, National Center for Research Resources, for Grant No. RR-08119 to Joseph R. Lakowicz and Grant No. RR-10955 and RR-14170 and matching funds from Genentech, Merck, and Pfizer to Govind Rao.

REFERENCES

1. *Advances in Fluorescence Sensing Technology III*, R. B. Thompson, Ed., *Proc. SPIE* **2980**, 582 (1997).
2. Proc. of 3rd European Conference on Optical Chemical Sensors and Biosensors, Europt(R)odeIII, *Sens. Actuators B*, R. E. Kunz, Ed., Vol. B38, pp. 1–188, and Vol. B39, pp. 1–468, Elsevier, New York (1997).
3. U. E. Spichiger-Keller, *Chemical Sensors and Biosensors for Medical and Biological Applications*, p. 413, Wiley-VCH, New York (1998).
4. M. E. Lippitsch, S. Draxler, and D. Kieslinger, ''Luminescence lifetime-based sensing: New materials, new devices,'' *Sens. Actuators B* **38, 39**, 96–102 (1997).
5. M. E. Lippitsch, J. Pusterhofer, M. J. P. Leiner, and O. S. Wolfbeis, ''Fibre-optic oxygen sensor with the fluorescence decay time as the information carrier,'' *Anal. Chim. Acta* **205**, 1–6 (1988).
6. H. Szmacinski and J. R. Lakowicz, ''Lifetime-based sensing,'' in *Topics in Fluorescence Spectroscopy: Vol. 4: Probe Design and Chemical Sensing*, J. R. Lakowicz, Ed., pp. 295–334, Plenum, New York (1994).
7. R. B. Thompson, ''Red and near-infrared fluorometry'' in Ref. 6, pp. 151–181.
8. G. A. Casay, D. B. Shealy, and G. Patonay, ''Near-infrared fluorescence probes,'' in Ref. 6, pp. 183–222.
9. E. Terpetschnig, H. Szmacinski, and J. R. Lakowicz, ''Long-lifetime metal-ligand complexes as probes in biophysics and clinical chemistry,'' *Methods in Enzymology*, pp. 295–321, Academic (1997).
10. H. Szmacinski, F. N. Castellano, E. Terpetschnig, J. D. Dattelbaum, J. R. Lakowicz, and G. J. Meyer, ''Long-lifetime Ru(II) complexes for the measurement of high molecular weight protein hydrodynamics,'' *Biochim. Biophys. Acta* **1383**, 151–159 (1997).
11. F. N. Castellano, X-Q. Guo, L. Li, H. Szmacinski, J. Sipior, and J. R. Lakowicz, ''A Long-lived, high luminescent rhenium (I) metal-ligand complex as a probe of biomolecules,'' *Proc. SPIE* **3256**, 223–233 (1998).
12. G. Neurauter, I. Klimant, G. Liebsch, U. Kosch, and O. S. Wolfbeis, ''A comparative study on different types of intensity independent optical pCO_2 sensors,'' Europt(r)ode IV, pp. 231–232, German Chemical Society (1998).
13. I. Klimant and O. S. Wolfbeis, ''Dual luminophore referenced optodes: A convenient way to convert the fluorescence intensity into a phase shift or time dependent parameter,'' 125–126, in Ref. 12.
14. L. Tolosa, I. Gryczynski, L. Eichhorn, J. D. Dattelbaum, F. N. Castellano, G. Rao, and J. R. Lakowicz, ''Glucose sensor for low cost lifetime-based sensing using a genetically engineered protein,'' *Anal. Biochem.* **267**, 114–120 (1999).
15. J. R. Lakowicz, F. N. Castellano, J. D. Dattelbaum, L. Tolosa, G. Rao, and I. Gryczynski, ''Low frequency modulation sensors using nanosecond fluorophores,'' *Anal. Chem.* **70**, 5115–5121 (1998).
16. J. R. Lakowicz, J. D. Dattelbaum, and I. Gryczynski, ''Quantitative intensity measurements in scattering media,'' Sens. Actuators (in press).
17. J. R. Lakowicz, I. Gryczynski, Z. Gryczynski, and J. D. Dattelbaum, ''Anisotropy-based sensing with reference fluorophores,'' *Anal. Biochem.* **267**, 397–405 (1999).
18. I. Gryczynski, Z. Gryczynski, and J. R. Lakowicz, ''Polarization sensing with visual detection,'' *Anal. Chem.* **71**, 1241–1251 (1999).
19. R. B. Thompson, B. P. Maliwal, and V. L. Feliccia, ''Determination of picomolar concentrations of metal ions using fluorescence anisotropy: Biosensing with a ''reagentless'' enzyme transducer,'' *Anal. Chem.* **70**, 4717–4723 (1998).
20. R. B. Thompson and B. P. Maliwal, ''Expanded dynamic range of free zinc ion determination by fluorescence anisotropy,'' *Anal. Chem.* **70**(9), 1749–1754 (1998).
21. J. S. Marvin and H. W. Hellinga, ''Engineering biosensors by introducing fluorescent allosteric signal transducers: Construction of a novel glucose sensor,'' *J. Am. Chem. Soc.* **120**, 7–11 (1998).
22. A. Kawski and Z. Gryczynski, ''On the determination of transition-moment directions from absorption anisotropy measurements,'' *Z. Naturforsch.* **42a**, 617–621 (1987).
23. A. Kawski, Z. Gryczynski, I. Gryczynski, J. R. Lakowicz, and G. Piszczek, ''Photoselection of luminescent molecules in anisotropic media in the case of two-photon excitation. Part II. Experimental studies of Hoechst 33342 in stretched poly-(vinyl alcohol) films,'' *Z. Naturforsch.* **51a**, 1037–1041 (1996).
24. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 2nd ed., p. 698, Plenum, New York (1999).
25. H. M. Heise, R. Marbach, Th. Koschinsky, and F. A. Gries, ''Noninvasive blood glucose sensors based on the near-infrared spectroscopy,'' *Artif. Organs* **18**(6), 439–447 (1994).
26. J. J. Burmeister, H. Chung, and M. A. Arnold, ''Phantoms for noninvasive blood glucose sensing with near infrared transmission spectroscopy,'' *Photochem. Photobiol.* **67**(1), 50–55 (1998).
27. H. M. Heise, R. Marbach, Th. Koschinsky, and F. A. Gries, ''Noninvasive blood glucose sensors based on near-infrared spectroscopy,'' *Artif. Organs* **18**(6), 439–447 (1994).
28. W. F. March, B. Rabinovitch, R. Adams, J. R. Wise, and M. Melton, ''Ocular glucose sensor,'' *Trans. Am. Soc. Artif. Intern. Organs* **28**, 232–235 (1982).
29. B. Rabinovitch, W. F. March, and R. L. Adams, ''Noninvasive glucose monitoring of the aqueous humor of the eye: Part I. Measurement of very small optical rotations,'' *Diabetes Care* **5**(3), 254–258 (1982).
30. G. M. Schier, R. G. Moses, I. E. T. Gan, and S. C. Blair, ''An evaluation and comparison of reflolux II and glucometer II, two new portable reflectance meters for capillary blood glucose determination,'' *Diabetes Res. Clin. Pract.* **4**, 177–181 (1988).
31. W. Clarke, D. J. Becker, D. Cox, J. V. Santiago, N. H. White, J. Betschart, K. Eckenrode, L. A. Levandoski, E. A. Prusinki, L. M. Simineiro, A. L. Snyder, A. M. Tideman, and T. Yaeger, ''Evaluation of a new system for self blood glucose monitoring,'' *Diabetes Res. Clin. Pract.* **4**, 209–214 (1988).
32. J. S. Shultz, S. Mansouri, and I. J. Goldstein, ''Affinity sensor: A new technique for developing implantable sensors for glucose and other metabolites,'' *Diabetes Care* **5**(3), 245–253 (1982).
33. J. R. Lakowicz and B. P. Maliwal, ''Optical sensing of glucose using phase-modulation fluorometry,'' *Anal. Chim. Acta* **271**, 155–164 (1993).
34. L. Tolosa, H. Malak, G. Rao, and J. R. Lakowicz, ''Optical assay for glucose based on the luminescence decay time of the long wavelength dye Cy5™,'' *Sens. Actuators B* **45**, 93–99 (1997).
35. J. A. Tamada, N. J. V. Bohannon, and R. O. Potts, ''Measurement of glucose in diabetic subjects using noninvasive transdermal extraction,'' *Nat. Med. (N.Y.)* **1**(11), 1198–1201 (1995).
36. S. L. Jacques, D. J. McAuliffe, I. H. Blank, and J. A. Parrish, ''Controlled removal of human stratum corneum by pulsed laser,'' *J. Invest. Dermatol.* **88**, 88–93 (1987).
37. W. Boos, A. S. Gordon, R. E. Hall, and H. D. Price, ''Transport properties of the galactose-binding protein of *Escherichia coli*,'' *J. Biol. Chem.* **247**(3), 917–924 (1972).