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Polarization Sensing of Fluorophores in Tissues for Drug Compliance Monitoring

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Abstract

We used a new method, polarization sensing, to monitor the concentration of the fluorophore rhodamine 800 in an intralipid suspension and in chicken tissue. Rhodamine 800 (Rh800) could be excited at 648 nm using a laser pointer. We developed a simple device for measuring the combined emission from a highly polarized reference film and the unpolarized or orthogonally polarized emission of Rh800 from the scattering intralipid or tissue. The concentration of Rh800 in this medium was revealed by large changes in the polarization (P) with values of P ranging from 0.8 to -0.9 . It is possible to vary the sensitive Rh800 concentration range by variation of the detected emission wavelengths, orientation of the excitation polarizer, or fluorophore concentration in the reference film. Polarization sensing of fluorophores in tissue requires only steady-state detection, and can be accomplished with simple and/or portable electronics. Such devices may find use in electronic detection of ingested medicines based on transdermal detection of nontoxic long-wavelength fluorophores.

Treatment of many chronic diseases often requires long-term administration and patient compliance in taking of medications. One important example is tuberculosis, which is the world's most deadly disease and appears even in developed countries (1). The World Health Organization has estimated that tuberculosis will kill 3.5 million people by the year 2000. Successful treatment of tuberculosis requires a 6-month treatment with antibiotics (2). Failure to complete the treatment results in failure to eliminate the infection, and in the emergence of drug-resistant strains of *Mycobacterium tuberculosis*. Such strains are difficult and expensive to treat even in developed countries. Drug compliance is difficult to monitor, particularly in less-developed countries. Additional examples of chronic conditions requiring long-term compliance include cancer, immunosuppression to prevent transplant rejection, cancer chemotherapy, and drug trials (3).

Several methods have been suggested to monitor injection of medication, including pill counting, microelectronic event monitoring system, and direct observation of therapy (4–6). The latter is expensive and inconvenient, but is often used for infectious diseases such as tuberculosis. In the present report, we describe the possibility of measuring drug compliance by polarization-based sensing. The method requires adding a tracer, a red or near-infrared fluorophore, to the medication. We show that the emission of such fluorophores can be detected through skin even at micromolar concentrations. In our approach, the sensing

device is placed against the skin. This device contains an oriented reference fluorophore in a stretched plastic film. The tissue is illuminated with light which passes through the reference film and appropriately oriented polarizer. The concentration of the fluorophore in the tissue can be estimated from the decrease in polarization which occurs when the tracer emission is comparable to the emission from the reference film. With modern electronics, we believe that such measurements can be accomplished with portable hand-held devices, or even a watch-sized device (Scheme 1).

MATERIALS AND METHODS

Rhodamine 800 (Rh800)¹ was obtained from Lambda Physik, and was used without further purification. For aqueous solutions, the probe was dissolved in water. Intralipid (20%) was obtained from KabiVitrum, Inc. (Clayton, NC). The intralipid was diluted 40-fold into buffer, to 0.5%, to provide a sample with scattering properties comparable to that of tissues like chicken or bovine muscles. The effective scattering coefficient (1-g) μ_s for 0.5% intralipid can be estimated as 7.25 cm⁻¹ (7). The concentration of Rh800 in water was determined from the extinction coefficient of 5.23×10^4 L mol⁻¹ cm⁻¹ at 687 nm.

All fluorescence measurements were performed using front-face illumination and detection, using the sample holder shown in Scheme 2. The incident light from the laser pointer was directed to the sample using two mirrors. The position of the sample could be adjusted with a movable stage. The reemergent light passed through one or more optical filters prior to reaching the detector. The sample consisted of either a cuvette containing the intralipid or a quartz slide covering the chicken muscle and skin. Excitation of 648 nm was provided using a red laser pointer of 5 mW power. The emission was observed using a monochromator and through two Corning cutoff filters (660 nm) to eliminate excitation light and/or attenuate the fluorophore emission relative to that of the long-lived reference.

The polarizer reference was made from polyvinyl alcohol (PVA) which contained the laser dye Styryl-7. This film was prepared as described previously (8, 9). The method consists of dissolving the PVA in water and polymerizing at ~360K. Styryl 7 in methanol was added to a 10–15% aqueous solution of polyvinyl alcohol. The PVA solution was then cast on a plate and dried over a period of several days in a dust-free atmosphere. The PVA films were physically stretched at about 350K up to sixfold to orient the Styryl 7 molecules. Such films display high polarization values when excited with polarized or unpolarized light.

THEORY

The theory for polarization sensing is relatively simple, and has been described recently (10–12). However, this concept is new and not widely understood. Hence, we describe the operating principles of the polarization sensor (Scheme 3). The various polarized intensities are shown on the schematic. The sample is positioned behind the oriented film. The stretch axis is oriented vertically. The excitation polarization may be oriented along the vertical

¹Abbreviations used: Rh800, rhodamine 800; IcG, iodocyanine green; PVA, polyvinyl alcohol.

axis, or rotated by an angle β relative to the axis. The emission is observed from the front surface, as is likely to occur in transdermal measurements.

For sensing, one measures the polarization of the combined emission from the reference film and from the intralipid or tissue sample (Scheme 3). The sample emission can be observed without a polarizer (Scheme 3b), or through a polarizer ($P_{\perp}$) which transmits the horizontal component of the emission from the sample (Scheme 3c),

$$P = \frac{I_{\parallel}^T - I_{\perp}^T}{I_{\parallel}^T + I_{\perp}^T}, \quad [1]$$

where the superscript T indicates the sum of the intensities from the sample and Styryl-7 reference. The subscripts indicate the parallel ($\parallel$) and perpendicular ($\perp$) components of the emission relative to the film orientation. The total parallel intensity is given by

$$I_{\parallel}^T = I_{\parallel}^R + I_{\parallel}^S \quad [2]$$

where the superscripts R and S refer to the reference film and sample, respectively. Similarly, the total emission in the perpendicular orientation is given by

$$I_{\perp}^T = I_{\perp}^R + I_{\perp}^S. \quad [3]$$

Each intensity depends on a number of chemical and instrumental factors, which we did not explicitly indicate to simplify the presentation. When the sensor is configured without the polarizer, $P_{\perp}$, in front of the sample the polarization is given by

$$P = \frac{I_{\parallel}^R - I_{\perp}^R + I_{\parallel}^S - I_{\perp}^S}{I_{\parallel}^R + I_{\perp}^R + I_{\parallel}^S + I_{\perp}^S}. \quad [4]$$

If the emission from the sample is zero, the polarization is determined by the reference film,

$$P_R = \frac{I_{\parallel}^R - I_{\perp}^R}{I_{\parallel}^R + I_{\perp}^R}. \quad [5]$$

If the emission from the sample dominates, the polarization tends toward that from the sample, which is near zero.

The use of a polarizer ($P_{\perp}$) in front of the sample results in elimination of the vertical component, so that $I_{\parallel}^S = 0$ and $I_{\perp}^T = I_{\perp}^R$. Substitution of Eqs. [2] and [3] into the definition of polarization (Eq. [1]) yields

$$P = \frac{I_{\parallel}^R - I_{\perp}^R - I_{\perp}^S}{I_{\parallel}^R + I_{\perp}^R + I_{\perp}^S}. \quad [6]$$

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

$$P = \frac{I_{\parallel}^R - I_{\perp}^R}{I_{\parallel}^R + I_{\perp}^R} = P_R \quad [7]$$

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

$$P = \frac{-I_{\perp}^S}{I_{\perp}^S} = -1. \quad [8]$$

The reference films typically display polarization values P_R near 0.8. Hence, one may expect a wide range of polarization values for this sensor configuration, from $+0.8$ to -1.0 .

RESULTS

Simulations

It is valuable to examine the range of polarization values expected for the polarization sensor (Scheme 3). For this simulation, we consider a parameter N which is the intensity of the sample relative to the reference, $N = (I_{\parallel}^S + I_{\perp}^S)/(I_{\parallel}^R + I_{\perp}^R)$ at the observation wavelength.

Larger values of N indicate larger intensities of the sample compared to the reference at a given concentration of fluorophores and observation wavelength. Figure 1 shows simulated values of the polarization for increasing concentrations of fluorophores. The starting value of the initial polarization was assumed to be 0.67, which is characteristic of a stretched film with the presence of a small amount of unpolarized sample emission. As the fluorophore concentration increases, the polarization approaches zero.

It is important to notice that the sensitivity to fluorophore concentration can be adjusted by changing the value of N . For instance, the value of N can be increased to over unity by selecting an observation wavelength that selects for the fluorophore relative to the reference. In this case, the polarization decreases more rapidly with fluorophore concentration. If a wavelength is chosen that selects for the reference emission, the polarization decreases more slowly with fluorophore concentration. The midpoints for the decreasing polarization can be varied over 10-fold for reasonable changes in N from 3 to 0.1. Hence, the sensitivity of the polarization sensor can be varied by a technically simple adjustment of the emission wavelength. One can also imagine a polarization sensor with two or more emission wavelengths to expand the measurable range of fluorophore concentrations.

Figure 2 shows similar simulations for the sensor with a horizontal polarizer in front of the sample ($P_{\perp}$ in Scheme 3c). In this case, only the horizontal component of the sample emission is observed, and this component contributes a negative component to the polarization. The use of $P_{\perp}$ increases the range of expected polarization values to -1.0 , which can improve accuracy. Once again, we note that the measurable concentration range

can be adjusted by changing N , which is typically dependent on the detected emission wavelength.

After configuration, we note that there is another way to vary N , which is by rotation of the polarization vector of the excitation beam. The absorption of oriented probes depend on polarization (8). If the stretch axis is oriented vertically, the absorption will be maximal when the excitation polarization is vertically polarized, and the reference absorption will decrease as the polarization is rotated. Since the sample is typically isotropic, the sample-to-reference ratio will typically be increased as the excitation polarization is rotated from the vertical. Of course, if the sensor contains $P_{\perp}$, the excitation must be rotated somewhat for transmission through $P_{\perp}$.

Spectral Properties of Styryl-7 and Rh800

Absorption and emission spectra of the Styryl-7 reference and Rh800 are shown in Fig. 3. Both fluorophores can be excited with the laser diode output at 648 nm. The emission spectra of Styryl-7 and Rh800 overlap but show slightly different emission maxima. This shift allows the relative intensities of the two fluorophores to be adjusted by selection of the observation wavelength. Observation wavelengths below 705 nm favor detection of Styryl-7, and wavelengths above 705 nm favor detection of Rh800. Most of our experiments were performed using detection at 710 nm, which was the approximate equivalence point for the peak-normalized emission spectra of Rh800.

Emission spectra of Rh800 in 0.5% intralipid are shown in Fig. 4. The emission maximum of Rh800 is essentially the same as that in water. Over the Rh800 concentration range of 0 to 1 μM , the intensity is proportional to the concentration. Hence, Rh800 shows no evidence of aggregation or self-quenching for this range of concentration. Examination of the emission spectra in the absence of Rh800 indicates the presence of background fluorescence below 700 nm. The effect of this nonzero background was seen in the polarization values (shown below), but the effect was minor.

Effect of Background Fluorescence

Prior to using our sensor to measure Rh800, we examined the effect of the sample background signal on the polarization values. These values are shown in Fig. 5 for various orientations of the excitation polarization. For the film alone ($\square$), the polarization is almost independent of the angle β . This unusual result is found because the elongated Styryl-7 molecules are strongly oriented in the vertical direction. Hence, the polarization remains high, above 0.7, independent of the orientation of the excitation polarization. This favorable property of the oriented film indicates that polarization sensing can be accomplished with an unpolarized excitation source.

Somewhat lower polarization values were observed when the intralipid was placed within the sensor, without Rh800. In this case, the polarization values decreased as the excitation polarization was rotated ($\circ$). This decrease is due to increased excitation of intralipid background with decreased excitation of the film, which results in increased observation of the intralipid background. Similar but somewhat larger decreases in polarization were observed when chicken tissue, without Rh800, was placed behind the reference film ($\triangle$).

For most of the remaining measurements, the excitation polarization was rotated about 45° from the vertical, resulting in initial polarization values near 0.6.

Next we examined the effects of micromolar concentration of Rh800 on the measured polarization, again without $P_\perp$ (Scheme 3b). The polarization decreased rapidly toward zero as the Rh800 concentration exceeded $2\ \mu\text{M}$ (Fig. 6). The decrease in polarization occurred more rapidly for observation at 710 nm ($\square$) than at 700 nm ($\circ$). This effect is due to the longer emission maximum of Rh800 and a larger value of N at 710 nm than at 700 nm.

Rh800 Sensing with $P_\perp$

We also measured the effect of Rh800 using the configuration with the perpendicular sample polarizer ($P_\perp$ in Scheme 3c). As predicted by the simulations, the polarization values now decrease toward -1.0 at high Rh800 concentration (Fig. 7). Hence, this sensor configuration provides a wide range of polarization values from $+0.8$ to -1.0 .

Using this configuration of the polarization sensor, we examined the effect of rotating the excitation polarization away from the vertical position ($\beta = 0$). Changing β from 25° to 45° results in shifting the polarization dependence toward lower Rh800 concentrations (Fig. 7). We have not attempted to determine the full range of Rh800 concentrations, but it seems clear that the concentration midpoints can vary 10-fold or larger with changes of the excitation polarization.

Rh800 in Chicken Tissue

The polarization sensor, including $P_\perp$ was used to examine Rh800 in chicken tissue. By chicken tissue, we mean raw chicken breast muscle with the skin intact. Pieces of this chicken were placed behind the reference film. The tissue was injected with about $50\ \mu\text{l}$ of different concentrations of Rh800 about 1 mm behind the skin. We estimate the tissue volume to be about 1.2 ml, so that Rh800 would be diluted 24-fold if mixing were complete within the tissue.

Polarization values for Rh800 in chicken tissue are shown in Fig. 8. The initial polarization value is decreased due to the background signal (Fig. 3). Nonetheless, the polarization changes over a wide range from about 0.5 to -0.4 for a reasonable range of Rh800 concentration. The sensitive range could be adjusted by selection of the emission wavelength with observation at 710 nm being more sensitive to Rh800 than observation at 700 nm.

DISCUSSION

In this study, we used polarization sensing (10–12) to detect and determine different concentrations of Rh800 in 0.5% intralipid and in chicken tissue. These samples mimic the scattering properties of the human skin. Fluorescence polarization was measured off the illuminated surface of these samples using the front face geometry (Scheme 2). This geometry represents measurements made directly off the surface of the human skin as would be performed in real-life situations.

Our technique of polarization sensing was found to be very sensitive. In 0.5% intralipid, concentrations as low as 10 nM Rh800 were detected with very good resolution (Figs. 6 and 7). In chicken tissue, concentrations as low as 1 μ M Rh800 were also detected (Fig. 8). Considering the dilution in the chicken tissue, the minimal detectable concentration was about 100 nM Rh800.

We were able to enhance the sensitivity of these measurements by selecting excitation wavelengths that are more sensitive to the sensing fluorophore, inserting an additional polarizer, $P_{\perp}$, with polarization orthogonally oriented to that of the reference film between the reference film and the sample (Scheme 3c). This increases the range of the measured polarization values from -1 to 1 (Fig. 7). The sensitivity could also be adjusted by making measurements at different excitation polarization angles (Scheme 3c and Fig. 7).

Can an optical compliance monitor be useful in real-world situations? The answer depends upon the severity of the medical condition and the probability that the patient may not take his or her medicine. For infectious diseases such as tuberculosis, individuals can be placed in custody to ensure compliance. This is done because the available methods of monitoring compliance are easily defeated. Under such circumstances, a portable compliance monitor may be acceptable. Other areas requiring rigorous compliance are in the testing of new drugs. In such cases, the cost of a compliance monitor may be modest compared to the overall cost of the drug trial.

Are fluorophores available that can be used *in vivo*? Fluorescein is presently used in retinal imaging (13, 14), but its absorption and emission spectra may be at short wavelengths, too short for transdermal measurements. Indocyanine green (IcG) absorbs and emits at wavelengths above 700 nm and is therefore suitable for transdermal measurements. Furthermore, IcG has already been detected through skin (15, 16). IcG is approved for use in humans for monitoring of burn severity (17, 18), ophthalmology, liver and kidney function, and to measure blood volume (19–23). Hence, at least one potential fluorophore is available. At present, we do not know if IcG is absorbed following ingestion because IcG is usually used by intravenous injection. If this occurs, ingestion of the prescribed medicine containing a tracer amount of IcG should be detectable by a transient decrease in the polarization.

It appears likely that modern optics and electronic technology will allow development of the device shown in Scheme 1. Excitation can be accomplished with LED, laser diode, or electroluminescent light sources. Such portable devices may find use in drug trials and in chronic medical conditions. However, IcG is less fluorescent than Rh800, and its emission is known to be quenched by aggregation in aqueous and macromolecular solutions (24–29). Hence, Rh800 would be a good candidate to validate our suggestion for a polarization compliance monitor.

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FIG. 1.

Theoretical dependence of observed polarization as a function of dye concentration for different values of the parameter N in the case of no additional polarizer (Scheme 3b). Initial polarization was 0.67. The horizontal line defines the midpoint of the total change. The inset shows a lower concentration range from 0 to 2 μM .

FIG. 2.

Theoretical dependence of observed polarization as a function of dye concentration for different values of the parameter N in the case of the additional polarizer. Initial polarization was 0.67. The horizontal line defines the midpoint of the total change. The inset shows the polarization for the concentration range of 0 to 2 μM .

FIG. 3.
Absorption and emission spectra of Styryl-7 (—) in the PVA film and rhodamine 800 (---) in water.

FIG. 4.

Emission spectra of Rh800 in 0.1% intralipid solution with laser pointer excitation. The Rh800 concentrations vary from 10 nM to 1 μ M.

FIG. 5. Observed fluorescence polarization from the film only (□), film with the 0.5% intralipid behind the film (○), and film with the chicken skin and about 2 mm of the tissue behind the film (△) for different orientations of the excitation polarization with respect to the film orientation.

FIG. 6.

Measured fluorescence polarization as a function of Rh800 concentration of 0.5% intralipid for different observation wavelengths 700 and 710 nm. According to Fig. 2, different observation wavelengths yield different emission ratios of the Styryl-7 and Rh800 fluorescence, and are expected to result in different values of the parameter N .

FIG. 7.

Measured fluorescence polarization as a function of Rh800 concentration in 0.5% lipid for two different polarizations of excitation beam, 25° and 45° in respect to the film orientation. The observation wavelength was 700 nm. Different polarization of the excitation beam changes the ratio of Styryl-7 and Rh800 fluorescence which results in a different value of the N parameter. The measurements included the emission polarizer $P_\perp$ (Scheme 3c). The inset is the expanded concentration range from 0 to 2 μM .

FIG. 8.

Measured fluorescence polarization from the device consisting of oriented PVA film, horizontal polarizer ($P_{\perp}$), and chicken tissue when different amounts of Rh800 were added. The injection of Rh800 was in the tissue about 1 mm behind the skin. The excitation polarization was rotated 35° from the vertical.

SCHEME 1.
Conceptual description of a polarization drug compliance monitor.

SCHEME 2.

Schematic of the experimental setup for measurements of fluorescence from the reference film and scattering media with laser pointer excitation.

SCHEME 3.

Optical system for anisotropy-based drug compliance monitoring. Top: Front-face configuration for drug monitoring with a reference-oriented film. Bottom: (a) Observe fluorescence intensity components for the film only, no sample. (b) Observe fluorescence intensity components for the sample and film. Fluorescence of the dye in the scattering medium is considered totally depolarized. (c) Observe fluorescence intensity components for the sample and film with an additional polarizer in front of the sample (polarizer oriented orthogonally to the film orientation). In this case, the sample contributes only an orthogonal component to the total fluorescence.