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Fluorescence Properties of Rhodamine 800 in Whole Blood and Plasma

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Abstract

We have characterized the fluorescence spectral properties of rhodamine 800 (Rh800) in plasma and blood in order to test the possibility of making clinical fluorescence measurements in whole blood without separation steps. Rh800 was used because of its absorption at red/near-infrared wavelengths away from the absorption bands of hemoglobin. We utilized the front-face illumination and detection to minimize the effects of absorption and/or scatter during measurements. The presence of Rh800 was detected in plasma and blood using steady-state fluorescence measurements. Absorption due to hemoglobin reduced the Rh800 intensity from the blood. Fluorescence lifetime measurements in plasma and blood showed that it is possible to recover lifetime parameters of Rh800 in these media. We obtained mean lifetimes of 1.90 and 1.86 ns for Rh800 in plasma and blood, respectively. Using the recently described modulation sensing method, we quantified the concentrations of Rh800 in plasma and blood. Rh800 was detected at a concentration of as low as 2 μM in both media. High anisotropy values were obtained for Rh800 in plasma and blood using steady-state and anisotropy decay measurements, implying the tight binding of this probe to the contents of these media. This binding can be exploited to monitor the concentrations of different blood components using already existing or new red-emitting probes that will be specially designed to bind to these components with high specificity. To test this possibility of direct measurements in blood, we used Rh800 to monitor albumin in the presence of red blood cells. Increase in the polarization of Rh800 as the concentration of albumin was increased in the presence of the red cells showed the feasibility of such measurements.

Fluorescence methods have been extensively used in the analysis of biological samples in clinical analysis and biomedical research because of their sensitivity, rapidity, and directness. However, direct fluorescence measurements in whole blood are almost nonexistent because of the strong background absorption, significant autofluorescence, and scatter observed from blood in the visible region. To date, we are aware of only one study in which fluorescence measurements have been made directly in blood, and this was with two-photon fluorometry with excitation in the near-infrared (NIR)² region (1).

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²Abbreviations used: Hb, hemoglobin; Hct, hematocrit; dppz, dipyrrophenazine; HSA, human serum albumin; bpy, 2,2'-bipyridyl; NIR, near infrared; PBS, phosphate-buffered saline; PVA, poly(vinylalcohol); RBC, red blood cell; Rh800, rhodamine 800.

Considering the need to determine different analytes (or components) in whole blood, the ability to routinely determine these components directly in blood will be an important development for clinical chemistry. Such measurements will require fewer manipulations, resulting in fewer sources of error, shorter times to results, and reduced health hazards due to the handling of samples.

In recent times, the use of red and NIR light has become increasingly popular in biomedical research. This is because at these wavelengths autofluorescence and absorption are minimal in biological samples. In this study, we have investigated the feasibility of making fluorescence measurements in whole blood using 610-nm excitation. We used front-face illumination and detection (2, 3) in order to minimize the effects of scatter and absorption. Rh800 was used as the dye of choice to show proof of principle because of its emission in the NIR, although it is not used for any specific medical test presently.

With the feasibility of performing fluorescence measurements in whole blood using red and NIR probes, it will then be possible to determine different blood components using probes that bind preferentially with high specificity. We would expect to monitor changes in concentrations of such components by observing changes in the intensity, lifetimes (phase or modulation), and/or polarization of the probes. Kessler and Wolfbeis (4) in a previous study demonstrated the use of two cyanine probes, AB633 and AB670, to monitor albumin in biological samples with high specificity at long wavelengths. Like these two probes, we believe that it is possible to identify or design other red or NIR probes that will bind preferentially to blood components of interest for clinical purposes.

In addition to blood, we have also made measurements in plasma. Although problems with autofluorescence, absorption, and scatter are highly minimized in plasma, they are nevertheless present and were found to affect our steady-state anisotropy and lifetime measurements.

MATERIALS AND METHODS

Rhodamine 800 (Rh800) was obtained from Lambda Physik (Fort Lauderdale, FL) and used without further purification. Human serum albumin (HSA) was obtained from Sigma (St. Louis, MO).

Whole blood was obtained either from one of the authors or from other colleagues in the same laboratory. For experiments in blood, whole blood was used as obtained, at a hematocrit (Hct) of about 41.5% (Hb = 13.5 g/dl). Plasma was obtained from the whole blood by centrifuging at 5000*g* using a Beckmann Avanti J-25 I centrifuge. At the end of the centrifugation, the plasma (supernatant) was carefully aspirated off and used for experiments as desired.

Red blood cell (RBC) ghosts were prepared by first washing the RBCs thrice in Dulbecco's phosphate-buffered saline, pH 7.4 (PBS), in order to remove buffy coat and plasma. This was followed by lysing the RBCs in 5 mM sodium phosphate buffer, pH 8.0, and then washing the hemoglobin (Hb) laced RBC membranes successively with the same buffer until

white. Each wash involved adding the sodium phosphate buffer to the red cell membranes and centrifuging at 19,000 rpm (48,000*g*).

Oxygenated (oxy-), deoxygenated (deoxy-), and oxidized (met-) Hb were prepared as follows. Washed red blood cells were lysed with water and the suspension spun at 19,000 rpm in order to separate the fragmented red cell membranes from the oxyHb-containing supernatant. To acquire the oxyHb spectrum, an aliquot of the oxyHb was suspended in PBS to a final concentration of about 40 μ M, and the spectrum obtained using a Hewlett Packard 8453 diode array spectrophotometer. DeoxyHb was obtained by slowly bubbling argon gas through the oxyHb solution while gently stirring in an airtight cuvette. MetHb was obtained by adding small crystals of potassium ferricyanide to the oxyHb solution to oxidize the Hb. Absorption spectra for these samples were also acquired using the Hewlett Packard 8453 diode array spectrophotometer.

Steady-state intensity and anisotropy measurements were performed using an SLM 8000 spectrofluorometer (SLM Instruments, Urbana–Champaign, IL) with an excitation source of either a xenon arc lamp or a 645-nm laser pointer. Steady-state anisotropy measurements were done on Rh800 in plasma and blood at ambient temperatures and in 95% glycerol:5% ethanol mixture at –60°C. Polarization was also determined for 5 mM Rh800 with 0.02–2.00 g/dl HSA in PBS and in 15% Hct washed RBCs at 705 nm. This was done to test the feasibility of directly measuring concentrations of analytes of interest (e.g., electrolytes, proteins, lipids, hormones, and lipoproteins) either in plasma or in whole blood using fluorescence probes with high specificity for these analytes.

Frequency-domain intensity decay measurements were performed as described previously (5–8). Excitation at 610 nm was provided by the fundamental output of a rhodamine 6G dye laser which was synchronously pumped by a mode-locked argon ion laser. The dye laser was cavity dumped at 3.77 MHz. Phase angle and modulation measurements at frequencies higher than 3.77 MHz were performed using the harmonic content of the picosecond pulses (9, 10). Phase angles and modulations were measured relative to scattered light at 610 nm using a 610-nm interference filter. Emission was observed at 710 nm using a 710-nm interference filter. Measurements were performed using magic-angle conditions.

Modulation-sensing measurements were performed in plasma and blood as previously described (2, 11). These measurements are based on observing the amplitude modulation of the emission from both a short-lifetime probe of interest, for example, Rh800, and a long-lifetime reference fluorophore pasted on the illuminated surface of our sample cuvette. At some low frequencies, the observed modulation of the combined emission is nearly equivalent to the fractional intensity of the short-lifetime probe. This method allows us to measure the concentration of our probe in our desired media and minimizes some of the problems associated with measuring fluorescence intensities in highly scattering media. However, we note that the low frequency modulation does not provide an absolute measurement of the intensity, but an apparent intensity as modified by the optical properties of the sample. In this study, we used [Ru(bpy)₂(dppz)](PF₆)₂ (Ru complex) in poly(vinyl alcohol) (PVA) film as the long-lifetime reference fluorophore. Excitation was at 610 nm while observation was at greater than 660 nm using a 660-nm Corning cutoff filter.

All fluorescence measurements were performed using front-face illumination and detection.

THEORY

The time-resolved intensity decay for Rh800 in plasma and blood was determined from the frequency-domain data using the multiexponential model,

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i), \quad [1]$$

where α_i and τ_i are the preexponential factors and decay times, respectively. The fractional contribution of each decay time to the steady-state intensity is given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j}, \quad [2]$$

while the mean lifetime is given by

$$\tau = \sum_i f_i \tau_i = \sum_i \alpha_i \tau_i^2 / \sum_j \alpha_j \tau_j. \quad [3]$$

The anisotropy decays were analyzed in terms of the multicorrelation time model,

$$r(t) = \sum_k r_{0k} \exp(-t/\theta_k), \quad [4]$$

where θ_k is the correlation time and r_{0k} is the corresponding amplitude of the anisotropy decay. For a fully resolved anisotropy decay, one expects $r_{0k} = r_0$, where r_0 is the fundamental anisotropy observed in the absence of rotational diffusion and other depolarization factors.

RESULTS

General Spectral Properties

In this study, we have characterized the photophysical properties of Rh800 (Fig. 1), a red-emitting probe, in plasma and whole blood. We have also used Ru complex (Fig. 1) in a PVA film as the long-lifetime reference fluorophore for the modulation-sensing measurements (2) in plasma and whole blood. Figure 2 shows the absorption and emission spectra of Rh800 and the Ru complex. One notices that Rh800 displays maximal absorption and emission above 650 nm, which is well removed from the absorption bands of hemoglobin in blood (Fig. 3). $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ displays shorter absorption and emission wavelengths which overlap more with those found in blood. This is not a serious problem because the Ru complex-PVA film is placed in front of the blood sample.

In Fig. 4, we see the emission spectra of plasma (top) and whole blood (bottom) with and without Rh800. While the background emission from the plasma is minimal (Fig. 4, top), we observe that the background emission from the whole blood (Fig. 4, bottom) is significant.

We attribute the autofluorescence from blood to the heme and other fluorescing moieties in whole blood. We also observe some scatter in the background emission of the whole blood. We believe this scatter arises from the presence of the RBC membranes in whole blood. Similar scatter has also been observed with intralipid, which is also a highly scattering medium (2). We also observed significant quenching of the emission of Rh800 in whole blood when compared to the plasma (Fig. 5). This quenching was attributed to the absorption of the Rh800 emission by the very large amounts of hemoglobin (about 20 mM) in the red cells. The extent of quenching has, however, been observed to depend on the nature of the dye and wavelength of excitation (unpublished results). The further away the excitation is to the red of the hemoglobin band, the smaller the absorption effect.

To minimize the contribution of autofluorescence and absorption to our measurements, we used 610-nm excitation for our lifetime measurements and 630 nm (xenon arc lamp)/645 nm (laser pointer) excitation for our steady-state measurements. In Fig. 3 we notice that, apart from metHb, absorption by the different Hb species above 600 nm is minimal. Contribution from metHb is, however, greatly minimized because of its low percentage of about 1–3% in whole blood.

In the plasma and blood, no appreciable shift was observed for Rh800 emission spectra in comparison to that in PBS (Fig. 6). However, a narrowing of the emission peak was observed in both blood and plasma, with that in plasma being more narrow. We determined the emission anisotropy for Rh800 in 9.50:0.05 glycerol:ethanol mixture at -60°C (Fig. 6) and obtained high fundamental anisotropy values of between 0.36 and 0.38 in the wavelength region of 690–720 nm. Anisotropy measurement in plasma in this region also yielded high anisotropy values of 0.36–0.38 (Fig. 6), suggesting that the Rh800 was completely bound to the components in plasma. In whole blood, however, we obtained lower anisotropy values of 0.29–0.32 in the same wavelength region of 690–720 nm (Fig. 6). This decrease in anisotropy in the whole blood could be a result of multiple scattering events due to the presence of the red cell membranes. Another possibility is that the RBCs could somehow be interfering with the binding of Rh800 to the plasma components.

Lifetime and Decay Anisotropy Measurements

We examined the frequency-domain intensity decay of Rh800 in PBS, plasma, albumin, whole blood, RBC ghosts, and RBC ghosts/albumin. Frequency responses of the emission from Rh800 in PBS, plasma, and albumin is shown in Fig. 7. In PBS, Rh800 displayed a single-exponential decay of 0.68 ns (Table 1). However, in plasma and albumin the intensity decay of Rh800 became more complex. The data were fit to a two decay time model. In plasma we obtained two lifetime values of 2.89 and 1.48 ns with a mean lifetime of 1.90 ns, while in albumin the lifetimes obtained were 3.30 and 0.95 ns with a mean lifetime of 1.80 ns (Table 1). Compared to the lifetime in PBS, these lifetimes suggest that Rh800 was bound to the plasma moieties and to albumin. Albumin is the major constituent of serum protein, usually over 50%. The closeness of the mean lifetime obtained in plasma to that of albumin would therefore indicate the reliability of our measurements in plasma. The slight increase in mean lifetime in plasma is probably due to the presence of other proteins (globulin etc.) and lipoproteins (LDL, HDL, etc.) in plasma.

Figure 8 shows the frequency responses of the emission from Rh800 in whole blood and in the RBC ghosts. As with plasma and albumin, the data required a two decay time model for a proper fit. Lifetimes of 5.17 and 1.67 ns with a mean lifetime of 1.86 ns and lifetimes of 4.36 and 1.19 ns with a mean lifetime of 1.69 ns were obtained for the whole blood and RBC ghosts, respectively (Table 1). Increased lifetimes obtained for Rh800 in the whole blood and in RBC ghosts when compared to that in PBS also suggest the binding of Rh800 in whole blood and in RBC ghosts, as was observed in plasma and albumin. As with albumin (used as reference for plasma), we measured the intensity decay of Rh800 in RBC ghosts as reference for measurements in whole blood. A reduced mean lifetime was expected for Rh800 in RBC ghosts in comparison to that for whole blood because of the weaker binding of Rh800 to the RBCs, as suggested by our steady-state anisotropy results and the lack of plasma in the system. This prediction we observed, suggesting that our lifetime results in whole blood are reasonable. This deduction is further supported by the increased mean lifetime of 1.81 ns observed when albumin is added to the RBC ghosts (Table 1).

In addition to the intensity decay measurements, we also measured the anisotropy decays of Rh800 in plasma (Fig. 9). Rotational correlation times of 62.3 and 0.17 ns, with corresponding r_{0k} values of 0.207 and 0.156, respectively, were recovered for Rh800 in plasma, with r_{0k} being 0.363. The recovered timezero anisotropy is in good agreement with that obtained from the steady-state measurement (Fig. 6), and this suggests that anisotropy/polarization measurements can be useful in plasma samples. In addition to the measurements in plasma, we also made anisotropy decay measurements in blood. Nevertheless, though we observed some anisotropy, these measurements were difficult to analyze.

Modulation Sensing

Recently, we developed the modulation-sensing technique in our laboratory (11) and showed that it can be used to monitor the concentration of fluorescent probes in scattering media and tissues (2). In order to test the feasibility of modulation sensing in blood, we determined Rh800 concentrations in plasma and blood using this method. The PVA film containing our long-lifetime reference was placed on the illuminated surface of our cuvette for these measurements. Representative emission spectra of Rh800 in plasma and whole blood in the presence of our Ru complex film are shown in Fig. 10. The peak near 710 nm (shoulder for whole blood) is due to Rh800, while the signal around 670 nm is due to the emission from the Ru complex.

Frequency responses of the combined emission from the Ru complex and Rh800 in plasma are shown in Fig. 11. As has been previously observed with Rh800 in intralipid (2), the modulation from 3 to 20 MHz was essentially independent of frequency and increased with increasing Rh800 concentration. The data were globally analyzed in terms of short and long decay times and the fractional intensity (f_i) of each component (Table 2). Two decay times of 2.33 and 661 ns were found to adequately fit all the Rh800 concentrations. We considered the decay time of 2.33 ns with its associated fractional intensity to represent Rh800, the short-lifetime component. As the concentration of Rh800 increased, the fractional intensities

of the short-lifetime component (Fig. 11, inset) also increased. At the frequency of 7.55 MHz, the observed modulation was similar to the fractional intensity of the short-lifetime Rh800 at the different concentrations of measurement (Fig. 11, Table 2). This shows that the low-frequency modulation values reflect the contribution of Rh800 to the total emission, and hence its concentration.

The frequency responses of the combined emission of the Ru complex and Rh800 in blood can be seen in Fig. 12. As was the case in plasma, the observed modulation was frequency independent in the low-frequency range but increased as Rh800 concentration increased. Acquired data were globally and adequately fit to three decay times of 0.83, 3.00, and 426 ns. Here, the decay times of 0.83 and 3.00 ns were taken to represent Rh800, while their fractional intensities were combined into a single fractional intensity for the short-lifetime component. The fractional intensity of Rh800 was found to increase with concentration (Fig. 12, inset). At 7.55 MHz, observed modulation values were also found to be similar to the calculated fractional intensities (Fig. 12, Table 3), as observed in plasma. This suggests that, like the plasma, measured modulation at low frequencies can be used as a measure of the concentration of fluorescent probes in whole blood using modulation sensing.

The nonlinearity of the relationship between the fractional intensities in plasma (Fig. 11, inset) and blood (Fig. 12, inset) and Rh800 concentration can be attributed to normalizing the fractional intensities of Rh800 and that of the long-lifetime component to unity. Therefore increasing concentrations of Rh800 increases its fractional intensity monotonically to one.

Polarization Measurements

Polarization measurements (12–16) have been extensively utilized in the determination of analyte concentrations in solutions. However, we have no knowledge of direct measurements of analytes in whole blood using this method. In this study, we have tried to determine different concentrations of albumin in PBS with or without RBCs using changes in the polarization of Rh800 in both media. This was done to test the feasibility of directly making measurements in plasma or whole blood samples using the polarization technique. Figure 13 shows polarization plots of Rh800 in albumin (top) and in albumin/blood (bottom). We observe that as the concentration of albumin increases, so does the polarization of Rh800 in the albumin until it levels off. In albumin/RBC, we also observe this increase and leveling off with increasing albumin concentration. In the presence of the RBCs, this increase and leveling off are more gradual and shifted to the right, suggesting an apparent weaker binding in this medium. Lower polarization values are also observed in the presence of the RBCs, as was the case with the plasma and whole blood (Fig. 6). As suggested earlier, this reduced polarization could be the result of multiple scattering events in the presence of the red cells. The smaller polarization range observed in the presence of the RBCs (Fig. 13) also indicates an effect due to the presence of the RBCs. This effect, which results in the observed decreased sensitivity, can be attributed to increased scatter in the presence of the RBCs. In general, however, these results show that it is possible to make fluorescence polarization measurements directly in plasma or whole blood using red or NIR probes.

DISCUSSION

In this study, we characterized the fluorescent spectral properties of Rh800 in order to test the feasibility of making direct fluorescence measurements in blood or plasma. These types of measurements have hitherto been almost nonexistent. We used Rh800 as the probe of choice to show proof of principle for these types of measurements because of its absorption and emission in the red/NIR regions. In these regions absorption and autofluorescence by Hb and other blood components are highly reduced.

We were able to make our steady-state and fluorescence lifetime measurements in plasma and whole blood utilizing the front-face illumination geometry. This allowed us to recover our emission directly from the surface of our cuvette. With steady-state intensity measurements we were able to detect the presence and concentrations of Rh800 in plasma and whole blood, although the absorption by Hb reduced appreciably the intensity and sensitivity of our measurements in the blood.

Lifetime measurements in plasma and whole blood showed that it is possible to recover lifetime parameters in these media despite the autofluorescence, absorption, and scattering present. Lifetime values obtained in plasma and whole blood (Table 1) indicated that Rh800 was bound to the blood contents, as indicated by the enhanced lifetime of the probe. Using lifetimes as a means of monitoring the concentration of Rh800 in these media was not practicable since observed changes were minimal. However, we were able to utilize our recently described modulation-sensing method (2, 11) to quantify the concentrations of Rh800 in the two media. We had previously proposed the use of modulation sensing of fluorescence dyes as tracer probes for noninvasive monitoring of drug compliance for tuberculosis, AIDS, and other diseases requiring strict compliance with medication and during clinical trials of new drugs (2). Our ability therefore to determine concentrations of Rh800 in the blood reinforces our previous assertions of being able to transdermally monitor potential long-wavelength tracer probes. It would only require making measurements on those parts of the body where blood veins are very close to the skin surface such as the wrists of the hand.

High anisotropy values obtained from the steady-state and anisotropy decay data suggested that Rh800 was bound to the plasma and whole blood contents. This binding of Rh800 to the blood contents like proteins, lipids, etc., which has also been observed with other dyes (2, 4, 17–20), can be exploited to monitor concentrations of blood components that can bind preferentially to selected dyes with high specificity. Kessler and Wolfbeis (4) have shown that AB633 and AB670, two long-wavelength cyanine dyes that bind to HSA preferentially, can be used to monitor the quantity of HSA in urine. With the identification of or design and synthesis of other long-wavelength probes that can bind preferentially to other blood components, it will be possible to monitor directly the concentration of these blood moieties using anisotropy or polarization measurements. Polarization measurements of Rh800 in albumin and albumin/RBCs show the feasibility of these types of proposed measurements. A change in the polarization of 5 μ M Rh800 is observed as the concentration of albumin changes in the presence of the RBCs.

How can we make measurements directly in blood? The use of red light away from the hemoglobin bands for the excitation of red-emitting dyes with preferential binding to desired blood components can be utilized for this purpose. Front-face illumination and detection can also be used to minimize scatter and absorption observed with blood. This will be particularly useful for dyes whose absorption is further removed from the hemoglobin bands, resulting in much reduced background. Red-emitting LEDs and laser diodes can be used as light sources for simple steady-state instruments. These light sources (LEDs and laser diodes) can also be used for lifetime measurements since they can be easily modulated to several megahertz (21–23). One can imagine the development of red/NIR probes which are specific for analytes of clinical interest such as pH, pCO₂, pO₂, and electrolytes. Then it will be possible to perform such measurements on whole blood without the need for handling or separation steps. Such capabilities will be valuable in emergency, bedside, and point-of-care testing.

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Rh800

$[\text{Ru}(\text{bpy})_2(\text{dppz})](\text{PF}_6)_2$

FIG. 1.
Structures of rhodamine 800 (Rh800) and bis(2,2'-bipyridine)(dipyridophenazine)ruthenium ($[\text{Ru}(\text{bpy})_2(\text{dppz})](\text{PF}_6)_2$).

FIG. 2. Absorption and emission spectra of Rh800 in PBS (top) and Ru complex ($[\text{Ru}(\text{bpy})_2(\text{dppz})](\text{PF}_6)_2$) in a PVA film (below).

FIG. 3. Absorption spectra of oxyHb (—), deoxyHb (---), metHb (- - -), and Rh800 (···) in PBS. The Rh800 spectrum was normalized to the 577-nm peak of the oxyHb spectrum. The concentration of the Hb species was about 40 μ M.

FIG. 4. Normalized emission spectra of Rh800 (—) in plasma (top) and whole blood (bottom). The dashed lines represent the background emission from plasma and whole blood. Normalization was with respect to the Rh800-containing plasma (top) and blood (bottom). Excitation was at 645 nm.

FIG. 5.
Quenching of Rh800 emission in whole blood as a result of Hb absorption.

FIG. 6. Normalized emission spectra of Rh800 in PBS (—), plasma (---), and whole blood (---) and the emission anisotropy in 95% glycerol:5% ethanol at 260°C (—), plasma at ambient temperature (---), and whole blood at ambient temperature (---). Excitation for anisotropy measurements was at 630 nm.

FIG. 7. Phase angle and modulation of Rh800 in plasma (■), albumin (●), and PBS (◆). Excitation was at 610 nm and observation at 710 nm.

FIG. 8. Phase angle and modulation of Rh800 in whole blood (■) and red blood cell ghosts (●). Excitation was at 610 nm and observation at 710 nm.

FIG. 9. Differential phase (●) and modulated anisotropy (■) plots for Rh800 in plasma. Excitation was at 610 nm and observation at 710 nm.

FIG. 10. Emission spectra of Rh800 with (---) and without (—) Ru complex film (reference) in plasma (top) and whole blood (bottom). Ru complex film was pasted on the illuminated surface of the sample cuvette. The short-dash lines represent the emission spectra of the Ru complex film alone. Excitation was at 645 nm using a laser pointer as light source.

FIG. 11. Phase angle and modulation plots for various Rh800 concentrations in plasma in the presence of the Ru complex long-lifetime reference. Inset shows the variation of the calculated fractional intensities of the short-lifetime component (Rh800) with concentration. Excitation was at 610 nm and observation at greater than 660 nm.

FIG. 12. Phase angle and modulation for various Rh800 concentrations in whole blood in the presence of the Ru complex long-lifetime reference. Inset shows the variation of the calculated fractional intensities of the short-lifetime component (Rh800) with concentration. Excitation was at 610 nm and observation at greater than 660 nm.

FIG. 13. Polarization of 5 μ M Rh800 with various concentrations of albumin in albumin solution (top) and albumin/washed RBC suspension (bottom). Washed RBCs were suspended in albumin at 15% Hct. Excitation was at 645 nm and observation at 705 nm.

TABLE 1

Lifetime Parameters of Rh800 in Whole Blood, Plasma, Red Blood Cell Ghosts, Albumin, and Mixture of Ghosts and Albumin

Medium	τ_i (ns)	α_i	f_i	$\langle \tau \rangle$	χ^2_R
PBS, pH 7.3	0.68	1.00	1.00	0.68	0.89
Plasma	2.89	0.18	0.30	1.90	1.71
	1.48	0.82	0.70		
Albumin	3.30	0.11	0.30	1.80	1.65
	0.95	0.89	0.70		
Whole blood	5.17	0.02	0.05	1.86	2.61
	1.67	0.98	0.95		
RBC ghosts	4.36	0.05	0.16	1.69	3.45
	1.19	0.95	0.84		
RBC ghosts	2.86	0.21	0.42	1.81	1.23
+ albumin	1.02	0.79	0.57		

TABLE 2

Global^a Intensity Decay Analysis of Rh800 in Plasma with the Ru-Complex PVA Film

Rh800 (μM)	τ_L^b (ns)	τ_S^c (ns)	α_S^d	f_S^e	$\langle \tau \rangle$ (ns)	m_S^f
0.0	661.32	2.33	0.963	0.085	605.4	0.096
2.0			0.986	0.261	508.2	0.259
5.0			0.995	0.428	379.5	0.423
10.0			0.998	0.613	257.6	0.605
15.0			0.999	0.783	145.0	0.766

^aFor the global intensity decay analysis, lifetimes were held constant at all dye concentrations.

^bLong lifetime.

^cShort lifetime, representing the intensity decay of Rh800.

^d $\alpha_L + \alpha_S = 1.0$.

^e $f_L + f_S = 1.0$.

^fObserved modulation values at 7.55 MHz.

TABLE 3

Global Intensity Decay Analysis of Rh800 in Whole Blood with the Ru-Complex PVA Film

Rh800 (μM)	τ_L (ns)	τ_{S1}^a (ns)	τ_{S2} (ns)	α_S^b	f_S^c	$\langle \tau \rangle$ (ns)	m_S^d
0.0	425.96	3.00	0.83	0.991	0.256	317.2	0.264
2.0				0.991	0.332	285.4	0.340
5.0				0.993	0.463	230.1	0.470
10.0				0.995	0.554	191.6	0.558
15.0				0.996	0.651	150.6	0.653

^a Short lifetime, representing the intensity decay of Rh800. This is a double-exponential with decay times of τ_{S1} and τ_{S2} in whole blood.

^b $\alpha_L + \alpha_S = 1.0$, where $\alpha_S + \alpha_{S1} = \alpha_{S2}$.

^c $f_L + f_S = 1.0$, where $f_S + f_{S1} + f_{S2}$.

^d Observed modulation values at 7.55 MHz.