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MODULATION SENSING OF FLUOROPHORES IN TISSUE: A NEW APPROACH TO DRUG COMPLIANCE MONITORING

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ABSTRACT

We describe a method to detect the presence of fluorophores in scattering media, including intralipid suspensions and chicken muscle covered with skin. The fluorophores were rhodamine 800 (Rh800) and indocyanine green (IcG), both of which can be excited at long wavelengths where there is minimal absorption by tissues. These fluorophores were dissolved in intralipid or in chicken muscle under skin. A method to approximate the fluorophore concentration in such samples was developed using a long lifetime reference fluorophore in a polymer film placed immediately on the illuminated surface of the sample. Because of the long lifetime of the reference film, the modulation of its emission at low frequencies near 2 MHz is near zero. Since the lifetime of Rh800 and IcG are below 2 ns the modulation of the combined emission is a measure of the intensity of the fluorophore (Rh800 or IcG) relative to the long lifetime reference. Using this method we were able to measure the concentration-dependent intensities of Rh800 and IcG in an intralipid suspension. Additionally, micromolar concentrations of these probes could be detected in chicken muscles, even when the muscle was covered with a layer of chicken skin. The presence of an India ink absorber in the intralipid had only a moderate effect on the modulation values. We suggest the use of this transdermal detection of long-wavelength fluorophores as a noninvasive method to monitor patient compliance when taking medicines used for treatment of chronic diseases such as AIDS or tuberculosis. [S1083-3668(99)00504-3]

1 INTRODUCTION

Successful treatment of many chronic diseases requires long term administration of medication and patient compliance in administration of the drugs. For example, tuberculosis is fast becoming the world's most deadly disease and is appearing with increasing frequency in developed countries.[1] It has been estimated that one third of the world's population is currently infected with mycobacterium tuberculosis with most of the infected individuals in the latent phase. The World Health Organization has estimated that tuberculosis will kill 3.5 million people in the year 2000. Treatment of tuberculosis requires a six month regimen of antibiotics, with a time course of up to 18 months sometimes required for HIV infected patients.[2] Lack of patient compliance with medication has been identified as the major cause of treatment failure in the emergence of drug resistant strains of mycobacterium tuberculosis.[3–5] These strains are difficult to treat even in developed countries. In light of the problems associated with poor patient compliance with long term administration of medication,[6,7] it has become important to identify methods by which compliance can be effectively monitored and enforced. Such a method would be expected to enhance compliance monitoring even in less developed countries where it is normally more difficult to monitor. Additional examples of chronic conditions requiring long term compliance include AIDS and a number of cancers.[8]

Presently, an electronic monitoring method [microelectronic event monitoring system (MEMS)][9,10] used in concert with an invasive marker method[11,12] has been highly recommended for objective monitoring of patient compliance. While the marker method provides dose ingestion, the electronic monitoring method provides a continuous record of timing of presumptive drug doses. As presently practiced, the marker method is laborious and time intensive, requiring the drawing of body fluids, sample preparation, and measurements in the laboratory. It is also associated with health hazards arising from the handling of these body fluids. These two methods can be defeated by the practice of whitewash compliance (as with the marker method) and the dubious operation of the electronic monitoring method.

In the present report we describe the possibility of using the modulation sensing method[13] for monitoring patient compliance noninvasively. The concept requires the coating of a drug with a red or near infrared (NIR) fluorophore. We show that the

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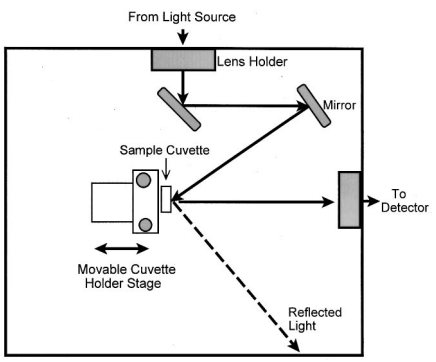
emission of such fluorophores can be detected through skin even at micromolar concentrations. In our approach a sensor will be placed against the skin. This sensor will contain a long lifetime fluorophore in a plastic film. The tissue will be illuminated with intensity modulated light at a frequency near 2 MHz. The presence of fluorophore in the tissue can then be detected from the modulation of the emission which represents the intensity of the short lifetime fluorophore in the tissue relative to that of the long lived reference.

With modern electronics and optics technology we believe such measurements can be accomplished with moderate cost, simple hand held devices in the doctor's office, or at the point of care. An interesting possibility is that these devices can also be designed to simultaneously determine the presence of the marker and continuously record the time of ingestion of medication.

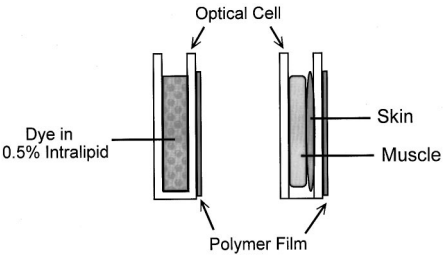
2 MATERIALS AND METHODS

Rhodamine 800 (Rh800) was obtained from Lambda Physik, and indocyanine green (IcG) from Sigma (St. Louis, MO), and were used without further purification. For aqueous solution the probes were dissolved in water. Intralipid (20%) was obtained from KabiVitrum, Inc. (Clayton, NC). The intralipid was diluted 40-fold into water, to 0.5%, to provide a sample with scattering properties comparable to that of tissues like chicken, bovine, or human muscles. The effective scattering coefficient (1 g) μ_s for 0.5% intralipid can be estimated as 7.25 cm^{-1}.[14] Higgins black India ink, No. 46 030, was obtained from Sanford (Bellwood, IL) and used as an absorber in the intralipid to determine the effects of tissue absorption on fluorescence measurements. Concentrations of Rh800 and IcG in water were determined from the extinction coefficients of 5.23×10^4 L mol^{-1} cm^{-1} at 687 nm and 1.39×10^5 L mol^{-1} cm^{-1} at 780 nm.

All fluorescence measurements were performed using front-face illumination and detection, using the sample holder shown in Scheme I. The incident light was redirected from the usual position usingtwo mirrors. The position of the sample could be adjusted with a movable stage. The reemergent



Scheme I Front face sample holder.



Scheme II Sample configuration for fluorescence measurements in intralipid (left) or in chicken tissue (right).

light passed through a 660 nm cutoff filter 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 (Scheme II). Excitation at 600 nm was provided by the fundamental output of a rhodamine 6G dye laser. This dye laser was synchronously pumped by a mode-locked argon ion laser. The dye laser was cavity dumped at 1.88 MHz.

Frequency domain intensity decay measurements were performed as described previously.[15–18] Phase angles and modulation measurements at frequencies greater than 1.88 MHz were accomplished using the harmonic content of the picosecond pulses.[19–21] Phase angles and modulations were measured relative to scattered light at 600 nm, which was isolated using a 600 nm interference filter. The excitation was polarized vertically, and the emission detected without an emission polarizer. The emission was observed through a 660 nm Corning cutoff filter in order to eliminate the excitation light and/or attenuate the fluorophore emission relative to that of the long lived reference. Steady state measurements were performed with an SLM 8000 spectrofluorometer (SLM Instruments, Urbana, IL). Excitation source was either a xenon arc lamp or a 645 nm laser diode.

A long lifetime reference signal was provided by $[Ru(bpy)_2(dppz)](PF_6)_2$ in a polyvinyl alcohol (PVA) film, where bpy is 2,2′-bipyridine and dppz is dipyrido[3,2-*a*:2′,3′-*c*]phenazine. Such metal-ligand complexes are known to display lifetimes from 100 ns to 13 μs.[22,23] This reference sample was prepared by dissolving the Ru complex in melted PVA, and allowing it to solidify into a film. This film was then pasted on the outer surface of the sample cuvette or holder. The mean lifetime of $[Ru(bpy)_2(dppz)](PF_6)_2$ in the PVA film was near 800 ns (Table 1). The intensity decay of the complex was somewhat heterogeneous, that is, multi-exponential. However, this does not affect its use in modulation sensing.

To avoid confusion it is valuable to explain the reasons for using the sample geometries shown in Schemes I and II. The goal of the measurements is

Table 1 Lifetime of ruthenium bis-2,2′-bipyridine dipyridophenazine in polyvinyl alcohol film.

τ_1 (μs)	τ_2 (μs)	α_1[a]	f_1[b]	$\bar{\tau}$ (μs)[c]
1.207	0.405	0.280	0.535	0.834

[a] $\alpha_1+\alpha_2=1.0$.

[b] $f_1+f_2=1.0$.

[c] $\bar{\tau}=\Sigma\alpha_i\tau_i^2/\Sigma\alpha_i\tau_i$

not to determine the lifetime of either the reference film or the probe in the scattering sample. In fact, the modulation frequence is selected so that there is little if any dependence of the lifetime of each fluorophore or moderate changes in their decay times. The optical geometry was chosen to mimic what could be accomplished with a device held against the skin of a patient. The reference film provides a convenient intensity reference which allows detection of a ns decay time fluorophore in the tissue from an increase in the modulation.

3 THEORY

Intensity decay of the nanosecond fluorophores (Rh800 and IcG) was determined from the frequency-domain intensity decay data, which were analyzed in terms of the multi-exponential model

$$I(t)=\sum_i \alpha_i \exp(-t/\tau_i). \tag{1}$$

In this expression α_i represents the pre-exponential factors associated with each lifetime τ_i. The fractional contribution of each decay time component to the steady state intensity is given by

$$f_i=\frac{\alpha_i\tau_i}{\Sigma_j\alpha_j\tau_j}. \tag{2}$$

The values of $\Sigma\alpha_i$ and Σf_i are typically normalized to unity and the mean lifetime given by

$$\bar{\tau}=\frac{\Sigma_i\alpha_i\tau_i^2}{\Sigma_i\alpha_i\tau_i}. \tag{3}$$

In the frequency-domain measurements the measured quantities are the phase shift of the emission (ϕ_ω) and its modulation (m_ω) at the light modulation frequency ω_i in rad/s. The values of α_i and τ_i are determined by nonlinear least squares fitting and minimization of the goodness-of-fit parameter χ_R^2

$$\chi_R^2=\frac{1}{\nu}\sum_{\omega,n}\left(\frac{\varphi_\omega-\varphi_{c\omega}}{\delta\varphi}\right)^2+\frac{1}{\nu}\sum_{\omega,n}\left(\frac{m_\omega-m_{c\omega}}{\delta m}\right)^2. \tag{4}$$

In this expression the subscript c refers to calculated values of ϕ_ω and m_ω for assumed values of α_i and τ_i, and ν is the number of degrees of freedom. The terms $\delta\phi$ and δm represent the uncertainties in the measured phase angle and modulation, respectively. In some cases we performed global analysis of data measured at more than one fluorophore concentration. In this case the sum in Eq. (3) extends over the multiple fluorophore concentrations (n).

Let us now assume that the system displays two decay times, one long (L) and one short (S) typical ns decay lifetime. The observed phase (θ) and modulation (m) for the system at any given frequency will be

$$\tan\varphi_\omega=N_\omega/D_\omega=\omega\tau_p, \tag{5}$$

$$m_\omega=(N_\omega^2+D_\omega^2)^{1/2}=(1+\omega^2\tau_m^2)^{-1/2}, \tag{6}$$

where

$$N_\omega=f_S m_S\sin\varphi_S+f_L m_L\sin\varphi_L \tag{7}$$

and

$$D_\omega=f_S m_S\cos\varphi_S+f_L m_L\varphi_L \tag{8}$$

and f_S, f_L, and ϕ_S, ϕ_L are the fractional intensities, phase angles, and modulation, respectively, for the short and long lifetime components. If the long lifetime component is much longer than the short lifetime component such that

$$\frac{\tau_L}{\tau_S}\geqslant 100 \tag{9}$$

then one could choose a modulation frequency such that the individual phase angles of the fluorophores, ϕ_S and ϕ_L, will fulfill the conditions

$$\varphi_S\approx 0°,\quad m_S\approx 1.0, \tag{10}$$

$$\varphi_L\approx 90°,\quad m_S\approx 0.0. \tag{11}$$

Substituting Eqs. (7) and (8) into Eq. (6) and applying conditions (10) and (11) we obtain

$$m_\omega\approx f_S m_S\cos\varphi_S\approx f_S. \tag{12}$$

Thus the observed modulation (m_{obs}) is

$$m_{\text{obs}}\approx f_S \tag{13}$$

and represents the fractional intensity of the fluorophore with the short lifetime.

It is well known that photon scattering in tissues results in change in the apparent fluorescence lifetime of dyes.[24–29] However, these effects do not invalidate the present measurements. The signal from the reference film is above the scattering sample, so this signal is not distorted. The phase and modulation of the ns fluorophore in the tissues may be altered, but such effects are only seen at high light modulation frequencies typically above 100 MHz. These effects occur at high frequency because the delay times due to photon migration are typically

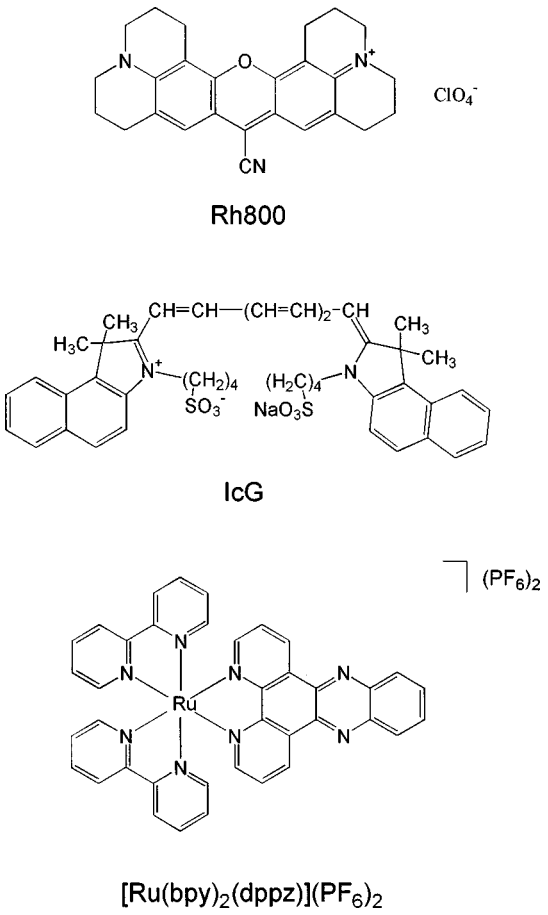


Fig. 1 The structures of rhodamine 800 (Rh800), indocyanine green (IcG) and ruthenium bis-2,2′-bipyridine dipyridophenazine, $[Ru(bpy)_2(dppz)]^{2+}$.

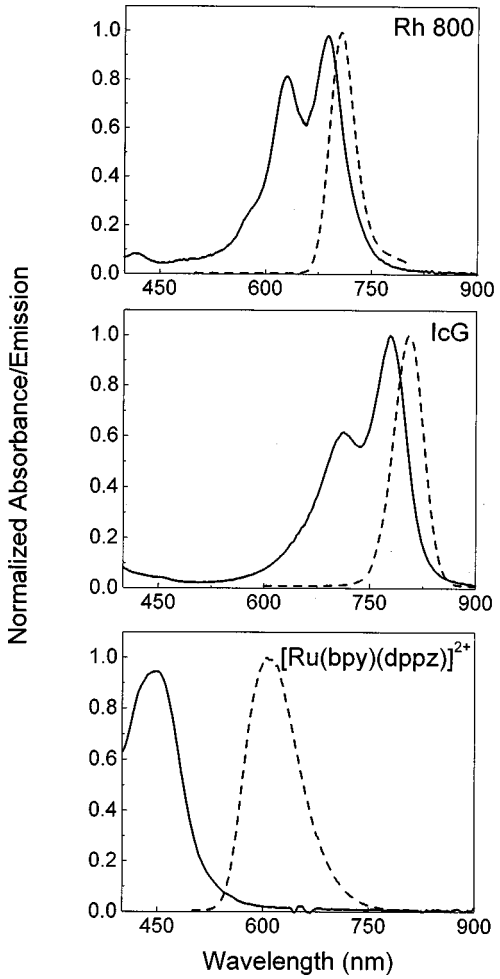


Fig. 2 Absorbance (———) and emission (– – –) spectra of rhodamine 800 and indocyanine green in water and ruthenium bis-2,2′-bipyridine dipyridophenazine complex, $[Ru(bpy)_2(dppz)]^{2+}$ in the PVA film.

200 ps or less.[28,29] Furthermore, changes in the decay time of the nanosecond probes will not affect the low frequency data. Stated mathematically, we select the two decay times and the modulation frequency so that Eq. (13) remains valid irrespective of the time delays due to photon migration or due to moderate changes in the decay times. Hence the observed modulation always reflects the fractional intensity of the short lifetime component.

4 RESULTS

4.1 SPECTRAL PROPERTIES OF Rh800 AND IcG

For the present studies we used two fluorophores with ns decay times, Rh800 and IcG as marker fluorophores, and a long lifetime metal ligand complex, $[Ru(bpy)_2(dppz)](PF_6)_2$, as the reference fluorophore. We refer to this long lifetime reference as the Ru complex (Figure 1). For use in compliance monitoring, a fluorescent marker probe needs to be safe for human consumption. For this reason, we have used IcG since it is already approved by the FDA for human use. Indocyanine green is widely used in ophthalmology, for studies of liver and kidney functions, to measure blood volume, and to estimate the severity of burns.[30–35] Because of its importance there have been several reports of its measurement in blood plasma[36] and even through skin.[37,38] We have also used Rh800 as a possible alternative to IcG because of its much higher quantum yield in aqueous media. Rh800 is structurally similar to fluorescein which is currently used in ophthalmology,[39] and it could be a good candidate for a marker fluorophore.

Absorption and emission spectra of these probes are shown in Figure 2. All three fluorophores can be excited at 600 nm. In water Rh800 emits maximally near 706 nm, and IcG near 805 nm. The Ru complex is essentially nonfluorescent in water,[40,41] but becomes fluorescent in nonpolar environments which prevent contact of the dppz ligand with water. The emission spectra of Rh800 in 0.5% intralipid are similar to those observed in water (Figure 3). In contrast, IcG displays a modest redshift of about 20 nm in the presence of intralipid (Figure 3). Additionally, the spectral properties of IcG are complex,

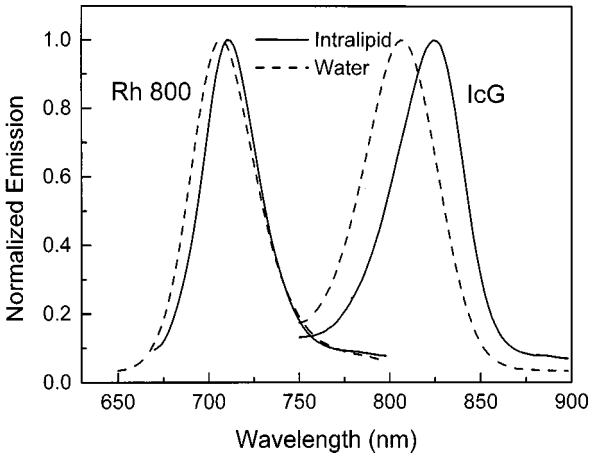


Fig. 3 Emission spectra of Rh800 and IcG in 0.5% intralipid solution. Also shown as dashed lines are the emission spectra in water.

and it appears to self-associate in water.

While the NIR absorption and emission of IcG in tissues can be readily detected, it is known that the intensity of IcG displays a complex dependence on its concentration and its interactions with biological macromolecules.[42–47] Indocyanine green appears to aggregate in aqueous solution with the aggregates being less fluorescent than the monomeric species. In biological samples IcG associates with proteins and lipids, with the intensity being dependent on the total concentration of IcG as well as on the concentration of macromolecules which bind IcG. Hence we examined the dependence of intensity on the concentrations of IcG and also Rh800.

The concentration-dependent intensities of Rh800 and IcG are shown in Figure 4. For both fluorophores the emission intensity initially increased, became constant, and then decreased as the probe concentration increased. For Rh800 the peak intensity was found to occur at much lower concentrations in 0.5% intralipid than in water. This observation suggests self-quenching of Rh800 in 0.5% intralipid. We believe this self-quenching to be due to self-association and/or energy transfer between aggregated Rh800 fluorophores in 0.5% intralipid. For IcG, the peak intensity in 0.5% intralipid occurred at slightly higher concentrations than that in water, implying reduced quenching of IcG in intralipid. This observation suggests a possible dispersion of the IcG molecules in intralipid as a result of becoming partly embedded in the lipid layer. This phenomena has been previously observed with IcG in micelles and liposomes,[46] and as observed in this study results in increased fluorescence and a redshift in spectra.

We examined the frequency-domain intensity decay of Rh800 and IcG in 0.5% intralipid. As the concentration of Rh800 increased the frequency response shifted to higher frequency (Figure 5), indicating a decrease in the mean lifetime of Rh800. These data were analyzed in terms of the multi-

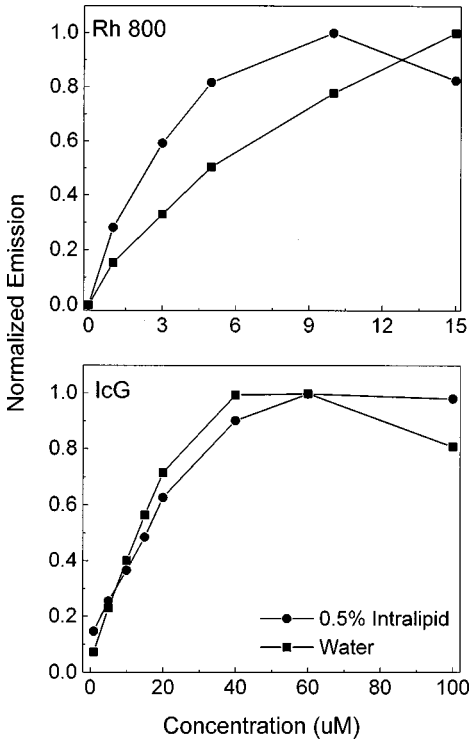


Fig. 4 Concentration dependence of fluorescence intensity of Rh800 and IcG in water (■) and 0.5% intralipid solution (●).

exponential model (Table 2). In water, in the absence of intralipid, Rh800 displayed a single exponential decay of 0.686 ns. In 0.5% intralipid the intensity decay of Rh800 became more complex. The data could be fit to a two decay time model. The individual decay times and the mean decay time decreased with increasing Rh800 concentrations (Table 2). Similar results are found for IcG, with the decay times and mean decay time decreasing with increasing IcG concentration (Table 3). While the precise values of the decay times depend on the probe concentration, the intensity decay of both Rh800 and IcG remains on the nanosecond or

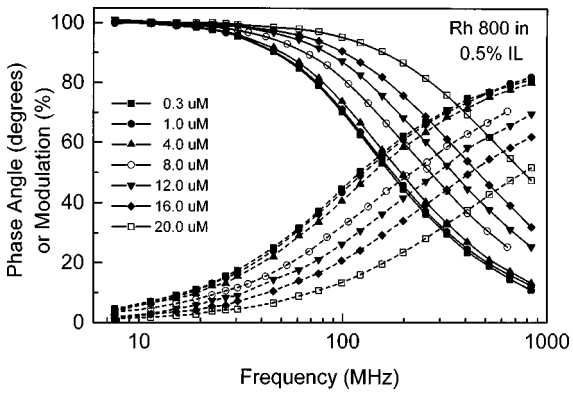


Fig. 5 Phase and modulation frequency responses for different concentrations of rhodamine 800 in 0.5% intralipid solution.

Table 2 Rhodamine 800 lifetimes in aqueous and 0.5% intralipid solutions.

Media	Rh800(μM)	τ_1 (ns)	τ_2 (ns)	α_1	f_1	$\bar{\tau}$ (ns)
Water	1.0–10.0	0.686		1.000	1.000	0.686
0.5% intralipid	0.3	1.650	0.351	0.892	0.975	1.618
	1.0	1.633	0.583	0.849	0.940	1.571
	4.0	1.511	0.487	0.810	0.930	1.439
	8.0	1.263	0.432	0.538	0.773	1.074
	12.0	0.982	0.316	0.522	0.772	0.830
	16.0	0.791	0.227	0.419	0.715	0.631
	20.0	0.582	0.173	0.265	0.547	0.397

subnanosecond time scale. This allows Rh800 and IcG to be measured by modulation sensing using a long lifetime reference fluorophore.

4.2 MODULATION SENSING OF Rh800 IN INTRALIPID

Concentrations of Rh800 in intralipid were determined by the modulation method. We initially used Rh800 because it displays a higher intensity than IcG, and was thus better suited to characterize this method. Rh800 was dissolved in 0.5% intralipid. The polyvinyl alcohol film containing the Ru complex was placed on the illuminated surface of the cuvette. Emission spectra are shown in Figure 6. The peak near 710 nm is due to Rh800 while the signal to the left of this is due to emission from the Ru complex. The excitation light was eliminated by observing the emission through a 660 nm cutoff filter. The emission filter also served to selectively attenuate the emission from the Ru complex relative to that of Rh800. This allowed the sensitivity to be adjusted without changing the concentration of Rh800 or of the Ru complex.

Frequency responses of the combined emission from the Ru complex and Rh800 are shown in Figure 7. The most dramatic features of these data are the modulation values from 2 to 20 MHz. The modulation is essentially independent of frequency, and increases as the Rh800 concentration increases. This result is due to the dramatic difference between the decay times of Rh800 in intralipid near 1.5 ns and of the Ru complex (834 ns). The data were globally analyzed in terms of short and long decay times and the fractional intensity (f_i) of each component (Table 4). Three decay times of 1.21, 1.62, and 545 ns were found adequate to fit the data of all Rh800 concentrations. We considered the two decay times of 1.21 and 1.62 ns to represent Rh800, and these two fractional intensities were combined into a single fractional intensity for the short lifetime component. As the concentration of Rh800 increased so did the fractional intensity of the short component (Table 4). This result can be understood as due to the combined measurements of the long lifetime standard and the short lifetime Rh800. At frequencies near 5 MHz the observed modulation is similar to the fractional intensity of the short lifetime Rh800. For instance, the modulation values are 0.185, 0.525, and 0.812 (Figure 7, Table 4), while calculated f_S values are 0.184, 0.534, and 0.803 (Table 4) for Rh800 concentrations of 0.05, 0.25, and 1.00 μM, respectively. Hence, the low frequency modulation values reflect the contribution of Rh800 to the total emission.

Table 3 Indocyanine green lifetimes in aqueous and 0.5% intralipid solutions.

Media	IcG(μM)	τ_1 (ns)	τ_2 (ns)	α_1	f_1	$\bar{\tau}$ (ns)
Water	7.0	0.430	0.071	0.494	0.855	0.378
0.5% intralipid	1.0	0.581	4.195	0.991	0.948	0.798
	2.0	0.584	4.502	0.995	0.960	0.739
	8.0	0.513	0.093	0.812	0.959	0.496
	16.0	0.286	0.042	0.514	0.879	0.256
	32.0	0.131	2e-4	0.010	0.788	0.103

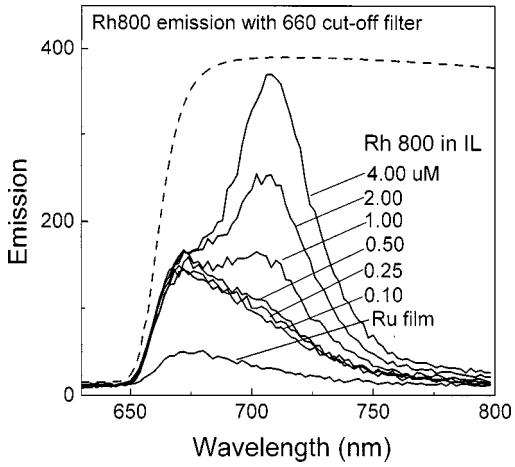


Fig. 6 Emission spectra of rhodamine 800 in 0.5% intralipid solution, in the presence of PVA film containing ruthenium metal ligand complex observed through a 660 cutoff filter. Excitation was at 600 nm. The dashed curve shows the transmission of the emission filter, which is near 80% above 700 nm.

The dependence of the modulation (m_S) and the recovered fractional intensities (f_S) on the Rh800 concentration is shown in Figure 8. For this concentration range the intensity of Rh800 is nearly linear with its concentration (Figure 4). However, the observed dependence of the fractional intensity is hyperbolic (Figure 8). This is the result of normalizing f_S+f_L to unity, so that increasing concentrations of Rh800 increase the fractional intensity f_S monotonically towards 1.0. Nonetheless, the value of m_S can be used to detect the presence of a short lifetime component and to estimate the probe concentration.

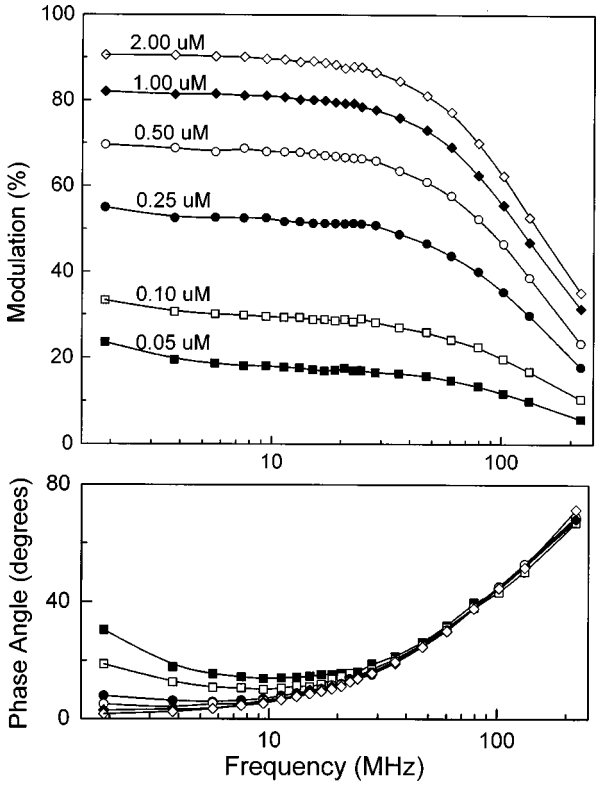


Fig. 7 Phase and modulation frequency responses for different concentrations of rhodamine 800 in 0.5% intralipid solution in the presence of PVA film containing ruthenium complex. Excitation was at 600 nm, and the emission above 660 nm was observed.

4.3 MODULATION SENSING OF IcG IN INTRALIPID

Similar experiments as with Rh800 were also performed with IcG in 0.5% intralipid. The emission of

Table 4 Global intensity decay analysis of Rh800 in 0.5% intralipid with the Ru-complex PVA film. For the global intensity decay analysis, lifetimes were held constant at all dye concentrations.

Rh800 (μM)	τ_L (ns)[a]	τ_{S1} (ns)[b]	τ_{S2} (ns)	α_S[c]	f_S[d]	$\bar{\tau}$ (ns)	m_S[e]
0.05	545	1.616	1.212	0.988	0.184	444	0.185
0.10				0.993	0.300	380	0.301
0.25				0.998	0.534	254	0.525
0.50				0.999	0.681	173	0.680
1.00				0.999	0.803	108	0.812
2.00				0.999	0.856	80	0.902

[a] τ_L=long lifetime.

[b] τ_{S1}=short lifetime. The intensity decay of Rh800 was a double exponential with decay times τ_{S1} and τ_{S2}.

[c] The terms $\alpha_L+\alpha_S=1.0$, where $\alpha_S=\alpha_{S1}+\alpha_{S2}$.

[d] The terms $f_L+f_S=1.0$, where $f_S=f_{S1}+f_{S2}$.

[e] The terms m_S=observed modulation values at 5.66 MHz.

Table 5 Global intensity decay analysis of IcG in 0.5% intralipid with the Ru-complex reference.

IcG (μM)	τ_L (ns)	τ_{S1} (ns)	τ_{S2} (ns)	α_S	f_S	$\bar{\tau}$ (ns)
0.25	584	1.494	0.428	0.993	0.130	508
0.50				0.996	0.200	467
1.00				0.997	0.299	409
2.00				0.998	0.437	329
4.00				0.999	0.537	270
6.00				0.999	0.572	250
10.00				0.999	0.601	233
20.00				0.999	0.659	199
40.00				0.999	0.719	164

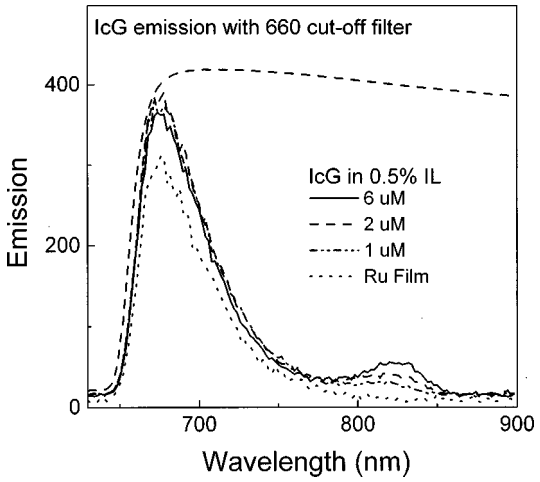


Fig. 9 Emission spectra of indocyanine green in 0.5% intralipid solution in the presence of the PVA film containing ruthenium metal ligand complex observed through a 660 cutoff filter. Excitation was at 600 nm.

IcG in intralipid occurs near 820 nm, and is considerably weaker than that of Rh800. Emission for the Ru complex is seen near 650 nm (Figure 9).

Frequency responses for IcG in intralipid are shown in Figure 10. These measurements were performed with the long lifetime reference. As seen for Rh800, the modulation increased with increasing concentrations of IcG. The modulation at 2 MHz and fractional intensity increase hyperbolically with IcG concentration (Figure 11). These concentration-dependent modulations are mostly consistent with the steady state data in Figure 4. However, there are some minor differences due to sample-to-sample variations in the IcG-intralipid samples. The important point is that the presence of IcG can be estimated from the relatively simple 2 MHz modulation measurement.

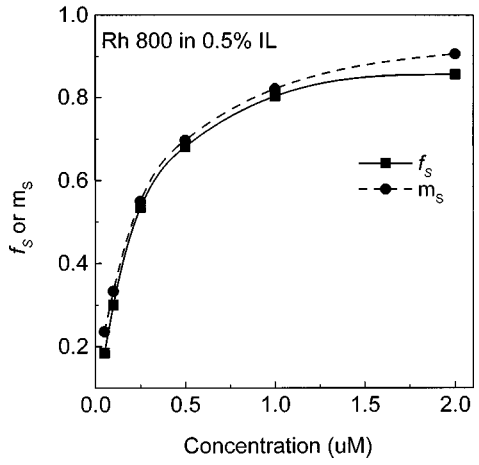


Fig. 8 Dependence of the fractional intensity of the short component (f_S) and the modulation at 1.88 MHz (m_S) on the concentration of Rh800.

4.4 ADJUSTMENT OF SENSOR SENSITIVITY

If our method is to be useful for compliance monitoring it must be useful over a range of fluorophore concentrations and skin types. Fortunately, the sensitivity of our method can be easily adjusted. This is shown in Figure 12, where the Rh800 concentration was held constant, and the concentration of the Ru complex in the PVA film altered. As the concentration and intensity of the reference was increased, the modulation decreased. This occurs because the fractional intensity of the long lifetime component

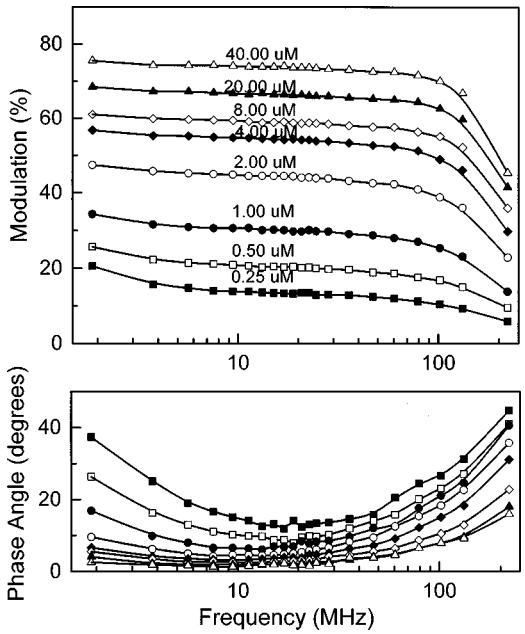


Fig. 10 Phase and modulation frequency response of different concentrations of indocyanine green in 0.5% intralipid solution in the presence of the PVA film containing ruthenium ligand complex. Excitation at 600 nm, and emission observed above 660 nm.

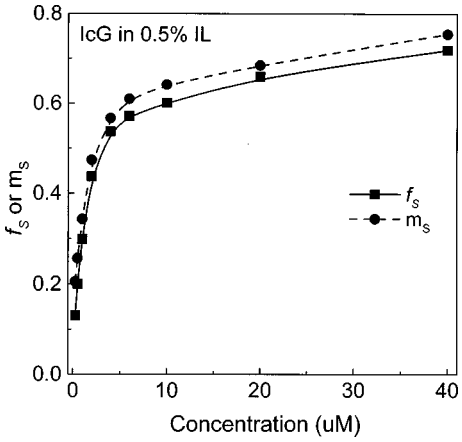


Fig. 11 Dependence of the fractional intensity of the short component (f_S) and the modulation at 1.88 MHz (m_S) on the concentration of IcG.

increases. In a real world application the sensor head could contain several reference films allowing the sensitivity to be adjusted as appropriate for a given individual.

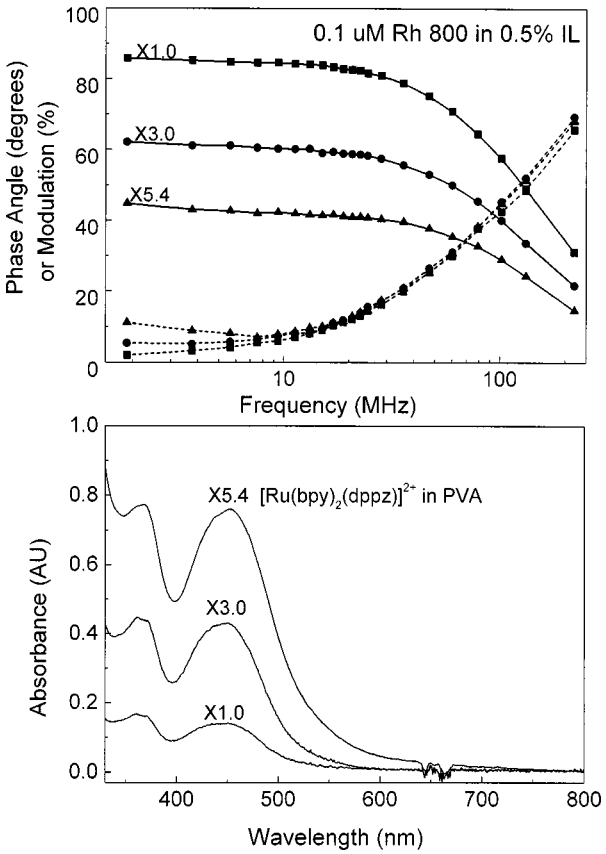


Fig. 12 Top: effect of different ruthenium metal ligand complex concentrations in the PVA film on phase and modulation response of 0.1 μM rhodamine 800 in 0.5% intralipid solution. Bottom: absorbance spectra of the films at the different ruthenium complex concentrations.

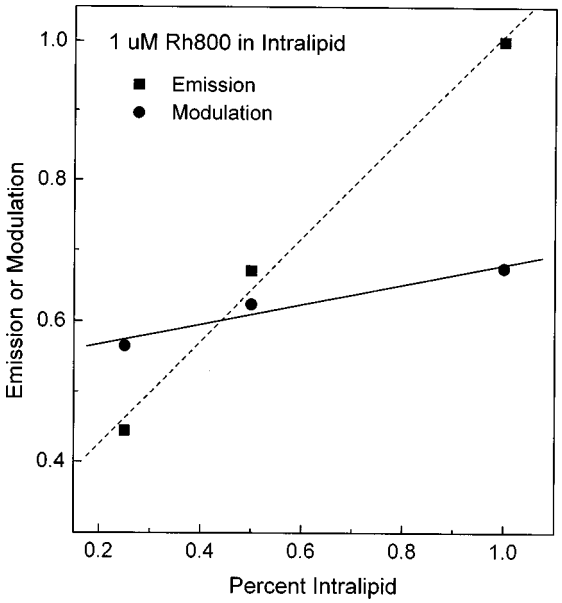


Fig. 13 Effect of variable intralipid concentration on the emission (– –) and modulation (———) of 1 μM Rh800 observed through PVA film containing ruthenium ligand complex. Modulation frequency was 3.67 MHz.

4.5 EFFECT OF RANDOM MEDIA SCATTERING AND ABSORPTION ON DETECTION OF FLUOROPHORES

Fluorescent lifetimes of fluorophores (especially short lived fluorophores) determined in highly scattering media-like tissues are usually distorted because of ''time of flight'' associated with the migration of the excitation and emission photons.[24–29] For this reason, deconvolution of the fluorophore lifetime from the photon migration time is normally required for lifetime based sensing within tissues. Absorption changes in tissues also alter photon migration,[48] and can distort the measured lifetime. To account for these effects, Patterson and Pogue[25] and Sevick-Muraca and Burch[26] have formulated mathematical models governing these processes, and recent work by Cerussi et al.[49] has verified these models experimentally. Hence we examine the effect of scattering and absorption processes on our measurements.

To determine the effects of scattering we examined the frequency domain intensity decay of 1 μM Rh800 at three (0.25, 0.50, and 1.00%) intralipid concentrations. For absorption effects, we also examined the frequency domain intensity decay of 1 μM Rh800 in 0.5% intralipid at different concentrations of black India ink. The frequency domain measurements were done in the presence of the Ru complex reference film. Steady state measurements were also performed on these samples in the presence of the Ru complex film, with a 645 nm laser diode excitation. Figure 13 shows the variation of the steady state intensity and modulation of the 1 μM Rh800 with intralipid concentration. We observe an

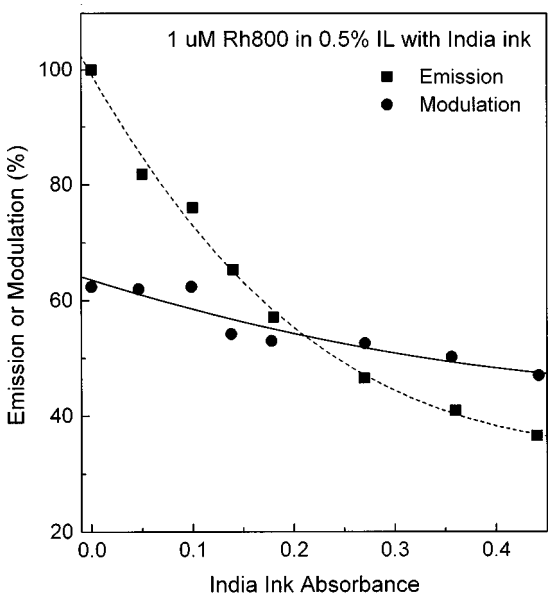


Fig. 14 Effect of variable black India ink (absorber) concentration on the emission (– –) and modulation (———) of 1 μM Rh800 in 0.5% intralipid observed through PVA film containing ruthenium ligand complex. Modulation frequency was 3.67 MHz.

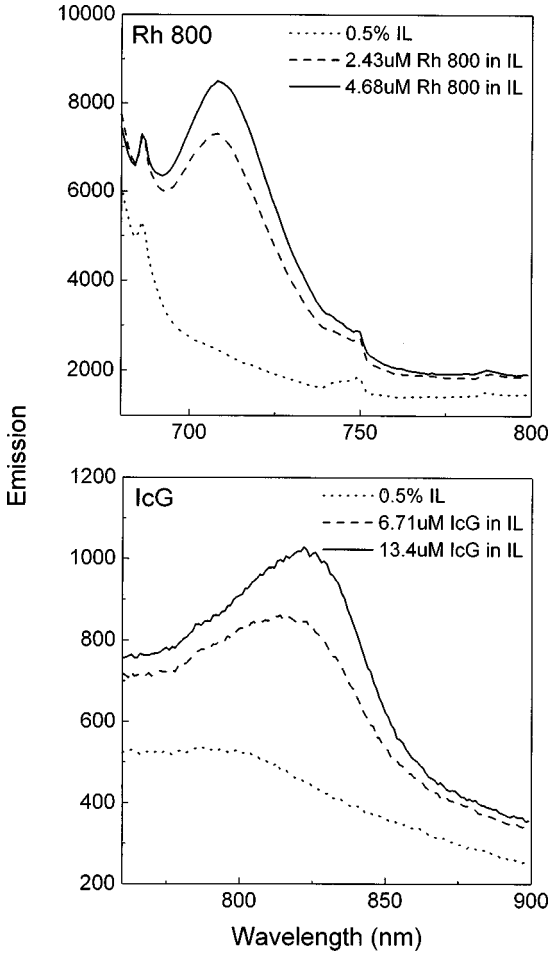


Fig. 15 Emission spectra of Rh800 and IcG in 0.5% intralipid solution in a cuvette covered with chicken skin.

increase in both the intensity and modulation with increased scattering, except that the increase with modulation was minimal for a large range of intralipid concentrations. An interesting observation with these measurements is that the phase angle change remained constant (not shown) at all concentrations of intralipid. Figure 14 shows the variation of the intensity and modulation of 1 uM Rh800 in 0.5% intralipid with the concentration of black India ink. Here we observe a decrease in both intensity and modulation with increased black India ink concentration, except that the decrease in modulation was also minimal.

In general these results show that though our measurements are affected by scatter and absorption in intralipid and tissue, the modulation changes are minimal. This is even interesting since the ranges of scatter that will be encountered in real measurements on skin are likely to be more narrow. It is also important to note that our modulation measurements under the chosen conditions [Eqs. (9)–(11)] of measurement are good estimates of the fractional intensities of our short lifetime components (IcG and Rh800) relative to the long lived reference [Eq. (13)]. The modulation values reflect the intensities (and hence concentration) of our fluorophores of interest relative to that of the reference background intensity. Since only an approximate intensity is needed for compliance monitoring, these results eliminate the need for a more complex analysis of our results.

4.6 DETECTION OF INDOCYANINE GREEN AND RHODAMINE 800 THROUGH SKIN AND/OR TISSUES

An actual compliance monitor would need to perform measurement of fluorophores in tissues through skin. We modeled this situation by placing chicken skin over the intralipid sample. Emission from Rh800 and IcG could be detected, as seen by peaks at 710 and 820 nm, respectively (Figure 15). To obtain a still more realistic situation the intralipid was replaced by chicken muscle (Figure 16). In this case the muscle with intact skin was placed into the sample holder. The IcG at the indicated concentrations was injected into the muscle. Typically about 100 μL of Rh800 or IcG at the indicated concentration was injected into an approximate tissue volume of 0.5–1.0 ml. Hence there was a substantial dilution of the IcG in the tissue. Nonetheless, peaks at 710 and 820 nm were still detectable.

We used the emission of Rh800 and IcG in the chicken muscle as a final test for the compliance monitor. Measurements were made as shown in Scheme 2. Frequency-domain data are shown for

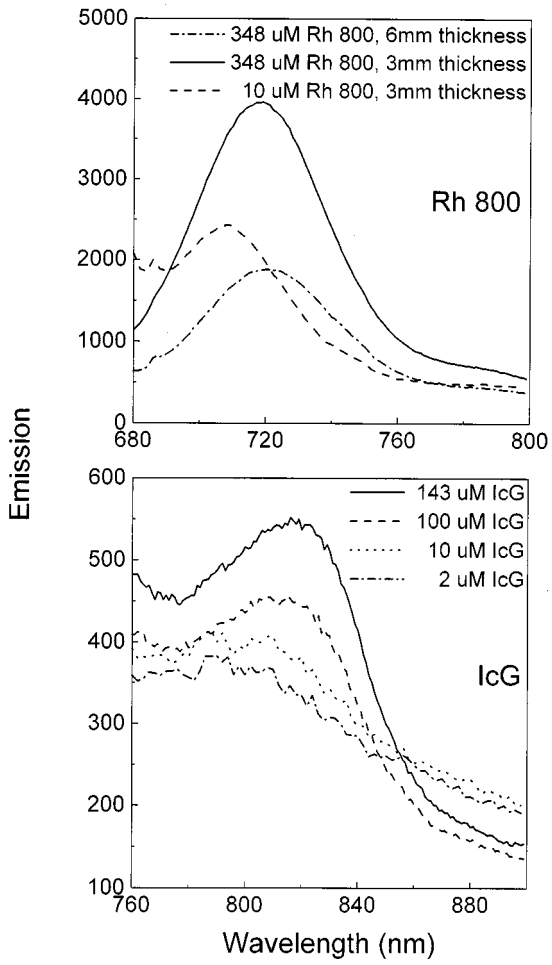


Fig. 16 Emission from chicken tissue of Rh800 (variable tissue thickness) and IcG (3 mm tissue thickness). Emission was observed through chicken skin over the muscle tissue.

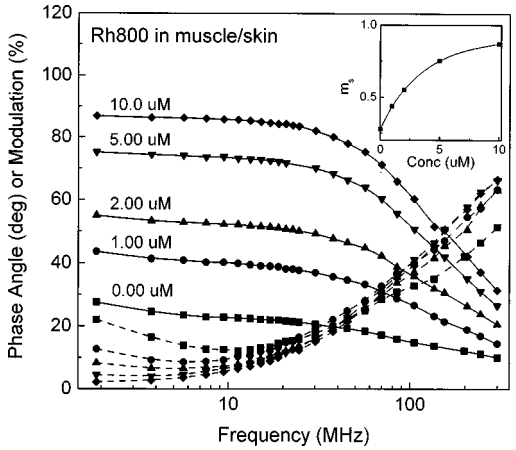


Fig. 17 Phase (– –) and modulation (———) response for different concentrations of Rh800 in chicken tissue observed through PVA film containing ruthenium ligand complex. Inset is the variation of the observed modulation with Rh800 concentration at 1.887 MHz.

Rh800 in Figure 17. As the Rh800 concentration increased, so did the modulation below 20 MHz. The low frequency modulation at 1.887 MHz was found to be sensitive to the concentration of Rh800 in the chicken tissue (Figure 17 and Table 6). Similar results were also obtained for IcG in chicken tissue (Figure 18 and Table 7). These results suggest that a relatively simple measurement of the modulation of the emission can be used to detect micromolar fluorophore concentrations in tissues.

5 DISCUSSION

In this study we estimated the concentrations of IcG and Rh800 in 0.5% intralipid and in chicken tissue using modulation measurements. When we began this study we expected to use the high frequency phase angles as a measure of the fluorophore concentration in tissues. However, while phase angle measurements may be useful, we chose the alternative method of measuring modulation in the presence of a long lifetime reference. For the combined emission of the reference and the nanosecond fluorophore, the modulation values at each dye concentration reveal the intensity of short lifetime emission. The modulation values are thus a measure of dye concentration. Our measurements show that these dyes can be detected noninvasively through skin and in muscle using the low frequency modulation. Concentrations of as low as 50 nM for Rh-800 (Figure 7) and 250 nM for IcG (Table 5) were detectable, showing the sensitivity of this technique.

It is well known that the apparent fluorescent lifetimes and intensity in highly scattering media are distorted because of migration of the excitation and emission photons.[24–29] We examined these effects (Figures 13 and 14) and found they are minimal for the ranges of our measurements. In our method, the measured modulation is dependent on the fractional intensity of the short lifetime fluorophore relative to that of the long lifetime reference used. The long lived reference imposes an ''artificial'' emission background on the media with a modulation close to zero. Consequently, any appropriate short lived fluorophore added to the system will contribute an amount of modulation that is proportional to its fractional intensity relative to that of the long lived reference, and hence its concentration. With this method, it is not necessary to recover an accurate lifetime since the measurement is simply

Table 6 Global intensity decay analysis of Rh800 in chicken muscle with the Ru-complex reference.

Rh800 (μM)	τ_L (ns)	τ_{S1} (ns)	τ_{S2} (ns)	α_S	f_S	$\bar{\tau}$ (ns)
1.00	502	1.748	0.485	0.997	0.391	306
2.00				0.997	0.518	242
5.00				0.999	0.738	132
10.0				0.999	0.865	69

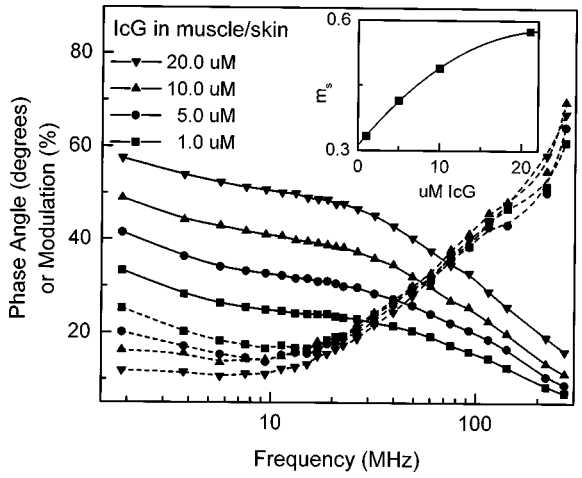


Fig. 18 Phase (– –) and modulation (———) response for different concentrations of IcG in chicken tissue observed through PVA film containing ruthenium ligand complex. Inset is the variation of the observed modulation with IcG concentration at 1.887 MHz.

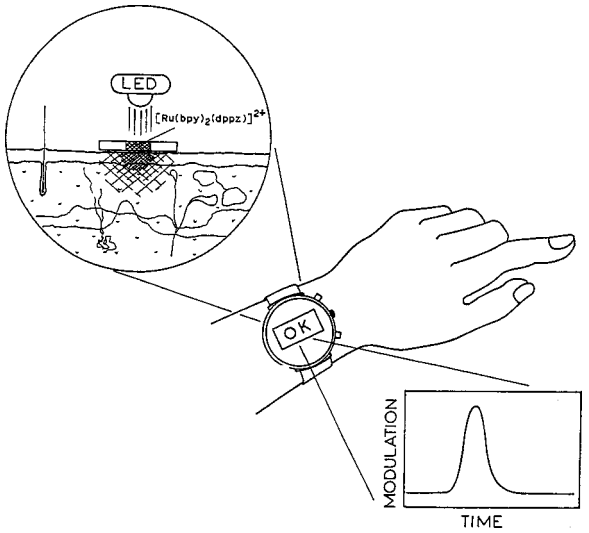


Fig. 19 Proposed watch-like device for noninvasive compliance monitoring.

reduced to a determination relative to an existing background. This eliminates complications associated with photon migration.

Another interesting aspect of this method is that by varying the concentration of the reference fluorophore in the PVA film (Figure 12), we can vary the background emission in order to increase or decrease the sensitivity of our measurements. The implication of this is that we will be capable of making modulation measurements on different skin types irrespective of variations in the composition of blood, melanin, water, tissue, and other factors present in the skin.

How can modulation measurements be used to monitor compliance? We suggest that an appropriate marker fluorophore be coated on the medication or be ingested as a placebo tablet with the medication. Some of the characteristics that will be required of this marker fluorophore will be low toxicity, short circulation lifetime, good intestinal absorption, noninteraction with medication, and a reasonable quantum yield. Indocyanine green is currently being used for different purposes in humans.[30–35,50] It is rapidly cleared from the circulation ($T_{1/2}$=4–5 min)[31] and its lethal concentration of ⩾150 μM is much higher than that required for optimum detection (1–5 μM based on our results) in the circulation. At the present time, we propose the use of IcG as a possible marker. Currently, IcG is intravenously injected when used. However, for the purpose of drug compliance monitoring it will need to be orally ingested. For this reason, it will be necessary to determine the dosage needed for oral ingestion in order to obtain optimal detection signals. This will also depend on IcG formulation and absorptivity in the intestine. We are currently proposing to study these various aspects *in vivo* using appropriate animal models.

Table 7 Global intensity decay analysis of IcG in chicken muscle with the Ru-complex reference.

IcG (μM)	τ_L (ns)	τ_{S1} (ns)	τ_{S2} (ns)	α_S	f_S	$\bar{\tau}$ (ns)
1.00	348	4.084	0.884	0.989	0.261	257
5.00				0.991	0.328	234
10.0				0.994	0.411	206
20.0				0.997	0.517	169

Evidence for the ingestion of medication will be determined noninvasively by a transdermal measurement of the modulation of the emission from the marker fluorophore using a compact modulation device. Presence of marker fluorophore will be detected as a transient increase in the modulation at a time chosen, after the moment of ingestion (Figure 19). As control for every measurement, modulation readings will be taken before the ingestion of medication.

We believe that current opto-electronics technology will make it possible for a battery powered hand modulation device to be readily built (Figure 19). The light source could be a light emitting diode (LED), laser diode, or electroluminescent device. The output of LEDs and laser diodes can be easily modulated to 50 MHz or higher[51–53] and electroluminescent devices have to be modulated to several MHz.[54] Such a portable drug compliance monitor should be widely useful in testing of new drugs as well as treatment of chronic diseases.

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