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. 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. 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. 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, 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.

Presently, an electronic monitoring method [microwire event monitoring system (MEMS)] used in concert with an invasive marker method 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 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
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 cm−1) 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 using two mirrors. The position of the sample could be adjusted with a movable stage. The reemergent light was redirected from the usual position using two mirrors. The position of the sample could be adjusted with a movable stage. The reemergent light was redirected from the usual position using two mirrors. The position of the sample could be adjusted with a movable stage. The reemergent light was redirected from the usual position using two mirrors. The position of the sample could be adjusted with a movable stage.

Scheme II Sample configuration for fluorescence measurements in intralipid (left) or in chicken tissue (right).
not to determine the lifetime of either the reference film or the probe in the scattering sample. In fact, the modulation frequency 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

\[
I(t) = \sum_{n} \alpha_n \exp(-t/\tau_n). \tag{1}
\]

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

\[
f_i = \frac{\alpha_i \tau_i}{\sum \alpha_j \tau_j}. \tag{2}
\]

The values of \(\Sigma \alpha_i\) and \(\Sigma f_i\) are typically normalized to unity and the mean lifetime given by

\[
\overline{\tau} = \frac{\Sigma \alpha_i \tau_i^2}{\Sigma \alpha_i \tau_i}. \tag{3}
\]

In the frequency-domain measurements the measured quantities are the phase shift of the emission (\(\phi_w\)) and its modulation (\(m_w\)) at the light modulation frequency \(\omega\) in rad/s. The values of \(\alpha_i\) and \(\tau_i\) are determined by nonlinear least squares fitting and minimization of the goodness-of-fit parameter \(\chi^2\)

\[
\chi^2 = \frac{1}{\nu} \sum_{\omega \in \Omega} \left( \frac{\phi_w - \phi_{\omega}}{\delta \phi} \right)^2 + \frac{1}{\nu} \sum_{\omega \in \Omega} \left( \frac{m_w - m_{\omega}}{\delta m} \right)^2. \tag{4}
\]

In this expression the subscript \(c\) refers to calculated values of \(\phi_{\omega}\) and \(m_{\omega}\) for assumed values of \(\alpha_i\) and \(\tau_i\), and \(\nu\) is the number of degrees of freedom. The terms \(\delta \phi\) and \(\delta m\) 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 \((\bar{\varphi})\) and modulation \((\bar{m})\) for the system at any given frequency will be

\[
\tan \bar{\varphi}_w = N_w / D_w = \omega \tau_p, \tag{5}
\]

\[
m_w = (N_w^2 + D_w^2)^{1/2} = (1 + \omega^2 \tau_m^2)^{-1/2}, \tag{6}
\]

where

\[
N_w = f_S m_S \sin \varphi_S + f_L m_L \sin \varphi_L \tag{7}
\]

and

\[
D_w = f_S m_S \cos \varphi_S + f_L m_L \varphi_L \tag{8}
\]

and \(f_S, f_L\), and \(\varphi_S, \varphi_L\) 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

\[
\frac{\tau_L}{\tau_S} \gg 100 \tag{9}
\]

then one could choose a modulation frequency such that the individual phase angles of the fluorophores, \(\varphi_S\) and \(\varphi_L\), will fulfill the conditions

\[
\varphi_S \approx 0^\circ, \quad m_S \approx 1.0, \tag{10}
\]

\[
\varphi_L \approx 90^\circ, \quad m_S \approx 0.0. \tag{11}
\]

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

\[
m_w \approx f_S m_S \cos \varphi_S \approx f_S. \tag{12}
\]

Thus the observed modulation \((m_{\text{obs}})\) 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

<table>
<thead>
<tr>
<th>(\tau_1 ) [(\mu)s]</th>
<th>(\tau_2 ) [(\mu)s]</th>
<th>(\alpha_1)</th>
<th>(f_1)</th>
<th>(\bar{\tau}) [(\mu)s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.207</td>
<td>0.405</td>
<td>0.280</td>
<td>0.535</td>
<td>0.834</td>
</tr>
</tbody>
</table>

\(a\) \(\alpha_1 + \alpha_2 = 1.0\).

\(b\) \(f_1 + f_2 = 1.0\).

\(c\) \(\bar{\tau} = \sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i\).
200 ps or less. 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)]${}^{2+}$, 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. Because of its importance there have been several reports of its measurement in blood plasma and even through skin. 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, 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, 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,
and it appears to self-aggregate 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. 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, 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-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
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 $\mu$M, respectively. Hence, the low frequency modulation values reflect the contribution of Rh800 to the total emission.

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

<table>
<thead>
<tr>
<th>Media</th>
<th>Rh800(μM)</th>
<th>$\tau_1$ (ns)</th>
<th>$\tau_2$ (ns)</th>
<th>$\alpha_1$</th>
<th>$f_1$</th>
<th>$\bar{\tau}$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.0–10.0</td>
<td>0.686</td>
<td>1.000</td>
<td>1.000</td>
<td>0.686</td>
<td></td>
</tr>
<tr>
<td>0.5% intralipid</td>
<td>0.3</td>
<td>1.650</td>
<td>0.351</td>
<td>0.892</td>
<td>0.975</td>
<td>1.618</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.633</td>
<td>0.583</td>
<td>0.849</td>
<td>0.940</td>
<td>1.571</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>1.511</td>
<td>0.487</td>
<td>0.810</td>
<td>0.930</td>
<td>1.439</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>1.263</td>
<td>0.432</td>
<td>0.538</td>
<td>0.773</td>
<td>1.074</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>0.982</td>
<td>0.316</td>
<td>0.522</td>
<td>0.772</td>
<td>0.830</td>
</tr>
<tr>
<td></td>
<td>16.0</td>
<td>0.791</td>
<td>0.227</td>
<td>0.419</td>
<td>0.715</td>
<td>0.631</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>0.582</td>
<td>0.173</td>
<td>0.265</td>
<td>0.547</td>
<td>0.397</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Media</th>
<th>IcG(μM)</th>
<th>$\tau_1$ (ns)</th>
<th>$\tau_2$ (ns)</th>
<th>$\alpha_1$</th>
<th>$f_1$</th>
<th>$\bar{\tau}$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>7.0</td>
<td>0.430</td>
<td>0.071</td>
<td>0.494</td>
<td>0.855</td>
<td>0.378</td>
</tr>
<tr>
<td>0.5% intralipid</td>
<td>1.0</td>
<td>0.581</td>
<td>4.195</td>
<td>0.991</td>
<td>0.948</td>
<td>0.798</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.584</td>
<td>4.502</td>
<td>0.995</td>
<td>0.960</td>
<td>0.739</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.513</td>
<td>0.093</td>
<td>0.812</td>
<td>0.959</td>
<td>0.496</td>
</tr>
<tr>
<td></td>
<td>16.0</td>
<td>0.286</td>
<td>0.042</td>
<td>0.514</td>
<td>0.879</td>
<td>0.256</td>
</tr>
<tr>
<td></td>
<td>32.0</td>
<td>0.131</td>
<td>2e-4</td>
<td>0.010</td>
<td>0.788</td>
<td>0.103</td>
</tr>
</tbody>
</table>
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 $f_S$ 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.

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...
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.

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

<table>
<thead>
<tr>
<th>IcG (μM)</th>
<th>τ₁ (ns)</th>
<th>τₛ₁ (ns)</th>
<th>τₛ₂ (ns)</th>
<th>αₛ</th>
<th>fₛ</th>
<th>μₛ</th>
<th>τ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>584</td>
<td>1.494</td>
<td>0.428</td>
<td>0.993</td>
<td>0.130</td>
<td>508</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td></td>
<td></td>
<td></td>
<td>0.996</td>
<td>0.200</td>
<td>467</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td>0.997</td>
<td>0.299</td>
<td>409</td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td></td>
<td></td>
<td></td>
<td>0.998</td>
<td>0.437</td>
<td>329</td>
<td></td>
</tr>
<tr>
<td>4.00</td>
<td></td>
<td></td>
<td></td>
<td>0.999</td>
<td>0.537</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>6.00</td>
<td></td>
<td></td>
<td></td>
<td>0.999</td>
<td>0.572</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>10.00</td>
<td></td>
<td></td>
<td></td>
<td>0.999</td>
<td>0.601</td>
<td>233</td>
<td></td>
</tr>
<tr>
<td>20.00</td>
<td></td>
<td></td>
<td></td>
<td>0.999</td>
<td>0.659</td>
<td>199</td>
<td></td>
</tr>
<tr>
<td>40.00</td>
<td></td>
<td></td>
<td></td>
<td>0.999</td>
<td>0.719</td>
<td>164</td>
<td></td>
</tr>
</tbody>
</table>

### 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

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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.

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.
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.

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. 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, and can distort the measured lifetime. To account for these effects, Patterson and Pogue and Sevick-Muraca and Burch have formulated mathematical models governing these processes, and recent work by Cerussi et al. 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
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 μM 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
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 Rh800 (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>
<thead>
<tr>
<th>Table 6 Global intensity decay analysis of Rh800 in chicken muscle with the Ru-complex reference.</th>
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<tbody>
<tr>
<td>Rh800 [µM]</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>1.00</td>
</tr>
<tr>
<td>2.00</td>
</tr>
<tr>
<td>5.00</td>
</tr>
<tr>
<td>10.0</td>
</tr>
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</table>
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 the circulation.\(^{30–35,30}\) It is rapidly cleared from the circulation \((T_{1/2} = 4–5 \text{ min})^{31}\) and its lethal concentration of \(>150 \mu \text{M}\) is much higher than that required for optimum detection \((1–5 \mu \text{M based on our results})\) 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.

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.

### References


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

<table>
<thead>
<tr>
<th>IcG (μM)</th>
<th>(\tau_1) (ns)</th>
<th>(\tau_{S1}) (ns)</th>
<th>(\tau_{S2}) (ns)</th>
<th>(\alpha_S)</th>
<th>(f_S)</th>
<th>(\bar{\tau}) (ns)</th>
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<tr>
<td>1.00</td>
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<td>0.261</td>
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<tr>
<td>5.00</td>
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<td></td>
<td></td>
<td>0.991</td>
<td>0.328</td>
<td>234</td>
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<tr>
<td>10.0</td>
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<tr>
<td>20.0</td>
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<td>0.517</td>
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<td></td>
<td>169</td>
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</table>

**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.