Frequency-domain method for measuring spectral properties in multiple-scattering media: methemoglobin absorption spectrum in a tissuelike phantom

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We have measured the optical absorption and scattering coefficient spectra of a multiple-scattering medium, i.e., a biological tissue-simulating phantom comprising a lipid colloid containing methemoglobin by using frequency-domain techniques. The methemoglobin absorption spectrum determined in the multiple-scattering medium is in excellent agreement with a corrected methemoglobin absorption spectrum obtained from a steady-state spectrophotometer measurement of the optical density of a minimally scattering medium. The determination of the corrected methemoglobin absorption spectrum takes into account the scattering from impurities in the methemoglobin solution containing no lipid colloid. Frequency-domain techniques allow for the separation of the absorbing from the scattering properties of multiple-scattering media, and these techniques thus provide an absolute measurement of the optical absorption spectra of the methemoglobin/lipid colloid suspension. One accurately determines the absolute methemoglobin absorption spectrum in the frequency domain by extracting the scattering and absorption coefficients from the phase shift $\phi$ and average light intensity $DC$ or $\phi$ and the amplitude of the light-intensity oscillations $AC$ data with relationships provided by diffusion theory, but one determines it less accurately by using the $\phi$ and modulation $M$ or $M = AC/DC$ data and the diffusion theory relationships. In addition to the greater uncertainty in the absorption and scattering coefficients extracted from the $\phi$ and $M$ data, the optical parameters extracted from the $\phi$ and $M$ data exhibit systematically inaccurate behavior that cannot be explained by random noise in the system. Possible reasons for the systematically lower accuracy of the methemoglobin absorption spectrum obtained from $\phi$ and $M$ data are discussed.

1. Introduction

The determination of the optical properties of turbid biological media is a challenging problem in several areas of medicine and biotechnology. The intent of this study is to determine the conditions in which the accurate and efficient determination of these optical properties is possible by frequency-domain techniques. Frequency-domain techniques consist of sinusoidally modulating the intensity of a light source (Fig. 1) and employing a phase-sensitive detection system to measure the amplitude of the light-intensity oscillations $AC$, average light intensity $DC$, and phase shift $\phi$ of the detected light-intensity signal relative to the source. We must remember that in the frequency-domain method, only the front of the light-intensity wave is considered, not the optical light front, which is multiply scattered in a turbid medium and typically has a frequency that is of the order of $10^6$ times greater than the light-source intensity-modulation frequency. Gratton et al. proposed using a frequency-domain diffusion model to describe light emitted into a turbid medium from a sinusoidally modulated point source. Fishkin et al. demonstrated that when intensity-modulated light is emitted from a point source into a quasi-infinite turbid medium, a spherically symmetric photon-density wave is launched. Since then, phase shift and/or demodulation data...
AC/DC_{detector} / [AC/DC_{source}] have been typically
in the frequency domain to determine the absorption
and scattering coefficients of turbid media.\textsuperscript{11-16} Thompson
et al.\textsuperscript{17} suggested using the phase-shift and DC
data as an alternative to the phase-shift and demodu-
lation data for determining these parameters when the
demodulation of the detected light-intensity sig-
nal is close to one. They show that in these circum-
stances, typical uncertainties in the demodulation
data and phase-shift data yield greater uncertainties in
the calculated absorption and scattering coeffi-
cients than those yielded by typical uncertainties in
the DC data and phase-shift data.

Our endeavor in this study is threefold: (1) Our primary
goal is to separate completely the light-absorbing from
the light-scattering properties of a tissue-like phantom containing a small concentration of methemoglobin. We cover a broad spectral range in this study, from the green to the red portion of the visible spectrum. (2) We wish to determine which combination of phase shift, AC, DC, and demodulation data yields the most accurate and precise calculation of the absorption and scattering coefficient spectra of this medium. (3) We wish to know the smallest data set that typically allows for accurate determination of these spectra, that is, at what minimum number of light-source–light-detector separations and light-source intensity-modulation frequencies can AC, DC, and phase-shift data be acquired to yield accurate absorption and scattering coefficients. The smaller the data set, the more efficient (i.e., rapid) the determination of the absorption and scattering coefficient spectra for a turbid medium. Fantini et al.\textsuperscript{18} used frequency-domain techniques in conjunction with a diffusion model for light propagation to obtain reasonably accurate absorption spectra for different concentra-
tions of the absorbing dye methylene blue in a
multiple-scattering medium. However, they were
constrained by the low power and limited response
time of their light-emitting diode source to a limited
range of source–detector separations and modulation
frequencies. We wish to know if the quality of the spectra may be improved through calculations from a data set at least 10 times larger than that obtained by Fantini et al. Our data were obtained at multiple modulation frequencies ranging across more than a decade of values and at multiple source–detector separations.

The data analysis we use to determine the absorption
and scattering spectra of a turbid medium is
based on a diffusion approximation to the Boltzmann
transport equation. We then determine the absorption
and scattering coefficient spectra of our macro-
scopically uniform turbid medium containing a known
concentration of methemoglobin (i.e., ferric hemoglo-
bin) by fitting different combinations of phase-shift,
AC, and DC data obtained at different light wave-
lengths to a frequency-domain diffusion model de-
duced by Fishkin and Gratton.\textsuperscript{19} At a given light
wavelength, these frequency-domain data were ac-
quired at multiple source–detector separations, with
multiple light-source intensity-modulation frequen-
cies at each source–detector separation. The light
wavelengths used covered the green to red region of
the visible spectrum, and the source intensity was
modulated at radio frequency in the 19.05–304.80-
MHz region. We obtained the contribution of the
methemoglobin to the absorption spectrum of this
turbid medium by comparing the absorption spec-
trum of the turbid medium to the absorption spec-
trum of a medium of the same turbidity containing no
methemoglobin (i.e., a blank or control turbid medium).
The absorption and scattering coefficient spectra of
the control turbid medium were obtained through the
same experimental protocol as the turbid medium
containing the methemoglobin.

The accuracy in determining the concentration
contribution of methemoglobin to the absorption spec-
trum of the uniform turbid medium was then evalu-
ated: The apparent absorption spectrum $\mu_{app}$ of an
equal concentration of methemoglobin in an aqueous
solution of minimal turbidity was compared with the
methemoglobin absorption spectrum obtained from
the turbid medium. We determined this apparent
absorption coefficient by a steady-state measurement
of the medium optical density, using a transmission
geometry and the Beer–Lambert relationship:

$$\mu_{app} = \mu_L + \mu_s = \frac{1}{L} \log_e \frac{I_0}{I} = \varepsilon C,$$

where $\mu_L$ is the inverse of the mean distance a photon travels before it is absorbed by the chromophore (the chromophore being methemoglobin in this case), $\mu_s$ is the inverse of the mean free path for elastic scattering of a photon by the chromophore, $L$ is the distance the photons traveled through the transporting medium before reaching the detector, $I_0$ is the incident light intensity, $I$ is the detected light intensity, $\varepsilon$ is the extinction coefficient of the chromophore at light
wavelength $\lambda$ (in units of cm$^{-1}$ $\mu$M$^{-1}$), and $C$ is the
concentration of the chromophore. Equation (1) is valid if the following assumption holds: \( 1 \mu_s \gg L \) so that the probability of multiple scattering of photons in the transporting medium is negligible; all photons reaching the detector thereby travel the same distance \( L \) through the medium. The steady-state expression in Eq. (1) may be derived from the Boltzmann transport equation\(^{20,21} \) if it is assumed that \( \mu_s \) is sufficiently small that the integral term in the Boltzmann transport equation can be neglected.

Equation (1) becomes an exact relationship when \( \mu_s = 0 \). Equation (1) is not applicable to the case of diffusive light transport through a turbid medium of width \( L \). In these circumstances, \( \mu_a \ll \mu_s \), and \( 1/\mu_a \ll L \) so that the probability of multiple scattering of photons in the medium is high. Hence we cannot assume that all the photons traversing the medium travel the same distance \( L \) from the source to the detector.

### 2. Theory

For an infinite, macroscopically uniform medium, Fishkin and Gratton\(^{19} \) solved the diffusion equation with a sinusoidally intensity-modulated point source for the photon density \( U(r, t) \) at a location \( r \) relative to the source at time \( t \) to yield (in photons per unit volume)

\[
U(r, t) = \frac{S}{4\pi V D r} \exp \left[ -\frac{r (\mu_a/3)^2}{D} \right] + \frac{SA}{4\pi V D r} \times \exp \left[ -\frac{r^2 (\mu_a^2 + \omega^2)^{1/4}}{V D^2} \cos \frac{1}{2} \tan^{-1} \left( \frac{\omega}{\mu_a} \right) \right] \times \exp \left[ -\frac{r^2 (\mu_a^2 + \omega^2)^{1/4}}{V D^2} \right] \times \sin \left( \frac{1}{2} \tan^{-1} \left( \frac{\omega}{\mu_a} \right) \right) - i(\omega t + \epsilon). \tag{2}
\]

The speed of each photon in the medium surrounding the scattering particles is given by \( v = 3.00 \times 10^{10} \) cm/s/n, \( v \) being the index of refraction of the transporting medium.

\[
D = \frac{1}{3\mu_a + \mu_s^\prime} \tag{3}
\]

is the diffusion coefficient in units of distance, \( \mu_a \) is the absorption coefficient [defined in Eq. (1)],

\[
\mu_s^\prime = (1 - g)\mu_s \tag{4}
\]

is the reduced scattering coefficient, where \( g \) is the average of the cosine of the scattering angle, and \( \mu_s \) is the scattering coefficient [defined in Eq. (1)]. \( S \) is the source strength (in photons per second), \( A \) is the modulation of the source, \( i = (-1)^{1/2} \), \( \omega \) is the angular modulation frequency of the source, and \( \epsilon \) is the phase of the source. From Eq. (2) we predict that the photon density \( U(r, t) \) generated by an isotropically emitting, sinusoidally intensity-modulated point source immersed in an infinite medium constitutes a scalar field that propagates at a constant speed in a spherical wave and attenuates as a decaying exponential in \( r \), divided by \( r \), as it propagates.

Figure 2 shows a typical geometry used to generate and detect the diffusive wave predicted by Eq. (2). Although the photons represented in Fig. 2 are injected into the multiply scattering medium in the direction \( -\Omega_a \), we assume in the manner of Patterson et al.\(^{22} \) that photons injected into the medium are initially scattered at a distance of \( 1/(\mu_a + \mu_s^\prime) \) (i.e., one mean free path) from the end of the source optical fiber. The assumption is that these first interactions are sufficiently localized that a simple Dirac-delta function accurately describes the light propagation when \( r \gg 1/\mu_s^\prime \). Fishkin and Gratton\(^{19} \) confirmed the \( r \) dependence of Eq. (2) for a quasi-infinite skim-milk medium containing an 810-nm light source modulated at 120 MHz, using the source/detector geometry shown in Fig. 2, with data acquired at \( r \) values varying from 2.5 to 9.6 cm in 0.115-cm increments.

The source terms \( S, A, \) and \( \epsilon \) are obviously independent of the quantities of interest, namely, the absorption and scattering coefficients \( [\mu_a(\lambda), \mu_s(\lambda)] \) of the medium at some light wavelength \( \lambda \). Ideally the source terms are also independent of \( \omega \), but in practice they are not. One possibility that eliminates these source terms from a measurement at a given \( \omega \) is to measure the properties of the photon density at two different source/detector separations, namely, \( r \) and \( r_0 \), and compare the quantities obtained at these two distances. Equation (2) yields expressions for quantities obtained at \( r \) relative to the corresponding quantities obtained at \( r_0 \), namely, the steady-state photon density \( D C \), the amplitude of the
photon-density oscillations $A_C$, and the phase shift of the photon-density wave $\Phi$. The relative quantities are given by

$$\text{DC}_{\text{rel}} = \frac{\text{DC}(r)}{\text{DC}(r_0)} = \frac{r_0}{r} \exp\left[-(r - r_0) \left(\frac{\mu_a}{D}\right)^{1/2}\right],$$  \hspace{1cm} (5)

$$\text{AC}_{\text{rel}} = \frac{\text{AC}(r)}{\text{AC}(r_0)} = \frac{r_0}{r} \exp\left[-(r - r_0) \left(\frac{\mu_s^2 + \omega^2}{v^2D^2}\right)^{1/2}\right] \times \cos \frac{1}{2} \tan^{-1} \left(\frac{\omega}{\nu \mu_a}\right),$$  \hspace{1cm} (6)

$$\Phi_{\text{rel}} = \Phi(r) - \Phi(r_0) = -(r - r_0) \left(\frac{\mu_s^2 + \omega^2}{v^2D^2}\right)^{1/2} \times \sin \frac{1}{2} \tan^{-1} \left(\frac{\omega}{\nu \mu_a}\right).$$  \hspace{1cm} (7)

The relative demodulation of the photon-density wave is given by

$$M_{\text{rel}} = \text{AC}_{\text{rel}} / \text{DC}_{\text{rel}}.$$  \hspace{1cm} (8)

Our frequency-domain data are obtained in a manner that allows for fitting these data (i.e., $\text{DC}_{\text{rel}}$, $\text{AC}_{\text{rel}}$, $\Phi_{\text{rel}}$, and $M_{\text{rel}}$) directly to Eqs. (5)-(8) to obtain the absorption and reduced scattering coefficients $\mu_a(\lambda)$, $\mu_s(\lambda)$ of the multiple-scattering medium at some light wavelength $\lambda$. Note that in the three equations Eqs. (5)-(7), there are only two unknowns, namely, $\nu \mu_a$ and $vD$, where $D$ is given by Eq. (3). We assume that the index of refraction of the transporting medium is known, so that explicit values for $\mu_a$ and $\mu_s$ can be recovered from the quantities $\nu \mu_a$ and $vD$. This means that in principle only two out of the three expressions in Eqs. (5)-(7) are needed to determine $\mu_a(\lambda)$ and $\mu_s(\lambda)$ at a single modulation frequency $\omega/2\pi$. Fantini et al.\textsuperscript{18} have calculated explicit expressions for $\mu_a$, $\mu_s$, and their relative uncertainties by using different combinations of Eqs. (5)-(7).

We may obtain explicit analytical expressions for $\mu_a$ in terms of $\mu_a$ from Eqs. (5)-(7) by employing the trigonometric identities

$$\sin \frac{\beta}{2} = \left(\frac{1 - \cos \beta}{2}\right)^{1/2}, \quad \cos \frac{\beta}{2} = \left(\frac{1 + \cos \beta}{2}\right)^{1/2},$$  \hspace{1cm} (9)

where

$$\beta = \tan^{-1} \left(\frac{\omega}{\nu \mu_a}\right).$$  \hspace{1cm} (10)

Equation (3) with Eqs. (5)-(10) yields the following:

$\text{DC}_{\text{rel}}$ equation,

$$\mu_s' = \frac{1}{3 \mu_a} \left[\ln(r/r_0 \text{DC}_{\text{rel}})^2 \left(1 + \frac{\omega^2}{v^2\mu_a^2} \right)^{1/2} \right] - \mu_a;$$  \hspace{1cm} (11)

$\Phi_{\text{rel}}$ equation,

$$\Phi_{\text{rel}} = \frac{2}{3 \mu_a} \left(1 + \frac{\omega^2}{v^2\mu_a^2} \right)^{1/2} - \mu_a;$$  \hspace{1cm} (12)

$M_{\text{rel}}$ equation,

$$M_{\text{rel}} = \frac{1}{3 \mu_a} \left[\ln(M_{\text{rel}})^2 \right] \times \left(1 - \frac{1}{2} \left(1 + \frac{\omega^2}{v^2\mu_a^2} \right)^{1/2} \right)^2 \mu_a;$$  \hspace{1cm} (13)

For an ideal system represented by Eqs. (11)-(14), a measurement of $\text{DC}_{\text{rel}}$, $\text{AC}_{\text{rel}}$, $\Phi_{\text{rel}}$, and $M_{\text{rel}}$ in a multiply scattering medium at a single modulation frequency $\omega/2\pi$ should be such that Eqs. (11)-(14), in the employment of these measured quantities, yield plots of $\mu_a'$ versus $\mu_a$ that intersect at the same point.

3. Experimental Apparatus and Method

3.A. Light Source, Fiber Optics, and Detectors for a Frequency-Domain Measurement

Two light sources, shown in Fig. 3, were used in the frequency-domain experiments. One of the light sources is a mode-locked Nd:YAG laser (Coherent Antares 76-5) that produces a train of equally spaced...
light pulses with a repetition rate of 76.20 MHz and with its output frequency doubled to a wavelength of 532 nm. The other light source is a dye laser (Coherent 700 dye laser, Palo Alto, Calif.) that is synchronously pumped by the 532-nm, 76.20-MHz output of the above-mentioned Nd:YAG system. The dye laser output is cavity dumped (by a Coherent 7200 cavity dumper) to yield a train of equally spaced light pulses with a repetition rate of 19.05 MHz, with the train of pulses from the dye laser supplying an average power of ~50 mW. Each pulse is ~5 ps full width at half-maximum. With the particular laser dyes utilized in the dye laser, namely, a Rhodamine 6G dye and a DCM dye (Exciton, Inc., Dayton, Ohio), the visible light obtained from the dye laser was continuously tunable over the wavelength range of 570–700 nm. Fourier analysis of the 19.05-MHz (76.20-MHz) light-pulse train yields a series of harmonic intensity-modulation frequencies with a spacing of 19.05 MHz (76.20 MHz) between each frequency. Cross-correlation techniques permit precise isolation of individual intensity-modulation frequencies. When measurements were performed with the 532-nm light, the average power of the 76.20-MHz pulse train was attenuated to 100 mW by transmission through a polarizer.

The 19.05-MHz (76.20-MHz) light-pulse train is coupled to a plastic bifurcated optical fiber, as shown in Fig. 3. One of the ends of this fiber conveys the laser light to the sample being studied (i.e., fiber Fs, the source, or sample optical fiber), and the other fiber end (i.e., fiber Fr, the reference optical fiber) conveys the light to a reference Hamamatsu R928 photomultiplier tube (PMTr). The bifurcation of the optical fibers is such that most of the laser light injected into the common end of the bifurcated optical fibers is transmitted to the scattering medium through fiber Fs with only a small fraction of the light going to PMTr through fiber Fr. The aperture diameter of optical fiber Fs is 0.15 cm, and the aperture diameter of optical fiber Fr is 0.1 cm. The detector optical fiber (i.e., fiber Fd) consists of a bundle of glass optical fibers with an overall aperture diameter of 0.3 cm whose output is detected by another Hamamatsu R928 photomultiplier tube (PMTd). PMTr is used as a reference for phase-shift measurements. A digital acquisition method processed the cross-correlated signal from the photomultiplier detector electronics.

In our measurements the cross-correlation frequency was $\Delta \omega / 2\pi = 40$ Hz. The geometrical configuration of the detector optical fiber with respect to the source optical fiber (Figs. 2 and 3) was such that most of the detected photons were scattered at right angles relative to the source/detector separation r. The multiply scattering media being studied were held in a glass container measuring 19 cm in diameter by 10 cm in height. The ends of optical fibers Fs and Fd (with a maximum separation distance of 3.0 cm) were immersed in the multiple-scattering medium as far as possible from the medium boundaries in order to best approximate the infinite medium boundary condition.

3.B. Measurement Technique in Multiple-Scattering Media

Frequency-domain measurements on the scattering media were made at 28 different light-source wavelengths $\lambda$, namely, at 532 nm and at wavelengths that range from 570 to 700 nm in 5-nm increments. At each light-source wavelength between 570 and 700 nm, measurements of phase shift $\Phi$, AC, and DC were made at five different intensity-modulation frequencies at source/detector separations of 2.0, 2.5, and 3.0 cm with the intensity-modulation frequencies ranging from 19.05 to 247.65 MHz. The source/detector separation r was controlled by a raster scanning device (Techno XYZ positioning table, New Hyde Park, N.Y.) with the uncertainty of a change in r equal to 10 µm. At the 532-nm wavelength the source/detector distances were the same but measurements were made at four different intensity-modulation frequencies ranging from 76.20 to 304.80 MHz. The DC, AC, and phase-shift $\Phi$ quantities measured at the r = 2.5-cm and r = 3.0-cm source/detector distances at a given intensity-modulation frequency $\omega / 2\pi$ were made relative to the corresponding quantities measured at the $r_0 = 2.0$-cm source/detector separation (at the same modulation frequency). As mentioned above, the measurement of the relative DC, AC, and phase-shift quantities (i.e., DC$_{rel}$, AC$_{rel}$, and $\Phi_{rel}$) at a given value of $\omega / 2\pi$ has the following advantage: Terms that are dependent on the source but independent of the parameters of the medium in which we are interested are eliminated, as are the spectral response factors of the phase-sensitive detection system. When fitting our frequency-domain data (DC$_{rel}$, AC$_{rel}$, and $\Phi_{rel}$) to Eqs. [5]–[8] to obtain the medium absorption and scattering coefficients $\mu_a(\lambda)$, $\mu_s(\lambda)$ at some light wavelength $\lambda$, we assume that $n = 1.33$ for the multiple-scattering media being investigated (which is the index of refraction of water in the spectral region considered). Typical instrumental uncertainties in a frequency-domain measurement are ±0.3% for both DC$_{rel}$ and AC$_{rel}$, ±0.4% for M$_{rel}$, and ±0.2° for $\Phi_{rel}$.

3.C. Absorbing Material and Scattering Medium

Methemoglobin (i.e., ferric hemoglobin) was selected as a test of a biologically important absorbing material. The advantage of using methemoglobin for these experiments is that it does not change into another form of hemoglobin while exposed to air at room temperature during a measurement. The methemoglobin solutions are brown, and the compound has a four-banded absorption spectrum with a band in the orange-red at 630 nm. We prepared a stock solution containing a 100-µM concentration of methemoglobin by dissolving equal concentrations of horse hemoglobin (Sigma Chemical Company, St. Louis, Mo.) and potassium ferricyanide into an aqueous solution buffered at a pH of 7.23. We prepared the buffered aqueous solution by dissolving a 50-mM concentration of sodium phosphate dibasic in water and then adding a sufficient amount of hydrochloric acid so that the scattering medium was buffered at a
The potassium ferrioxalate converted the balance of the hemoglobin that was not already in the methemoglobin form into methemoglobin. The resultant methemoglobin form was stable during all the experiments. A quantity of 0.075 mL of the 100-µM stock methemoglobin solution was then combined with 2.925 mL of the 7.23 pH buffer to give a 2.5-µM concentration of methemoglobin. We then measured the apparent absorption spectrum of this sample at room temperature between wavelengths of 532 and 700 nm by employing a transmission geometry, where the width of the sample-holding cuvette was 1 cm. The $|\mu_a|_{\text{app}}$ spectrum shown in Fig. 4 is obtained by inserting the measured optical density and the width of the sample-holding cuvette into Eq. (1). Optical density, which is defined as $\log_{10}(I_0/I)$, must be converted to $\log_{10}(I_0/I)$ to employ Eq. (1). A standard steady-state spectrophotometer (Perkin-Elmer Lambda 5, The Perkin-Elmer Corporation, St. Louis, Mo.) was used to obtain this spectrum, which is used for quantitative comparison with the methemoglobin absorption spectrum measured in a multiple-scattering medium.

We prepared 2300 mL of the scattering medium containing no methemoglobin (a blank scattering medium) by combining 177 mL of Liposyn III 20% with 2123 mL of the above-mentioned 7.23 pH buffer. This mixture of Liposyn and buffer yields a medium scattering coefficient of ~20 cm$^{-1}$, which is typical for soft tissues.27 The solids content of this medium was 1.54% Liposyn. The absolute absorption and scattering coefficient spectra of this medium were determined through the frequency-domain measurement technique described in Subsection 3.B and Section 4, and these spectra are presented in Fig. 5. We then uniformly mixed 59 mL of the above-mentioned 100-µM stock solution of methemoglobin with 2300 mL of the scattering medium to give a 2359-mL solution containing a 2.5-µM concentration of methemoglobin with a solids content of 1.50% Liposyn. The absolute absorption and scattering coefficient spectra of this medium were then determined through our frequency-domain methodology, and the absorption spectrum obtained from the above-mentioned blank scattering medium was then subtracted from the absorption spectrum of this 2.5-µM methemoglobin/1.50% Liposyn/7.23 pH aqueous buffer medium. Thus the absolute absorption spectrum of 2.5 µM of methemoglobin was recovered from the multiply scattering medium. This absorption spectrum along with the measured reduced scattering coefficient spectrum of the medium is shown in Figs. 6 and 7.

4. Results

The absolute absorption coefficients $\mu_a(\lambda)$ and absolute reduced scattering coefficients $\mu_s(\lambda)$ represented, respectively, by the solid and open circles in Figs. 5–7 are extracted from the frequency-domain data obtained at each light-source wavelength $\lambda$ through a nonlinear least-squares fitting routine designed to fit multiple sets of data simultaneously.28 This fitting routine, originally designed to fit frequency-domain fluorescence decay data, has been specifically modified to fit the data obtained at a given $\lambda$ at multiple modulation frequencies $\omega/2\pi$ and multiple relative source detector separations $r - r_0$ to different pairings of Eqs. (5)–(8). The $\mu_a(\lambda)$ and $\mu_s(\lambda)$ parameters were extracted from frequency-domain data sets obtained at given $\lambda$ values in three different ways: $\text{DC}_{\text{rel}}$ and $\Phi_{\text{rel}}$ data were simultaneously fit to Eqs. (5) and (7), respectively; $\text{AC}_{\text{rel}}$ and $\Phi_{\text{rel}}$ data were simultaneously fit to Eqs. (6) and (7), respectively; and finally $\Phi_{\text{rel}}$ and $\text{M}_{\text{rel}}$ data were simultaneously fit to Eqs. (7) and (8), respectively. All the fitting routines we used are part of the commercial...
Fig. 6. (a) Frequency-domain-determined scattering and absorption of the 2.5-µM methemoglobin/1.50% Liposyn/7.23 pH aqueous buffer medium. Absolute absorption coefficients \( \mu_a \) and absolute reduced scattering coefficients \( \mu_s' \) were determined by simultaneously fitting DC\(_{rel}\) and F\(_{rel}\) data, obtained at two relative distances (i.e., \( r = 2.5 \) and 3.0 cm relative to \( r_0 = 2.0 \) cm) at multiple modulation frequencies ranging from 19.05 to 304.80 MHz, to Eqs. 1 and 2, respectively. The uncertainties in the \( \mu_a \) and \( \mu_s' \) values recovered from the data analysis are of the order of \( 5 \times 10^{-4} \) and 0.1 cm\(^{-1} \), respectively. The dashed curve is the same curve as shown in Fig. 4. The solid curve is the Rayleigh-scattering-corrected methemoglobin absorption spectrum that we obtained by subtracting scattering values determined by Eq. 15 from the dashed curve. (b) Same sample as in (a) except that we determined the medium optical parameters represented by the circles by simultaneously fitting AC\(_{rel}\) and F\(_{rel}\) data to Eqs. 6 and 7, respectively. The uncertainties recovered from the data analysis here are the same as in (a). (c) Same sample as in (a) except that we determined the medium optical parameters represented by the circles by simultaneously fitting F\(_{rel}\) and M\(_{rel}\) data to Eqs. 7 and 8, respectively. The uncertainties in the \( \mu_a \) and \( \mu_s' \) values recovered from the data analysis are of the order of \( 8 \times 10^{-4} \) and 0.2 cm\(^{-1} \), respectively.

Fig. 7. (a) Same sample as in Fig. 6(a) except that the DC\(_{rel}\) and F\(_{rel}\) data simultaneously fit to Eqs. 5 and 7, respectively, were obtained at a single relative distance (i.e., \( r = 2.5 \) cm relative to \( r_0 = 2.0 \) cm) and a single modulation frequency \( \omega = 2\pi \). At a wavelength of 532 nm, \( \omega = 228.6 \text{ MHz} \), and at wavelengths of 570–700 nm, \( \omega = 114.3 \text{ MHz} \). The uncertainties in the \( \mu_a \) and \( \mu_s' \) values recovered from the data analysis are of the order of \( 16 \times 10^{-4} \) and 0.3 cm\(^{-1} \), respectively. (b) Same as Fig. 6(a). Shown for comparison.
package Globals Unlimited software (Laboratory for Fluorescence Dynamics, Department of Physics, University of Illinois at Urbana–Champaign).

4.A. Blank Multiple-Scattering Medium

Figure 5 shows spectra of the absolute absorption coefficient and absolute reduced scattering coefficient (i.e., $\mu_a$ and $\mu_s^r$, respectively) obtained for the blank multiple-scattering medium (i.e., no methemoglobin, solids content of 1.54% Liposyn). The $\mu_a$ and $\mu_s^r$ values at a given light-source wavelength $\lambda$ were extracted from the $\Phi_{rel}$ and $\varphi_{rel}$ data obtained at $\lambda$ from this blank scattering medium. The uncertainties of the $\mu_a$ and $\mu_s^r$ values are given in the caption of Fig. 5. The $\mu_a$ spectrum of the blank multiple-scattering medium (i.e., the solid circles) is compared with values of $\mu_a$ for water (i.e., the crosses) given at several wavelengths by Hale and Querry. The order of magnitude of $\mu_a$ is the same for both quantities, and their spectral dependence is qualitatively comparable. The measured $\mu_s^r$ spectrum of Fig. 5 (i.e., open circles) is compared with the solid $\mu_s^r(\lambda)$ curve predicted by van Staveren et al. for the same amount of solids content on the basis of Mie theory calculations. Although van Staveren et al. considered a slightly different scattering medium (i.e., Intralipid 10%, Kabivitrum, Stockholm), it is clear that both the order of magnitude and the spectral dependence of our measured $\mu_s^r$ values are reproduced by the Mie theory calculations of van Staveren et al. Wilson et al. obtained results similar to ours when comparing their frequency-domain determined values of $\mu_s^r$ of a 1% Liposyn solution with the predictions of the Mie theory calculations of van Staveren et al.

4.B. Multiple-Scattering Medium Containing 2.5 µM of Methemoglobin

Figures 6 and 7 show spectra of the absolute absorption coefficient $\mu_a$ (i.e., the solid circles) resulting from 2.5 µM of methemoglobin uniformly mixed with a 1.50% Liposyn solution and the absolute reduced scattering coefficient $\mu_s^r$ (i.e., the open circles). The $\mu_a$ and $\mu_s^r$ values shown in Figs. 6a, 6b, and 6c were obtained, respectively, from $\Phi_{rel}$ and $\varphi_{rel}$, $\Phi_{rel}$ and $\varphi_{rel}$, and $\Phi_{rel}$ and $M_{rel}$. The uncertainties of the $\mu_a$ and $\mu_s^r$ values are given in the captions of Figs. 6 and 7. We obtained the values of $\mu_a$ represented by the solid circles by subtracting the values of $\mu_a$ measured in the blank medium from the values of $\mu_a$ determined for the 2.5-µM methemoglobin/1.50% Liposyn/aqueous buffer solution.

4.B.1. Analysis of $\Phi_{rel}$ and DC$_{rel}$ Measurement

We determined the $\mu_a$ values shown in Fig. 6a at given $\lambda$ values by extracting the absolute absorption coefficient (and scattering coefficient) of the 2.5-µM methemoglobin/1.50% Liposyn/7.23 pH aqueous buffer solution from the $\Phi_{rel}$ and $\varphi_{rel}$ data acquired from this medium at $\lambda$ and then subtracting the $\mu_a$ value at the same $\lambda$ in Fig. 5 from this absorption. The values of $\mu_a$ represented by the dashed curve in Fig. 6a are also given in Fig. 4; i.e., the dashed curve represents the steady-state-determined methemoglobin absorption spectrum $\mu_a^{app}$. Note that the solid circles and the dashed curve follow the same general trend, although a systematic discrepancy between the frequency-domain-determined absorption spectrum and the steady-state-determined absorption spectrum grows larger at lower wavelengths. We believe that this systematic discrepancy is due to Rayleigh scattering. Note that by employing Eq. [1] to determine the dashed curve from the measured optical density, one determines $\mu_a^{app} = \mu_a + \mu_s$. Although methemoglobin is an almost spherical molecule of 5.5-nm diameter and scatters light at the wavelengths used, it is likely that the large discrepancy between the steady-state- and frequency-domain-determined absorption spectra is due to scattering from impurities rather than from methemoglobin molecules. Further purification of our methemoglobin samples have provided steady-state-determined spectra that are coincident with the spectra reported in Figs. 6 and 7 that were determined from frequency-domain data. We obtained the solid curve in Fig. 6a by subtracting an assumed amount of Rayleigh scattered light from the steady-state-determined spectrum represented by the dashed curve. The amount of Rayleigh scattering subtracted from the dashed curve is given by

$$\mu_s = \frac{\alpha}{\lambda^4},$$

where $\alpha$ is a constant that is chosen so that the subtraction yields a solid curve, i.e., a Rayleigh-scattering-corrected methemoglobin absorption spectrum, that best fits the solid circles. The solid curves in Figs. 6b, 6c, and 7 were determined in an identical fashion, with $\alpha$ chosen to best fit the solid circles in a given figure. The value of $\alpha$ used to obtain the solid curves shown in Figs. 6a and 6b is $5.3 \times 10^{-19}$ cm$^3$, and the value of $\alpha$ used in Fig. 6c is $5.7 \times 10^{-19}$ cm$^3$. The good agreement between the solid curve (i.e., the Rayleigh-scattering-corrected absorption spectrum) and the solid circles in Fig. 6a indicates that the methemoglobin absorption spectrum obtained from the $\Phi_{rel}$ and $\varphi_{rel}$ data is accurately determined. However, unlike for the calculation of the solid curve, we made no assumptions about the type of scattering in calculating the $\mu_a$ and $\mu_s^r$ values from the frequency-domain data, other than that the light transport through the methemoglobin/Liposyn/buffer medium was diffusive and that $\mu_a \ll \mu_s^r$ for this medium. The assumption of diffusive light transport allows for the complete separation of all absorption processes (contained in the values of $\mu_a$ represented by the solid circles) from all scattering processes (contained in the values of $\mu_s^r$ represented by the open circles), including Mie scattering from Liposyn particles and Rayleigh scattering from impurities in the methemoglobin sample. The difference between the dashed curve and the solid circles of Fig. 6a indicates that in the measurement performed with a steady-state technique and a transmission geometry a significant fraction of the light traversing
the 1-cm cuvette fails to reach the detector because of Rayleigh scattering in the 532–700-nm spectral region.

We emphasize that an independent evaluation of the scattering present in the methemoglobin solution (in the absence of Liposyn) was obtained. This scattering was determined from the comparison of the methemoglobin spectrum determined from the steady-state method (in the presence of Liposyn) with the methemoglobin spectrum determined from the frequency-domain method (in the absence of Liposyn). The significant scattering detected in the methemoglobin solution (in the absence of Liposyn) should serve as a caveat to researchers who employ the Beer–Lambert relationship [i.e., Eq. 1] to determine the absorption spectra of hemoglobin solutions from steady-state measurements of the optical density of these solutions.

4.B.2 Analysis of the $F_{rel}$ and $AC_{rel} Measurement

Figure 6b shows the values of $\mu_a$ and $\mu_s'$ that were extracted from the $F_{rel}$ and $AC_{rel}$ data. The $\mu_a$ and $\mu_s'$ values shown in this figure are almost identical to their respective counterparts in Fig. 6a, which were calculated from $F_{rel}$ and $DC_{rel}$ data. The good agreement between the solid curve (i.e., the Rayleigh-scattering-corrected absorption spectrum) and the solid circles in Fig. 6b indicates that the methemoglobin absorption spectrum obtained from the $F_{rel}$ and $AC_{rel}$ data is accurately determined.

4.B.3 Analysis of $F_{rel}$ and $M_{rel}$ Measurement

Figure 6c shows the values of $\mu_a$ and $\mu_s'$ that were extracted from the $F_{rel}$ and $M_{rel}$ data. The $\mu_a$ values extracted from the $F_{rel}$ and $M_{rel}$ data at any given $\lambda$ with the exception of the $\mu_s$ and $\mu_s'$ values obtained at $\lambda = 620, 625, 630, \text{ and } 635 \text{ nm}$ are all smaller than their counterparts that were extracted from the $F_{rel}$ and $DC_{rel}$ ($AC_{rel}$) data in Figs. 6a and 6b. At wavelengths between 700 and 640 nm the $\mu_a$ and $\mu_s'$ values shown in Fig. 6c are smaller than the $\mu_s$ and $\mu_s'$ values shown in Fig. 6a (or Fig. 6b) by $\sim 5\%$. At wavelengths between 570 and 615 nm the $\mu_a$ and $\mu_s'$ values shown in Fig. 6c are smaller than the $\mu_a$ and $\mu_s'$ values shown in Fig. 6a (or Fig. 6b) by $\sim 15\%$. This deviation increases to $\sim 50\%$ at 532 nm. This systematic effect cannot be explained by random noise in the frequency-domain measurement. Also, unlike in Figs. 6a and 6b, the $\mu_s$ spectrum extracted from the $F_{rel}$ and $M_{rel}$ data compares relatively poorly with the Rayleigh-scattering-corrected $\mu_s$ spectrum represented by the solid curve. The comparison is particularly poor at wavelengths smaller than 620 nm, with the most dramatic deviation occurring at 532 nm, where the absorption of the methemoglobin determined from the steady-state measurement is largest. In addition, at wavelengths smaller than 660 nm, the fluctuations in the $\mu_s'$ spectrum follow the same trend as the fluctuations in the $\mu_a$ spectrum.

Clearly the simultaneous nonlinear least-squares fit of the $F_{rel}$ and $M_{rel}$ data to Eqs. (7) and (8), respectively, yields a systematically inaccurate description of the scattering and absorption properties of the medium when the absorbing properties of the medium become sufficiently large. This result is surprising because the relative demodulation is given by $M_{rel} = AC_{rel} / DC_{rel}$, and the $AC_{rel}$ and $DC_{rel}$ data yield reasonable results when used separately in conjunction with the $F_{rel}$ data for the calculation of $\mu_a$ and $\mu_s'$ (see Figs. 6a) and 6b). The greater contribution to the uncertainty in $\mu_a$ and $\mu_s'$ from the $M_{rel}$ data (used with the $F_{rel}$ data) compared with the contribution to the uncertainty in these quantities from the $AC_{rel}$ or $DC_{rel}$ data (used with the $F_{rel}$ data) does not explain the phenomena shown in Fig. 6c, namely, why the values of $\mu_s$ represented by the solid circles show a significant systematic deviation from the solid curve at larger absorption values or why the fluctuations in the $\mu_s'$ spectrum correlate with the fluctuations in the $\mu_s$ spectrum at larger absorption values. We mention here that the values of $\mu_a$ and $\mu_s'$ that were extracted from the $DC_{rel}$ and $AC_{rel}$ data by simultaneously fitting these quantities to Eqs. (5) and (6), respectively, also show systematically inaccurate behavior when compared with any Rayleigh-scattering-corrected, steady-state-determined absorption spectrum.

4.C. Single-Modulation-Frequency Measurement Versus Multiple-Modulation-Frequency Measurement

Figure 7a shows values of $\mu_a$ and $\mu_s'$ extracted from $DC_{rel}$ and $F_{rel}$ data acquired at a single intensity-modulation frequency in the 2.5–µM methemoglobin. 1.5% Liposyn / 7.23 pH aqueous buffer solution. Equations (5) and (7) were used for this calculation with $r = 2.5$ cm, $r_0 = 2.0$ cm and with $\omega / 2\pi = 228.6$ MHz at $\lambda = 532$ nm and 114.3 MHz at $\lambda = 570$–700 nm. Figure 7b shows $\mu_a$ and $\mu_s'$ values that were determined by simultaneously fitting from $DC_{rel}$ and $F_{rel}$ data obtained at multiple modulation frequencies to Eqs. (5) and (7), respectively. The $\mu_a$ and $\mu_s'$ values obtained in this manner as well as the solid curve are also shown in Fig. 6a. The results in Fig. 7a are almost identical to the results in Fig. 7b, albeit the $\mu_a$ and $\mu_s'$ values (particularly the $\mu_s'$ values) in Fig. 7a are slightly noisier and their uncertainties are approximately three times larger than the uncertainties in the points in Fig. 7b (see the Fig. 6a caption for the errors in the Fig. 7b parameters). The greater noise and larger uncertainties are not surprising, given that only one-tenth of the data set that was used in calculating the $\mu_a$ and $\mu_s'$ values in Fig. 7b was used to calculate the $\mu_a$ and $\mu_s'$ values in Fig. 7a. The greater noise notwithstanding, a comparison of the spectra in Fig. 7a with the spectra in Fig. 7b indicates that a single, properly chosen light-intensity-modulation frequency $\omega / 2\pi$ will suffice to determine accurately the absorption spectrum of a tissue-like phantom from the $F_{rel}$ and $DC_{rel}$ ($AC_{rel}$) data. (A properly chosen modulation frequency is such that for a given value of $r - r_0$ the signal-to-noise ratio is maximized for the particular pair of variables used.) Comparatively rapid acquisition of the absorption and scattering spectra of tissue-like phantoms is thereby possible, because mea-
measurement at a single modulation frequency is sufficient.

5. Discussion
To obtain some feeling for how different values of \( \mu_a \) and \( \mu_s' \) are extracted from different combinations of \( \text{DC}_{\text{rel}} \), \( \text{AC}_{\text{rel}} \), and \( \Phi_{\text{rel}} \) data, contours of constant \( \text{DC}_{\text{rel}} \), \( \text{AC}_{\text{rel}} \), \( \Phi_{\text{rel}} \), and \( M_{\text{rel}} \) are plotted in Fig. 8 from Eqs. [11]–[14]. Frequency-domain data acquired from the 2.5-\( \mu \)M methemoglobin, 1.50% Liposyn, 7.23 pH aqueous buffer medium with a light-source wavelength of 605 nm at light-intensity modulation frequencies of 92.25 and 190.50 MHz were used in these calculations. These 605-nm data exhibit behavior that is the same as the behavior of the data at most other wavelengths and modulation frequencies. The values of the data (i.e., \( \text{DC}_{\text{rel}} \), \( \text{AC}_{\text{rel}} \), \( \Phi_{\text{rel}} \), and \( M_{\text{rel}} \)) and the source-detector separations (i.e., \( r \) and \( r_0 \)) used in these calculations are given in the caption of Fig. 8. The intersection of a pair of curves in Fig. 8 yields a value of \( \mu_a \) and a value of \( \mu_s' \) that together satisfy the two equations used to calculate those curves. Ideally in a noiseless system a diffusive medium with specific values of \( \mu_a \) and \( \mu_s' \) at a given \( \lambda \) would yield specific frequency-domain data (i.e., specific values for \( \text{DC}_{\text{rel}} \), \( \text{AC}_{\text{rel}} \), \( \Phi_{\text{rel}} \), and \( M_{\text{rel}} \)) at given values of \( r \), \( r_0 \), and \( \omega/2\pi \) that would in turn yield four curves generated from Eqs. [11]–[14], all intersecting at the same point. Our frequency-domain data do not yield this result, as can be seen in Fig. 8. Regardless of the modulation frequency, our data yield intersection points of the contours of constant \( \text{DC}_{\text{rel}} \), \( \text{AC}_{\text{rel}} \), \( \Phi_{\text{rel}} \), and \( M_{\text{rel}} \), which always have the same relative orientation on the \( \mu_a-\mu_s' \) plane. For example, in Fig. 8(a), the intersection of the \( \Phi_{\text{rel}} \) and \( M_{\text{rel}} \) curves yields \( \mu_a \) and \( \mu_s' \) values that are 0.051 and 14.4 cm\(^{-1} \), respectively, whereas the intersection of the \( \Phi_{\text{rel}} \) curve with the \( \text{AC}_{\text{rel}} \) or \( \text{DC}_{\text{rel}} \) curve yields \( \mu_a \) and \( \mu_s' \) values that are 0.075 and 20.5 cm\(^{-1} \), respectively. The intersection of the \( \text{AC}_{\text{rel}} \) curve with the \( \text{DC}_{\text{rel}} \) curve in Fig. 8(a) yields \( \mu_a \) and \( \mu_s' \) values that are 0.062 and 24.0 cm\(^{-1} \), respectively. However by comparing Fig. 8(a) with Fig. 8(b), we see that the ideal case of a common intersection point for all the contours is more closely approximated at the higher light-intensity-modulation frequency. The systematic behavior of the intersection points in Fig. 8 illustrates the consistently low (and inaccurate) values obtained for \( \mu_a \) and \( \mu_s' \) in Fig. 6(c) compared, respectively, with the \( \mu_a \) and \( \mu_s' \) values shown in Figs. 6(a) and 6(b). The systematic deviation between the intersection point of the \( \Phi_{\text{rel}} \) and \( \text{DC}_{\text{rel}} \) curves and the intersection point of the \( \Phi_{\text{rel}} \) and \( \text{AC}_{\text{rel}} \) curves is relatively small, and in any case both of these data sets yield reasonably accurate absorption spectra, as can be seen in Figs. 6(a), 6(b), and 7. The relatively gross systematic inaccuracy of the spectra in Fig. 6(c) compared with the spectra in Figs. 6(a) and 6(b), which increases with increasing absorption, appears to arise from the relative behavior of the \( \text{DC}_{\text{rel}} \) and \( \text{AC}_{\text{rel}} \) data, with the size of this effect being magnified at lower modulation frequencies, where the \( \text{DC}_{\text{rel}} \) and \( \text{AC}_{\text{rel}} \) data are correspondingly closer in value. The intersection point of the \( \Phi_{\text{rel}} \) and \( \text{M}_{\text{rel}} \) contours is affected by the relative behavior of the \( \text{DC}_{\text{rel}} \) and \( \text{AC}_{\text{rel}} \) data because \( \text{M}_{\text{rel}} = \text{AC}_{\text{rel}} / \text{DC}_{\text{rel}} \). Because \( \text{M}_{\text{rel}} = \text{AC}_{\text{rel}} / \text{DC}_{\text{rel}} \), the contours of constant \( \text{AC}_{\text{rel}} \), \( \text{DC}_{\text{rel}} \), and \( \text{M}_{\text{rel}} \) intersect at the same point. A small systematic shift in the orientation of the \( \text{AC}_{\text{rel}} \) contour relative to the \( \text{DC}_{\text{rel}} \) contour in the \( \mu_a-\mu_s' \) plane leads to a relatively large shift of the intersection point of the contours of constant \( \text{AC}_{\text{rel}} \), \( \text{DC}_{\text{rel}} \), and \( \text{M}_{\text{rel}} \), which in turn leads to a large systematic shift in the location of the intersection point between the contours of constant \( \Phi_{\text{rel}} \) and \( \text{M}_{\text{rel}} \).

The systematic inaccuracy of the spectra extracted from the \( \Phi_{\text{rel}} \) and \( \text{M}_{\text{rel}} \) data or from the \( \text{DC}_{\text{rel}} \) and \( \text{AC}_{\text{rel}} \) data has several possible origins:

(a) An unaccounted for instrumental artifact in our frequency-domain spectrophotometer introduces a slight systematic deviation in the \( \text{DC}_{\text{rel}} \) data relative to the \( \text{AC}_{\text{rel}} \) data, which becomes less evident in our frequency-domain diffusion model at higher modulation frequencies, as shown in Fig. 8. One possible effect that we considered was connected to the 0.3-cm
diameter of the detector optical fiber (i.e., optical fiber Fd in Fig. 3). We have assumed in our measurements that the detector optical fiber with the 0.3-cm-diameter aperture measures the properties of a photon-density wave (i.e., DCRef, ACRef, \( \Psi \), and MR) at a precise distance r from an isotropically emitting point source in an infinite medium. Assuming for the moment that our model of the light source and medium boundaries is completely accurate, the measurement geometry that we employ (see Fig. 2) actually permits the detector optical fiber to sample a continuously distributed light intensity that decays exponentially with r, divided by r, across the 0.3-cm-diameter fiber aperture and is continuously shifted in phase across this aperture according to Eq. [2]. With this premise in mind, we considered the possibility that our assumption of a single r value for a given set of frequency-domain data in these circumstances could cause the results from our diffusion model to be skewed in one particular direction and skewed slightly differently for the AC data compared with the DC data. We disregarded this possibility after we reduced the diameter of our collection optics to 0.1 cm and observed that no significant change occurred in the above-mentioned systematic behavior.

(b) The analytical model we employ fails to describe our system with complete accuracy. Specific inaccuracies within the analytical model may be the following: (1) With the model that we use we assume that we have an isotropically emitting point source of light, with this light source located approximately one mean free path from the end of the source optical fiber (i.e., optical fiber Fs in Fig. 3). This may not be a completely accurate assumption, given that the distance of the end of the detector optical fiber ranges from 2.0 to 3.0 cm from the end of the source optical fiber. In this region the source may be more accurately modeled as a distributed entity that injects light in one direction into the scattering medium. (2) The scattering medium in which we perform our measurements is not infinite, but with our model we assume an infinite medium. Escaping light from the scattering medium is visible by eye, and the application of the infinite geometry diffusion model to this medium is thereby not completely accurate. We disregard this as a possible source of inaccuracy of our spectra when we note that the largest systematic error is observed at a wavelength where the mean free path of a photon in the medium is smallest.

(c) The diffusion approximation that is represented by Eq. [2] has some limits. In Eq. [2] we give a most accurate description of the light transport when the albedo of the medium [i.e., \( \mu_s/\mu_a + \mu_s \)] is close to unity, i.e., \( \mu_s \gg \mu_a \). In Fig. 6 the ratio \( \mu_s/\mu_a \) decreases from \( \mu_s/\mu_a \approx 900 \) at 700 nm to \( \mu_s/\mu_a \approx 200 \) at 532 nm. This result indicates that it may not be coincidental that the magnitude of the systematic behavior observed in the \( \mu_s' \) and \( \mu_s'' \) spectra obtained from the demodulation data increases with increasing absorption.

Figure 9 demonstrates how a frequency-domain measurement at a single properly chosen modulation frequency suffices to extract accurately the optical properties of a tissue-like phantom by simultaneously fitting the DCRef and \( \Phi_{rel} \) data to Eqs. [5] and [7], respectively. The five contours of constant \( \Phi_{rel} \) and the one contour of constant DCRef in this figure are calculated from the data acquired from the 2.5-\( \mu \)M methemoglobin / 1.50% Liposyn / 7.23 pH aqueous buffer medium at a 605-nm wavelength, with r = 2.5 cm, \( r_0 = 2.0 \) cm and with \( \omega/2\pi = 95.25, 114.30, 152.40, 190.50, \) and 247.65 MHz. The \( \Phi_{rel} \) value associated with each \( \omega/2\pi \) value, as well as the DCRef value, is given in the caption of Fig. 9. We performed the calculations for this figure using Eqs. [11] and [13]. A simultaneous fit of all the multifrequency \( \Phi_{rel} \) data to Eq. [7] yields optical parameters from our data set (obtained in the modulation-frequency range of 19.05–304.80 MHz), which are less well determined than those extracted from a simultaneous fit of the DCRef and \( \Phi_{rel} \) data acquired at a single modulation frequency to Eqs. [5] and [7], respectively. A simultaneous fit of the DCRef and multifrequency \( \Phi_{rel} \) data to Eqs. [5] and [7], respectively, yields smaller confidence limits than those that we obtained by fitting the DCRef and \( \Phi_{rel} \) data acquired at a single modulation frequency to Eqs. [5] and [7], respectively [see Figs. 6a and 7a]. If a sufficiently large modulation-frequency range is used (for example, ~100 MHz to 1 GHz), a simultaneous fit of multifrequency \( \Phi_{rel} \) data to Eq. [7] might yield optical parameters that are as well determined as those determined by a simultaneous fit of our DCRef and \( \Phi_{rel} \) data acquired at a single modulation frequency to Eqs. [5] and [7], respectively. However, note that at modulation frequencies of the order of or greater than 1 GHz, Eq. [2] no longer provides an accurate description of photon-density waves in a tissue-like phantom, and a higher-order diffusion approximation is needed to describe more accurately the propagation of photon-density waves in tissue-like media.\(^{19,33}\)

Figure 9. Plots of Eqs. [11] and [13] with frequency-domain data obtained from the 2.5-\( \mu \)M methemoglobin / 1.50% Liposyn / 7.23 pH aqueous buffer medium at \( \lambda = 605 \) nm and at \( r = 2.5 \) cm relative to \( r_0 = 2.0 \) cm. These data are as follows: DCRef = 0.278 at \( \omega/2\pi = 95.25 \) MHz, \( \Phi_{rel} = 10.74^{\circ} \); at \( \omega/2\pi = 114.30 \) MHz, \( \Phi_{rel} = 12.71^{\circ} \); at \( \omega/2\pi = 152.40 \) MHz, \( \Phi_{rel} = 16.53^{\circ} \); at \( \omega/2\pi = 190.50 \) MHz, \( \Phi_{rel} = 20.56^{\circ} \); at \( \omega/2\pi = 247.65 \) MHz, \( \Phi_{rel} = 25.89^{\circ} \). The \( \Phi_{rel} \) curves are generated by Eq. [13] and become more horizontal with increasing modulation frequency.
6. Conclusion

In this frequency-domain study we have presented evidence that the relative phase shift (i.e., $\Phi_{rel}$) data used in conjunction with the DC$_{rel}$ data or alternatively the $\Phi_{rel}$ data used in conjunction with the AC$_{rel}$ data yield accurate absolute absorption coefficient spectra of turbid media. The $\Phi_{rel}$ data used in conjunction with the relative demodulation (i.e., $M_{rel} = AC_{rel}/DC_{rel}$) data yield relatively less accurate absolute absorption coefficient spectra of turbid media. An infinite medium, frequency-domain diffusion model with an isotropically emitting point source of light was employed to extract the absolute optical properties of the turbid medium from our frequency-domain data. We confirmed the accuracy of the model by comparing the frequency-domain-determined methemoglobin absorption spectrum in a tissue-like phantom to a steady-state-determined methemoglobin absorption spectrum in a minimally scattering medium, which is corrected for Rayleigh scattering. We have demonstrated that simultaneously fitting data acquired at multiple modulation frequencies ranging from 19.05 to 304.80 MHz offers no significant improvement in the noise or accuracy of the measured absorption spectra.

Figures 6a and 6b show that we can separate Rayleigh scattering that contributes to the apparent absorption spectrum from a steady-state measurement in the visible spectral region from the absorption of the methemoglobin molecules by using frequency-domain techniques, provided that the methemoglobin is uniformly distributed in a multiple-scattering medium. Paradoxically, we have corrected the steady-state-determined methemoglobin absorption spectrum for scattering by impurities in the methemoglobin by adding more scattering.

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