Functional optical coherence tomography: spatially resolved measurements of optical properties
Faber, D.J.

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We report on the scattering properties of oxygenated and de-oxygenated whole
blood from 250-1000 nm. We determine the complex refractive index of oxygenated
and de-oxygenated hemoglobin using Kramers Kronig analysis and Optical
Coherence Tomography measurements. Combining these data with Mie theory, the
scattering properties are calculated. The strong oxygen saturation dependent
scattering effects should be taken into account in data analysis of optical oxymetry.
6.1 Introduction

Hemoglobin (Hb), responsible for the transport of oxygen from the lungs to tissues, is primarily contained inside red blood cells (RBCs) and can reversibly bind up to 4 oxygen molecules to form oxygenated hemoglobin (HbO₂). The oxygen saturation (SO₂) is the statistical average of all oxygen bound to hemoglobin, relative to the total amount that can be bound. SO₂ dependent absorption of light by blood has been studied extensively [1-4] because optical methods allow for non-invasive and continuous monitoring of saturation. Studies have in particular focused on the wavelength range 250-1000 nm. Here, absorption by blood is mainly determined by the concentration and SO₂ level of hemoglobin since the absorption of water is low. Moreover, the Hb and HbO₂ spectra exhibit characteristic features (Soret and Q-bands) in this wavelength range. Currently, the clinical use of optical methods to measure bulk tissue SO₂ is widespread. These measurements are based on light extinction, which includes absorption and scattering losses along the path traveled by the light in the tissue. Scattering losses are not explicitly included in the data analysis, but are dealt with using calibration factors since the optical path length of the detected light is unknown and scattering properties vary from tissue type to tissue type and between individuals. Consequently, accurate quantitative SO₂ measurements are not possible. Recently developed techniques based on low-coherence interferometry, e.g. Optical Coherence Tomography (OCT) [5], allow for control over the optical path length traveled by the detected light and may thus resolve part of the before mentioned problem. Moreover, it may enable highly localized SO₂ measurements [6] of the microcirculation. To enable quantitative extinction based SO₂ measurements, inclusion of scattering from RBCs in the data analysis is required. The main cause of scattering by blood is the refractive index mismatch between the hemoglobin solution inside the cell, and its plasma environment. From the principle of causality (Kramers Kronig relations) and given the strong SO₂ dependent absorption spectra of hemoglobin, the refractive index of hemoglobin is also expected to be SO₂ dependent and, consequently, so is scattering by RBCs. However, SO₂ dependent whole blood scattering data are currently not available.

We present a novel method for retrieving the scattering properties of whole blood. From accurately known absorption spectra of Hb and HbO₂ we derive their complex refractive index by the Kramers Kronig (KK) relations in the wavelength range 250-1000 nm similar to the approach taken by Shumilina [7]. The obtained spectra are calibrated using OCT at 800 nm; and subsequently used to calculate the scattering properties of oxygenated and de-oxygenated whole blood with Mie theory.

6.2 Theory

Kramers Kronig Relations

Knowledge of the complex refractive index \( n(\omega) = n(\omega) + i\kappa(\omega) \) of Hb and HbO₂ is required to calculate the optical properties of oxygenated and de-oxygenated blood. The imaginary part \( \kappa(\omega) \) relates to absorption coefficient \( \mu_a \) of Hb or HbO₂ through:

\[
\kappa(\omega) = \frac{c\mu_a(\omega)}{2\omega}
\]  

(6-1)
where \( c \) is the speed of light in vacuum and \( \omega \) the angular frequency of the light. As for any causal system, \( n(\omega) \) and \( \kappa(\omega) \) are related through KK relations. For convenience we use a subtractive KK analysis [8], defined by:

\[
n(\omega) = n(\omega_0) + \frac{2}{\pi} \left( \omega^2 - \omega_0^2 \right)^{1/2} \int_{\omega_0}^{\omega} \frac{\omega' \kappa(\omega')}{\left( \omega^2 - \omega_0^2 \right)^{1/2} \left( \omega^2 - \omega'^2 \right)^{3/2}} d\omega'
\]

where \( n(\omega_0) \) is the refractive index measured at reference frequency \( \omega_0 \). The reference value \( n(\omega_0) \) provides scaling of the calculated curves. In addition, this integral offers greater convergence than the conventional expression when data are available only on a limited frequency range. \( P \) represents the Cauchy principle value of the integral. Thus, knowledge of the absorption spectrum of the hemoglobin solution in combination with one measurement at a reference frequency allows determination of the refractive index spectrum of the hemoglobin solution.

REFERENCE POINT MEASUREMENT WITH OCT

To obtain reliable refractive index measurements it is vital to keep the hemoglobin samples in physiological conditions. Optical Coherence Tomography is used to measure \( n(\omega_0) \) [9,10] of hemoglobin solutions in a closed circulation where \( \text{SO}_2 \) can be precisely controlled. The interference signal \( i_A(z) \) on the photodetector in an OCT setup can be expressed as the convolution of the source autocorrelation \( R_s(z) \) and the sample's (complex) reflectance profile \( h(z) \)

\[
i_A(z) = R_s(z) \otimes h(z)
\]

where \( z \) is the position of the coherence gate inside the sample. The amplitude of \( i_A(z) \) measured from a cuvette consists of two interference fringe envelopes corresponding to the glass-sample boundaries, separated by the cuvette's optical thickness \( OT = n_g d \). Here, \( n_g \) is the group refractive index of the sample and \( d \) is the geometrical thickness of the cuvette lumen. The group refractive index at any frequency can be written as:

\[
n_g(\omega) = n(\omega) + \frac{\partial n(\omega)}{\partial \omega}
\]

where \( n(\omega) \) is the refractive index at \( \omega \) as it appears in equation 6-2. The second right-hand side term of equation 6-4 can be obtained from equation 6-2 by differentiation to \( \omega \), hence measurement of \( n_g(\omega) \) suffices to determine \( n(\omega) \).

MIE THEORY

To calculate the scattering properties of whole blood, the RBC is assumed to be a homogeneous sphere as in most theoretical analyses on interaction of light with blood. Scattering by homogeneous spheres is described by Mie theory, which has been shown to describe single backscattering of light by RBCs well [11]. The basic results are the extinction and scattering cross sections (\( \sigma_e \) and \( \sigma_s \)) and the scattering anisotropy factor \( g \) (their formulas can be found e.g. in ref [12]) which are complex functions of the size parameter \( x \) and relative refractive index \( m \) of the sphere, given by:

\[
x = km_{\text{medium}} \quad \text{and} \quad m = m_{\text{sphere}} / m_{\text{medium}}
\]
where $m_{\text{spheres}}$ and $m_{\text{medium}}$ are the complex refractive index of the sphere and medium respectively; $a$ is the sphere radius and $k$ is the illuminating wavenumber in vacuum. The cross sections $\sigma_{e,s}$ can be scaled to macroscopic coefficients using [12]:

$$\mu_{e,s} = \frac{Hct}{V} \sigma_{e,s}$$  \hspace{1cm} (6-7)

Here, $Hct$ is the particle volume fraction (for blood called hematocrit); and $V$ is the sphere volume. The extinction coefficient accounts for both scattering and absorption losses. The absorption coefficient $\mu_{a}$ is therefore obtained by subtracting $\mu_{e}$ from $\mu_{e,s}$.

### 6.3 materials and methods

The OCT setup includes a Ti:Sapphire laser source (FemtoSource Pro, Femtolasers, Vienna), operating at center wavelength 800 nm ($\omega_0 = 2.36 \times 10^{15}$ rad/sec) with a 125 nm FWHM bandwidth in a standard Michelson interferometer. Dispersion in both interferometer arms is matched using a folded grating-lens pair in the reference arm. In-depth scanning is performed by moving the reference mirror mounted on a voice coil translator (QuickScan V-102.2L, Physik Instrumente, 150 nm resolution), driven with a 1 Hz round-off triangular waveform. In the sample arm, the focusing lens ($f$=25 mm) is mounted on a similar but independently driven voice-coil translator to provide continuous matching of the position of the focus with the measurement position. The axial resolution is 3 $\mu$m with a dynamic range of 110 dB. The detector signal is band-pass filtered and coherently demodulated by a Lock-In amplifier, digitized (8192 points per depth scan) and stored. The amplitude of $i(z)$ measured from a cuvette filled with a hemoglobin solution consists of two interference fringe envelopes corresponding to the glass-sample boundaries, separated by the cuvette’s optical thickness $OT = n_g d$. Here, $n_g$ is the group refractive index of the hemoglobin solution and $d$ is the geometrical thickness of the cuvette lumen (2.865 mm). $OT$ is determined by least-squares fitting $R_g$ (Gaussian functions) to the boundaries and calculating the separation of their centers; $n_g$ is obtained from $OT/d$. The accuracy with which $OT$ is determined depends on the width of $R_g$ and its shape. Therefore a light source with broad, Gaussian shaped spectral bandwidth is required.

The hemoglobin solutions used in the experiments are obtained from fresh porcine blood, which is anti-coagulated before use. Non scattering hemoglobin solutions are obtained by cell lysis and filtration as described before in chapter 5 (and ref [6]), resulting in a reduction of total hemoglobin concentration to 33%; which is measured to be 93 g/l using a Radiometer OSM3 blood gas analyzer. Any residual scattering in the solutions would result in deterioration of the signal corresponding to the cuvette boundaries, reducing the accuracy of the $n_g$ measurement. However, this is not observed in the corresponding OCT images. The solutions are maintained at 37 °C and oxygen saturated at 0% and 100% using a Minimax plus hollow fiber clinical oxygenator (Medtronic). Before applying the subtractive KK analysis, the measured group indices of refraction are scaled to physiological hemoglobin concentration using

$$n_g(HbX) = n_g(H_2O) + \Delta n_{g,HbX} \cdot [HbX]$$  \hspace{1cm} (6-8)
where \( n_g(H_2O) \) is the group refractive index of water (1.3409 at 800 nm; from ref [13] and using eq. 6-4), \( \Delta n_g[HbX] \) the refractive increment and \([HbX]\) the hemoglobin species' concentration.

6.4 results and discussion

GLUCOSE MEASUREMENTS

The accuracy of the setup to do the reference point measurements is tested using glucose-water solutions ranging from 1 g/l to 10 g/l.

![Figure 6-1: group refractive index measured of glucose solutions measured by OCT at varying glucose concentrations.](image)

The extrapolated group index of refraction of water (at zero glucose concentration) is \( n_g = 1.343 \pm 0.001 \) which corresponds well to the literature value of \( n_g=1.3409 \) [13] (using equation 6-4). From the data, the group refractive increment is determined as \( \Delta n_g = (1.8 \pm 0.2) \times 10^{-4} \) g/l which corresponds well to \( \Delta n_g = (1.67 \pm 0.01) \times 10^{-4} \) g/l at 850 nm found by combining data from [14] and [15].

HEMOGLOBIN MEASUREMENTS

The measured indices of refraction of HbO\(_2\) and Hb solutions at 800 nm are 1.392 ± 0.001 and 1.388 ± 0.002 respectively, average ± SD of 1000 measurements. These values are in very close agreement with values reported in the literature, re-calculated at 800 nm when appropriate [16-21] as summarized in table 6-1.

In reference [7], using a similar KK analysis, but without experimental scaling, and in reference [21] higher values of the refractive index are reported. The differences with our results are most likely due to differences in hemoglobin concentration of the used samples, and the fact that these experiments were performed on intact erythrocytes rather than hemoglobin solutions.
Since the measured values are used as reference points for scaling the real refractive index (obtained by equation 2), we verified that the means of the two data sets are significantly different at a (p<0.0001 level) using an unpaired t-test [22]. The imaginary part of the complex refractive index is calculated using equation 6-1 with $\mu_a$ obtained from ref [4]. The Kramers Kronig analysis is performed by numerical evaluation of equation 6-2 over the frequency range $1.88 \times 10^{15}$ rad/sec to $7.53 \times 10^{15}$ rad/sec (250 nm to 1000 nm wavelength range). The resulting real part of the refractive index is shown in figure 6-2.

**Table 6-1: real refractive indices reported in literature compared to our measured values.**

<table>
<thead>
<tr>
<th>reference</th>
<th>Hemoglobin or blood oxygen saturation</th>
<th>difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>This work</td>
<td>1.392±0.001 1.388±0.002</td>
<td>-</td>
</tr>
<tr>
<td>Orttung [16]</td>
<td>1.381 -      -</td>
<td>+0.8%</td>
</tr>
<tr>
<td>Stoddard [17],</td>
<td>-          - 1.382</td>
<td>+0.7% HbO2</td>
</tr>
<tr>
<td>Hammer [20]</td>
<td>-          - 1.407</td>
<td>-1.1% HbO2</td>
</tr>
<tr>
<td>Khairullina [21]</td>
<td>-          - 1.3934</td>
<td>-0.1% HbO2</td>
</tr>
<tr>
<td>Reynolds [19]</td>
<td>-          - 1.400±0.001</td>
<td>-0.6% HbO2</td>
</tr>
<tr>
<td>MacRea [18]</td>
<td>-          - 1.419</td>
<td>-1.9% HbO2</td>
</tr>
<tr>
<td>Shumilina [7]</td>
<td>-          - 1.407</td>
<td>-0.4% Hb</td>
</tr>
</tbody>
</table>

**Figure 6-2. real part of the complex refractive index of HbO$_2$ and Hb obtained from a subtractive KK analysis of the data of ref [4].**

**CALCULATED SCATTERING PROPERTIES OF BLOOD**

For the Mie calculations the code by Zijp et al [23] was modified to take complex arguments. In equation 6-6 we use $n_{medium}=1.340$ [24] as the refractive index of plasma at 800 nm; and $a=2.78$ µm, corresponding to a typical RBC volume of $V=90$ µm$^3$. For scaling to macroscopic properties (equation 6-7) the hematocrit in the calculations is taken as $Hct = 0.5$. 

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60
Figure 6-3 shows the calculated scattering coefficient and figure 6-4 the scattering anisotropy vs. wavelength of oxygenated and de-oxygenated whole blood. In the wavelength range 400-500 nm, corresponding to the location of large absorption peaks in the Hb/HbO₂ absorption spectra, large scattering changes are observed varying from 200 mm⁻¹ to 425 mm⁻¹. For λ > 600 nm scattering by oxygenated blood is about 10% higher than scattering by de-oxygenated blood; in this wavelength range the scattering coefficient decreases with increasing wavelength. The scattering anisotropy g is slightly lower for oxygenated blood for λ > 600 nm. The effect of the uncertainty in the reference measurement on the scattering properties was assessed by repeating the Mie calculations with \( n(\omega) \) now scaled by \( n(\omega) \pm \text{SD} \). The deviation at 800 nm and at the clinically relevant wavelengths 405 nm, 580 nm and 910 nm is depicted by error bars in figures 6-3 and 6-4. For the whole spectral range, deviations in \( \mu_s \) are less than 4% (8%) for oxygenated (de-oxygenated) whole blood; deviations in g are less than 1%. Consistency of the method was tested by calculating the absorption coefficient by subtracting \( \mu_s \) from \( \mu_e \). This calculated spectrum shows good agreement with the absorption data of ref [4] (figure 6-5).
Much care should be taken with regard to the absolute values of the scattering coefficient. The scaling with particle concentration assumes independent scattering for the whole medium, however, with volume concentrations of 50% this assumption is not obvious and has been performed for illustration purposes only. Alternative scaling factors have been proposed in literature [25]. These however do not change the shape of the presented curves, only their amplitude. For low hematocrits, our calculated data show good agreement with the experimental optical data of oxygenated diluted blood by Roggan et al. measured using a double integrating sphere technique [26]. However, contrary to our results, variations of scattering coefficient and anisotropy with SO$_2$ did not reach significance. Measurements of the scattering coefficient of oxygenated whole blood using the same technique by Yaroslavsky et al. [27] showed qualitative agreement with our data in the wavelength range 700-1000 nm. However, an increase in the anisotropy factor with wavelength was found. The highly forward scattering of blood, with $g$ reported between 0.985 and 0.997 [24,26-28], makes it difficult to separate scattered from non-scattered light, e.g. required in integrating sphere measurements, implying that the optical properties of whole blood are difficult to determine experimentally. Moreover, aggregation and biochemical responses, such as clotting, make it especially challenging to perform measurements of ex vivo whole blood samples under controlled physiological conditions. Extraction of optical properties from such measurements is complex, and usually requires an inverse Monte Carlo technique [26,27]. Consequently, a wide variety of experimental values have been reported [19, 24, 26-28]. The approach outlined in this Letter avoids the necessity of measurements on whole blood and using complex reconstruction algorithms. Rather, we determine the refractive index of non-scattering hemoglobin solutions using the Kramers Kronig relations, and calculate the scattering properties of oxygenated and de-oxygenated whole blood. The choice of Mie theory to perform the latter calculations is not essential. Other, possibly more accurate scattering theories (for an overview, see ref [20,24]) also take the complex refractive index as input, and it is its SO$_2$ dependence that determines the SO$_2$ dependent scattering properties. Our method therefore provides a template for accurate evaluation of the blood scattering properties.
6.5 conclusions

Summarizing, in this Letter we present experimental values for the refractive index of oxygenated and de-oxygenated hemoglobin at 800 nm. The former is significantly larger than the latter; which has not been identified before. The scattering properties of oxygenated and de-oxygenated whole blood are determined by a physically straightforward novel method based on accurately known hemoglobin absorption spectra and Kramers Kronig relations to obtain the complex refractive index as input for Mie theory. This methodology is particularly advantageous for retrieving scattering properties of whole blood since it does not require keeping whole blood under controlled physiological conditions; uses non scattering hemoglobin solutions instead of highly (forward) scattering blood samples; and does not require complex inverse strategies. It is shown that not only the absorption coefficient but also the scattering properties strongly depend on SO$_2$. Therefore, to enable quantitative (regional) tissue SO$_2$ measurements, it is vital to include both in data analysis.
ACKNOWLEDGEMENTS
We thank Alexei Kharine for translating the reference 7 from Russian.

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