

# Determination of the complex refractive index of highly concentrated hemoglobin solutions using transmittance and reflectance measurements

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**Abstract.** The complex refractive index of highly concentrated hemoglobin solutions as they appear in red blood cells are determined in the wavelength range of 250 to 1100 nm using transmittance and Fresnel reflectance measurements. The determined real parts of the refractive indices are on average 0.02 units higher than the values found in the literature. The wavelength dependence of the measured data in the UV region differs from the calculated data using the Kramers-Kronig relation. © 2005 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2138027]

**Keywords:** complex refractive index; hemoglobin; transmittance; Fresnel reflectance.

Paper 05036R received Feb. 9, 2005; revised manuscript received Jun. 6, 2005; accepted for publication Jun. 21, 2005; published online Dec. 8, 2005.

## 1 Introduction

Knowledge of the optical properties of blood is important not only for many diagnostic and therapeutic applications in laser medicine, but also for routine medical diagnostics. The optical properties of blood are required for a number of methods to calculate the light distribution in blood perfused tissues, e.g., optical tomography, fluorescence diagnosis, near infrared (NIR) diaphanoscopy, photodynamic therapy (PDT), and laser-induced thermotherapy (LITT). They can be described by the optical parameter absorption coefficient  $\mu_a$ , scattering coefficient  $\mu_s$ , and anisotropy factor  $g$ . The optical parameters of red blood cells for circulating human blood have been determined using integrating sphere measurements, followed by inverse Monte Carlo simulations.<sup>1,2</sup> Other methods calculate the optical properties from the complex refractive index of the red blood cell using Mie's theory or the Rayleigh-Gans theory.<sup>3,4</sup> The complex refractive index differs from the refractive index of water or the surrounding plasma, because of the high absorption behavior of hemoglobin combined with its very high concentration (up to 35 g/dL) within the cells. For the calculation of the optical properties, the absorption and the real part of the refractive index is required, dependent on wavelength for physiological concentrations of hemoglobin within the cells.

To our knowledge, there are no experimental studies where the refractive index of hemoglobin solutions has been measured for a continuous spectral region, either for diluted solutions or at the high concentration found in red blood cells. Barer and Joseph<sup>5</sup> presented a formula for the real part of the refractive index of different substances at 589 nm, including hemoglobin. The required refractive index values for other wavelengths, especially where the absorption of hemoglobin

is very high, were calculated using approximate formulas according to the Kramers-Kronig relation.<sup>6</sup> Faber<sup>4</sup> made similar calculations using his own measurements for the real part of refractive index values of hemoglobin solutions up to 9.3 g/dL at 800 nm. Giving reliable results for a wide wavelength range by using the Kramers-Kronig relation is problematic if the absorption behavior of the adjacent spectral areas is not known. The absorption coefficient within the erythrocyte was previously extrapolated from molar  $\mu_a$  values measured at much lower hemoglobin concentrations, neglecting the question whether the Beer-Lambert law is still valid for such highly concentrated dye solutions.

In this work, the real and imaginary parts of the refractive index of highly concentrated hemoglobin solutions of about 30 g/dL were measured in the wavelength range of 250 to 1100 nm. The real part was determined by measuring the Fresnel reflectance remitted by the surface of a hemoglobin solution; the imaginary part was determined by transmittance measurements.

## 2 Material and Methods

### 2.1 Blood Preparation

Fresh erythrocyte concentrates from healthy human donors with high mean corpuscular hemoglobin concentration (MCHC) values were selected, washed with phosphate buffer, and concentrated by centrifugation to hematocrit values of 75 to 80%. The hemoglobin concentration was measured using a clinical blood analyzer (Micros 60 OT 18, ABX Diagnostics, Montpellier, France). The blood samples were completely hemolyzed by freezing ( $-60^\circ\text{C}$ ) and thawing the sample for a total of three times. The cell membranes were removed using ultra-centrifugation (Allegra 64 R, Beckman Coulter GmbH, Krefeld, Germany) at 62,000 g for 120 min. Finally, the highly concentrated hemoglobin solution was filtered

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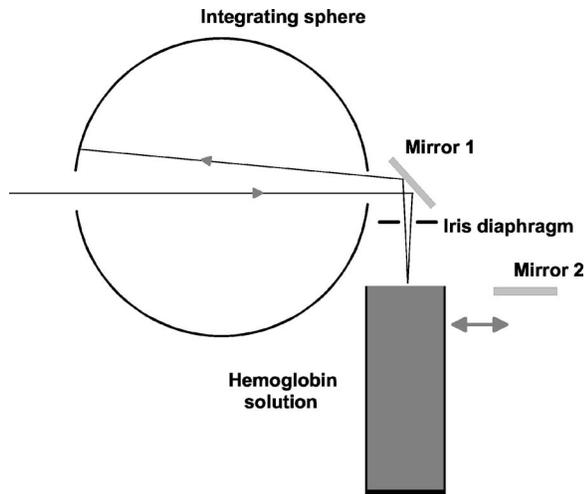


Fig. 1 Principle setup of the measurement.

through a 0.2- $\mu\text{m}$  microfilter to ensure the removal of residual cell membranes. This technique enables the preparation of cell-free hemoglobin solutions with concentrations of 28.7 and 30.6 g/dL, which are close to the physiological hemoglobin concentration within the erythrocyte (31 to 36 g/dL) without adding chemicals. Lower concentrations (2.67 and 15.3 g/dL) of hemoglobin were prepared by diluting the hemoglobin (Hb) solution of known total hemoglobin content with physiological saline solution. The prepared Hb solutions were adjusted to pH 7.4, and all measurements were carried out at 22 °C. 100% oxygen saturation of the Hb was achieved by equilibration with oxygen for 15 min before measurement. Deoxygenation (0% oxygen saturation) was effected by diluting the concentrated Hb solution with sodium dithionite in phosphate buffer (0.3% w/w) maintaining the pH at 7.4.

## 2.2 Experimental Setup

Transmittance of the diluted hemoglobin solution (2.67 g/dL) was measured for oxygenated and deoxygenated solutions using a two-beam VIS-NIR spectrometer (Lambda 900, Perkin Elmer, Rodgau-Jugesheim, Germany). The sample thickness was varied from 1 to 10 mm within the wavelength region, depending on the absorption. To prove the validity of Beer-Lambert's law at physiological hemoglobin concentrations, solutions of 15.2 and 30.6 g/dL were measured using a sample thickness of 0.02 to 1.0 mm.

A modified integrating sphere spectrometer (Lambda 900, Perkin Elmer, Rodgau-Jugesheim, Germany) was used to determine the Fresnel reflectance (Fig. 1). The illuminating light beam is reflected on mirror 1 slanted at 45 deg and reaches the surface of the hemoglobin solution at an angle close to 90 deg. The Hb solution is filled into a 50-mm-deep cylindrical vessel with a black inner surface to avoid diffuse reflectance from the depth of the sample, and an iris diaphragm is mounted to block any possible backscattered light. The Fresnel reflectance goes back vertically through the diaphragm and is reflected on mirror 1, then reaches the inner surface of the integrating sphere for detection. For the calculation of the Fresnel reflectance, the measurement was repeated with a precision mirror 2 of exact known reflectance,

which is interchangeable with the vessel of Hb solution. To evaluate the method, pure water was measured, for which the refractive index is known. No deoxygenated Hb was prepared for the Fresnel reflectance measurements, because the Hb in the highly concentrated solution is at a level of near saturation. The addition of solid sodium dithionite to 0.3 % w/w would lead to precipitation, and as a consequence causes light backscattering, which would interfere with the Fresnel reflectance.

## 2.3 Theoretical Background

For transmittance measurements on diluted absorbing solutions, the transmittance is given by Beer-Lambert's law:

$$T = \exp(-\mu_a d), \quad (1)$$

with the absorption coefficient  $\mu_a$  and the thickness  $d$  of the sample.

If a light beam is reflected rectangularly on the surface of an absorbing material, the relationship between the amplitude of the reflected beam  $\tilde{E}_r$  and the incident beam  $\tilde{E}_i$  is given by the Fresnel formula:

$$\frac{\tilde{E}_r}{\tilde{E}_i} = \frac{\tilde{n} - 1}{\tilde{n} + 1} = \frac{n - 1 - ik}{n + 1 - ik}, \quad (2)$$

with the complex refractive index  $\tilde{n}$ .

$$\tilde{n}(\lambda) = n(\lambda) - i \cdot k(\lambda), \quad (3)$$

where  $n$  is the real part of the refractive index and  $k$  is the absorption constant

$$k = \mu_a \lambda / 4\pi, \quad (4)$$

with absorption coefficient  $\mu_a$  and the corresponding wavelength  $\lambda$ .

Since the energy is proportional to the square of the amplitude, the reflectance  $R$  can be defined as the relationship  $\tilde{E}_r^2 / \tilde{E}_i^2$ . Then  $R$  is given by:

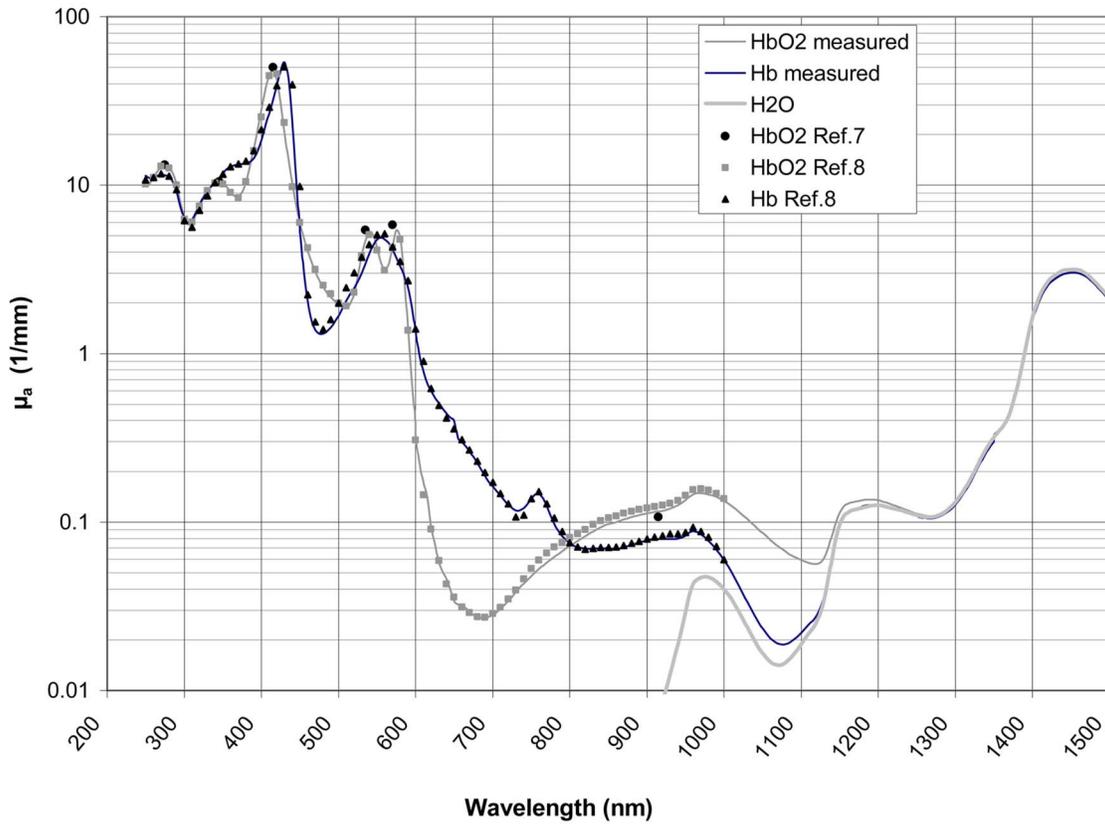
$$R = \frac{(n - 1)^2 + k^2}{(n + 1)^2 + k^2}. \quad (5)$$

Therefore, the real part of the refractive index can be calculated from the transmittance and the reflectance measurements.

## 3 Results

### 3.1 Transmittance Measurements

The absorption coefficients of an Hb solution calculated using Eq. (1) at a concentration of 2.67 g/dL from 250 to 1100 nm are shown in Fig. 2 for oxygenated and deoxygenated hemoglobin compared with reference data.<sup>7,8</sup> The Soret absorption band appears at 415 nm with an absorption coefficient of about 48  $\text{mm}^{-1}$ . The other typical absorption maxima are at 274, 344, 542, 577, and 915 nm for oxygenated hemoglobin. At 1130 nm, the values approach those of the water absorption, and above 1250 nm the absorption curves are equal. The absorption of deoxygenated hemoglobin shows the expected

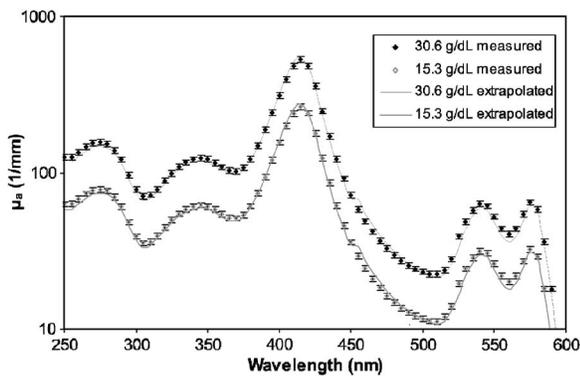


**Fig. 2** Absorption coefficient of oxygenated and deoxygenated hemoglobin solution at a concentration of 2.67 g/dL (measured with different sample thicknesses and calculated using Beer-Lambert's law) compared with some reference data and the absorption spectrum of water.<sup>7,8</sup>

shift of the Soret peak, the fusion of the two maxima at 542 and 577 nm to one peak at 555 nm, and an enhanced absorption in the red light. The absorption of the deoxygenated hemoglobin already approximates that of the water absorption at about 1000 nm and is identical above 1130 nm. The isosbestic point, where absorption is independent of the oxygenation level, is clearly visible at 805 nm.

The results of the investigations using highly concentrated hemoglobin solutions, close to the concentration inside the

red blood cell, are shown in Fig. 3. The absorption spectra of Hb solutions of 15.3 and 30.6 g/dL in the wavelength range of 250 to 1100 nm are compared with absorption values measured at a concentration of 2.67 g/dL (shown in Fig. 2) linearly extrapolated to concentrations of 15.3 and 30.6 g/dL. The curves show a high correlation with almost no differences within the range of the error limits, indicating that Beer-Lambert's law is still valid, even at such high concentrations.

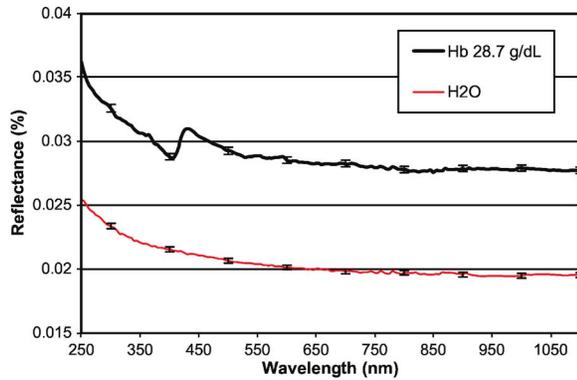


**Fig. 3** Measured absorption coefficients of highly concentrated hemoglobin solutions of 30.6 and 15.3 g/dL (sample thickness 0.02 mm) compared to the extrapolated data calculated from the 2.67 g/dL concentration.

### 3.2 Reflectance Measurements

The measured Fresnel reflectance of Hb solutions at 28.7 g/dL and pure water for control are shown in Fig. 4. The reflectance generally decreases with increasing wavelength. The shape of the Hb curves at the point of highest absorption (approximately 415 nm) is notable, indicating anomalous refraction.

In Fig. 5, the upper diagram shows the absorption constant  $k$  in the wavelength range of 250 to 1100 nm calculated using Eq. (4), and the absorption values extrapolated to the Hb concentration 28.7 g/dL from the spectra of the hemoglobin concentration level 2.67 g/dL. Using this data, the real part of refractive index  $n$  for hemoglobin (28.7 g/dL) is calculated from the Fresnel reflectance according to Eq. (5), and is shown in the lower diagram of Fig. 5. The measured reference values of the refractive index of water are also shown.<sup>9,10</sup> Similar to the Fresnel reflectance,  $n$  generally decreases with increasing wavelength with a maximum at the UV region



**Fig. 4** Measured Fresnel reflections of Hb solutions with a concentration of 28.7 g/dL and of pure water, as illustrated in Fig. 1.

(normal diffraction). The wavelength dependence of the real part of the refractive index of the hemoglobin solution is similar to the one of water, but parallel shifted by about 0.08 units to higher values. In contrast to the water curve, the slope of the Hb curve in the UV region from 250 to 310 nm is steeper. In the range of 360 to 510 nm, the real part of the refractive index shows the typical S-shape of an anomalous diffraction with a point of inflexion at 415 nm, corresponding to the absorption maximum of hemoglobin, where  $k$  is at a maximum. Furthermore, at 545 and 580 nm, small relative maxima are visible, which correspond to the relative absorption maxima. These small maxima are within the error limits and are therefore not significant.

## 4 Discussion

### 4.1 Imaginary Part of the Refractive Index

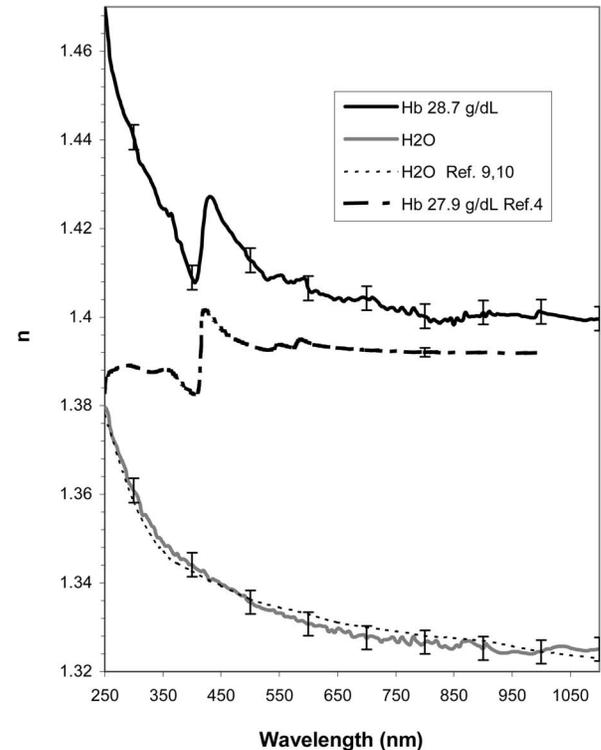
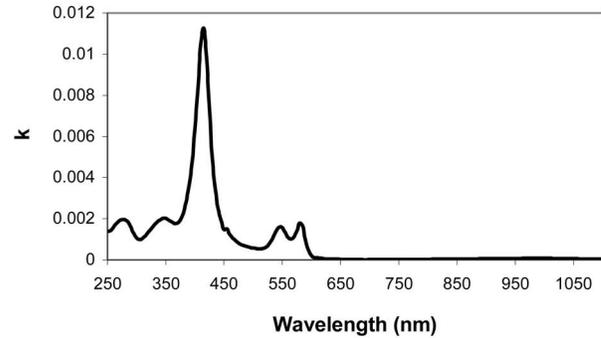
The imaginary part of the refractive index, characterized by the absorption constant  $k$ , is proportional to the absorption coefficient. The absorption coefficients determined in the present study are in agreement with most of the values found in the literature. Figure 2 shows a comparison of the measured absorption spectra of hemoglobin at a concentration of 2.67 g/dL, as well as some values from other authors and the absorption spectrum of water.

The results depicted in Fig. 3 show that the absorption coefficient is linearly correlated to the hemoglobin concentration up to 30.6 g/dL. This means that the absorption cross section is constant in this range. For the first time, these direct measurements show definitely that Beer-Lambert's law is valid for Hb concentrations that are close to the physiological values within the red blood cells (about 31 to 36 g/dL). This verifies the current extrapolations.

### 4.2 Real Part of the Refractive Index

For pure water, the determined refractive index is equal to the literature values of water within the error limit, as shown in Fig. 5, lower diagram, indicating that the method using reflectance measurements to determine the refractive index is valid.

This curve of the refractive index is different in shape and height compared to other investigations. It has to be noted that, to our knowledge, no studies exist where the refractive index of hemoglobin in physiological high concentration were



**Fig. 5** Top: Absorption constant  $k$  depends on the wavelength calculated using Eq. (4) and the absorption values extrapolated to the Hb concentration 28.7 g/dL. Bottom: Real part of refractive index  $n$  calculated from the Fresnel reflectance, following Eq. (5) compared to the refractive index of water<sup>9,10</sup> and  $n$  values obtained by subtractive Kramers-Kronig analysis.<sup>4</sup>

directly measured, whereas some authors have extrapolated or calculated this data. Based on the work of Stoddard and Adair,<sup>11</sup> who did measurements with a refractometer using white light, Barer's formula  $n_{\text{Hb}} = n_{\text{H}_2\text{O}} + 0.001942 c_{\text{Hb}}$ , a Hb concentration of 28.7 g/dL would result, for example, in a  $n_{\text{Hb}}$  of 1.388 at 589 nm. Faber et al.<sup>4</sup> presented a refractive index at 800 nm of  $1.392 \pm 0.001$  for a 27.9 g/dL Hb concentration using optical coherence tomography. Using transmittance measurements of erythrocyte monolayer, Khairullina<sup>12</sup> gives  $n$  values for intact erythrocytes with physiological Hb concentration (31 to 35 g/dL) of 1.406 to 1.409 in the wavelength range 380 to 800 nm and without any distinct wavelength dependence.

The refractive index for the hemoglobin solution of 28.7 g/dL in this investigation is  $1.408 \pm 0.003$  at 589 nm

and  $1.403 \pm 0.003$  at 800 nm, where the concentration of hemoglobin is a little lower than a normal physiological concentration. Therefore, our value is higher than values from previous investigations. This could be due to the practical problems involved in preparing such high hemoglobin concentrations and its correct determination. Stoddard and Adair used highly purified hemoglobin, whereas in our investigations, the refractive index of a hemoglobin solution was measured as it naturally occurs in human red blood cells, i.e., including salts ( $\sim 0.7$  g/dL), and other organic compounds ( $\sim 0.2$  g/dL).<sup>13</sup> This is essential, as methods used to calculate the light distribution in blood or blood perfused tissues require the refractive index data that relate to the situation *in situ*, and it is known that the refractive index of multicomponent solutions can be influenced by nonabsorbing components.<sup>14</sup> Therefore, the additional compounds will lead to an increase in the refractive index compared to pure hemoglobin solutions of identical concentration. Another problem is the tendency of highly concentrated biomolecules to adsorb on nonphysiological surfaces,<sup>15</sup> such as the optical glass of a refractometer, which can lead to falsified results.

The wavelength dependence of the real part, obtained from subtractive Kramers-Kronig analysis of the absorption data of Prahl,<sup>7</sup> as presented by Faber et al.<sup>4</sup> (see Fig. 5), exhibits the same maximum amplitude in the anomalous dispersion ( $\Delta n = 0.018$  units). The two maxima at 545 and 580 nm also have the same range of amplitude ( $\Delta n = 0.002$ ), even though they are near the error limit or within it. The increase of 0.008 from 800 nm to 430 nm is low compared to our studies with an increase of 0.024, and the curve in the UV region has lower values than at higher wavelengths, which also differ from our results. When calculating the refractive index for a defined wavelength range, using the Kramers-Kronig relation, it is necessary to know, if not the whole spectrum, at least the adjacent spectral areas, which is not the case for the spectral range below 250 nm. However, a high increase in absorption of hemoglobin in the UV range below 250 nm is probable and would, if neglected, lead to incorrect results in the Kramers-Kronig calculation. This would also explain the generally low increase with decreasing wavelength in these calculations.

Similarly, Hammer et al.<sup>3</sup> calculated the refractive index of a Hb solution with a concentration of 34 g/dL in the wavelength range of 400 to 700 nm using Tycko's real part for some wavelengths,<sup>16</sup> Latimer's approximate formulas<sup>6</sup> for dispersion of liquids, and absorption spectra from van Assendelft.<sup>17</sup> The general form of the curve is qualitatively similar to Faber's and to our results. The refractive index is about 1.402 at 700 nm; the two maxima at 545 and 580 nm show only half the range of amplitude (0.001). The increase from 700 to 430 nm of 0.002 is less than 10% of the observed increase in our study. The maximal amplitude for the anomalous dispersion between 430 and 400 nm has only half the value compared with our results. These deviations can also be explained by the previously mentioned problems found with calculating wavelength-dependent refractive indices.

## 5 Conclusion

In this study, the complex refractive index of a highly concentrated hemoglobin solution is determined in the wavelength range of 250 to 1100 nm. Absorption spectra of concentrated and diluted hemoglobin solutions are measured with regard to the imaginary part of the refractive index. It could be proved by transmittance measurements, using highly concentrated hemoglobin solutions, that Beer-Lambert's law is valid for physiological hemoglobin concentrations within the red blood cell, making it possible to extrapolate absorption coefficients determined at lower concentrations.

Using Fresnel reflectance measurements, the real part of the refractive index of a hemoglobin solution of a concentration of 28.7 g/dL could be directly determined without using extrapolations or Kramers-Kronig calculations. The wavelength dependence in the UV region of the measured real part differs from that calculated by the Kramers-Kronig relation. The determined real parts of the refractive indices are on average 0.02 higher than the values calculated with Barer's formula from 1954, which gave the basic data for a number of further calculations.

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