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Abstract. Because direct measurements of the refractive index of hemoglobin over a large wavelength range are challenging, indirect methods deserve particular attention. Among them, the Kramers-Kronig relations are a powerful tool often used to derive the real part of a refractive index from its imaginary part. However, previous attempts to apply the relations to solutions of human hemoglobin have been somewhat controversial, resulting in disagreement between several studies. We show that this controversy can be resolved when careful attention is paid not only to the absorption of hemoglobin but also to the dispersion of the refractive index of the nonabsorbing solvent. We present a Kramers-Kroning analysis taking both contributions into account and compare the results with the data from several studies. Good agreement with experiments is found across the visible and parts of near-infrared and ultraviolet regions. These results reinstate the use of the Kramers-Kronig relations for hemoglobin solutions and provide an additional source of information about their refractive index. [DOI: 10.1117/1.JBO.17.11.115002]

Keywords: hemoglobin; refractive index; Kramers-Kronig relations.

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1 Introduction

Study of optical properties of hemoglobin is important for the development of diagnostic and treatment techniques where considering blood optical properties is critical. Various optical methods—such as visible and near-infrared spectroscopy, optical coherence tomography, and fluorescence spectroscopy—are used to investigate biological tissues and blood and their components.[1,2] Strong light scattering and absorbing properties of blood are determined by the optical parameters of hemoglobin. Hemoglobin in erythrocytes can exist in the oxygenated and deoxygenated forms, in the form of methemoglobin and in some other forms. Different forms of hemoglobin have distinct optical properties; for example, conversion of oxygenated into deoxygenated form leads to the deformation of the Soret band and shifting of the position of its maximum.[3]

Various disease conditions have an impact on the chemical and optical properties of hemoglobin. For example, as Maximov et al.[4] showed, increase of glucose concentration in blood plasma at diabetes mellitus changes the hemoglobin affinity to oxygen, which results in decrease of the efficiency of oxygen transport and can lead to tissue hypoxia. Hemoglobin interacts chemically with glucose in blood plasma forming the so-called glycated (glycosylated) hemoglobin.[5] The knowledge of the amount of glycated hemoglobin in blood is widely used as a long-term diagnostic marker of the quality of the plasma glucose control in patients with diabetes mellitus. As Mazarevica et al. reported,[6] high concentrations of glucose and consequent increase of the amount of glycated hemoglobin leads to an increase of the refractive index of erythrocytes in diabetic patients. The increase of the refractive index of hemoglobin upon glycation was also observed for hemoglobin solutions mixed with various concentrations of glucose.[7]

Hence the refractive index of hemoglobin can be a sensitive marker of dysfunctions and pathologies caused by various diseases. Hemoglobin absorbs strongly in the visible range, and its refractive index is complex-valued, $n + ik$. To characterize the refractive index completely, both the real and the imaginary parts should be known. The imaginary part can be obtained from absorption measurements whose results between 250 and 1000 nm are known and were carefully tabulated by Prahl.[3]

Because of the strong absorption, direct measurements of the real part, n, using conventional refractometers (for example, Abbe refractometer) have proven to be difficult, and data are available at a few wavelengths only. In an early study, for

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example, Barer measured[8] n for solutions of oxygenated hemoglobin at 589 nm. He also discussed its dependence on the hemoglobin concentration and presented the expression

$$n = n_{H_2O} + \alpha C, \tag{1}$$

where n_{H_2O} is the refractive index of distilled water, C is the concentration, and α is the specific refraction increment. Faber et al.[9] measured the refractive indices of solutions of oxygenated and deoxygenated hemoglobin at 800 nm. Friebel and Meinke[10] measured directly the refractive index of solutions of oxygenated hemoglobin at 633 nm for several concentrations, and Jin et al.[11] measured it at 633 and 532 nm. Recently, we presented[12] direct measurements of oxygenated and deoxygenated hemoglobin at nine wavelengths across the visible range. These measurements were done with a refractometer based on total internal reflection and for the hemoglobin concentrations up to 140 g/l. We then derived, at each wavelength, expressions analogous to Eq. (1).

The limitations of the direct measurements have attracted attention to indirect methods. Friebel and Meinke[10,13] calculated the real part of the refractive index of solutions of oxygenated hemoglobin at 84 wavelengths between 250 and 1100 nm from measurements of its imaginary part and reflectance. They also derived a model function to calculate n depending on the hemoglobin concentration. These studies are to date the most complete reference for the refractive index of solutions of oxygenated hemoglobin covering the visible and parts of both the near-infrared and ultraviolet regions. They did not, however, consider deoxygenated hemoglobin. Both forms of hemoglobin are present in blood and both are of interest. In addition, experimental data for the refractive index differ across publications,[8–13] which is probably due to different sample preparation. It this situation, it is helpful for researchers to have a tool to analyze experimental results or even to derive the refractive index of hemoglobin solutions theoretically.

Kramers-Kronig relations are a good candidate for such a tool. They are widely used in optics of solids, liquids, and gases to derive the real part of the refractive index from its imaginary part.[14] Shumilina[15] and, more recently, Faber et al.[9] applied the Kramers-Kronig relations to solutions of oxygenated and deoxygenated hemoglobin. To derive the real part of the refractive index in the range of 250 to 1000 nm, Faber et al. used the absorption data from Ref. 3 and their own measurements of n at 800 nm. They demonstrated anomalous dispersion around the Soret band close to 430 nm.

However, in a subsequent study, Friebel and Meinke[13] found disagreement between their measurements and the results of the Kramers-Kronig analysis. The refractive index obtained from the experiments was larger and showed steeper increase toward ultraviolet than the refractive index obtained from the Kramers-Kronig relations.

Here we report on a Kramers-Kronig analysis of the refractive index of hemoglobin solutions and show how the controversy over the results of the earlier studies can be resolved. Our numerical formulation used the tabulated[3] and measured data for the imaginary part of the refractive index of hemoglobin and the tabulated data for the refractive index of water.[16] It allowed us to obtain good agreement with the experimental results of Friebel and Meinke[10] in the range 250 to 1000 nm and Zhernovaya et al.[12] in the range 440 to 700 nm.

Section 2 formulates the problem and describes how the imaginary part of the refractive index was measured. Section 3 compares the results with the studies of Faber et al.,[9] Friebel and Meinke,[10] and Zhernovaya et al.[12] Section 4 draws conclusions.

2 Materials and Methods

This section first formulates the Kramers-Kronig relations and describes numerical calculations and then describes measurements of the imaginary part of the hemoglobin refractive index.

2.1 *Theoretical*

The standard Kramers-Kronig relation for the real part of the refractive index has the form[14]

$$n(\omega) = 1 + \frac{2}{\pi} P \int_0^\infty \frac{\omega'}{\omega'^2 - \omega^2} k(\omega') \mathrm{d}\omega', \tag{2}$$

where ω is the angular frequency, and P denotes the Cauchy principal value. The total absorption of a hemoglobin solution can be presented as a sum of the absorption of hemoglobin itself and the absorption of the solvent, which we assume to be water, leading to

$$k = k_{Hb} + k_{H_2O}. \tag{3}$$

Substituting Eq. (2) into Eq. (3) we get

$$n(\omega) = 1 + \frac{2}{\pi} P \int_0^\infty \frac{\omega'}{\omega'^2 - \omega^2} k_{H_2O}(\omega') \mathrm{d}\omega' + \frac{2}{\pi} P \int_0^\infty \frac{\omega'}{\omega'^2 - \omega^2} k_{Hb}(\omega') \mathrm{d}\omega'. \tag{4}$$

The sum of the first two terms on the right-hand side yields the refractive index of pure water, so that

$$n(\omega) = n_{H_2O}(\omega) + \frac{2}{\pi} P \int_0^\infty \frac{\omega'}{\omega'^2 - \omega^2} k_{Hb}(\omega') \mathrm{d}\omega'. \tag{5}$$

As long as the integrals in Eqs. (2) and (5) are taken over all frequencies, both expressions give the same results. In practice, however, the imaginary part of the refractive index in known only in a finite frequency region, between ω_1 and ω_2, and one assumes

$$P \int_0^\infty \frac{\omega'}{\omega'^2 - \omega^2} k(\omega') \mathrm{d}\omega' \approx P \int_{\omega_1}^{\omega_2} \frac{\omega'}{\omega'^2 - \omega^2} k(\omega') \mathrm{d}\omega'. \tag{6}$$

In the region 250 to 1000 nm, water absorbs much weaker than hemoglobin, so that $k \approx k_{Hb}$. As a result, both integrals in Eq. (2) and Eq. (5) evaluated according to Eq. (6) will have equal values. The total refractive indices given by Eqs. (2) and (5) will, however, differ; the difference being the refractive index of water, which changes from about 1.33 at 1000 to about 1.43 at 200 nm.[16] In other words, while Eq. (2) evaluated according to Eq. (6) is able to describe the refractive index of hemoglobin as a constituent of a solution,[17] it cannot describe the solution's overall refractive index. As we show below, the change of the refractive index of water is able to explain the discrepancy found by Friebel and Meinke[13] between the experimental data and the data obtained by the Kramers-Kronig analysis.

The imaginary part of the hemoglobin refractive index is proportional to the hemoglobin concentration, C, and can be presented as

$$k_{\mathrm{Hb}} = C \times 535211 \frac{e_{\mathrm{Hb}}}{\omega}, \tag{7}$$

where e_{Hb} is the hemoglobin molar extinction coefficient[3] in $1/(\mathrm{cm} \cdot \mathrm{mol})$, and the angular frequency ω is in rad/s. Substituting Eq. (7) into Eq. (5) and comparing the result with Eq. (1), we obtain an analytical expression for the specific refraction increment in the form

$$\alpha = 340726 \times P \int_0^\infty \frac{1}{\omega'^2 - \omega^2} e_{\mathrm{Hb}}(\omega')\mathrm{d}\omega'. \tag{8}$$

We were thus able to derive Barer's formula, Eq. (1), theoretically.

If the value of the real part of the refractive index is known at the frequency ω_0, the subtractive Kramers-Kronig relation can be used that has the form[14]

$$n(\omega) = n(\omega_0) + \frac{2}{\pi}(\omega^2 - \omega_0^2) P \int_0^\infty \frac{\omega'}{(\omega'^2 - \omega^2)(\omega'^2 - \omega_0^2)} k(\omega')\mathrm{d}\omega'. \tag{9}$$

Following the above procedure to separate the refractive index of water, we get

$$n(\omega) = n_{\mathrm{H_2O}}(\omega) + n(\omega_0) - n_{\mathrm{H_2O}}(\omega_0) + \frac{2}{\pi}(\omega^2 - \omega_0^2) P \int_0^\infty \frac{\omega'}{(\omega'^2 - \omega^2)(\omega'^2 - \omega_0^2)} k_{\mathrm{Hb}}(\omega')\mathrm{d}\omega'. \tag{10}$$

The subtractive relations are expected to have better convergence[14] and also enable one to match the calculated results exactly to a known value at the frequency ω_0. The latter is particularly valuable for hemoglobin solutions, for which it is easy to measure the real part of the refractive index at a single wavelength[8,9,11] and its imaginary part in a wide wavelength range. Numerical integration of Eq. (10) was done by the trapezoidal rule outside the poles and by the method of Ref. 18 in their vicinity. The water refractive index was calculated from the analytical expression of Ref. 16. The imaginary part of the refractive index was taken from Ref. 3 to compare with the results of Refs. 9 and 13 and was measured as described below to compare with the results of Ref. 12. The data from Refs. 9, 12, and 13 were used without modification.

2.2 *Experimental*

The hemoglobin samples were prepared as described in Ref. 12. Briefly, hemoglobin solutions (lyophilized powder, Sigma-Aldrich) were made using phosphate buffered saline to maintain pH at 7.4. To convert dry methemoglobin into deoxygenated and oxygenated forms of hemoglobin, sodium dithionite (concentration 10 g/l), and sodium bicarbonate (concentration 15 g/l), respectively, were added to the solutions. The hemoglobin concentration in the solutions was 140 g/l, and absorption was measured with a spectrophotometer (UV-3600, Shimadzu, Japan). All experiments were done at 20°C. Since sodium dithionite has absorption bands in the ultraviolet region, values of the absorbance of oxygenated and deoxygenated hemoglobin were obtained in the range of 370 to 1000 nm (see Fig. 1). For comparison, Fig. 1 also shows (gray lines) the absorption data for oxygenated and oxygenated hemoglobin from Ref. 3. There

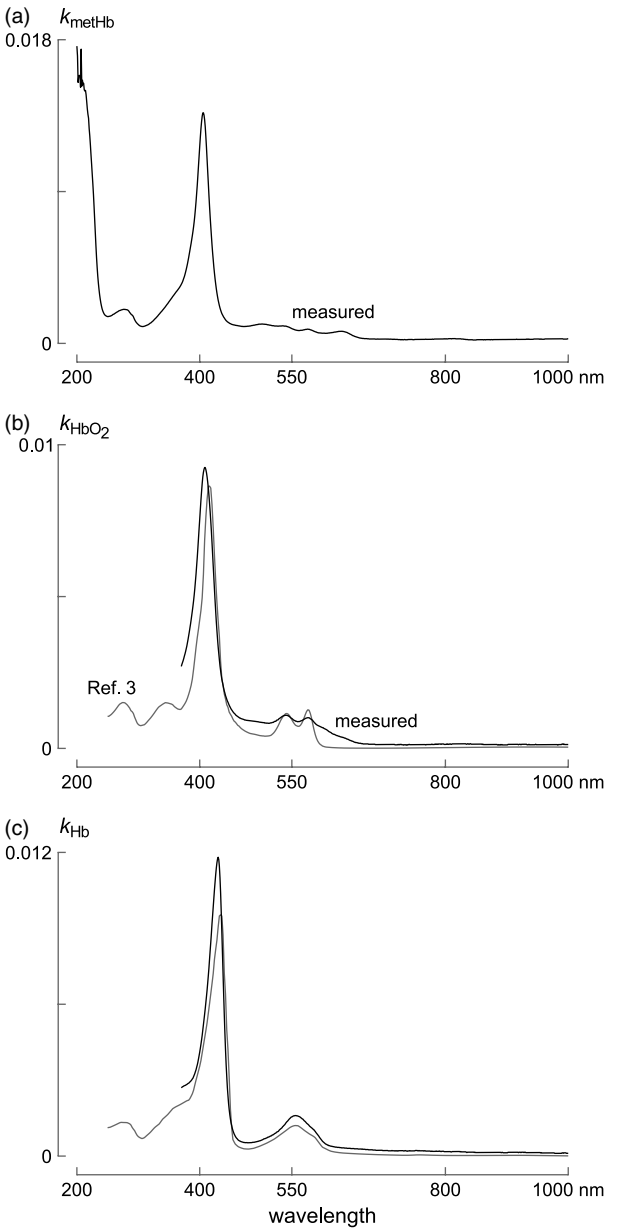

Fig. 1 Measured values (black lines) of the imaginary part of the refractive index of (a) methemoglobin; (b) oxygenated; and (c) deoxygenated hemoglobin solutions. The hemoglobin concentration is 140 g/l. Gray lines are calculated from the tabulated data of Ref. 3.

is a minor disagreement between the absorption curves, most probably due to different sample origins (fresh hemoglobin in Ref. 3 and lyophilized reagent in our experiments).

3 Results and Discussion

This section applies the Kramers-Kronig relations to the real part of the refractive index of hemoglobin solutions, compares numerical calculations with experiments, and analyzes the results.

The first problem that needs to be addressed is the effect of the refractive index of water. As discussed in the previous section, Eqs. (2) and (5) and their counterparts Eqs. (9) and (10), all being forms of the Kramers-Kronig relations, give different results when the integrals are evaluated between 250 and 1000 nm, where the water absorption is negligible. This is

demonstrated in Fig. 2, where the refractive indices obtained with (black lines) and without (gray lines) water refractive index are shown for (a) oxygenated and (b) deoxygenated hemoglobin. The hemoglobin concentration was 281 g/l. Using the subtractive Kramers-Kronig relations, Eqs. (9) and (10), we matched the refractive indices to 1.392 for the oxygenated and to 1.389 for the deoxygenated hemoglobin at 800 nm (the experimental values from Ref. 9). The imaginary part of the refractive index was calculated from the absorption data of Ref. 3 between 250 and 1000 nm.

The above parameters are the same as those reported by Faber et al.,[9] and our results obtained ignoring the water contribution (gray lines in Fig. 2) are almost identical to theirs (dashed black lines in Fig. 2). The refractive index of water, makes, however, a large quantitative difference. Although the curves intersect at 800 nm, the refractive indices taking water contribution into account (solid black lines) are larger, especially toward the ultraviolet region. The values are also different in the region of anomalous dispersion around 430 nm, which corresponds to the Soret absorption band of hemoglobin. When the water refractive index is taken into account, the differences between the maximum and minimum values of n in this region are smaller by a factor of 0.03 for both types of hemoglobin.

To further demonstrate the importance of the water contribution, we compared the results of the Kramers-Kronig relations with the model function for the refractive index of oxygenated hemoglobin obtained by Friebel and Meinke.[10] These functions were derived from reflectance measurements at 84 wavelengths between 250 and 1100 nm. We first chose the hemoglobin concentration to be 140 g/l and calculated the imaginary part of the refractive index, again, from the data tabulated by Prahl[3] [see Fig. 1(b)]. We then matched, using Eq. (10), the real part calculated from the Kramers-Kronig relations to that calculated from the model function of Friebel and Meinke at 800 nm.

The results of the model function and of the Kramers-Kronig analysis agree at almost all wavelengths [see Fig. 3(a)]. The agreement is excellent between 500 and 1000 nm. Notably, the shapes of the curves in the region of anomalous dispersion, around 430 nm, also agree. In this region, the curve calculated from the Kramers-Kronig relation gives a slightly smaller difference between the maximum and minimum values than the curve calculated from the model function. Below 400 nm, the Kramers-Kronig analysis also gives lower values of the refractive index, although both curves have similar shapes. We then repeated the same calculations for the hemoglobin concentration of 281 g/l [see Fig. 3(b)]. The Kramers-Kronig relations and the model functions of Friebel and Meinke give similar results, although the agreement between them is less good than for 140 g/l.

Despite these differences, the Kramers-Kronig analysis clearly matches much closely the model function of Friebel and Meinke when it takes the water contribution into account than it does ignoring the water contribution (the curves without

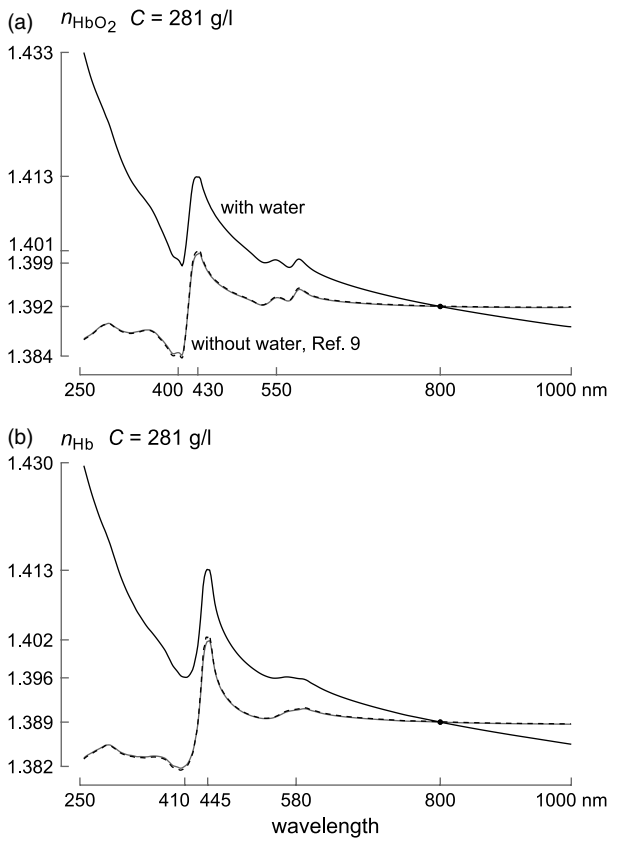

Fig. 2 The refractive index for (a) oxygenated and (b) deoxygenated hemoglobin solutions obtained from the Kramers-Kronig relations. The calculations took the water contribution into account for the solid black lines and ignored it for the gray lines. The gray lines almost coincide with the dashed black lines that are taken from Ref. 9. The hemoglobin concentration is 281 g/l.

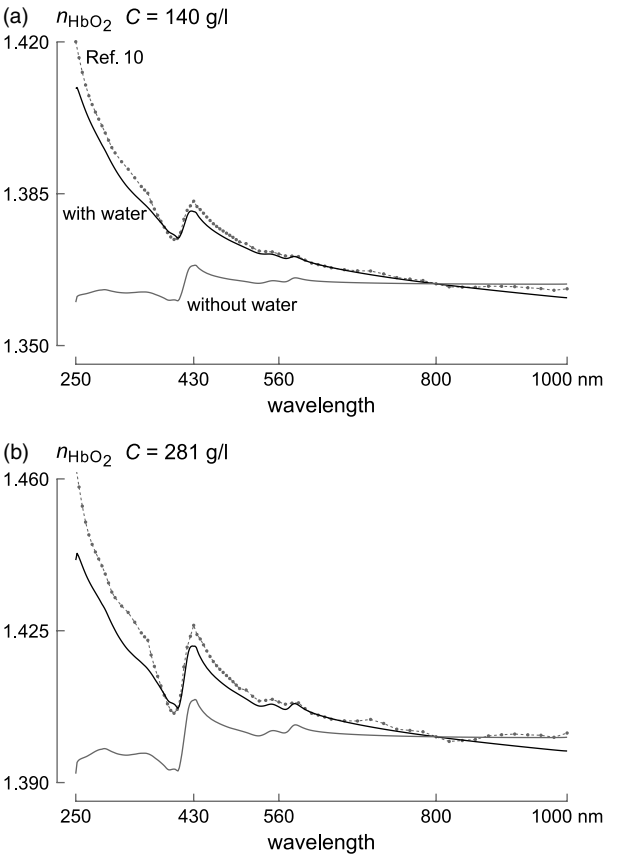

Fig. 3 The real part of the refractive index obtained by the Kramers-Kronig analysis with the water contribution taken into account (black lines) agrees with the results of the model functions of Friebel and Meinke (gray dots).[10] They disagree when the water contribution is ignored (gray lines). The hemoglobin concentration is 140 g/l in (a) and 281 g/l in (b).

the water contribution are plotted by gray lines in Fig. 3). It can explain the discrepancy Friebel and Meinke found when comparing their measured results[13] with the results of the Kramers-Kronig relations.[9]

We then compared the results of the Kramers-Kronig analysis with our recent direct measurements for solutions of met-, oxygenated- and deoxygenated hemoglobin at nine wavelengths between 400 and 700 nm.[12] The hemoglobin concentration was 140 g/l, and the measurements of the imaginary part of the refractive index were presented in the previous section. The curves were matched at 546 nm. For three hemoglobin solutions, the theoretical and experimental data agree for all but the shortest wavelength (see Fig. 4). For deoxygenated hemoglobin, Fig. 4(c), the theoretical values lie above the experimental ones

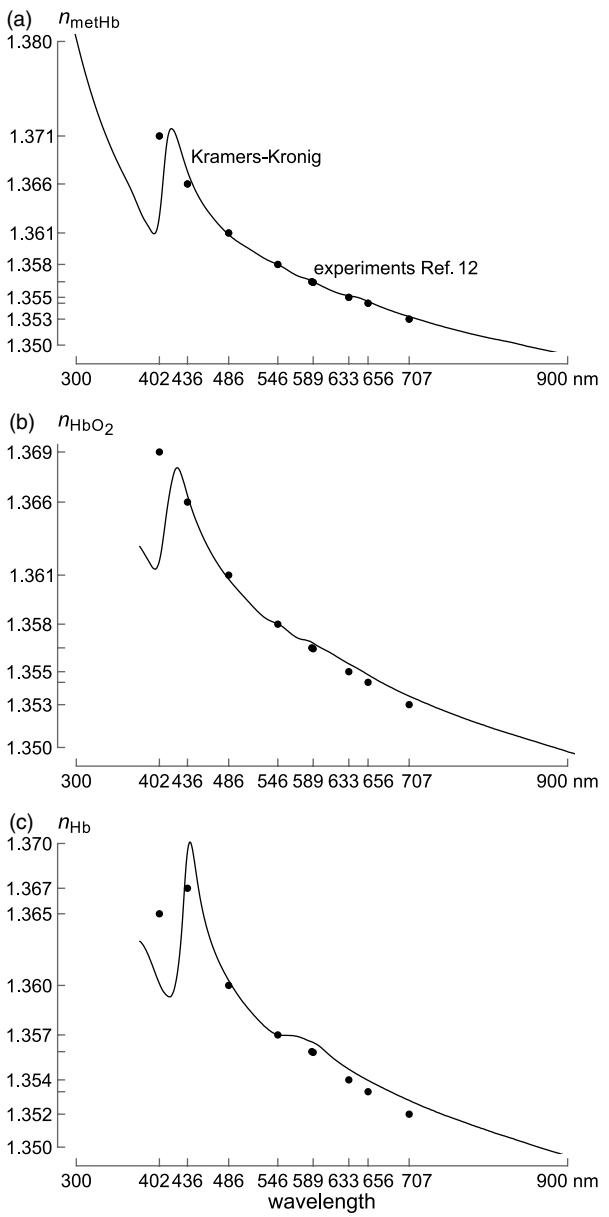

Fig. 4 The real part of the refractive index obtained by the Kramers-Kronig analysis (black lines) agrees with the measured results of Ref. 12 (circles) at all but the shortest wavelength for (a) methemoglobin; (b) oxygenated; and (c) deoxygenated hemoglobin. The hemoglobin concentration is 140 g/l

for longer wavelengths, but the difference is still within the experimental error of 0.001.[12]

The measurements at 402 nm disagree with the theoretical calculations. The reason for it is not clear; it might originate from the finite wavelength range for which the absorption was measured. This wavelength lies close to the edge of the measurement range, especially for the oxygenated and deoxygenated hemoglobin (see Fig. 1), where the numerical error is expected to be large. In favor of this explanation speaks the smaller disagreement for the methemoglobin solution, where absorption was measured in a wider range. Further investigations are needed to clarify this point; particularly valuable would be measurements of the hemoglobin absorption in the ultraviolet range, below 200 nm. A further reason for the disagreement could be the contributions from sodium dithionite and sodium bicarbonate (see Sec. 2.2).

We have so far compared separately the results of Kramers-Kronig analysis with the three experimental studies, by Faber et al.,[9] Friebel and Meinke,[10] and Zhernovaya et al.[12] One notices, however, differences when comparing the curves at the same concentrations for different studies [Fig. 2(a) against Fig. 3(b) and Fig. 3(a) against Fig. 4(b)]. Experimentally these differences are, as noted above, probably due to different sample preparations. A strong advantage of the Kramers-Kronig relations, which emerges from this comparison, is their versatility. The subtractive forms of these relations, Eqs. (9) and (10), allow us to match the theoretical curve to a measured value at a single wavelength. The Kramers-Kronig analysis can, therefore, be tailored to a particular experiment and thus accommodate various techniques for preparing hemoglobin solutions.

4 Conclusions

Employing the Kramers-Kronig relations, we analyzed the refractive index of hemoglobin solutions between 250 and 1000 nm. The total real part of a hemoglobin solution is a sum of the contributions from hemoglobin itself and from water, the solvent in our study. Because water does not absorb in this region, its refractive index is not given by the Kramers-Kronig integral evaluated at these wavelengths and should be considered separately. Comparing our results with the experiments of Friebel and Meinke[10,13] and Zhernovaya et al.,[12] we found good agreement at almost all wavelengths, which previous attempts[9,13] could not achieve. Our results reinstate the Kramers-Kronig relations as a useful tool to investigate refractive index of hemoglobin solutions. They provide the values of the real part of the hemoglobin refractive index at wavelengths where no direct measurements exist. In addition, they are concentration independent and can, therefore, augment the model functions for the refractive index derived by alternative methods.[8,10,12]

Acknowledgments

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