

Measurement of the refractive index of hemoglobin solutions for a continuous spectral region

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Abstract. Determination of the refractive index of hemoglobin solutions over a wide wavelength range remains challenging. A famous detour approach is the Kramers-Kronig (KK) analysis which can resolve the real part of complex refractive index from the imaginary part. However, KK analysis is limited by the contradiction between the requirement of semi-infinite frequency range and limited measured range. In this paper, based on the Multi-curve fitting method (MFM), continuous refractive index dispersion (CRID) of oxygenated and deoxygenated hemoglobin solutions are measured using a homemade symmetrical arm-linked apparatus in the continuous wavelength range with spectral resolution of about 0.259nm. A novel method to obtain the CRID is proposed.

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1. Introduction

As a strongly chromophoric protein, hemoglobin existed in erythrocytes play an important role for blood optical property. The Soret band shift of blood is highly sensitive to the physiological and pathological status of the hemoglobin solution, for example, porphyria. Normally, the absorption spectrum of porphyrins has its Soret band at the UV–VIS edge of 400 nm [1]. Exploiting the related changes in the refractive index (RI) of hemoglobin may provide useful information for diagnostic and therapeutic applications [2], while determination of the continuous refractive index dispersion (CRID) of hemoglobin solution over a wide wavelength range remains challenging. Although insights have been gained through these studies, previous studies have largely focused on several separated wavelengths [3–6]. The Fresnel reflectance measurement [5, 6] can calculate the RI of oxygenated hemoglobin (HbO₂) solution, while for high concentrated deoxygenated hemoglobin (Hb) solution, the light is almost saturated. For high concentrated Hb solution, traditional Abbe refractometer also failed.

Spectrometer is now routinely used to resolve the absorption coefficient of hemoglobin [7–10]. A famous roundabout approach to calculate RI is the Kramers-Kronig (KK) analysis [11–13]. With the aid of a half Fourier transform, the real part of complex refractive index can be solved from the imaginary part by measuring the absorption coefficient. As a brilliant method to deal with the causality of the optic field, KK analysis still has some disadvantages. One of the main drawbacks is the contradiction between the requirement of semi-infinite frequency range of KK analysis and limited measured range [5]. Addition information of RI at anchor point may compensate for this drawback, at the expense of more time-consuming and costly measurements. For example, the optical coherence tomography (OCT) at certain wavelength was carried out when using KK analysis [12].

In this work, based on the multi-curve fitting method (MFM), the CRID of Hb and HbO₂ solutions are measured using a homemade symmetrical arm-linked apparatus. The spectral resolution is about 0.259nm in the wavelength range of 400-750nm. We compare the RI values at a certain wavelength measured by CRID measurement with our previous study of derivative total reflection method (DTRM) [14]. Then comparisons among CRID measurement, the Fresnel reflectance measurement [6] and KK analysis [12, 13] are made. CRID measurement greatly contributes as a new characterization technique for its great advantage of for automatic, high resolution, short-time and convenient.

2. Material and method

2.1 Materials

The Hb and HbO₂ solutions (Sigma-Aldrich Company Ltd) were prepared by the same procedures as described in Ref.4. Briefly, lyophilized powder of bovine hemoglobin was dissolved in phosphate buffered saline (PBS) to maintain pH at 7.4. Then 10 g/L sodium dithionite and 15 g/L sodium bicarbonate solutions were added to get Hb and HbO₂ solutions with concentrations of 20, 40, 60, 80, 100, 120, 140, 280, 320 g/L, respectively. All experiments were taken at room temperature.

2.2 CRID measurement and multi-curve fitting method (MFM)

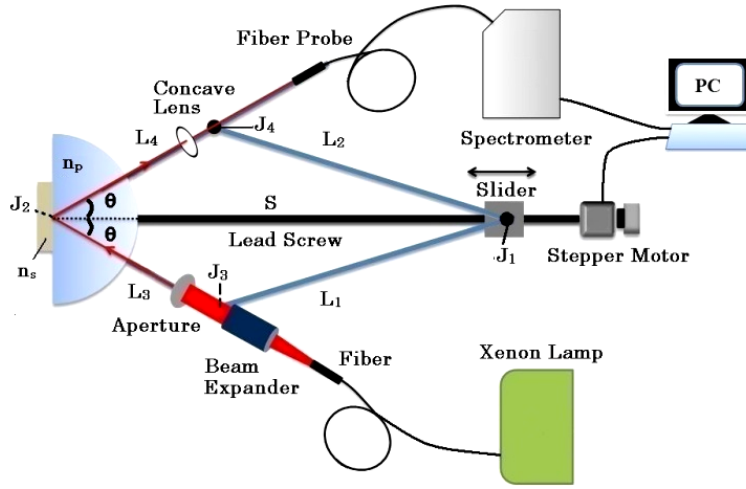


Fig. 1. Experimental setup for reflectance measurement.

The homemade symmetrical arm-linked apparatus is schematically shown in Fig. 1, which is similar to Ref. 15. Four arms (labeled L_1 , L_2 , L_3 , and L_4 , $L_1 = L_2$; $L_3 = L_4$) are connected by high precision bearings with intersection points J_1 , J_2 , J_3 , and J_4 , respectively. A slider fixed on J_1 can move along the lead screw S , driven by a stepper motor. J_2 coincides with the center of the semi-cylindrical prism. L_1 and L_2 function as towed arms. The incident arm L_3 is used to mount a light source fiber, a beam expander and an aperture. Light from L_3 is first reflected by the prism, then passes through the concave lens ($f = -50\text{mm}$) and finally reaches the spectrometer (HR4000, Ocean Optics) on the reflector arm L_4 . The triangle labeled $J_1J_2J_3$ should satisfy the Law of Cosines, as

$$a^2 = b^2 + s^2 - 2bs \cos(\theta). \quad (1)$$

where a , b , and s denote the lengths of $|J_1J_3|$, $|J_2J_3|$ and $|J_1J_2|$, respectively. $a = 160\text{mm}$, $b = 90\text{mm}$.

The complex refractive index of sample is defined as $n_j = n_{r,j} + in_{i,j}$. The CRID of water is used for system calibration [16]. As the slider moves along S , totally i^*j th reflectance data were collected at i th incident angle and j th wavelength. A computer is used to record all the movement and reflection spectrum information. At a fixed j th wavelength, a Matlab program is used to pick up all the reflectance data for the entire i th incident angle. For unpolarized light at a fixed j th wavelength of i th incident angle, the theoretical reflectance $R_{i,j}$ is

$$R_{i,j} = (R_{p,i,j} + R_{s,i,j}) / 2 \quad (2)$$

here, according to the Fresnel Formula [17], $R_{s,i,j}$ and $R_{p,i,j}$ are the reflectance for TE and TM

wave, respectively. $R_{s,i,j} = \left| \frac{n_{p,j} \cos \theta_{i,j} - n_j \cos \varphi_{i,j}}{n_{p,j} \cos \theta_{i,j} + n_j \cos \varphi_{i,j}} \right|^2$ and $R_{p,i,j} = \left| \frac{n_j \cos \theta_{i,j} - n_{p,j} \cos \varphi_{i,j}}{n_j \cos \theta_{i,j} + n_{p,j} \cos \varphi_{i,j}} \right|^2$.

$n_{p,j}$ is the RI of prism. $\varphi_{i,j} = \arcsin(n_{p,j} \sin \theta_{i,j} / n_{r,j})$ is the reflective angle at the prism-sample interface.

A Multi-curve fitting method (MFM) based on Nelder–Mead simplex algorithm is adopted. For the nonlinear fitting program, from each of the angle-dependent reflectance curve at j th wavelength, $n_{r,j}$ can be resolved by minimizing the sum

$$S(N)_j = \sum_{i=1}^{i_{\max}} [R_{m,i,j} - R_{i,j}]^2 \quad (3)$$

here, $R_{m,i,j}$ is the measured reflectance. Here, $i_{\max} = 250$, $j_{\max} = 1353$.

$E_s^2 = 1 - \frac{\sum_{j=1}^M (R_{m,i,j} - R_{i,j}^+)^2}{\sum_{j=1}^M (R_{m,i,j} - \bar{R}_{m,i,j})^2}$ is used to calculate the reliability of the fit.

$\bar{R}_{m,i,j}$ is the mean value of measured reflectance over all incident angles. E_s^2 ranges from 0 to 1 and is closer to 1 for a reliable fitting. First, we can obtain the angle-dependent reflection curves and calculate $n_{r,j}$ by Eq. (3) at each wavelength. Then we get the CRID of sample over the whole wavelength.

2.3 Kramers-Kronig (KK) analysis

For comparison, KK analysis is used to calculate the RI of Hb and HbO₂ solutions. The standard KK analysis can deduce the real component $n(\omega)$ from the imaginary component $k(\omega)$ [13], which is

$$n(\omega) = n_{pBS}(\omega) + \frac{2}{\pi} P \int_0^{\infty} \frac{\omega'}{\omega'^2 - \omega^2} k(\omega') d\omega' \quad (4)$$

where ω is the angular frequency and P denotes the Cauchy principal value. $k(\omega) = c\mu_a(\omega)/2\omega$, where c stands for the speed of light in vacuum and $\mu_a(\omega)$ is the absorption coefficient of sample. $n_{pBS}(\omega)$ is measured by CRID measurement. $\mu_a(\omega)$ is measured by a spectrometer (U4100, Hitach).

3. Results and discussion

The CRID of Hb and HbO₂ solutions with different concentrations are shown in Fig. 2, which vary almost linearly with concentration. We can find the Soret band clearly, which is 438nm for Hb and 421nm for HbO₂ solution. For comparison, we measured the RI of some HbO₂ solutions at the incident wavelength of 632.8nm by DTRM [14]. According to the Snell's Law, the RI of sample n_r can be calculated by $n_r = n_p \sin \theta_c$. n_p is the RI of the prism. By derivative of the reflectance curve, we can obtain θ_c at the peak position and then calculate n_r . As shown in Fig. 2(b), the RI values measured by DTRM and CRID measurement fit very well, with a discrepancy of less than 0.002.

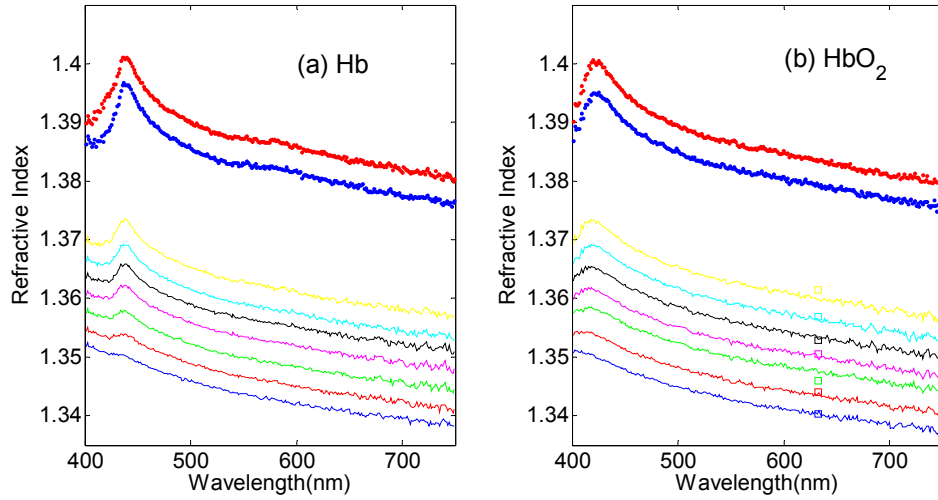


Fig. 2. Plots of the CRID of (a) Hb and (b) HbO₂ solutions with concentrations of 20, 40, 60, 80, 100, 120, 140, 280 and 320g/L (from lower to upper lines), respectively. Squares in (b) indicate RI values measured by DTRM at 632.8nm.

In Fig. 3, we compare CRID of 280g/L HbO₂ and Hb solutions calculated by CRID measurement and KK analysis. The Soret band appears almost at the same location for the two methods and the CRID results give fair agreement, with a shift of no more than 0.002. By the way, we have proved the rightness of the work of O. Sydoruk [13] for amendment of Ref.12, which emphasize that the influence of the substrate material should be taken into account when doing the KK analysis. We compare the measured CRID with the Fresnel reflectance measurement [6] and KK analysis [12], and find that our values are much smaller. We speculate that these differences are mainly contributed by the sample difference. Samples in Ref.6 are not highly purified, and other compounds may increase the RI of the solution.

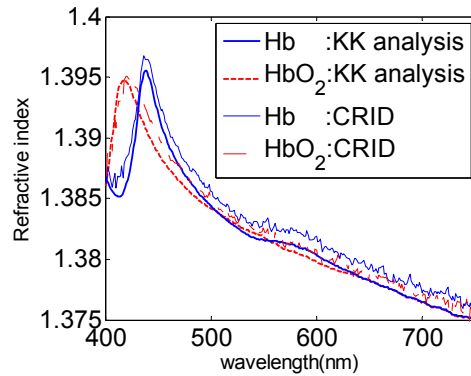


Fig. 3. The measured CRID of 280g/L HbO₂ and Hb solutions, compared with KK analysis.

For bovine hemoglobin we used here and human hemoglobin used in Ref.4 were bought from the same Company, we compared the 140g/L and 320g/L solutions with Ref.4 in Fig. 4. For 140g/L Hb solution, the Soret band appears almost at the same position in Fig. 4(a), which is about 438nm for CRID measurement and 436nm for Ref.4. For both 140g/L HbO₂ and Hb solutions, we get RI of 1.374 at the peak position of CRID curves. For a fixed wavelength of 140g/L solution, the RIs of bovine HbO₂ and Hb solutions are about 0.01 higher than human. For 320g/L solutions, the CRID measurement and Ref.4 fit better, as

shown in Fig. 4(b). For HbO₂ solution, we can also clearly recognize the Soret band near 421nm for CRID measurement, while for measurement taken at discrete wavelengths in Ref.4, obviously, the position of Soret band was lost just as shown in Fig. 4(b). The results have proved the great advantage of CRID measurement for continuous spectrum.

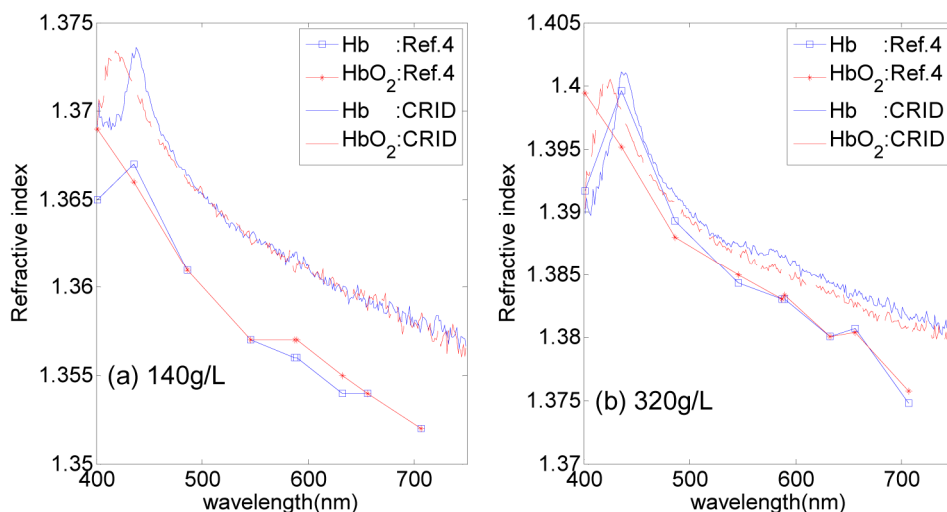


Fig. 4. CRID of HbO₂ and Hb with concentration of (a) 140g/L, (b) 320g/L, compared with Ref.4

For providing the detailed RI information over a continuous spectral range, CRID measurement shows its great advantage compared with previous studies focused on several separated wavelengths, which may cause the loss of some important information, such as the Soret band [3–6]. As an automated technique, CRID measurement is more convenient compared with Fresnel reflectance measurement [5, 6] and KK analysis [11–13]. Especially, for highly concentrated Hb solutions, CRID measurement is superior to the Fresnel reflectance measurement, for avoiding the problem of light saturation. Compared with KK analysis, we don't need the spectrometer to measure the absorption coefficient or other approaches to matching the theoretical RI curve to a measured value at a single wavelength. The system error of the CRID measurement is detailed in Ref.15, which is about 0.001.

4. Conclusion

For this study, a homemade symmetrical arm-linked apparatus in combination with the multi-curve fitting method are used to determine the CRID of Hb and HbO₂ solutions. CRID measurement is another promising method to resolve the RI of samples over a continuous spectral region, which overcomes the shortages of the existing methods, such as Fresnel reflectance measurement and KK analysis. The setup of CRID measurement shows its great advantage for automatic, high resolution, short-time and convenient.

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