Hemoglobin Heme Conformation in Patients with Different Oxygen Saturation Values

Alexander I. Yusipovich[1], Elvin S. Allakhverdiev[2], Evgeniia Yu. Parshina[1*], Sergey K. Pirutin[1,3], Margarita A. Silicheva[1], Oleg V. Rodnenkov[2], Tamila V. Martynyuk[2], and Georgy V. Maksimov[1,4]

[1] Lomonosov Moscow State University, GSP-1, Leninskie Gory, Moscow 119991, Russian Federation

[2] Russian National Medical Research Center of Cardiology, 15A 3rd Cherepkovskaya str., Moscow 121552, Russian Federation

[3] School of Biology, Shenzhen MSU–BIT University, No. 1, International University Park Road, Dayun New Town, Longgang District, Shenzhen, Guangdong Province 518172, PRC

[4] University of Science and Technology MISIS and MISIS University, 4 Leninskiy Prospekt, bulding 1, Moscow 119049, Russian Federation

*** e-mail:** parshinae@gmail.com

Abstract. The characteristics of prosthetic group of hemoglobin – heme at different values of the oxygen saturation (sO_2) was studied in human whole blood of patients with various clinical forms of coronary heart disease using the method of Raman spectroscopy. Correlation between the values of ratios of Raman spectra bands (1375 and 1355 & 1588 and 1552 cm^{-1}, correspondingly) and oxygen saturation was shown. Based on the analysis of the Raman spectra, it is directly shown that at low sO_2 the streatching of the heme ring comes before the binding of the bivalent iron atom to the oxygen molecule. It is probably caused by the cooperative effect (the attachment of a ligand to one heme leads to a change in of the protein and therefore the conformations of other hemes without ligands). © 2022 Journal of Biomedical Photonics & Engineering.

Keywords: Raman Spectroscopy; whole blood; erythrocytes; heme; oxygen saturation.

Paper #3529 received 14 Sep 2022; revised manuscript received 11 Nov 2022; accepted for publication 27 Nov 2022; published online 13 Dec 2022. doi: 10.18287/JBPE22.08.040505.

1 Introduction

Currently, the significant attention is payed to the studies of moieties conformation in a living cell. This approach allows both to investigate the molecules isolated from cells and to compare the results of molecular biology, biochemistry, and biophysics with specific molecular processes in a functional cell. In this work, we investigated the conformational state of hemoglobin prosthetic group, the heme, in the blood of patients with different clinical forms of coronary heart disease under the different values of oxygen saturation using Raman spectroscopy (RS). This method produces the information directly from whole blood. It is important because the composition of blood plasma can determine the oxygen-binding properties of hemoglobin.

A hemoglobin molecule consists of four protein subdomains linked together, each contains a prosthetic group – Heme B (Fig 1). Whereby the intensity and position of the RS bands of hemoglobin, both isolated and in erythrocytes, depends on the Heme B conformation, in particular, on the oxidation and the spin state of the iron atom in heme [1–4]. Earlier, the RS spectroscopy was shown to be suitable for a comparative analysis of the hemoglobin properties in blood of healthy donors and patients with various cardiovascular diseases [5–7], diabetes mellitus type 1 [8, 9], growth hormone deficient children [10], astronauts after the space missions of long duration [11, 12]. However, the matter of the relationship between the RS components and the parameters of the oxygen transport process in the body still remains open.

Consequently, the main goal of this article is the identification of the molecular mechanism of the conformation changes in heme associated with level of sO_2 in the whole blood.

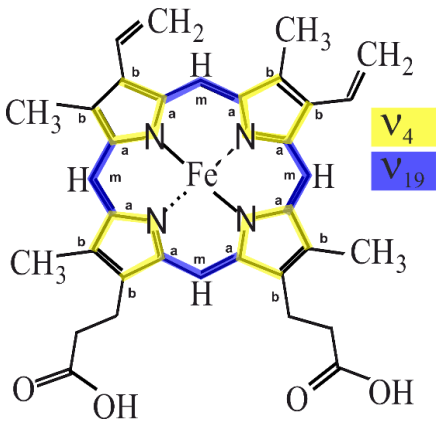

Fig. 1 Structure formula of the prosthetic hemoglobin group – the heme B. The groups of chemical bonds corresponding to vibrational modes are shown by color: ν_4 (yellow), ν_{19} (blue).

2 Materials and Methods

2.1 Ethics Statement and Patients

All experiments were performed on donor blood of patients with various clinical forms of coronary heart disease (CHD) obtained in accordance with the requirements of the ethical committee of Federal State Budgetary Institution National Medical Research Center of Cardiology Ministry of Health of the Russian Federation (protocol № 267). The study involved 31 patients, the median of age (median [1 quartile; 3 quartile]) was 63 [50.25; 66.25] years.

2.2 Blood Gas Analysis

The blood was sampled from the cubital vein into vacutainers containing heparin (20–50 U/ml blood). In samples of venous blood, the values of the oxygen partial pressure pO_2, pH, pCO_2, HCO_3, K^+, Na^+ and (hemoglobin) oxygen saturation (sO_2, %) were monitored using a GEM Premier 3000 analyzer of blood gases and electrolytes (Instrumentation Laboratory, USA). All procedures were performed at room temperature (25 ºC).

2.3 Raman Spectroscopy and Data Processing

The properties of Hb in whole blood were examined by Raman Spectroscopy. DPSS laser Ventus 473 (Laser Quantum, Germany, $\lambda = 473$ nm) and a DFS-24 spectrometer (LOMO, Russia) were used for the measuring of Raman spectra; output power on the sample (1 mm^3) was 18–20 mW. The spectral resolution was 7 cm^{-1}. All samples were measured at room temperature (25 ºC). Blood was collected from human donor in tubes containing 20–30 IU/ml heparin and stored in closed tube on ice for no more than 2 h. Before measurement the glass was filled with blood until 90% full from tube and sealed to avoid gas exchange with air. The capillary was placed in the spectrometer. Single spectrum was recorded for 100 sec. The measurements were performed in quadruplicates, the obtained values were averaged. The standard deviation of averaged peaks intensities did not exceed 5% from sample.

The background luminescence was eliminated by subtracting the baseline, then the intensity of the Raman spectrum bands was determined using the original program (Pyraman, https://bitbucket.org/alexeybrazhe/pyraman). The baseline was calculated using a cubic spline at any curve segments of spectrum lone. The borders of a curve segments are shown in the Fig. 2b as blue dots. A typical image with a subtracted baseline is shown in Fig. 2.

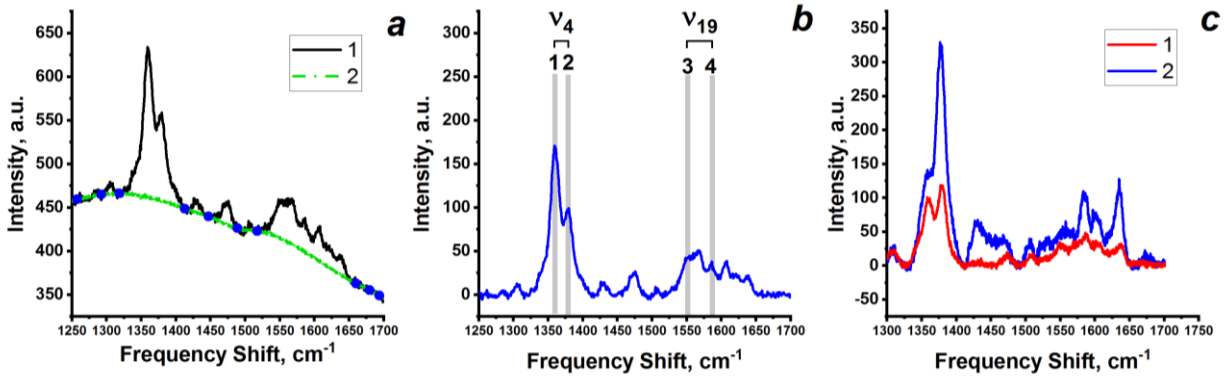

Fig. 2 The typical Raman spectra of whole blood. (a) The raw Raman spectra (1) and the Raman spectra baseline (2). The baseline was calculated using the cubic spline interpolation, the areas for spectrum approximation limited by points are shown by blue dots. (b) The location of ν_4 (1, 2) and ν_{19} (3, 4) Raman spectral bands in "deoxy"(1, 3) and "oxy"(2, 4) hemoglobin forms. (c) The typical Raman spectra of normally (1) and high (2) oxygenated blood. $\lambda = 473$ nm, P = 18–20 mW.

Table 1 The meaning of some Raman spectrum bands.

Mode	I_{deoxi}, cm^{-1}	I_{oxi}, cm^{-1}	Intensity ratio	Meaning
ν_4	1355	1375	$I_{1375}/(I_{1355} + I_{1375})$	Correlate with ratio of oxyhemoglobin and the total amount of hemoglobin in oxi- and deoxy- form
ν_{19}	1552	1588	$I_{1588}/(I_{1588} + I_{1552})$	

Table 2 Positions and assignments of bands in Raman spectra of whole blood, erythrocytes, and hemoglobin [2].

Maximum of wavenumber, cm^{-1}	Bond of heme*	Sensitivity of vibration (The intensity of vibration depends from)
1355	CaCb, CaN	Redox state of Fe, presence of ligand
1375		
1552	CaCm, CaCmH	The spin state of Fe, the diameter of heme
1588		

2.4 Statistics

The obtained results were statistically processed using the trial version of Graphpad Prism 9.00. The correlation between the parameters was estimated using the non-parametric Spearman test. The statistical significance of differences between the ratios of the spectral band amplitudes of the RS blood spectrum was performed using the Wilcoxon test. Changes were considered significant at $p < 0.05$.

The $I_o/(I_o + I_d)\cdot(sO_2)$ data approximation was performed using GraphPad Prism version 9.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3 Results

3.1 Raman Spectra of Whole Blood

Under specific conditions, the RS of blood is an equivalent of RS erythrocytes and a superposition of spectra of hemoglobin Heme molecules in different conformational states (usually in the oxy- (oHb) and deoxy-forms (dHb)). In this work, we focus on the analyzing of changes in the ν_4 and ν_{19} modes of the RS spectrum (Fig. 2). The dHb oxidation leads to a shift of the afore cited bands to the long-wavelength region of the spectrum, thus it allows to distinguish the oxy- and deoxy forms of hemoglobin. In the deoxy form, hemoglobin is characterized by the presence of the ν_4 and ν_{19} modes in the 1355 (I_{1355}) and 1552 (I_{1552}) cm^{-1} regions, correspondingly, and in the oxy form, ν_4 and ν_{19} modes reside in 1375 (I_{1375}) and 1588 (I_{1588}) cm^{-1}, correspondingly (Table 1). Using the ratio: the intensity of the Raman band specific to oHb, I_{oxi}, divided by the sum of the intensities of the peaks in oxy and deoxy form ($I_{oxi} + I_{deoxi}$) of the same mode (ν_4 or ν_{19}, respectively), we could evaluate a hemoglobin oxygen saturation (the percent of oxyhemoglobin in the blood), sO_2 [3, 13].

The amplitude of the ν_4 mode is defined by symmetrical vibrations of the pyrrole rings and changes during the oxidation of Fe^{2+} to Fe^{3+} or if a ligand (for example, O_2) is linked to the iron atom (Table 2). In the oxyhemoglobin molecule, oxygen "pulls" a pair of electrons from the iron atom, and as a result, there is a shift of ν_4 to 1375 cm^{-1} in RS spectrum.

Both for the ligated ferrous heme and the non-ligated ferric (Fe^{3+}) forms of Hb (methemoglobin) the mode ν_4 occurs at about 1375 cm^{-1} [14]. However, the concentrations of the Hb non-ligated ferric form in blood do not normally exceed 1–2%, and I_{1375} value normally depends on the presence of a ligand, mainly oxygen.

The amplitude of 1552 and 1588 cm^{-1} bands depends on the vibrations in the methine bridges of the heme pyrrole rings, which, in turn, depend on the spin state of the heme iron atom. It is known that in the dHb molecule the iron atom is in the high-spin state [15] and localize outside the plane of the heme. In this case, the peak of 1552 cm^{-1} will be more pronounced in the RS spectrum of hemoglobin (Fig. 2b). In the case of attachment of any ligand to the heme, the iron atom switches in a low-spin state, becomes smaller in diameter and changes its localization in order to locate closer to the plane of the heme ring. As a result, the heme molecule becomes more "compact", and the 1588 cm^{-1} peak will be more pronounced in the Raman spectrum.

However, at present, the relationship between the parameters of the Hb Raman spectrum, the sO_2 value (blood oxygenation is determined by the alternative methods) and heme conformation is not sufficiently investigated. Up to the present day, the assessment of interrelation between the sO_2 and any RS bands ratio in the Raman spectrum has so far been conducted on isolated human or animal erythrocytes, but not on human whole blood [3, 13]. Obviously, the study of whole blood is more in line with the "real environment" in the body, where the red blood cells are surrounded by specific ions, proteins and other blood cells. The proposed approach is

significantly more adequate to be used in the clinical diagnostics.

3.2 Result of Blood Gas Analysis

During the study, we obtained correlation of pH, pCO_2, pO_2, K^+, Na^+ and HCO_3^- values from the sO_2 values (see supplementary materials Fig. S1). The value of oxygen saturation of venous blood in 19 out of 31 patients was less than 50% (see Fig. 3). It is believed that oxygen saturation of venous blood less than 50% indicates an increase in oxigen demand or a decline in oxygen delivery [16], in other words, hypoxia develops. Thus, in most of the examined patients, various forms of hypoxia were observed. It was shown that pO_2 and pH values increased, and the levels of pCO_2, HCO_3^-, K^+ and Na^+ decreased during increase of the sO_2. Also, the positive correlations were found between the ratio of intensities of the Raman spectra bands ($I_o/(I_o+I_d)$ for ν_4, ν_{19}, and sO_2: for sO_2 vs ν_4 $p = 4.2 \times 10^{-5}$, $r = 0.861$; for sO_2 vs ν_{19} $p = 0.011$, $r = 0.625$; for ν_4 vs ν_{19} $p = 2.7 \times 10^{-4}$, $r = 0.809$. The dependencies of the $I_o/(I_o+I_d)$ for ν_4 and ν_{19} from sO_2 are shown in Fig 3. Moreover, the significant differences between the ratios ν_4 and ν_{19} were obtained ($p < 0.0001$).

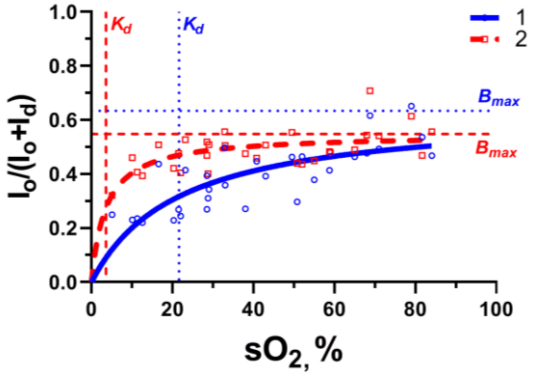

Fig. 3 The experimental dependency between oxygen saturation (sO_2) and Raman bands ratio ($I_o/(I_o+I_d)$). (1) ν_4, (2) ν_{19}; the experimental values of Raman bands ratios from sO_2 shown as open squares (ν_4) and open circles (ν_{19}); lines – the approximate curves for ν_4 (solid) and ν_{19} (dash) data, correspondingly.

3.3 $I_o/(I_o + I_d)$ (sO_2) Data Approximation

We used several assumptions in our work. Firstly, in the erythrocytes the hemoglobin is in oxy- or deoxy- forms (the fraction of other Hb forms is extremely negligible):

$$oHb+dHb = 1 \text{ or } dHb = 1 - oHb, \quad (1)$$

where dHb and oHb are fractions of deoxy- and oxyhemoglobin, correspondingly.

Second, at the first approximation the intensity of the Raman spectrum band (I) linearly depends on the incident light intensity (I_L), the concentration of the substance in the sample (C), and constant (σ) which magnitude depends on the excitation wavelength, collection geometry and other parameters defined by the specimen properties and its microenvironment [17]:

$$I = \sigma I_L C. \quad (2)$$

Therefore, the ratio of intensities of the deoxy- and oxyhemoglobin bands (I_d and I_o, respectively) could be represented as:

$$\begin{aligned} &I_o/(I_o + I_d) = \\ &= \sigma_o I_L oHb / (\sigma_o I_L oHb + \sigma_d I_L (1 - oHb)) = \\ &= [(\sigma_o/(\sigma_o - \sigma_d))oHb] / [(\sigma_d/(\sigma_o - \sigma_d)) + oHb]. \end{aligned} \quad (3)$$

This makes it possible to approximate the $I_o/(I_o + I_d)\cdot(sO_2)$ dependencies by hyperbolic function (Fig. 3) very similar to the specific binding model:

$$I_o/(I_o + I_d) = (B_{max} \cdot sO_2)/(K_D + sO_2), \quad (4)$$

where $B_{max} \cdot \sigma_o/(\sigma_o - \sigma_d)$ and $K_D \cdot \sigma_d/(\sigma_o - \sigma_d)$ are constants; B_{max} is the maximum value of $I_o/(I_o + I_d)$ extrapolated to maximum values of sO_2, K_D is the value of sO_2 at 50% of $I_o/(I_o + I_d)$. The values of B_{max} and K_D for experimental data are shown in Table 3.

4 Discussion

In this work, we used a hyperbolic dependence to describe the dependency between blood sO_2 and RS spectrum bands, while in other works the linear dependence between these parameters was used [3, 18]. Admittedly, for $I_o/(I_o + I_d)\cdot sO_2$ function in narrow range of sO_2, the linear dependence matches well with experimental data. However, in whole sO_2 range, the linear dependence does not give a reasonable fit; moreover, in our opinion, the hyperbolic dependence more correctly describes the "physic" interaction between the sO_2 (oxygen in blood) and ratio of RS spectrum bands.

Table 3 The values of B_{max} and K_D in different $I_o/(I_o + I_d)$ ratios of RS hemoglobin spectrum bands.

Mode	B_{max} Mean ± SE	95% CI B_{max}	K_D Mean ± SE	95% CI K_d
ν_4	0.63 ± 0.07	0.51 to 0.86	21.62 ± 6.99	9.6 to 45.32
ν_{19}	0.55 ± 0.02	0.51 to 0.60	3.59 ± 1.20	1.48 to 6.57

In our experiments, we measure the $I_o/(I_o+I_d)\cdot sO_2$ dependence. In this model the peak magnitudes (value of $I_o/(I_o+I_d)\cdot sO_2$) is determined by both the presence of oxygen (the number of oxyhemoglobin molecules) and the conformation of the heme. The physiological temperature of the blood is about 37 ºC, we carried out measurements at room temperature (t = 25 ºC), therefore, based on the results of work [19], we do not expect noticeable changes in the values of $I_o/(I_o+I_d)\cdot sO_2$ in our experimental system from blood *in vivo*.

The ν_4 is sensitive to an oxidation of Fe^{2+} or a ligand binding to the iron atom. The value of ν_{19} depends on the spin state of the heme iron atom, but also can be changed by interaction of the porfirin ring with protein surroundings [20]. Thus, using the Eq. 4 with calculated B_{max} and K_D for $I_{1375}/(I_{1375}+I_{1355})$ we could determine the value of sO_2 in whole (venous) blood from the measured RS spectrum. Moreover, in case of erythrocytes (the model is designed for use on erythrocytes), this procedure should not depend on the condition of the patient or erythrocytes, and can also be carried out not only in blood, but also in other solutions (the main thing is that there is no destruction of the hemoglobin molecule). The value of ν_{19} is more sensitive to changes in the heme surroundings, so it is reasonable to use it in conjunction with ν_4, comparing these values with each other, and with other methods, in order to better explain the observed changes in conformation.

It could be observed that in the case of the ν_4 band, the K_D value of the approximating hyperbolic functions significantly exceeds the K_D value for the ν_{19} mode approximating function, in contrast to B_{max}. It means that over the same sO_2 values (especially low), the $I_o/(I_o+I_d)\cdot sO_2$ value for ν_{19} is always higher than that for ν_4 (Fig. 3).

Consequently, in the blood at low sO_2 values, the number of prosthetic hemoglobin groups with altered conformation (estimated by $I_o/(I_o+I_d)\cdot sO_2$ ratio for ν_{19} and occurred as a decrease of the distance between the iron atom and heme plane) exceed the number of prosthetic groups with associated ligand (estimated by $I_o/(I_o+I_d)\cdot sO_2$ ratio for ν_4). In other words, at low sO_2, there is a rather considerable amount of non-ligated heme molecules with altered conformation in whole blood.

Therefore, in patients with various clinical forms of CHD, since the hemoglobin molecule is an assembly of four globular protein subunits and each subunit is associated with heme, it can be assumed that an increase of the I_{1588} Raman spectrum band intensity is the result of a collective change in the conformation of all these hemes in subdomains initiated by a conformational shift of some protein part of the hemoglobin molecule subdomains as a result of ligand association to heme (known as the "cooperative effect"). Unfortunately, it is not possible to assess whether this feature of the heme is typical of any blood or is observed only in CHD patients.

5 Conclusion

Thus, using Raman spectra, we can both estimate the concentration of oxyhemoglobin molecules in whole blood (using the ν_4 band intensity and position), and evaluate the conformation change in the prosthetic hemoglobin group, heme (using the ν_{19} band intensity and position). By monitoring ν_{19} ratios the cooperative transitions under various pathological conditions and drugs impact can be studied.

Disclosures

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to Dr. M. V. Rodionova and N. A. Brazhe for their valuable help in manuscript preparation.

The study was financially supported by the Russian Science Foundation (project no. 19-79-30062). For A. I. Yusipovich, E. Yu. Parshina, and G. V. Maksimov. This research also has been supported by the Interdisciplinary Scientific and Educational School of Moscow University "Molecular Technologies of the Living Systems and Synthetic Biology".

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