Micro-Raman scattering and infrared spectra of hemoglobin

,M.Polakovs[a] N. Mironova-Ulmane[a], N.Kurjane[b], E.Reinholds[c], M. Grube[d]
[a]Institute of Solid State Physics, University of Latvia, Riga, Latvia
[b]Centre of Occupational and Radiological Medicine of P.Stradins Cl. University Hospital, Latvia
[c]Nuclear Medicine Dep. P.Stradins Clinical University Hospital, Riga, Latvia
[d]Institute of Microbiology and Biotechnology, University of Latvia, Riga, Latvia

ABSTRACT

Confocal micro-Raman and FT-IR spectroscopies have been used for detection of radiation influence of hemoglobin of patients examined by radio-isotopes diagnosis (Tc^{99m}). After irradiation we observed some little changes of the Raman scattering bands which connected with out of plane porphyrine bending vibrations, also we observed additional band due to methemoglobin. Radiation of blood lead to the transition from hemoglobin (Fe^{2+}) to methemoglobin (Fe^{3+}) with a delocalization of iron from porphyrine plane . It was shown that FT-IR spectra indicate the radiation effects on hemoglobin.

Keywords: Raman scattering, FT-IR spectroscopy, hemoglobin, blood

1. INTRODUCTION

Raman spectroscopy or inelastic light scattering from vibrational modes is a fruitful optical technique for studying of biological molecules. Resonance Raman scattering occurs when the wavelength of the exciting light of laser falls within an electronic absorption band of the investigated materials and leads to a strong enhancement of the Raman scattering intensity.

Resonance Raman spectroscopy has long provided useful information about electronic state of iron in haemoglobin (Hb) [1-6]. The hemoglobin is a tetramer of two α-hemoglobin and two β-˜ hemoglobin chains, each containing a heme group or porphyrin ring with ion of iron required for oxygen binding. Resonance Raman spectroscopy offers promise as a sensitive structural probe for heme proteins. Resonance Raman scattering involves an interaction of vibrational and electronic transition with the consequence that certain vibrational modes of the hemoglobin are greatly enhanced in the spectrum. The prominent bands in the resonance Raman spectrum should therefore arise from those vibrations which affect the conjugation in the porphyrin ring.

The resonance Raman spectra of Hb provide information about the oxidation and spin state of the heme irons [1]. Resonance Raman scattering from Hb occurs only at its surrounding ligands group because only this part of the molecule absorbs in the visible and near ultraviolet region. Thus it is possible to investigate exclusively vibrations of the four heme groups (Fig. 1) of Hb without interference by scattering of the surrounding globin or other parts of the red blood cell (RBC) or erythrocyte. In this technique, laser excitation within an electronic absorption band produces selective enhancement of Raman bands associated with vibrations of the protein. In the case of heme proteins, vibrations of the porphyrin ring are enhanced due to resonance with the π-π* transitions which dominate near ultraviolet absorption spectra. (the Soret band).and the visible (the α-β band). Different modes are brought out by excitation in the regions of the Soret absorption band or of the α-β absorption bands [7].

The ion of iron in the heme commonly occurs in one of two different electronic states in the haemoglobin ferric Fe^{3+} (methemoglobin) with electronic configuration Ar ($3d^5$) and ferrous Fe^{2+} Ar($3d^6$) (haemoglobin). Each of these ions of iron can exist in two different spin states, which reflect the distribution of electrons within the d orbital - high-spin and low-spin configuration.

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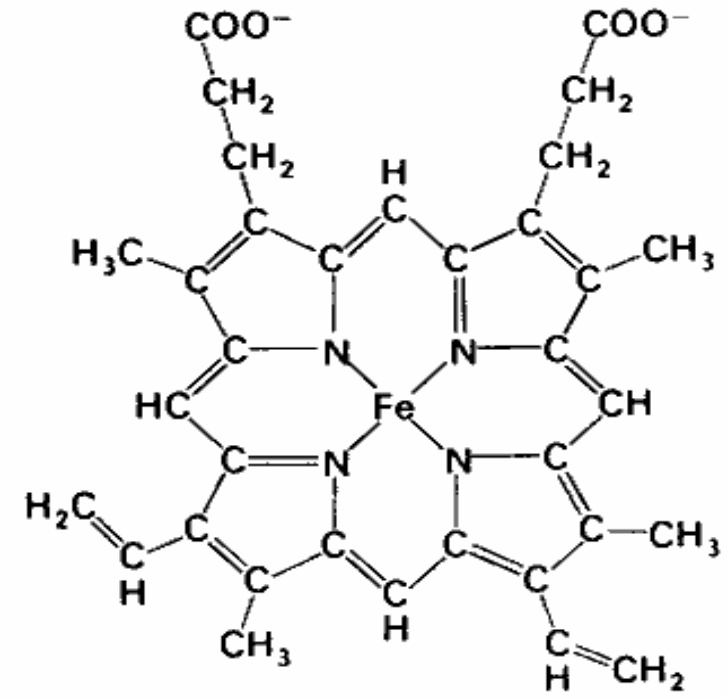

Fig. 1. Structure of heme

Methemoglobin or (ferric form of hemoglobin) is the form of hemoglobin, which is oxidized to the ferric (Fe^{3+}) state, this form of hemoglobin is not able to bind oxygen. In the low-spin ferric state the porphyrin is more planar because the Fe ion translocates into the porphyrin plane. However, the movement of the Fe ion into the porphyrin plane can distort the macrocycle, further reducing this planarity and increasing no totally symmetric components.

The Raman spectra of hemoglobin are well known for several excitation lines of laser (488.0, 514.5, and 568.2 nm) [1-5]. The Raman spectrum strongly depends on the wavelength of the excitation light of laser [1-5]. Because different modes are brought out by excitation in the two regions: of the Soret absorption band (415nm) and of the α-β absorption bands or Q-bands.

The Raman scattering technique shows potential as diagnostic probe for a myriad of erythrocyte disorders including thalassemia, sickle cell anemia, altitude sickness, and met-Hb disorders. Moreover, the in vivo nature of the measurements implies that drug studies could be performed on diseased or impaired erythrocytes [3].

The study of hemoglobin radiation damage is very important in order to understand the biological effects of ionizing radiation. [8-17] Various physical techniques were used to determine structural damages of hemoglobin after irradiation: absorption spectroscopy [17], IR spectroscopy [9], EPR [9] and Mössbauer spectroscopy [15].

In the present work, we studied for the first time the Raman scattering and FT-IR spectroscopy the hemoglobin of patients before and after radio-isotopes diagnosis

2. MATERIALS AND METHODS

Raman spectra were collected at RT using a confocal microscope with spectrometer "Nanofinder-S" (SOLAR TII, Ltd.) (Fig.2). The “Nanofinder-S” system consists of an inverted Nikon ECLIPSE TE2000-S optical microscope connected simultaneously to a laser confocal microscope unit with Hamamatsu R928 photomultiplier tube (PMT) and to a monochromator-spectrograph (SOLAR TII, Ltd., Model MS5004i, 520 mm focal length) with attached Hamamatsu R928 PMT detector and Peltier-cooled back-thinned CCD camera (ProScan HS-101H, 1024×58 pixels). The colour video CCD camera (Kappa DX20H) is used for optical image detection. All measurements were performed through Nikon Plan Fluor 40x (NA=0.75) optical objective. The Raman spectra were excited by a He-Cd laser (441.6 nm, 50 mW cw power) and dispersed by 600 or 1800 grooves/mm diffraction grating. The elastic component of the laser light was eliminated by the edge filter (Omega, 441.6AELP-GP). More details can be found in [18,19].

Fig. 2. "Nanofinder S" 3D Scanning Confocal icroscope with Spectrometer

Frequency shifts in the Raman spectra were calibrated using CaWO4 as a reference. The laser power at the sample was regulating by the neutral filter (OD=0.5) to prevent sample heating.

FT-IR absorption spectra of blood samples were recorded on the HTS-XT micro plate reader (BRUKER). 10 µl of sample were dropped on a 96 place silicon plate, dried at 50o C and spectra registered in 500-4000 cm-1 region, absorption mode.

The spectra were baseline corrected and vector normalized. Data were processed by OPUS 6.0 integration mode "K"- vertical to the oblique line for a semi-quantitative evaluation of the radiation caused changes in cell.

The mciro-FT-IR measurements of single erythrocyte were performed at room temperature using a Bruker Vertex 70 spectrometer equipped with the Hyperion 2000 IR microscope. The absorption FT-IR spectra were registered in the range from 400 to 4000 cm-1 by liquid nitrogen cooled MCT detector. The measurements were performed using attenuated total reflection (ATR) and IR (15x) objectives for point and mapping acquisition modes, respectively

Venous blood was donated by consenting patients before and after radio-isotopes diagnosis and collected under air in glass tubes containing a small amount of heparin used as an anticoagulant. Patients have been injected with a radioactive pharmaceutical Tc^{99m} and the venous blood was taken after injection in two hours.

3. EXPERIMENTAL RESULTS AND DISCUSSION

3.1. **Raman scattering**

Raman spectra of hemoglobin in erythrocyte using 441.6 nm excitation are shown in Fig. 3.

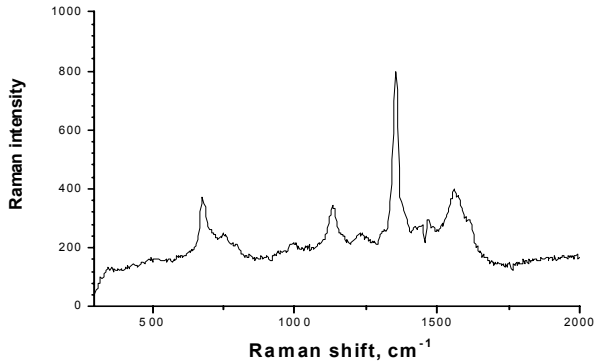

Fig. 3. Micro-Raman spectra of hemoglobin in erythrocyte recorded before radio-isotopes diagnosis

The Raman spectra of the hemoglobin in the single erythrocyte were recorded on ”Nanofinder-S” using the 441.6 nm excitation line. The Raman excitation line used in 441.6 nm cause resonance enhancement with absorption band 415 nm. The power of the Raman excitation laser 441.6 nm at the sample was regulating by the neutral filter (OD=0.5) to prevent sample heating and damage of erythrocyte by the light of laser [20]. Observation and imaging of the erythrocyte was made by an external CCD camera connected with additional screen (Fig. 4).

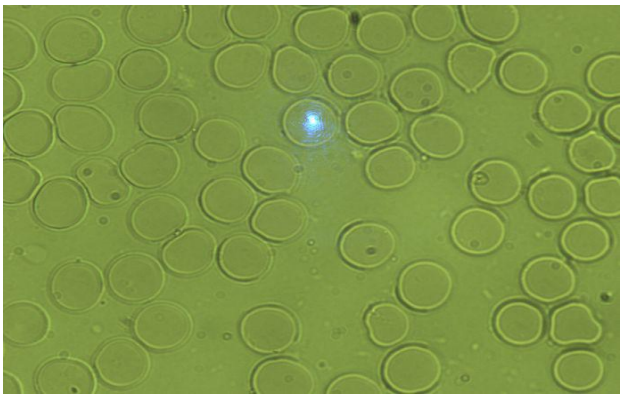

Fig. 4. Bright-field image of RBC taken by the CCD camera, with point of the laser Raman excitation beam.

The Raman scattering spectra obtained for the 441.6 nm excitation wavelengths which near (pre-resonance) at Soret absorption band with center about 415 nm are in good agreement [1-5].

Table 1. Symmetry, local coordinates and position for Hb

Sym.[1,2]	Local coordinates [1,2]	Assignments	Position, cm^{-1}
A_1	δ(deform)sym	ν_7	676
B_{1g}	ν(pyr breathing)	ν_{15}	743
E_u	δ(pyr deform)asym	ν_{46}	795
E_u	ν(CbC1)asym	ν_{47}	996
A_{2g}	ν(pyr half-ring)asym	ν_{22}	1136
B_{2g}	ν(pyr half-ring)asym	ν_{30}	1230
A_{1g}	δ(CmH)	$\nu_5+\nu_{18}$	1280
A_{2g}	δ(CmH)	ν_{21}	-
E_u	ν(pyr half-ring)sym	ν_{41}	1300
A_{1g}	ν(pyr half-ring)asym	ν_4	1358
A_{2g}	ν(pyr quarter-ring)	ν_{20}	1450
B_{2g}	ν(CaCm)sym	ν_{28}	1556
B_{1g}	ν(CbCb)	ν_{11}	1558
E_u	ν(CbCb)	ν_{37}	1664
A_{2g}	n(CaCm)asym	ν_{19}	1800

Raman spectra of hemoglobin in erythrocyte for patient after examined by radio-isotopes diagnosis (Tc99m) using 441.6 nm excitation are shown in Fig. 5. The Raman scattering spectra of patient after radio-isotopes diagnosis have changes detected in the high-frequency region. The high-frequency region of the resonance Raman spectrum of hemoproteins contains a number of modes that are sensitive to the π-electron density of the heme.

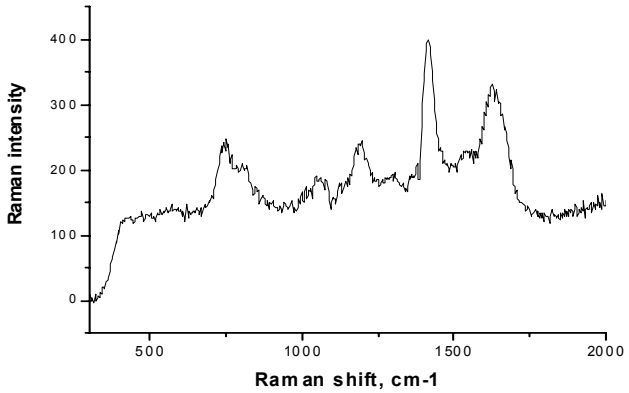

Fig. 5. Micro-Raman spectra of hemoglobin in erythrocyte recorded after radio-isotopes diagnosis

The most intense of them and little acted upon by other factors is the mode of symmetric stretching vibrations ν4. For ferri-forms of hemoglobin or methemoglobin and also for liganded ferro-forms (a 6-coordinated heme), the frequency of the mode ν4 is in the range 1370-1380 cm^{-1}, whereas for desoxygenated (reduced) hemoglobin (a 5-coordinated heme), it is 1353-1358 cm^{-1} [2]. The large difference in the frequency of the mode ν4 for the liganded and deliganded forms of hemoglobin and their practically equal intensity make it possible to use it for detecting the liganding state of hemoproteins [2-3]. Although the Raman spectra of hemoglobin of patients before and after radio-isotopes diagnosis are similar, some of the bands (1360 cm-1 and 1556 cm-1) do show changes in their relative intensities.

After radiation we observed of some little changes of the band which connected with out of plane porphyrine bending vibrations. Also we observed additional band near 1360 cm^{-1} shoulder 1370 cm^{-1}due to methemoglobin. Radiation of blood lead to the transition from hemoglobin (Fe^{2+}) to methemoglobin (Fe^{3+}) with a delocalization of iron from porphyrine plane.

3.2. FT-IR spectroscopy

This was our first attempt to study the influence of radiation on blood cells by means of FT-IR spectroscopy. The absorption spectra of initial/control sample and after irradiation are shown in Fig.6.

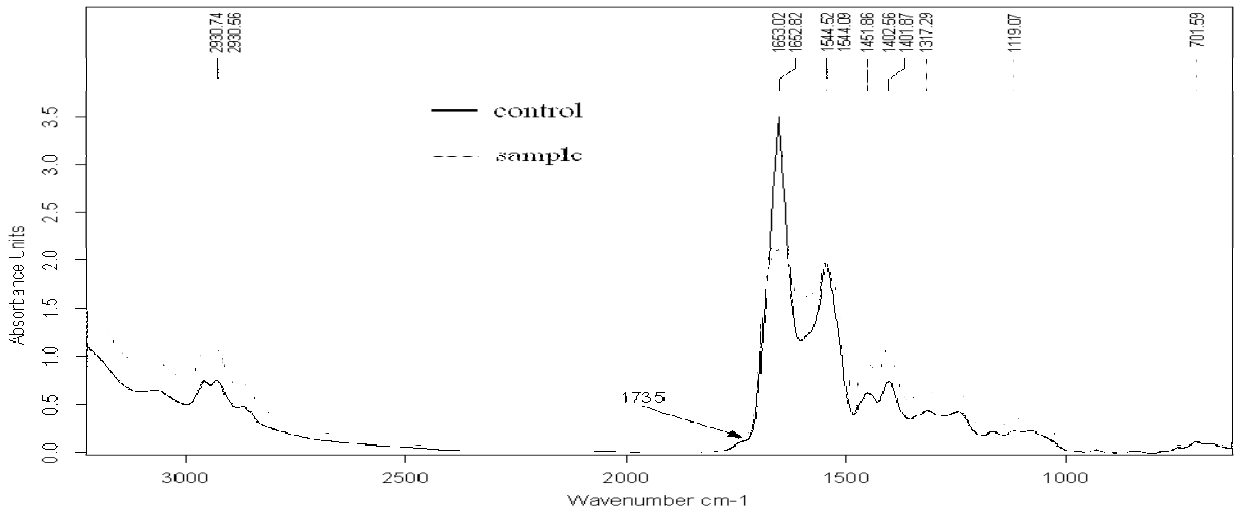

Fig. 6. FT-IR absorption spectra of hemoglobin: 1- initial/control. 2- irradiated.

The spectra are similar to usual bio-sample but it must be pointed out that absorption in “carbohydrate” region 900-1200 cm^{-1} is negligible. Comparison of blood spectra with published erythrocyte FT-IR spectra [14,21] are very

similar and the main absorption regions are from stretching vibrations of C=O and bending vibrations of N-H and NH_2 in proteins and amino acids (1653 cm^{-1}); stretching vibrations of C=H, and asymmetric stretching vibrations CH_2 and CH_2 of fatty acids and cholesterol (2930 cm^{-1}); and stretching vibrations of P-O, P=0 and C-O, of phospholipids and cholesterol (1119 cm^{-1}).

Evaluation of spectral profiles of the initial/control and treated sample spectra clearly shows changes in proteins: 1) the ratio of Amid I (1653 cm^{-1}) and Amid II (1544 cm^{-1}) is significantly higher in a control sample 2) minimum between Amid II (1544 cm^{-1}) and Amid I is much less pronounced (higher) that can indicate the change of protein configuration – dominant of α or β sheet confirmations. Holman [22] in studies of blood cell responses to chemicals showed that the protein Amide I peak cancroids shifts down indicates a change in the overall protein conformational state within the cell.

A semi-quantitative integral-based method was used for demonstration of differences between the control and irradiated samples (Fig .7).

Evaluation of spectra after baseline correction and normalization showed that the absorption in phosphate and nucleic acid region 1191-1359 with maximum at 1243 cm^{-1} is equal in both hemoglobin samples and thus can be used as internal standards for evaluation of quantitative changes caused by irradiation. The ratio of integrals: Amid I/1243; 1402/1243 and 2930/1243 showed well pronounced differences in both samples not only in proteins and amino acids but fatty acids as well.(Fig .8) It can be seen that irradiation causes the decrease of protein content in cells but the fatty acid content increases that can be an indication of the cell response to the stress.

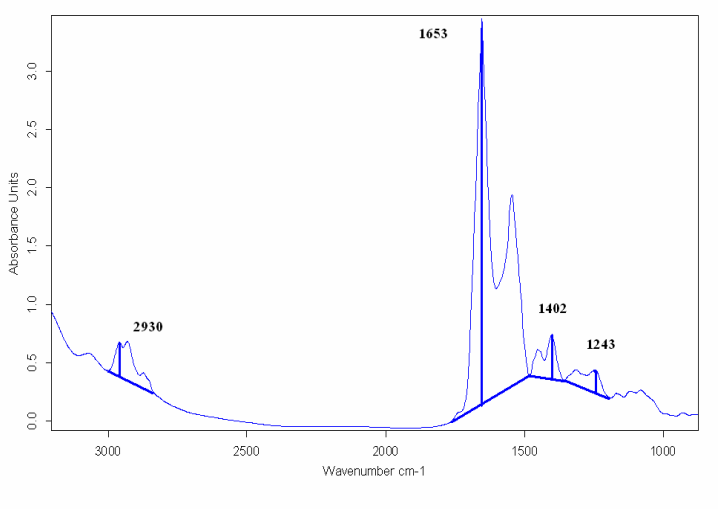

Fig. 7. Integration absorption spectra of hemoglobin

Integration was used in four regions: 1) 1191-1359 cm^{-1} with maximum at 1243 cm^{-1} (phospholipids, and nucleic acids); 2) 1359-1481 cm^{-1} with maximum at 1402 cm^{-1} (stretching of COO^-); 3) 1481-1780 cm^{-1} with maximum at 1653 cm^{-1} (Amid I); and 4) 2769-3005 cm^{-1} with maximum at 2930 cm^{-1} (fatty acids).

The integral meanings of those bands are shown in Table 2.

Table 2. Integrals of the principal absorption bands in the FT-IR spectra of hemoglobin

Absorption bands	Initial/control sample	After radiation
1243 cm^{-1}	0.005	0.005
Amid I - 1653 cm^{-1}	0.107	0.055
1402 cm^{-1}	0.012	0.016
Fatty acids -2930 cm^{-1}	0.011	0.014

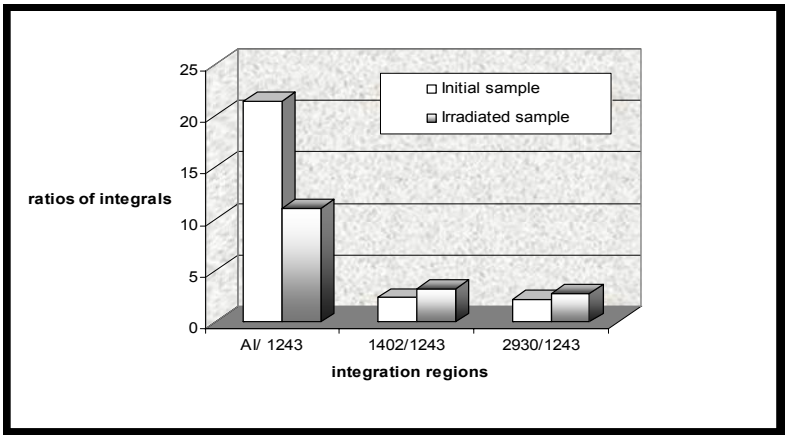

Fig. 8. Semi-quantitative analysis of initial/control and irradiated hemoglobin samples

4. Conclusions

After radiation we observed of some little changes of the Raman scattering bands, which connected with out of plane porphyrine bending vibrations. Also we observed additional band 1370 cm^{-1}. Radiation of blood lead to the transition from hemoglobin (Fe2+) to methemoglobin (Fe3+) with a delocalization of iron from porphyrine plane.

It was shown that FT-IR spectra indicate the radiation effects on hemoglobin. Taking into account that FT-IR spectroscopy is a quick and highly informative method that needs small amounts of sample (in our case even few micro liters) and when using micro plate reader even no sample pretreatment is necessary it is very promising for studies of irradiation effects on blood cells.

This study could be continued applying priorities of infrared micro spectroscopy that hopefully can speed up the evaluation of influence of irradiation and other environmental effects on single erythrocyte cells. Results showed that the combined application of resonance Raman scattering and FT-IR spectroscopy a fruitful tool for determining structural modification hemoglobin by ionizing.

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