Excitation wavelength-dependent changes in Raman spectra of whole blood and hemoglobin: comparison of the spectra with 514.5-, 720-, and 1064-nm excitation

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Abstract. Raman spectra of whole blood and oxy-hemoglobin (Hb) were measured under the same conditions with visible (514.5 nm) and near-infrared (NIR; 720 and 1064 nm) excitation, and the obtained spectra were compared in detail. The Raman spectrum of blood excited with visible light is dominated by very intense bands due to carotenoids, so that it was difficult to obtain information about Hb from the spectrum. The Raman spectra of whole blood and oxy-Hb excited with 720 nm light are very close to each other; both spectra are essentially Raman spectra of the heme chromophore that is preresonant with Q bands. Qualitative spectral analysis including band assignment and investigation of nature of resonance effect were carried out for the Raman spectra with 720 nm excitation. The spectra of whole blood and oxy-Hb excited with 1064 nm light contain contributions from nonresonance Raman spectra of the heme chromophore and Raman spectra of proteins. The 1064 nm excited spectra of blood and oxy-Hb are similar to each other but different in some features. For example, bands due to protein appear stronger in the spectrum of whole blood than in that of oxy-Hb which does not contain protein except globin part. The comparison between the 514.5, 720, and 1064 nm excited Raman spectra reveal that the excitation wavelength of 720 nm is more practical than that of visible light and 1064 nm in the Raman analysis of Hb, such as oxygenation, specially *in situ* measurement. © *2001 Society of Photo-Optical Instrumentation Engineers.*
[DOI: 10.1117/1.1380668]

Keywords: Raman spectroscopy; near-infrared; NIR; blood; hemoglobin; tunable lasers.

Paper 012103 received Sep. 20, 2000; revised manuscript received Jan. 24, 2001; accepted for publication Feb. 9, 2001.

1 Introduction

Raman spectroscopy has considerable promise as a diagnostics and analytical tool in medicine.[1–14] However, practically, the medical application of Raman spectroscopy has long been limited to *in vitro* studies, because in many cases instrumental performance of Raman spectrometers was not enough for *in vivo* measurements. Much effort has recently been made for development of more practical Raman instruments. Near-infrared (NIR)-Fourier transform (FT)-Raman spectroscopy that became popular about 10 yr ago is regarded as a powerful tool for studies of biomedical materials.[4–8] The use of 1064 nm excitation allows us to circumvent two major drawbacks of Raman spectroscopy, fluorescence and photodecomposition, which often obstructed the biomedical applications. FT-Raman spectroscopy has one problem; usually, it takes at least several minutes to obtain Raman spectra of biomedical samples with a high signal-to-noise ratio. The recent rapid development of NIR-Raman spectrometers with a charge coupled device (CCD) detector has received keen interest from many researchers in the biomedical field because they allow one to measure a Raman spectrum much quicker than the NIR-FT-Raman spectrometers with high sensitivity.[5,9–14] In the CCD-NIR-Raman technique, lasers with the emission wavelength of 700–860 nm are employed as excitation light sources because generally CCD has sensitivity up to about 1100 nm that corresponds to a Raman shift of about 2500 cm^{-1} from the excitation wavelength of 860 nm. Indeed, some pioneering works have shown the usefulness of CCD-NIR-Raman spectroscopy for *in situ* measurements of human tissues.[5,9–14]

We have been interested in Raman studies of blood and hemoglobin (Hb) partly because Raman spectroscopy may be useful for laboratory blood analysis, and partly because blood exists in all over human body and thus the Raman studies of blood cannot be avoided for *in situ* medical diagnostics using Raman spectroscopy.[6] Moreover, *in vivo* analysis of oxygenation of Hb and production of NO-, CO-, and met-Hb may be

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useful for monitoring pathological and/or functional changes in a human body. Raman spectra of whole blood with the 830 nm excitation were reported by Berger et al.[11] and Buschman et al.,[13] but they are different from each other, specially in the 800–850 cm^{−1} region. Both research groups demonstrated the potential of NIR-Raman spectroscopy in quantitative analysis of biomedical materials. Berger et al. analyzed many important constituents, such as glucose, cholesterol, triglyceride, urea, total protein, and alubumin, in serum and whole blood samples at physiological concentration *in vitro*. They employed a 66-patient data set and partial least squares regression to make calibration models. Bushman et al. succeeded in measuring a Raman spectra of artery wall *in vivo* with miniaturized fiber-optic probes. They analyzed the chemical composition of a blood vessel wall by adopting a multiple least-squares fitting method. Since the methods they employed to analyze the spectra did not require detailed analysis of spectra of constituents, they did not need to make band assignments and investigate origin of resonance effect of the blood spectra. Hence, the spectral analysis of the blood spectra with 830 nm excitation was insufficient in terms of the band assignments and the elucidation of the mechanism of the resonance effect.

The purpose of the present study is to measure Raman spectra of oxy-Hb and whole blood with visible (514.5 nm) and NIR (720 and 1064 nm) excitation wavelengths under the same condition, to compare the spectral features observed in the 514.5, 720, and 1064 nm excited Raman spectra systematically. The present study also aims at detailed qualitative analysis including band assignment and investigation of resonance effect with NIR excitation, for the first time. Raman spectra of whole blood, serum, and Hb with the visible and NIR excitation have been reported.[6,7,11–17] However, thus far, there is no report that compares Raman spectra of whole blood and Hb with visible excitation, excitation in the 700–860 nm region, and 1064 nm excitation.

2 Experiment

2.1 Blood and Hemoglobin (Hb) Samples

Blood samples were drawn fresh from healthy male adults and treated with heparin sodium. Hb sample was purified from the blood. Erythrocyte was separated by centrifugation at 10 000 g for 30 min at 4°C. Obtained erythrocyte was washed with isotonic sodium chloride solution three times. 1% of Triton-X solution was added to the erythrocyte at 1:1 in volume and centrifuged at 10 000 g for 30 min. Supernatant was carefully separated and used as the Hb sample. All the procedure was carried out under air. Absorption spectra of the obtained sample showed that oxy-Hb was dominant (data not shown). The Raman spectra of the blood and Hb were measured using a quartz cell without spinning or stirring during the measurements.

2.2 Spectroscopic Measurements

For Raman measurements, three types of Raman spectrometers were employed. A NRS-2100 triple-polychrometer (JASCO, Tokyo) equipped with a liquid nitrogen cooled CCD detector (Princeton Instruments, USA) and an Ar ion laser (Spectra Physics, USA) were used for the visible excited Raman measurements. The spectral resolution is 5 cm^{−1} and the
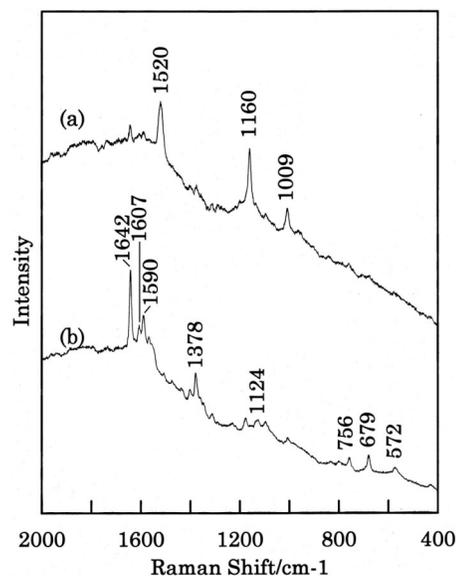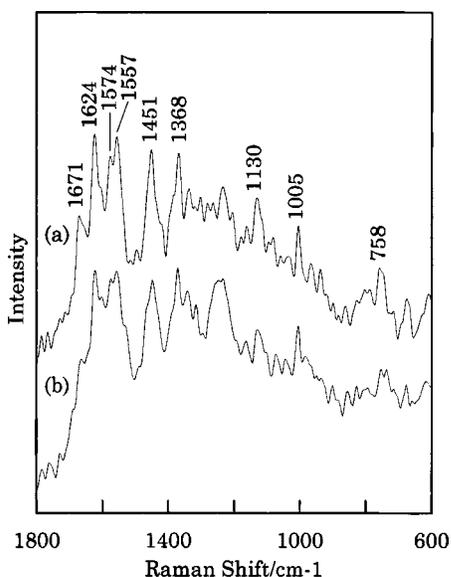

Fig. 1 The 514.5 nm excited Raman spectra of blood (a) and Hb (b) measured with a CCD-based dispersion spectrometer.

total spectral acquisition time was 20 min. Laser power at the sample was about 30 mW for the 514.5 nm excitation. Raman measurements with the 1064 nm excitation were carried out with a JRS-6500N NIR-FT-Raman spectrometer (JEOL, Tokyo, Japan). Laser power was 100 mW at the sample for the 1064 nm excitation. The sampling interval is 8 cm^{−1} and the 514 scans were accumulated. For Raman measurements with the 720 nm excitation, a newly developed excitation wavelength-tunable NIR Raman system was utilized. The system was composed of a triple-polychrometer made by JASCO (Tokyo, Japan), liquid nitrogen cooled CCD detector (Princeton Instruments, USA) and a background free electronically tuned Ti:sapphire (BF-ETT) laser which was developed in our group. Detailed system configuration and the performance of the BF-ETT laser were reported elsewhere.[18,19] Laser power at the sample was about 7 mW for the 720 nm excitation. The spectral resolution is 10 cm^{−1} and the total spectral acquisition time was 30 min. Relative band intensity in each spectrum exhibited in the present study was corrected by dividing the law spectra by that of a white tungsten lamp.

3 Results and Discussion

Figures 1(a) and 1(b) depict Raman spectra of whole blood and purified oxy-Hb measured with the 514.5 nm excitation, respectively. Bands due to amide modes of proteins that are major components of whole blood are totally missing in the spectrum of whole blood. Intense bands at 1520, 1160, and 1009 cm^{−1} observed in Figure 1(a) are assigned to carotenoids in the blood.[20] On the other hand, spectrum (b) in Fig. 1 is a resonance Raman spectrum of Hb.[15] Lack of the band at 1520 cm^{−1} due to carotenoids reveals that the oxy-Hb sample was free from blood plasma components. Strong bands at 1642, 1590, and 1378 cm^{−1} are assignable to oxy-Hb, indicating that oxy-Hb is predominant in the sample.[15] Weak appearance of a band at 1607 cm^{−1} may be due to deoxygenated (deoxy-) Hb that was produced by photodissociation of bind-
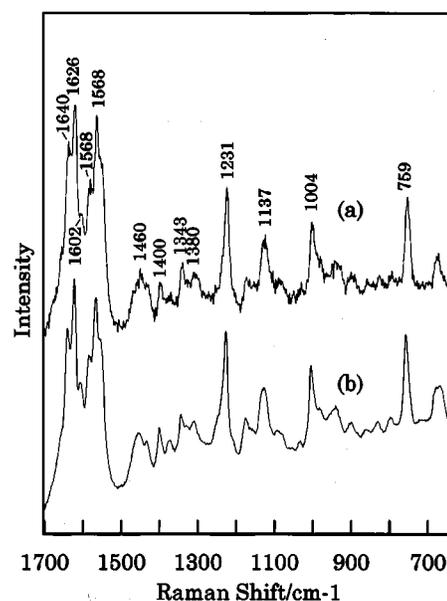
Fig. 2 The 1064 nm excited FT-Raman spectra of blood (a) and Hb (b).

Fig. 3 The 720 nm excited Raman spectra of blood (a) and Hb (b) measured with a CCD-based dispersion spectrometer.

ing oxygen. Comparison of Figure 1(a) with 1(b) reveals that it is difficult to analyze the oxygenation and spin state of Hb in the Raman spectrum of whole blood excited with 514.5 nm light. Even if the quantity of Hb overwhelms that of carotenoids in whole blood and the spectrum of Hb is resonance enhanced, the spectrum of carotenoids that shows much stronger resonance enhancement covers up the contribution of Hb.

The FT-Raman spectra of whole blood and oxy-Hb with the 1064 nm excitation are shown in Figures 2(a) and 2(b), respectively. Note that the 1064 nm excited spectra are largely different from the 514.5 nm excited spectra. Bands in the $1650-1400$ cm^{-1} region of the 1064 nm excited spectra are assigned to modes involving largely double-bond stretching vibrations, such as the methin-bridge (CaCm) and pyrrole rings (CbCb) stretching vibrations of the heme.[6] The two spectra in Figure 2 are similar to each other but there are minor differences. A band at 1671 cm^{-1} due to amide I mode of protein is observed to be stronger in the spectrum of whole blood than in that of Hb. Judging from the frequency of the amide I band, proteins in the blood sample are denatured by the strong laser irradiation during the Raman measurement. In fact, the sample after the Raman measurement was coagulated around the laser irradiation point. As the globin protein of Hb takes largely α-helix structure in an aqueous solution, it is expected to show an amide I band at 1653 cm^{-1}.[19] This is not the case. The spectral shift of amide I mode of blood due to laser irradiation was discussed in more detail in our previous paper.[6] A band at 1451 cm^{-1} is assigned to a CH$_2$ bending mode of the protein part.[20] A band at 1368 cm^{-1} is referred to as an oxidation maker band of the heme iron and the frequency indicates that Hb is in an oxidized (Fe^{3+}) state.[17] A band at 1005 cm^{-1} is assigned to phenylalanine residues in the protein part.[20]

Figures 3(a) and 3(b) show the 720 nm excited Raman spectra of whole blood and oxy-Hb, respectively. It is noted that these spectra are very close to each other. Lack of amide bands due to proteins in both spectra indicates that the spectra

reflect mainly resonance Raman spectra of heme groups in Hb. Difference in signal-to-noise ratio seems to be brought about by aberration at the illuminating and/or focus point by changing the sample cells. The bands due to carotenoids are not observed, suggesting that the 720 nm excited spectra are no longer resonant with absorption bands of carotenoids. The resemblance of the two spectra in Figure 3 suggests that the Raman measurement with the 720 nm excitation is very suitable to selectively probe Hb in whole blood. A band at 1626 cm^{-1} is assigned to a vinyl C=C stretching mode of the heme groups. Bands at 1640, 1568, and 1231 cm^{-1} are assigned to ν_{10}, ν_{11}, and ν_{13} of B_{1g} modes, respectively, following assignments and numbering scheme by Kitagawa et al.[21] A band at 1583 cm^{-1} is assigned to ν_2 of A_{1g} modes. Resonance with the B band ($\sim$450 nm) of heme enhances its Raman bands associated with totally symmetric vibrations A_{1g}, while the excitation in the Q_0 and Q_1 band region (the $500-550$ nm region) yields selective enhancement of nontotally symmetric modes, such as A_{2g}, B_{1g}. and B_{2g}.[17,21] In the spectra shown in Figure 3, many of observed bands belong to the B_{1g} modes, suggesting that the Raman spectra of Hb measured with the 720 nm excitation are resonant with Q_0 and/or Q_1 bands. Comparison between Figure 3 and Figure 2 reveals interesting aspect about the band at 1004 cm^{-1}. Relative intensity of the band at 1004 cm^{-1} is weaker than that of the band at 1450 cm^{-1} assigned to the CH$_2$ bending mode of protein in the 1064 nm excited spectra but the former is stronger than the latter in the 720 nm excited spectra that is pre-resonance Raman spectra of heme groups. These observations suggest that the band at 1004 cm^{-1} in Figure 3 includes contribution from heme groups as well as phenylalanine residues.

Recently, Berger et al.[11] and Buschman et al.[13] reported Raman spectra of whole blood with 830 nm excitation. Those spectra show different features from each other. At first, they are different in relative intensity of bands in whole region. Second, broad bands in the $800-850$ cm^{-1} region are ob-

served only in the spectrum of whole blood reported by Berger et al. Our spectrum of whole blood with the 720 nm excitation shows closer overall features to the Raman spectrum measured by Buschman et al.[13] However, relative intensity among the bands seems slightly different between ours and that of Buschman et al.[13] It is hard to judge whether this slight change is due to the difference in excitation wavelength or in oxygenation rate of Hb because they did not mention the oxygenation rate of the sample and did not label frequency of the bands in their figures. The broad bands in the 800–850 cm^{-1} region are not observed in the spectra in Figures 3(b) as well as 3(a). This fact implies that the broad band observed in the spectra of Berger et al.[11] does not arise from oxy-Hb, at least.

Venkatesh et al.[7] assumed that the 1064 nm excitation could very well give rise to resonance enhancement of heme groups to a certain degree with an electronic transition band in the 800–1300 nm region. However, in our previous work, it was suggested that the Raman spectra of hemin and met-Hb with the 1064 nm excitation are nonresonant Raman spectra. We have ascribed relatively strong appearance of the bands arising from the heme chromophore compared with those from the protein part to its highly polarizable nature.[6] In the present comparative study, bands arising from the B_{1g} modes that are enhanced via nonsymmetric electronic transitions, such as Q bands, observed in the spectra with 720 nm excitation disappear or become very weak in those with the 1064 nm excitation. Hence, it seems that the resonance enhancement via Q bands is very weak or absent in the FT-Raman spectra. The nature of absorption bands in the 700–1000 nm region is electronic transitions between the central iron ion and porphyrin ring and/or between the ion and the coordinating oxygen.[7] Therefore, the resonance effect may appear in the lower frequency region (~ 600 cm^{-1}) of the Raman spectra with the excitation in the 700–1000 nm region where stretching modes arising from the central ion and coordinating ligands appear.

4   Conclusions

In the present study, three excitation wavelengths, 514.5, 720, and 1064 nm, were compared in the Raman studies of whole blood and oxy-Hb. Those three excitation wavelengths gave typical B band resonance, Q band preresonance, and nonresonance spectra of blood and oxy-Hb, respectively. The overall features of the Raman spectra changed dramatically among those excitation wavelengths, suggesting the difference in mechanism of resonance enhancement with B and Q bands. With the 1064 nm excitation, it is possible to probe protein secondary structure as well as the structure of heme groups but it takes a longer time to obtain the Raman spectra with a good signal-to-noise ratio partly because the sensitivity of the FT-Raman spectrometer is lower than that of the Raman spectrometer with a CCD detector and partly because the spectra are not resonance enhanced. With the 514.5 nm excitation, the bands arising from carotenoids conceal Raman features from any other components including Hb, so that visible-excitation Raman spectroscopy is not suitable for *in situ* Hb studies. For the whole blood sample, only the 720 nm excitation yielded the Raman spectrum of the heme chromophore that is free from the interference from fluorescence, strong Raman scat-

tering of carotenoids, and protein bands. Excitation wavelengths of 785 and 830 nm are frequently used in the previous literatures of biomedical application of the NIR-Raman spectroscopy, except for FT-Raman.[9–14] The present study is the first report of biomedical Raman study of blood and Hb with 720 nm excitation to the best of our knowledge, and has suggested that it is possible to avoid the interference from fluorescence and carotenoids by using a shorter excitation wavelength of 720 nm, which is advantageous for CCD detection. Raman spectra of whole blood and oxy-Hb were obtained with 700 nm excitation by us (data not shown). The overall features of the spectra with the 700 nm excitation are similar to those in Figure 3, but the increase in background emission that may be ascribed to fluorescence was observed. Thus, we concluded that 720 nm is the shortest excitation wavelength to obtain satisfactory Raman spectra of whole blood and oxy-Hb for biomedical applications. We have measured Raman excitation profiles for oxy- and deoxy-Hb in the 700–860 nm region including the lower frequency region, which will provide much information about the resonance effect of oxy- and deoxy-Hbs with NIR excitation. However, to make the results and discussion clear, we limited the Hb sample and the excitation wavelength to oxy-Hb and 720 nm, respectively, in the present study. Those will be published elsewhere.

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