Measurement of hemoglobin oxygen saturation using Raman microspectroscopy and 532-nm excitation

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The resonant Raman enhancement of hemoglobin (Hb) in the Q band region allows simultaneous identification of oxy- and deoxy-Hb. The heme vibrational bands are well known at 532 nm, but the technique has never been used to determine microvascular Hb oxygen saturation (SO2) in vivo. We implemented a system for in vivo noninvasive measurements of SO2. A laser light was focused onto areas of 15–30 μm in diameter. Using a microscope coupled to a spectrometer and a cooled detector, Raman spectra were obtained in backscattering geometry. Calibration was performed in vitro using blood at several Hb concentrations, equilibrated at various oxygen tensions. SO2 was estimated by measuring the intensity of Raman signals (peaks) in the 1,355- to 1,380-cm−1 range (oxidation state marker band v19), as well as from the v10 and v10 bands (1,500- to 1,650-cm−1 range). In vivo observations were made in microvessels of anesthetized rats. Glass capillary pathlength and Hb concentration did not affect SO2 estimations from Raman spectra. The Hb resonances observed in blood were consistent with earlier Raman studies using Hb solutions and isolated cells. The correlation between Raman-based SO2 estimations and SO2 measured by CO-oximetry was highly significant for v14, v10, and v10 bands. The method allowed SO2 determinations in all microvessel types, while diameter and erythrocyte velocity could be measured in the same vessels. Raman microspectroscopy has advantages over other techniques by providing noninvasive and reliable in vivo SO2 determinations in thin tissues, as well as in solid organs and tissues in which transillumination is not possible. Intravital microscopy; resonance Raman spectroscopy; microcirculation; rat

Raman spectroscopy is a form of vibrational spectroscopy, where the energy transitions arise from molecular vibrations. Because these vibrations involve identifiable functional groups, when the energies of these transitions are plotted as a spectrum, they can be used to uniquely identify a molecule. Raman spectroscopy has been shown to be an attractive optical technique to provide direct access to the state of hemoglobin (Hb). Shifts to higher frequency occur upon oxidation of ferrous derivatives to ferric derivatives, and, consequently, Raman scattering provides information about the oxygenation state, as well as the oxidation and spin state, of the heme irons (35). Several authors have reported Raman scattering of laser radiation from vibrational modes in the heme group of oxy- and deoxy-Hb in aqueous solution (3, 4) and in isolated red blood cells (RBCs) (25, 31, 32). Excitation within the α-, β-, and Soret bands of Hb results in enhancement of numerous Raman peaks between 100 and 1,700 cm−1, whose relative intensities show a dramatic dependence on the excitation wavelength (33). The respective Soret maxima of oxy- and deoxy-Hb occur at 415 and 430 nm, and excitation within the Soret band of heme proteins provides strong enhancement in the 1,350- to 1,380-cm−1 range, also known as v14 (1, 36). While the oxidation state marker band v14 is the highest intensity heme Raman band under Soret excitation, the v19 and v10 bands (1,500–1,650 cm−1 range) are usually more prominent under 532-nm excitation (35, 37, 39).

The determination of Hb O2 saturation (SO2) using Raman spectroscopy has the advantage that the oxygenation information is only related to the specific interaction of the photons, with the Hb molecules generating a unique Raman spectrum (4, 5, 9, 34, 38, 39). Our laboratory has recently reported that this technology can be adapted to the measurement of SO2 in flowing blood of single microvessels of living tissues, in a configuration that provides the measurement of other microvascular parameters, like diameter and blood flow, using other noninvasive optical techniques (42). These studies have shown that 406-nm excitation is useful to estimate SO2 using resonance Raman peak intensity changes of the v14 band and affords differential resonance Raman signals from oxy- and deoxy-Hb in living tissue that are sufficiently strong to overcome interference from background fluorescence (46, 47).

Although our previous work utilized a 406-nm diode laser system, better laser performance is provided by 532-nm YAG lasers. Doubling of the 1,064-nm fundamental to 532 nm under differential resonance Raman scattering provides information about the oxygenation state, as well as the oxidation and spin state, of the heme irons (35). Several authors have reported Raman scattering of laser radiation from vibrational modes in the heme group of oxy-and deoxy-Hb in aqueous solution (3, 4) and in isolated red blood cells (RBCs) (25, 31, 32). Excitation within the α-, β-, and Soret bands of Hb results in enhancement of numerous Raman peaks between 100 and 1,700 cm−1, whose relative intensities show a dramatic dependence on the excitation wavelength (33). The respective Soret maxima of oxy- and deoxy-Hb occur at 415 and 430 nm, and excitation within the Soret band of heme proteins provides strong enhancement in the 1,350- to 1,380-cm−1 range, also known as v14 (1, 36). While the oxidation state marker band v14 is the highest intensity heme Raman band under Soret excitation, the v19 and v10 bands (1,500–1,650 cm−1 range) are usually more prominent under 532-nm excitation (35, 37, 39).

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Although our previous work utilized a 406-nm diode laser system, better laser performance is provided by 532-nm YAG lasers. Doubling of the 1,064-nm fundamental to 532 nm under low current effectively eliminates side bands, since they do not have sufficient peak power to be properly doubled. The availability of components for wavelengths such as 532 nm is presently less limited than for 406 nm, and most commercially available systems employ 532-nm lasers. In addition, Raman spectra with 532-nm excitation are also well known and distinctive for oxy- and deoxy-Hb (39). Finally, the penetration of 532-nm excitation is deeper than that using a 406-nm laser, allowing a larger fraction of a blood vessel (or vessels located deeper within the tissue) to be probed for SO2 levels. Considering all of these factors, the aim of this study was to evaluate...
the utility of 532-nm laser excitation and Raman microspectroscopy to estimate $SO_2$.

MATERIALS AND METHODS

Raman Intravital Microscope

The main elements of the system were similar to those described previously (42) and included an upright microscope (Axioplan Imaging 2, Zeiss) equipped with transillumination and epi-illumination optics, dry and immersion objectives. Unless otherwise specified, all Raman spectra being reported were obtained using a $\times40$ water immersion objective (Zeiss, numerical aperture = 0.80).

The 532-nm excitation was provided by a diode pumped solid-state, continuous-wave laser connected to a controller (LCS-DTL-317, Power Technology, Little Rock, AR). The laser beam was directed onto the epi-illumination optical path of the microscope, and the laser intensity was varied by neutral density filters and by adjusting the laser current using the controller. A 2-mm mirror in the microscope then reflected the laser light down onto the preparation. Backscattered light from the sample was collected and collimated by the objective and directed back into the instrument along the same path as the incident laser beam. A high-quality, long-pass filter was used to attenuate the Rayleigh line, while providing good transmission of the Raman scattered light in the desired range ($>540$ nm). The light was projected into the entrance slit (set at an aperture of 20–40 $\mu$m in this study) of a 150-mm focal length lens spectrometer (SpectraPro, Acton Research, Acton, MA) via a mechanical adapter. Inside the spectrometer, the light was reflected to a diffraction grating (2,400 grooves/mm) and directed to the detector. A back-illuminated charge-coupled device detector (Pixis: 400B, Princeton Instruments) was selected to maximize quantum efficiency and resolution (1,340 $\times$ 400 pixels). The detector was Peltier cooled to $55^\circ$C to reduce thermal noise and connected to a controller interfaced to a personal computer, where spectra were stored and analyzed.

Wave number calibration. To precisely assign a wave number to each individual detector channel, two types of external sources were used: calibration lamps and a reference compound whose Raman peak positions are known. Emission lines of low-pressure argon-mercury and mercury calibration lamps (Oriel Instruments, Stratford, CT), as well as indene, were used for calibration (6). Calibration points distributed over the whole spectral region of interest were fitted by a polynomial (WinSpec Software, Roper Scientific, Trenton, NJ). The wavelength of the incident laser light was determined at the beginning of each experiment to transform the wavelength calibration into a wave number calibration. The final spectral resolution was 2 cm$^{-1}$ in the range of interest (1,300–1,700 cm$^{-1}$). The power of the incident laser at the $\times40$ microscope objective focal point was also determined (PD300-TP and PD300–3W Power Meter, Ophir Optronics, Wilmington, MA) and kept below 2 mW.

In vitro measurements. Calibration of the $SO_2$ apparatus was performed in vitro with Hb solutions, as well as with whole blood, equilibrated with known oxygen tensions. Prolonged equilibration of blood with pure nitrogen was used to obtain measurements under anaerobic conditions. Pigs and Sprague-Dawley rats were used for collection of blood, which was withdrawn under sterile conditions from the exposed carotid artery of anesthetized animals into sterile syringes containing heparin. Outdated human blood was also used in some experiments. The blood was used either unclotted or diluted with normal saline to yield different Hb concentrations. Hb solutions were prepared by osmotic rupture of the RBC membrane followed by ultra-centrifugation. Each solution (or blood) was placed in a temperature-controlled (37°C) extracorporeal blood circuit system composed of a pump, a gas mixer, an oxygenator, and interconnected tubing that allowed the flowing solution to be exposed to various levels of $O_2$ and $CO_2$, yielding controlled levels of $SO_2$. A segment of the circuit that could be adjusted to include glass capillary tubes with different path lengths (50–800 $\mu$m) was attached to the microscope stage. Both round and flat capillaries were tested, and the cross-sectional shape did not influence the results. The path length value did not affect the results, and most of the data reported were obtained using a 10-mm long, 200-$\mu$m path length demountable glass chamber (Warner Instruments). Raman spectroscopy of sample solutions was then performed using various laser excitation areas (spot sizes 10–160 $\mu$m in diameter). Immediately before or after Raman measurements, samples were collected from the circuit to measure (within 2 min) the total Hb concentration, met-Hb, and $SO_2$ using a CO-oximeter adjusted for each species’ Hb absorption spectra (OSM3, Radiometer, Copenhagen, Denmark). An additional 0.06-ml sample was used for hematocrit determination. The tested Hb concentration of the samples used for the Raman measurements ranged from 5 to 16 g/dl.

In vivo measurements. This study was approved in advance by the Institutional Animal Care and Use Committee of Virginia Commonwealth University Health System and conforms to the Public Health Service Policy on Humane Care and Use of Laboratory Animals (August 2002) and the American Physiological Society’s Guiding Principles in the Care and Use of Animals. In vivo experiments were performed in anesthetized male Sprague-Dawley rats (300–450 g body wt) spontaneously breathing room air or 100% oxygen. Anesthesia was provided by isoflurane (1–3%), followed by constant intravenous infusion (0.24–0.36 mg·kg$^{-1}$·min$^{-1}$) of alfaxalone/alfadalone acetate (Saffan, Schering-Plough Animal Health, Welwyn Garden City, UK). The rats had acute cannulas implanted into the femoral (or carotid) artery and the jugular vein to measure blood pressure and to collect blood samples. The rat mesentery was prepared for microvascular studies, as previously described (40, 43). Briefly, an ileal loop and the associated mesentery were carefully exteriorized and placed on top of a temperature-controlled Plexiglas viewing platform (18), without excessive stretch. Any exposed gut was covered with swabs soaked in saline. After exposure, the preparation was covered with a thin plastic film (Saran Wrap, Dow Corning, Midland, MI) to minimize both desiccation of the tissue and gas exchange with the atmosphere. Using a similar approach, the spinotrapezius muscle was prepared for intravital microscopy, as described previously (19). We did not observe any obvious deleterious effects of Saran (e.g., altered microvascular patency, platelet aggregation, or increased numbers of leukocytes) on these preparations or changes in the Raman spectra.

Each animal was positioned over the stage of the microscope, and the tissue image was projected onto a TV camera (DC-330, Dage-MTI, Michigan City, IN) connected to a video timer, a digital recorder (DVR-533H, Pioneer, Japan), and a video monitor (SSM 175A, Sony, Japan). Each field was recorded under transmitted light before and after each $SO_2$ determination. Vessel diameter was measured from the digitzed video images at a resolution of 0.12 $\mu$m/pixel. RBC velocity was determined online using an Optical Doppler Velocimeter (Texas A&M University, College Station, TX) (2, 10). The method gives an average RBC velocity that can be digitally displayed on a meter and allows for calculation of microvessel blood flow. The temperature of the area under study was periodically measured using a thermocouple (YSI, Dayton, OH). Total Hb concentration, met-Hb, and $SO_2$ were measured as described above in blood samples obtained from the femoral artery.

To obtain a wide range of $SO_2$ levels, arterioles, capillaries, and venules were evaluated in situ in animals under different conditions: 1) control (normovolemia and room air breathing); 2) hypoxia (normovolemia and 10% $O_2$ breathing); 3) hyperoxia (normovolemia and 100% $O_2$ breathing); and 4) hemorrhagic hypotension (room air breathing and stepwise hemorrhage until death). Hemorrhagic hypotension was induced by withdrawing blood from the carotid artery using a heparinized syringe. Blood pressure was simultaneously recorded using a data-acquisition system (Biopac Systems, Goleta, CA). We compared the values of $SO_2$ of arterioles and venules obtained using Raman spectroscopy and the main six peaks at 532-nm
excitation (1,350- to 1,650-cm⁻¹ range) with those obtained using the microspectrophotometric Hb absorption method (also known as the 3-wavelength method) of Pittman and Duling (27, 28). This method was used as an in vivo SO₂ reference. A digital video-based version of this method using a high-resolution digital charge-coupled device camera (CoolSnap cf; Roper Scientific) was implemented to calculate SO₂, as described in our laboratory’s earlier study (42). Wavelengths in the green region of the spectrum were used for microvessels up to 100 μm in diameter. Raman spectra and digital images were captured with the room light turned off to eliminate noise caused by stray light. For each microvessel, one to two Raman spectra and four snapshots (for 3-wavelength evaluation) were recorded sequentially, but in random order. During SO₂ measurements, the tissue was exposed to only one light source at a time. The tested laser excitation spot sizes were in the range of 10 to 100 μm in diameter, but the Raman data reported were obtained using an epi-illuminated area of ~30 μm in diameter (relative to the actual microscopic field) that was placed in the interstitium (close to the vessel wall) or in the center of the vessel under study.

Analysis
Each Raman spectrum was the cumulative signal of one to four exposures, each one with 20- to 30-s duration. Each spectrum was processed for removal of cosmic ray spikes and normalized to the same baseline level by subtracting each spectrum from the average intensity obtained from ranges of the spectrum where no Raman peaks were present. No further processing of the Raman spectra was performed, and the Raman spectra are presented without smoothing.

A Raman spectrum is a plot of the intensity of Raman scattered radiation as a function of its frequency difference from the incident radiation. This difference is called the Raman shift (usually expressed in relative wave number units, cm⁻¹):

\[
\text{Raman shift} = \frac{1}{\lambda_{\text{inc}}} - \frac{1}{\lambda_{\text{scatt}}} \quad (1)
\]

where \(\lambda_{\text{inc}}\) and \(\lambda_{\text{scatt}}\) are the incident and Raman scattered light wavelengths (in cm), respectively. Because it is a difference value, the Raman shift is independent of the frequency of the incident radiation.

For each spectrum, the relative absolute peak intensities of the Raman bands for oxygenated (\(I_{\text{oxy}}\)) and deoxygenated Hb (\(I_{\text{deoxy}}\)) were measured for \(v_4\) (\(I_{\text{oxy}}\) at 1,375 cm⁻¹ and \(I_{\text{deoxy}}\) at 1,360 cm⁻¹), \(v_{19}\) (\(I_{\text{oxy}}\) at 1,585 cm⁻¹ and \(I_{\text{deoxy}}\) at 1,555 cm⁻¹), and \(v_{10}\) (\(I_{\text{oxy}}\) at 1,640 cm⁻¹ and \(I_{\text{deoxy}}\) at 1,605 cm⁻¹). As a first step in the estimation of the SO₂, nine peak ratios (PR) were computed using the intensities of the six Raman peaks for oxy- and deoxy-Hb, using the general formula:

\[
\text{PR}_n = \frac{I_{\text{oxy}}}{I_{\text{deoxy}} + I_{\text{oxy}}} \times 100 \quad (2)
\]

where \(I_{\text{oxy}}\) and \(I_{\text{deoxy}}\) are the respective \(I_{\text{oxy}}\) and \(I_{\text{deoxy}}\) at each \(n\) Raman band. We also verified whether average intensity or integral area over specific spectral ranges would improve the estimations. Since no clear improvement was found, all data presented in this report were calculated using peak intensities at the Raman shift positions specified above.

The SO₂ was then estimated (in %) using each \(\text{PR}_n\), according to the general formula:

\[
\% \text{SO}_2 = \text{PR}_n \times a_n + b_n \quad (3)
\]

The coefficients \(a\) and \(b\) were obtained from calibration experiments where PR was measured from the Raman spectra and SO₂ was independently measured using the reference device (CO-oximeter), as described above. The spectra were stored and analyzed with commercially available software (WinSpec software, Roper Scientific, Trenton, NJ; GRAMS/AI, Thermo-Electron; Origin Pro, Origin Lab), as well as with customized software specifically developed to streamline processing of Raman spectra and estimate SO₂.

Statistics
Values are reported as means ± SE. Differences among the SO₂ estimated under different conditions (different laser spot sizes, different glass capillary path lengths, etc.) were analyzed using analysis of variance. For correlation analysis, linear least squares regressions were performed, and significance of the correlation coefficients was tested. In addition to performing linear regressions between the Raman-estimated SO₂ and oximeter-estimated SO₂ for each band (e.g., \(v_4\), \(v_{10}\), or \(v_{19}\)), we used multiple linear regression to test whether using more than one band (e.g., \(v_4\) and \(v_{10}\)) would improve SO₂ estimation. The statistical tests were performed using commercial computer software (Origin 7, OriginLab and Excel 2003, Microsoft). All \(P\) values correspond to two-tailed tests, with significance set at 0.05.

RESULTS

In Vitro Measurements
Raman spectra were obtained with Hb solutions, as well as with blood, equilibrated with known O₂ tensions. When the SO₂ (measured by the OSM3 CO-oximeter) was lower than 3% or above 98%, one Raman peak was observed for each of the investigated bands (\(v_4\), \(v_{10}\), and \(v_{19}\)). For intermediate levels of SO₂, the Raman spectra of blood in vitro showed six peaks in the 1,350- to 1,650-cm⁻¹ range, as shown in Fig. 1. The amplitude of each peak varied according to the oxygenation level. Each Raman peak was always in the same position among spectra (±2 cm⁻¹), independent of the source and concentration of the Hb. Keeping the SO₂ constant, similar Raman spectra were obtained from solutions using glass capillary tubes with different path lengths (50–800 μm) and using different laser excitation areas (15–150 μm).

In vitro calibration was performed with blood with 11 different Hb concentrations (range: 4.7–15.7 g/dl) equilibrated with known O₂ tensions. The fractions of met-Hb and CO-Hb were 1.4 ± 0.1 and 2.4 ± 0.3%, respectively. We checked the assumption that the intermediate oxygenation states yield peak heights that were proportional to the concentrations of oxy-Hb and deoxy-Hb by measuring those heights under similar conditions, except for the SO₂ levels. Figure 2 presents data from one experiment, where the peak amplitudes of \(v_4\) for Hb and HbO₂ were measured at 18 different SO₂ values (range: 5–80%, measured by an oximeter) and at three Hb concentrations (5, 10, and 12 g/dl). The absolute value of the intensity of the Raman bands for oxy-Hb was always directly proportional to the SO₂ measured by oximetry with statistically significant correlation coefficients. Similarly, the absolute value of the intensity of the Raman band for deoxy-Hb was negatively correlated to the SO₂ measured by oximetry. Both findings were confirmed at various laser intensities and at various Hb concentrations. The PR was calculated for each set of measurements at a given Hb concentration band and was related to the SO₂ measured by CO-oximetry using a least squares linear regression. The coefficients obtained from the regressions between PR and SO₂ (by oximetry) were used to estimate SO₂ from the Raman spectra. Figure 3 illustrates another experiment showing that the correlation between Raman-based SO₂ estimations and SO₂ measured by CO-oximetry was highly significant for \(v_4\), \(v_{10}\), and \(v_{19}\) bands. Similar results were
obtained from over 100 Raman spectra in 11 experiments using the oxy and deoxy peaks for $v_4$, $v_{10}$, and $v_{19}$ bands. These results are presented in Table 1.

Even when the oxy peak from one band ($I_{4\text{ oxy}}$ of $v_4$, for instance) was used, together with the deoxy peak from another band ($I_{10\text{ deoxy}}$ of $v_{10}$, for instance) to estimate $SO_2$, the correlations with $SO_2$ by oximetry were also significant. This is illustrated in Table 2 (simple regression). In summary, all approaches resulted in significant correlations between $SO_2$ values measured with the oximeter (the standard of reference) and $SO_2$ values estimated from Raman spectra. However, using more than one band to estimate $SO_2$ did not improve the correlation, as also shown in Table 2 (multiple regression).

In Vivo Measurements

In microvascular preparations from anesthetized animals, extravascular signals did not seem to interfere with Raman signals from intravascular $Hb$, and typical $Hb$ Raman emission

Successive determinations of $SO_2$ in a given area of constant oxygen level provided results varying ±1–5%. The detection of all physiological $SO_2$ levels was possible. The mean difference between $SO_2$ values estimated from Raman spectra (using $I_{4\text{ oxy}}$ and $I_{10\text{ deoxy}}$) and $SO_2$ values measured using CO-oximetry was 4.5 ± 1.5%. Similar results were found when other Raman peaks were used.

Innovative Methodology

1812 RAMAN MICROSCOPY USING 532 nm
was detected only when pointing the laser excitation over a microvessel. Therefore, it was not critical to keep the laser excitation exclusively over the vessel under study, and, in practice, the excitation spot used was 30 μm, which gave an adequate signal-to-noise ratio in the whole range of microvessels. The SO₂ estimated from spectra obtained in vivo using different laser excitation sizes were not significantly different from each other (data not shown). We did not observe any adverse reactions of the animals to the laser radiation, either acutely or after 4–6 h of experimentation. Neither arteriolar vasoconstriction nor increased venular leukocyte adhesion was observed when the same tissue area was illuminated with the same laser excitation.

Fig. 3. Results from one in vitro calibration experiment. Raman spectra were collected using the intravital microscope system, and flowing blood (at the same [Hb]) was exposed to different levels of oxygen. Each point represents one measurement at a given Hb SO₂. Each panel present 6 measurements used to calculate each least squares regression line. The correlation coefficients (r) are shown and were all statistically significant. I₄ oxy, I₄ deoxy, I₁₉ oxy, I₁₉ deoxy, I₁₀ oxy, and I₁₀ deoxy are the absolute peak intensities of oxy- and deoxy-Hb at each one of the bands (v₄, v₁₉, and v₁₀), respectively, and are shown in the left and middle columns. Intensity is expressed in au. Note that y-axis scale is different between oxy and deoxy measurements. Right: SO₂ calculated using the respective peak intensities of oxy- and deoxy-Hb at each one of the bands. The SO₂ measured by CO-oximetry is shown on the horizontal axis.

Table 1. Summary data for the in vitro individual experiments

<table>
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<th>Experiment No.</th>
<th>[Hb], g/dl</th>
<th>n</th>
<th>v₄</th>
<th>v₁₀</th>
<th>v₁₀</th>
<th>COHb, %</th>
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<td>0.990</td>
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<td>0.983</td>
<td>0.970</td>
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</table>

[Hb], mean hemoglobin concentration; n, number of measurements; COHb, mean CO-hemoglobin concentration; MetHb, mean methemoglobin concentration. The correlation coefficients refer to least squares linear regressions between Raman-estimated saturations (using the corresponding r band) and CO-oximeter measured saturations. All coefficients were statistically significant (P < 0.01).

Table 2. Linear regression analysis for 113 measurements in vitro

<table>
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<tr>
<th>Raman Peaks Used</th>
<th>Simple Regression</th>
<th>Multiple Regression</th>
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</tbody>
</table>

I₄ oxy and I₄ deoxy: absolute peak intensity of Raman band for oxygenated and deoxygenated Hb, respectively; I₁₉ oxy, I₁₉ deoxy, and I₁₀ oxy; I₁₉ deoxy, and I₁₀ deoxy: I oxy for v₄, v₁₉, and v₁₀, respectively; I oxy and I deoxy: for v₄, v₁₉, and v₁₀, respectively; r, correlation coefficient; R, coefficient of multiple correlation. All values are statistically significant (P < 0.001).
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laser for uninterrupted periods of up to 3 min. However, adhesion of platelets and leukocytes was observed in microvessels exposed continuously to >5 min of laser excitation. The fraction of met-Hb was always <2%.

Raman spectra were obtained from mesenteric microvessels of anesthetized rats before and during hemorrhagic hypotension. Under these conditions, a substantial reduction in perfusion is observed throughout the mesenteric network. In addition, by supplying different amounts of oxygen in the breathing air mixture, a wide range of intravascular SO2 was induced in microvessels. Intravascular SO2 was estimated from Raman spectra in 12 arterioles and in 21 venules under normal conditions and following hemorrhage. Arteriolar (mean control diameter 22.3 ± 2.2 μm, range = 14–35 μm) and venular (mean control diameter 27.1 ± 2.1 μm, range = 15–50 μm) SO2 varied 20.8–91.6 and 4.1–88.7%, respectively. Similar recordings were obtained from vessels located in the serosal surface of the exposed ileum and in the spinotrapezius muscle.

When SO2 values estimated from Raman spectra (using the oxy and deoxy peaks for ν4, ν19, and ν19 bands) were compared with SO2 values measured using the 3-wavelength in the same microvessels, a statistically significant correlation was always found (r > 0.9, P < 0.001). Figure 4 shows SO2 estimated from Raman spectra (using the ν19 band) compared with SO2 measured using the 3-wavelength in 33 microvessels. The mean absolute difference (in %saturation) was calculated between SO2 values estimated from Raman spectra (using I19 oxy and I19 deoxy) and SO2 values measured using the 3-wavelength. The values obtained for arterioles and venules were 11.0 ± 1.5 and 10.1 ± 1.4%, respectively. Slightly lower values were found when other Raman peaks were used. Since the absolute differences were similar between the two types of vessels and the mean SO2 was higher for arterioles, the relative difference (in %) was lower for arterioles than venules, but this difference did not reach statistical significance. The differences were independent of vessel size in the studied range of microvessels (14–50 μm).

Since extravascular signals did not seem to interfere with Raman signals from intravascular Hb, it was not critical to keep the laser excitation exclusively over the vessel under study. The same estimated SO2 was obtained when a 30-μm diameter laser spot was centered over a 15-μm diameter vessel and when the laser spot was reduced to 15 μm. On some occasions, it was possible to observe the tissue (using transillumination) during the period of measurement, to check for movements of the preparation as potential sources of error. The position of the laser excitation spot was always checked before and after each measurement, and the measurement was repeated in case the two positions were different. The laser was the only light source to which the tissue was exposed during the SO2 measurements reported here using Raman signals. The use of transillumination concomitantly with the laser excitation and signal collection increased the background noise level, but did not alter the calculations using the Raman Hb peaks.

DISCUSSION

Intravital Raman Microscopy

Resonance Raman spectroscopy has been applied to detect heme perturbations caused by the quaternary R-T transition in oxy-Hb. The combination of Raman spectroscopy and optical microscopy allows noninvasive acquisition of detailed biological data with good spatial resolution. In vivo applications are broad and continuously expanding. Our results show, for the first time, the application of Raman spectroscopy using 532-nm excitation to evaluate dynamic changes in blood oxygenation in vitro and in individual microvessels under physiological conditions. Similar to nearly all micro-Raman instruments currently available, we employed a single, wide-aperture objective in the backscattering configuration. The laser beam coaxially illuminated the sample through the objective that transmitted the backscattered radiation toward the spectrometer. In addition, water immersion objectives were used to increase numerical aperture and thus the resolution of the system. The system was implemented to allow the acquisition of Raman spectra and other microcirculatory variables in vivo, while systemic parameters (such as blood pressure) were simultaneously recorded. While several Raman microscope systems are commercially available, they do not allow a complete evaluation of other physiological parameters in vivo. The system described is integrated and versatile, allowing digital imaging at a wide range of sensitivity levels and measurements of several microcirculatory parameters. The phosphorescence quenching method for PO2 determinations (41) is part of the system, but was not used in the experiments reported here.

In agreement with previous studies (4), we found that Hb spectra from RBCs (in whole blood) did not differ from purified Hb spectra. This is advantageous, since it allows the study of Hb in its natural environment, allowing chemical characteristics of descriptions obtained from the previous spectroscopic studies of the Hb molecule to be carried over to in vivo studies. In this regard, it is important to note that the Hb Raman peaks observed in flowing blood using the intravital microscopy are identical to those reported in previous in vitro studies (3).

In vitro measurements and data analysis. Metalloporphyrins, carotenoids, and other classes of biologically important molecules strongly allow electronic transitions in the visible range.
The spectrum of the chromophoric moiety is resonance enhanced and that of the surrounding protein matrix is not. This allows probing the chromophoric site (often the active site) without spectral interference from the surrounding protein. Therefore, Raman spectra obtained with Hb solutions, as well as with blood, showed that the oxidation state marker band, known as ν4, appeared as two clearly defined Hb peaks in the range of 1,350–1,400 cm⁻¹. The position of these peaks in this range is within the values previously published by our laboratory (42, 46, 47) and others (3). One peak is at maximum when Hb is fully deoxygenated, and the other one is at maximum when Hb is fully oxygenated. This marker band is preferentially enhanced by blue/violet excitation wavelengths (46), but also appeared using 532-nm excitation (Fig. 1). We have previously demonstrated that the peak heights of ν4 are proportional to the concentrations of deoxy-Hb and oxy-Hb, allowing the estimation of SO2 over a wide range of physiologically relevant Hb concentrations, microvessel diameters, and SO2. In the present study, we found similar results for the ν4, υ19, and ν10 bands using 532-nm excitation. However, the absolute signal intensity of the Raman signal is influenced by a number of factors, such as focal plane, tissue absorption, laser intensity, and fluorescence background. Therefore, it is necessary to apply corrections to account for signal variations that are not related to changes in concentration. In our laboratory’s previous study (42), the intensity of the ν4 oxy peak was divided by the sum of the oxy- and deoxy-Hb peaks. This approach was also employed in this study and extended to the ν10 and υ19 bands. The resulting ν4-, υ19-, and ν10-based SO2 estimations showed excellent correlation with SO2 values measured with the OSM3 oximeter. Further internal consistency was demonstrated when oxy and deoxy peak intensities of different bands were used to compute SO2 and yielded similar results (Table 1). Raman spectra were not affected by the path length of the glass capillary or different laser excitation areas (except for changes in overall Raman band intensity). Successive determinations of SO2 (from Raman signals) showed good reproducibility.

In vivo measurements. Our approach was directed to an in vivo evaluation of blood oxygenation states in microvessels. Most previous Raman spectroscopic investigations did not focus on oxygenation state and/or were performed on single RBCs (48–51), sometimes immobilized by methanol fixation on a glass slide in air (25) and adsorbed on polylysine-coated glass surfaces (31). In addition, Raman spectra have been obtained from individual optically trapped cells using different excitation lines (32). In the few studies where oxygenation was investigated, the RBCs were not studied under flowing conditions (45, 49–51). Nevertheless, those studies demonstrate the potential for Raman spectroscopy in the study of RBC disorders, such as thalassemia, sickle-cell disease (21), and malaria (30).

As observed for 406-nm excitation (42), intravascular SO2 measured using 532-nm excitation in mesenteric arterioles and venules from anesthetized rats under normal conditions and after hemorrhage were similar to those reported in the same species, using the 3-wavelength method. A good correlation was found when SO2 values estimated from Raman spectra were compared with SO2 values measured using the 3-wavelength method in the same microvessels. The relatively large variability for the in vivo data comparisons was probably due to inherent inaccuracies on both methods. In addition, since Raman and 3-wavelength measurements were not performed simultaneously, some temporal variability was also present. An excitation laser spot of 30-μm diameter gave an adequate signal-to-noise ratio to measure SO2 in the whole range of microvessels in vivo. The measurement of all physiological SO2 levels was possible.

Limitations and Applications

Previous studies on isolated RBCs have shown that the type of glass coating and the laser irradiation are important factors to be considered, since cells may show morphological changes and photo-induced effects may be detected on the Raman spectra (29, 31, 32). These artifacts are known to be reduced when higher wavelength excitation is employed. In our experiments, these problems were further avoided by using very low laser energy (0.5 mW) and by studying flowing RBCs in microvessels. To further minimize light-related tissue reactions, illumination was restricted to periods of <60 s. We did not observe photo-induced effects in the studied microvessels reported here.

Laser safety limits for the application of ultrashort pulses on human tissues are more stringent than those with continuous wave lasers (23). Therefore, we used low-power continuous laser beams to avoid harmful effects associated with intense pulsed laser beams. Using <0.5 mW of laser power at the preparation level and 30-s exposures, the minimum excitation spot diameter that provided reliable data was ~15 μm. Smaller sizes could be used in situations where longer collection times are possible to increase the signal-to-noise ratio. An improved signal-to-noise ratio could also be achieved by averaging a larger number of spectra. However, such a procedure would lead to a significant increase in the time of laser exposure of the tissue, increasing the possibility of photo damage (24). Another approach is the use of objectives with increased light-gathering power. Our measurements confirm that a superior lens (Zeiss, ×40, numerical aperture = 1.3) provided considerably better Raman signals (unpublished results). Other techniques of signal processing would also enhance the capabilities of the system. Further refinements of the system will allow faster SO2 determinations.

A potential problem using intravital Raman spectroscopy is that Raman signals generated in tissue compartments other than blood could interfere with the analysis of the Hb. This problem was approached by considering only the characteristic and strong Raman Hb bands. Therefore, it was easy to distinguish the spectrum of Hb from any other Hb-free compartment. In fact, intravascular areas filled with plasma studied in vivo never showed the Raman bands, whereas these bands could be observed when the excitation spot was placed over extravasated erythrocytes located in the fluid above avascular areas. In skeletal muscle, myoglobin could be considered one of these interfering compartments. However, our preliminary observations in the spinotrapezius muscle showed that SO2 could be measured using the proposed technique. The contribution from areas below the vessel under study is probably small, because the signal coming from those regions is absorbed by the intravascular blood column. Considering the extinction coefficient of Hb at the excitation wavelength used in our experiments (532 nm), the penetration depth probably varies between
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120 and 150 μm (44). Moreover, due to the excitation wavelength and the high numerical aperture (0.80) of the objective used, the depth of field in our optical system was ≈2 μm, which resulted in a significant decrease in excitation and emission from regions outside the focal plane. However, using the current configuration, the data are spatially averaged, and the system may not allow the detection of small SO2 gradients within a blood vessel. Since collection times of 20–60 s were used to obtain a spectrum, the data also included a temporal averaging of the SO2 in the sampled region. In vivo, variations of SO2 during this interval would not be detected.

The ability to determine microvessel SO2 will depend on the amount of tissue present in the light path. If this layer is too thick, the optical signal may be too weak to be detected by the system. However, since most preparations used in microvascular research rely on the direct visualization of the microvessels, the thickness of this layer is minimal, the corresponding absorption is minimal, and the technique should be applicable in most circumstances. The methodology described here extends the range of applicability of the resonance Raman spectroscopy technique and allows SO2 determinations in vivo and in situ. Resonance Raman spectroscopy may be used to study individual RBC behavior in living microvessels or overall tissue oxygenation when larger areas, comprising many microvessels, are evaluated. Therefore, the method allows oxygenation analysis at the tissue level or in a single RBC. In addition, this implementation allows the measurement of SO2 in solid organs. The possibility of measuring important hemodynamic (e.g., microvascular blood flow) and oxygenation (PO2) variables also increases the ability to more accurately assess O2 transport in living systems.

Previous studies have already shown the suitability of Raman spectroscopy in human skin and eye (7, 8, 15–17, 20, 22). For transcutaneous measurements, both elastic and Raman scattering from the skin are potential concerns. Elastic scattering limits the photon penetration depth, while increasing the Raman spot size. A consequence may be an increase in the error of estimating SO2. Thus understanding Raman scattering in complex environments, such as the blood-skin matrix, is critical for noninvasive, transcutaneous blood analysis by Raman spectroscopy. Implementing alternative collection geometries may also be important to allow measurements in very heterogeneous tissues. Further research is required to make this technique practical in clinical laboratories and to develop transcutaneous SO2 monitoring schemes.

There are advantages of using 532-nm excitation to estimate SO2 using Raman spectroscopy. Currently, better laser performance is provided by 532-nm YAG lasers than by 406-nm based systems. The availability of components for wavelengths such as 532 nm is limited by the 406 nm, and most commercially available systems employ 532-nm lasers. The penetration of 532-nm excitation is deeper than using a 406-nm laser, allowing a larger fraction of a blood vessel (or vessels located deeper within the tissue) to be probed for SO2 levels. Finally, one could argue that 532 nm represents a “safer” wavelength than 406 nm, and it is possible that implementation of a 532-nm-based application for human use might present fewer concerns than using a wavelength closer to the ultraviolet. At longer wavelengths, photodissociation and hemolysis are minimized compared with the shorter excitation wavelengths (50). One challenge of using 532-nm excitation is that the overall Raman signal is less intense compared with the selective enhancement of the porphyrin vibrations present at 406 nm, when the frequency of the exciting light falls within the electronic absorption band of the molecule (38).

In summary, we assessed the adequacy of resonance Raman spectroscopy to measure SO2 noninvasively in flowing blood using 532-nm excitation. The Hb oxidation state marker band, known as ν4, as well as the bands ν19 and ν20, could be used to accurately estimate SO2 in vitro and in vivo. The system was implemented in the same optical system used for RBC velocity and microvessel diameter measurements, providing noninvasive measurements from all vessels in the microvascular network. This methodology can be used to study SO2 in thin tissues, as well as in solid organs and tissues such as liver, brain, and tumors, which are unsuitable for techniques that require transillumination.

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