

# NIR Raman spectra of whole human blood: effects of laser-induced and in vitro hemoglobin denaturation

P. Lemler · W. R. Premasiri · A. DelMonaco · L. D. Ziegler

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**Abstract** Care must be exercised in the use of Raman spectroscopy for the identification of blood in forensic applications. The Raman spectra of dried whole human blood excited at 785 nm are shown to be exclusively due to oxyhemoglobin or related hemoglobin denaturation products. Raman spectra of whole blood are reported as a function of the incident 785-nm-laser power, and features attributable to heme aggregates are observed for fluences on the order of  $10^4$  W/cm<sup>2</sup> and signal collection times of 20 s. In particular, the formation of this local-heating-induced heme aggregate product is indicated by a redshifting of several heme porphyrin ring vibrational bands, the appearance of a large broad band at  $1,248$  cm<sup>-1</sup>, the disappearance of the Fe–O<sub>2</sub> stretching and bending bands, and the observation of a large overlapping fluorescence band. This denaturation product is also observed in the low-power-excitation Raman spectrum of older ambient-air-exposed bloodstains (2 weeks or more). The Raman spectrum of methemoglobin whole blood excited at 785 nm is reported, and increasing amounts of this natural denaturation product can also be identified in Raman spectra of dried whole blood particularly when the blood has been stored prior to drying. These results indicate that to use 785-nm-excitation Raman spectra as an identification method for forensic applications to maximum effect, incident laser powers need to be kept low to eliminate variable amounts of heme aggregate spectral components contributing to the signal and the natural aging process of hemoglobin denaturation needs to be accounted for. This also suggests that there is a

potential opportunity for 785-nm-excitation Raman spectra to be a sensitive indicator of the age of dried bloodstains at crime scenes.

**Keywords** Raman spectroscopy · Hemoglobin · Human blood · Forensics

## Introduction

Raman spectroscopy is a technique that has just recently been recognized as a potentially useful method in the field of forensic science. This optical method has proven successful for the identification of a variety of materials relevant to forensic investigations, such as inks [1], paints [2], fibers [3, 4], illicit drugs [5], explosives [6], and gunshot residue [7]. In the past few years, the use of Raman spectroscopy to identify human body fluids, important components in many crime scene investigations, has been proposed and demonstrated [8–17]. As shown in these prior preliminary reports, Raman spectroscopic analysis is particularly attractive for forensic purposes because it allows nondestructive, rapid, on-site, specific, and low-cost identification of body fluids, and requires only minimal sample size. Identification is accomplished by comparison of the in situ acquired Raman spectrum of the interrogated potential fluid with a library of previously obtained Raman spectra of human body fluids. Statistical analysis procedures can then be used to provide a match to the suspected body fluid type for confirmatory identification [10, 11, 15]. Thus, the ability to acquire robust and reproducible Raman vibrational signatures is essential for the successful application of this method for forensic purposes. Although it is not essential for identification of body fluids, it is useful to know the chemical origins of these vibrational spectral signatures so that any fundamental limitations for identification purposes arising from

P. Lemler · W. R. Premasiri · L. D. Ziegler (✉)  
Department of Chemistry and The Photonics Center, Boston  
University, Boston, MA 02215, USA  
e-mail: lziegler@bu.edu

A. DelMonaco  
Department of Biomedical Engineering, Boston University, Boston,  
MA 02215, USA

biochemical activity or subsequent degradation processes are properly recognized.

Human blood is probably the body fluid most commonly encountered at a crime scene. In prior studies of the Raman spectrum of dried whole blood excited with near-infrared (NIR) radiation, several molecular components were identified via principal component analysis, and a corresponding method based on statistical analysis techniques was developed allowing identification of human blood [12, 14, 15]. The three main components consistently found to contribute to the observed Raman spectrum of dried blood excited at 785 nm were identified as hemoglobin (Hb), fibrin, and a broad fluorescence background [10–12, 14]. The Hb contribution is expected given that 33 % of red blood cells (RBCs) are Hb by volume. The second molecular component of the dried blood spectrum identified in the prior work was attributed to the Raman bands of fibrin, the protein involved in the clotting of blood. The basis of this assignment was the resemblance of this component to the spectrum of fibrin and the reported spectral differences between the Raman spectra of liquid blood and dried blood [12]. Furthermore, the Raman spectra of whole blood samples excited at 785 nm showed a large heterogeneity in the relative contributions of these three components to the dried blood spectrum in these prior studies, thus complicating the identification of this body fluid by Raman spectroscopy. However, a statistical procedure was developed to account for this inhomogeneity and thus provide identification via Raman spectroscopy of this body fluid type [10–12].

In a previous study primarily describing surface-enhanced Raman spectroscopy (SERS) of whole human blood and RBCs, we compared normal (non-SERS) Raman spectra of whole blood with SERS spectra of the same blood samples excited at 785 nm [18]. For low incident laser powers, we showed that the normal (non-SERS) Raman spectrum of dried fresh whole blood is essentially identical to the Raman spectrum of RBCs. Every vibrational feature observed in the Raman spectra of whole blood excited at 785 nm could be found in the corresponding Raman spectra of RBCs [18]. Thus, this prior work already called into question the interpretation of the Raman spectrum of whole blood excited at 785 nm as arising from non-heme contributions [12]. More specifically, we demonstrate here that the second component tentatively identified in the earlier Raman studies of whole human blood as due to fibrin [12] is the signature of a photodegradation heme aggregation product produced by high excitation fluences.

Furthermore, as for any biological sample removed from its *in vivo* environment, naturally occurring degradation or denaturation processes *in vitro* may contribute to the heterogeneity of the observed Raman signals of a bloodstain sample. The effects of ambient exposure and aging could contribute a time-dependent heterogeneous component in

blood that could also offer challenges for identification of blood by a variety of physical techniques. Whole blood components arising from the auto-oxidation of oxyhemoglobin (oxyHb) to the non-dioxygen-binding methemoglobin (metHb) and additional denaturation products known as hemichromes have been described recently [19]. A steady-state population corresponding to approximately 2 % of the Hb in normal human blood is in the high-spin metHb form. Outside the body and in the ambient environment, the relative amount of this heme oxidation product changes with time [19]. Changes in the Raman spectrum of whole blood with time were noted in a previous 532-nm excitation study [17]. However, the NIR excitation used here is further from regions of electronic resonances of Hb and its denaturation products. Thus, the spectral changes due to Hb degradation or denaturation may contribute very differently at these NIR Raman excitation wavelengths.

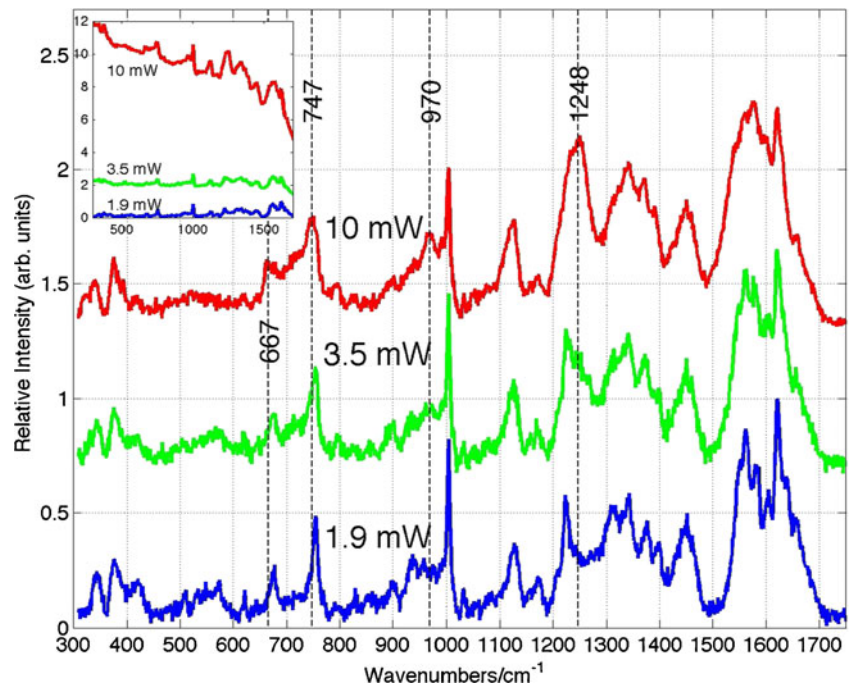
The results described here illustrate that care must be taken to use reduced incident powers to avoid optically induced thermal degradation effects in whole blood, and the products of natural Hb aging in the ambient environment can be detected in dried whole blood. However, these effects do not preclude the use of Raman spectroscopy as an effective platform for identification of whole human bloodstains when low laser power conditions are used and the signatures of the expected *in vitro* “aging” products are known and incorporated into data analysis methods.

## Experimental

### Sample preparations

Following standard protocols, 5 mL of blood was collected by venipuncture from healthy donors into Vacutainer blood collection tubes containing EDTA as an anticoagulant (BD, Franklin Lakes, NJ, USA). Blood samples were stored in an 8 °C refrigerator immediately after collection until required for measurements or additional separation steps. MetHb whole blood was prepared by mixing 1 mL of whole blood with 10 mL of 0.1 wt % sodium nitrite in phosphate-buffered saline and incubating the mixture for 1 h at room temperature. Conversion to metHb was confirmed by UV/vis absorption change. Following previous procedures [18, 20], metHb RBCs were prepared from an RBC sample concentrated by centrifugation (1 min at 10,000 rpm). For this, concentrated RBCs (10  $\mu$ L) were suspended in 10 mL of 0.1 % (by weight) sodium nitrite in phosphate-buffered saline and incubated for 1 h at room temperature. MetHb RBCs were recovered after centrifugation and washed three times with saline before they were resuspended in 0.5 mL of saline prior to Raman data acquisition. The dried RBC pellet was made by drying 50  $\mu$ L of packed RBCs on a silicon wafer.

**Fig. 1** Power/fluence dependence of Raman spectra of dried whole human blood excited at 785 nm. The Raman spectrum on top of the observed fluorescence as a function of incident laser power is shown in the *inset*. Spectral changes at 667, 747, 970, and 1,248  $\text{cm}^{-1}$  are highlighted by *vertical lines*. The whole blood was stored at 8  $^{\circ}\text{C}$  for about 24 hours before being dried in ambient air for approximately 1 hour. The Raman illuminated area was 100  $\mu\text{m}^2$ , corresponding to incident laser fluences of  $1.9 \times 10^3$  (1.9 mW),  $3.5 \times 10^3$  (3.5 mW), and  $1.0 \times 10^4$   $\text{W}/\text{cm}^2$  (10 mW) at 785 nm for the three spectra



#### Acquisition of Raman spectral data

An RM-2000 Renishaw Raman microscope using a  $\times 20$  (short working distance) objective and 785-nm excitation was used to obtain all of the spectra shown here. The Raman illumination area was approximately 100  $\mu\text{m}^2$  (5  $\mu\text{m} \times 20 \mu\text{m}$ ). Spectra of dried whole blood were typically obtained from a drop of blood that had been placed on a silicon wafer, exposed to the ambient air, and allowed to dry for about 1 h. The 520- $\text{cm}^{-1}$  band of the silicon wafer was used for frequency calibration.

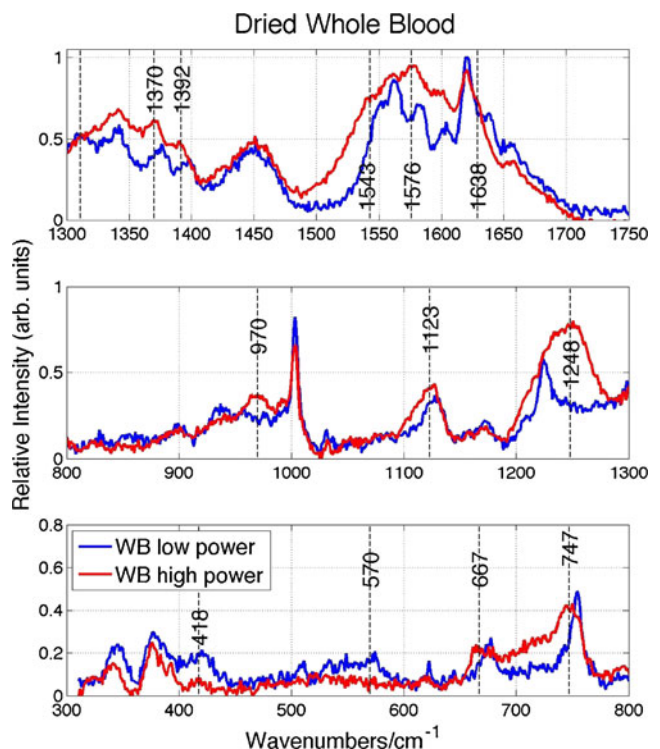
We used 1.8 mW of incident 785-nm-laser power and 20-s data accumulation times to acquire Raman spectra in order to avoid any photoinduced thermal effects as described further below.

## Results

#### Dependence on incident 785-nm-laser power

The Raman spectra of whole blood dried for 1 h after being stored at 8  $^{\circ}\text{C}$  for approximately 24 h as a function of incident 785-nm-laser power are shown in Fig. 1. Each spectrum displayed is the average of five scans and has been background-corrected, i.e., any broad overlapping fluorescence component has been removed. Changes in the appearance of the dried whole blood Raman spectrum are evident as the incident laser power is increased from 1.9 mW, corresponding to a fluence of approximately  $1.9 \times$

$10^3$   $\text{W}/\text{cm}^2$ , to 10 mW, or a fluence of approximately  $1.0 \times 10^4$   $\text{W}/\text{cm}^2$ . A more direct comparison of the relative vibrational band positions and intensities of the dried whole blood spectrum at the low (1.9 mW) and high (10 mW) Raman excitation powers is shown in Fig. 2. New vibrational



**Fig. 2** Expanded view of the spectra of dried whole blood excited at 785 nm at low and high laser power shown in Fig. 1. *WB* whole blood

bands at 970 and 1,248  $\text{cm}^{-1}$  are observed, and several other vibrational bands shift by 5–10  $\text{cm}^{-1}$  to lower frequency as the incident fluence at 785 nm is increased to the  $10^4 \text{ W/cm}^2$  level. For example, bands at 677  $\text{cm}^{-1}$  ( $\nu_7$ ), 754  $\text{cm}^{-1}$  ( $\nu_{15}$ ), 1,128  $\text{cm}^{-1}$  ( $\nu_5$ ), 1,374  $\text{cm}^{-1}$  ( $\nu_4$ ), 1,398  $\text{cm}^{-1}$  ( $\nu_{20}$ ), 1,549  $\text{cm}^{-1}$  ( $\nu_{11}$ ), 1,582  $\text{cm}^{-1}$  ( $\nu_{37}$ ), and 1,639  $\text{cm}^{-1}$  ( $\nu_{10}$ ) at 1.9 mW downshift to 667, 747, 1,123, 1,370, 1,392, 1,543, 1,576, and 1,629  $\text{cm}^{-1}$  when the incident power is 10 mW. These are all vibrational modes that are due to the porphyrin moiety of oxyHb, and their vibrational assignments [21–23] are indicated above. In addition, the bands associated with Fe–O<sub>2</sub> stretching (570  $\text{cm}^{-1}$ ) and bending (418  $\text{cm}^{-1}$ ) are greatly reduced in intensity in the high-fluence spectrum (Figs. 1 and 2). The observed frequency changes, vibrational assignments, and corresponding local coordinate motion for the modes affected by the high-power laser excitation are summarized in Table 1. Furthermore, as seen in the inset in Fig. 1, a large increase in the fluorescence background that overlaps the Raman emission region is also observed in the dried whole blood spectrum excited at 785 nm as the laser excitation power is increased to 10 mW.

We have previously shown that all the bands observed in the Raman spectrum of dried human blood excited at 785 nm with low power were also observed in the corresponding Raman spectra of isolated RBCs and could be assigned to oxyHb more specifically [18]. This was also the conclusion of an earlier Raman study of whole blood excited at 720 nm [24]. Further evidence that all the features of the NIR spectrum of

whole blood are attributable to oxyHb result from a comparison of the incident Raman excitation power dependence of dried whole blood and RBCs. As shown in Fig. 3, the spectral changes observed in the Raman spectrum of RBCs excited at 785 nm as the incident power is raised from 1.9 to 10 mW are identical to those seen for whole blood (Fig. 2). Specifically, the appearance of bands at 970 and 1,248  $\text{cm}^{-1}$ , the disappearance of the Fe–O<sub>2</sub> stretching band (570  $\text{cm}^{-1}$ ) and the Fe–O<sub>2</sub> bending band (418  $\text{cm}^{-1}$ ), and the redshift of the  $\nu_7$ ,  $\nu_{15}$ ,  $\nu_4$ ,  $\nu_{20}$ ,  $\nu_{11}$ ,  $\nu_{37}$ , and  $\nu_{10}$  oxyHb bands are analogously seen in the Raman spectra of RBCs excited with high incident (10-mW) laser power. Thus, these features can have nothing to do with blood components other than Hb, as discussed further below.

The effects of high incident laser power and/or prolonged laser excitation of RBCs at red, 632.8-nm [25], and NIR, 785-nm [21], wavelengths have been previously reported. In agreement with the spectral changes described above, vibrational bands at 1,396, 1,365, 1,248, 972, and 662  $\text{cm}^{-1}$  were identified as features due to prolonged laser excitation, high excitation power, or sample heating (above 42 °C) in these previous studies of RBCs [21, 25]. These vibrational changes were attributed to the formation of Hb aggregates resulting from thermal or photoinduced Hb denaturation. As shown in Figs. 1 and 2 and Table 1, bands at these nearly identical frequencies are seen in the 10-mW spectra of whole

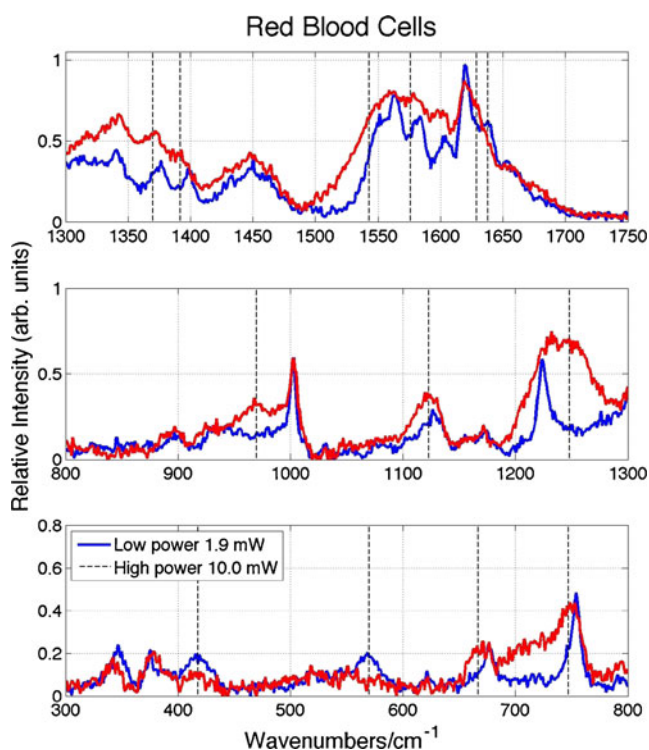
**Table 1** Vibrational bands of whole blood and red blood cells affected by high laser fluence

Low power spectrum ( $\text{cm}^{-1}$ ) <sup>a</sup>	High power spectrum ( $\text{cm}^{-1}$ ) <sup>a</sup>	Vibrational assignment <sup>b</sup>	Local coordinate
419	–	$\delta(\text{Fe-O}_2)$	Fe–O <sub>2</sub> bend
570	–	$\nu(\text{Fe-O}_2)$	Fe–O <sub>2</sub> stretch
677	667	$\nu_7$	$\nu(\text{pyr deform})_{\text{sym}}$
754	747	$\nu_{15}$	$\nu(\text{pyr breathing})$
–	970		$\gamma(\text{C}_\alpha\text{H=})?$
1,128	1,123	$\nu_5$	$\nu(\text{C}_\beta\text{-methyl})$
–	1,248	$\nu_{13}$ or $\nu_{42}$	$\delta(\text{C}_m\text{H})$
1,311	–	$\nu_{21}$	$\delta(\text{C}_m\text{H})$
1,374	1,370	$\nu_4$	$\nu(\text{pyr half-ring})_{\text{sym}}$
1,398	1,392	$\nu_{20}$	$\nu(\text{pyr quarter-ring})$
1,549	1,543	$\nu_{11}$	$\nu(\text{C}_\beta\text{C}_\beta)$
1,582	1,576	$\nu_{37}$	$\nu(\text{C}_\beta\text{C}_m)_{\text{asym}}$
1,638	1,629	$\nu_{10}$	$\nu(\text{C}_\beta\text{C}_m)_{\text{asym}}$

$C_\alpha$ ,  $C_\beta$ ,  $C_m$ , and *pyr* represent the carbon atoms at the alpha, beta, and meso positions of porphyrins and the pyrrole ring respectively

<sup>a</sup> The estimated band peak frequency precision is  $\pm 1 \text{ cm}^{-1}$

<sup>b</sup> Assignments based on [21–23]

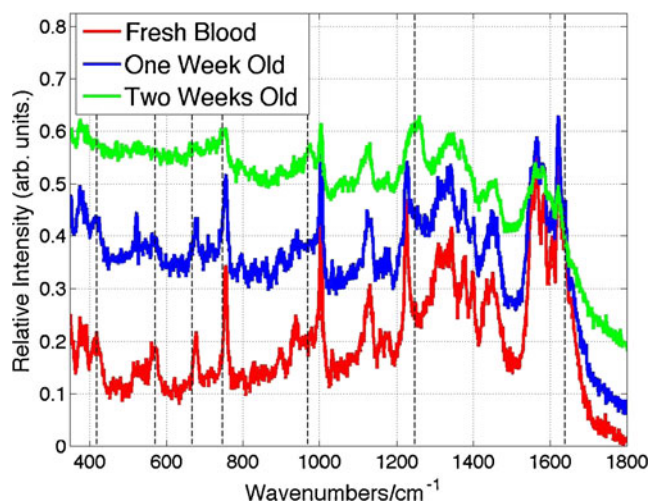


**Fig. 3** Raman spectra of red blood cells excited at 785 nm at low (1.9 mW) and high (10 mW) laser power. These spectra are essentially identical to those corresponding to the power-dependent Raman spectra of dried whole blood excited at 785 nm (Figs. 1 and 2)

blood and indicate that these same heme aggregation products are formed in response to high Raman excitation fluences in dried whole blood samples. In the prior reports on Raman spectra of RBCs, the appearance of new bands and the changes in intensity of several others were highlighted [21, 25]; however, this study more carefully shows that spectral redshifts are noted for a number of these bands in addition to the appearance of these new vibrational features and some changes in relative intensities. The concurrent appearance of a dramatically increasing fluorescence component in the spontaneous emission resulting from 785-nm excitation (Fig. 1, inset) and the redshifting of the heme vibrational bands described above is consistent with changes in electronic structure due to aggregation. Furthermore, the significant decrease in the observed intensity of the Fe–O<sub>2</sub> stretching and bending bands at 570 and 418 cm<sup>-1</sup>, respectively, indicates that O<sub>2</sub> is no longer ligated to Fe in the thermally produced heme aggregates. The  $\nu_{10}$  band at 1,638 cm<sup>-1</sup> in oxyHb, which has  $\nu(C_{\alpha}C_m)_{\text{asym}}$  local coordinate character [21], is an O<sub>2</sub> marker band and is known to weaken and redshift in deoxygenated Hb [21]. Similarly, the  $\nu_7$  band at 677 cm<sup>-1</sup> is another vibrational band that redshifts when O<sub>2</sub> is no longer ligated to Fe [25]. Thus, these observations are consistent with the loss of O<sub>2</sub> in this heme aggregate photo/thermal complex resulting from excessive 785-nm laser irradiation.

#### Blood aging effects

In addition to the consequences of high incident NIR laser fluence for acquisition of Raman spectral signals of dried blood, the effects of the age of whole blood have not been fully considered or carefully demonstrated for the use of Raman spectroscopy as a sensitive identification method in forensic applications [8, 9, 12, 14, 15]. As noted earlier, some changes in the spectrum with the age of blood were reported in a prior 532-nm Raman study [17]. The age of whole blood may be considered both in terms of the length of time a dried fresh whole blood stain has been exposed to ambient air and the age of a stored liquid whole blood sample before drying for 1–2 h prior to data acquisition. The effects of exposure to ambient conditions for a dried blood sample that has been prepared from fresh whole human blood (approximately 1 h after acquisition) is shown in Fig. 4 for exposure times of up to 2 weeks. After approximately 2 weeks, the spectrum of a dried whole blood sample excited at 785 nm exhibits the same vibrational features as are observed at high laser powers; new bands at 667, 747, and, most obviously, 1,248 cm<sup>-1</sup> appear. In addition, bands at 419, 570, and 1,638 cm<sup>-1</sup>, all Fe–O<sub>2</sub> markers, disappear in the Raman spectrum of the 2-week-old blood excited at 785 nm. All of the dried blood spectra shown in Fig. 4 were obtained with low laser power (1.9 mW,  $\times 20$ ). Thus, the same oxyHb denaturation product



**Fig. 4** Fluorescence and Raman emission of dried whole blood excited at 785 nm at low power (1.9 mW) as a function of the time of exposure to ambient air. Vertical lines highlight the appearance of bands at 667, 747, 970, 1,248, and 1,629 in these low-power spectra. These same features are seen in the high-power Raman spectra of dried “fresh” whole blood (see Fig. 1)

attributed to heme aggregates resulting from high laser power incident on dried fresh whole blood samples results when dried blood is exposed to ambient conditions for about 2 weeks.

Furthermore, Hb undergoes chemical changes as soon as it leaves the human body [19]. About 1–2 % of oxyHb (low-spin Fe<sup>2+</sup>) in the blood of normal healthy human bodies is auto-oxidized to metHb (high-spin Fe<sup>3+</sup>) and water takes the place of O<sub>2</sub>. MetHb does not bind O<sub>2</sub> and its concentration is maintained at this low level in vivo by the reduction of metHb back to Hb by the reductase protein cytochrome *b*<sub>5</sub> [26]. On blood exiting the body, contact with oxygen in the ambient environment and the decreasing availability of cytochrome *b*<sub>5</sub> for reduction back to Hb lead to an increase in the concentrations of metHb and other Hb denaturation products such as hemichrome [19]. Thus, it can be anticipated that the Raman signature of whole blood could exhibit an additional inhomogeneity due to the relative amount of metHb in the dried sample accumulated during the liquid blood storage period, in addition to the auto-oxidation and subsequent denaturation occurring in the dried blood sample as a function of the drying time. Depending on the extent of the Raman spectral differences between oxyHb and metHb at 785 nm, this denaturation process could have an impact on the use of Raman spectroscopy for identification of body fluids. In our prior study of blood, small differences were clearly recognizable when the 785-nm-excitation normal Raman spectra of oxyHb and metHb were compared [18].

The low-power (1.9-mW), 785-nm-excitation Raman spectrum of dried whole blood, with its oxyHb fully converted to metHb, is shown in Fig. 5. This Raman spectrum is

compared with the corresponding spectrum of a dried normal oxyHb whole blood sample in this figure. Both of these dried blood samples were exposed to the ambient atmosphere for approximately 1–2 h before signal acquisition. Firstly, comparison of the 785-nm-excitation Raman spectrum of metHb whole blood (Fig. 5) with our previously obtained spectrum of metHb RBCs [18] shows that the two Raman signatures are virtually identical. This further verifies that the Raman spectrum of whole blood is derived exclusively from its Hb population. Although many of the vibrational features of oxyHb and metHb whole blood are the same, a number of clearly distinguishable features are evident (Fig. 5) as found for the RBCs [18]. A metHb whole blood and oxyHb whole blood difference spectrum is shown in the lower panel in Fig. 5. For example, a vibrational band at  $377\text{ cm}^{-1}$  in the dried metHb whole blood (and metHb RBC) spectrum is perhaps the most distinctive change in the 785-nm-excitation Raman spectrum, indicating the presence of the high-spin ferric heme protein. Additional spectroscopic differences in the metHb whole blood spectra are the increased intensity and 2–3- $\text{cm}^{-1}$  redshift of the  $\nu_4$  band appearing at  $1,372\text{ cm}^{-1}$  in the spectrum of metHb whole blood. Additional spectral changes in the spectrum of dried metHb whole blood are an increase in the intensity of the  $1,213\text{-cm}^{-1}$  band, decreases in the intensities of the  $1,603\text{-cm}^{-1}$  [ $\nu(\text{C}=\text{C})_{\text{vinyl}}$ ] and  $1,224\text{-cm}^{-1}$  ( $\nu_{13}$  or  $\nu_{42}$ ) bands, and the absence of the Fe–O<sub>2</sub> stretching band ( $570\text{ cm}^{-1}$ ) and the Fe–O<sub>2</sub> bending band ( $419\text{ cm}^{-1}$ ) relative to the whole blood spectrum containing primarily oxyHb (see Fig. 5).

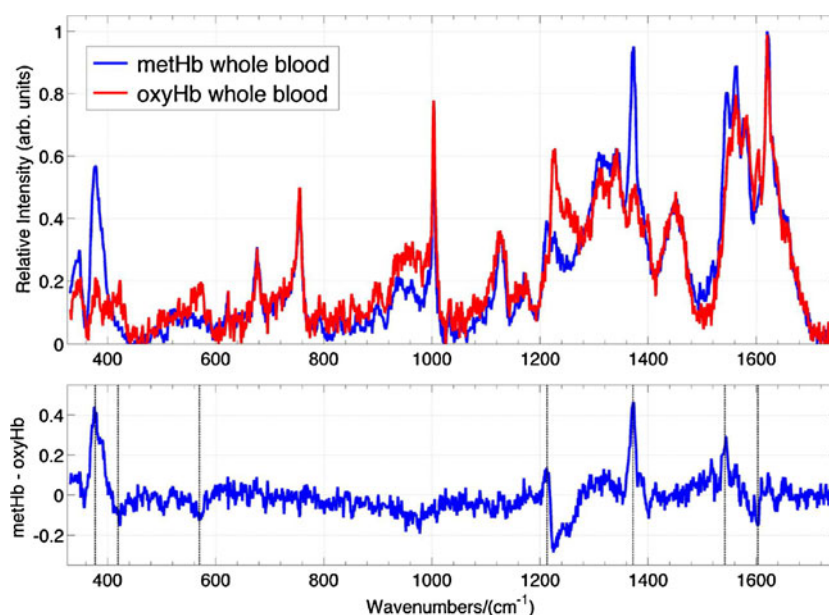
The consequences of this naturally occurring auto-oxidation process resulting in the conversion of oxyHb to metHb are illustrated in Fig. 6, which shows 785-nm-excitation Raman spectra of whole human blood that had been

stored in liquid form at  $8\text{ }^\circ\text{C}$  for 1 h, 24 h, and 2 weeks and then dried in the ambient environment for approximately 1–2 h. These spectra were acquired with low incident laser power ( $1.9\text{ mW}$ ,  $\times 20$ ). Relatively subtle but clearly distinguishable changes are evident in these spectra as a function of the storage time. An increase in intensity of the characteristic metHb feature at  $377\text{ cm}^{-1}$  is observed. This change and the small increases in the intensities of the 1, 372- and  $1,213\text{-cm}^{-1}$  bands and the slight decrease in the intensity in the  $1,603\text{-cm}^{-1}$  region are all consistent with increasing concentrations of metHb (Fig. 5) in the whole blood as a function of storage time.

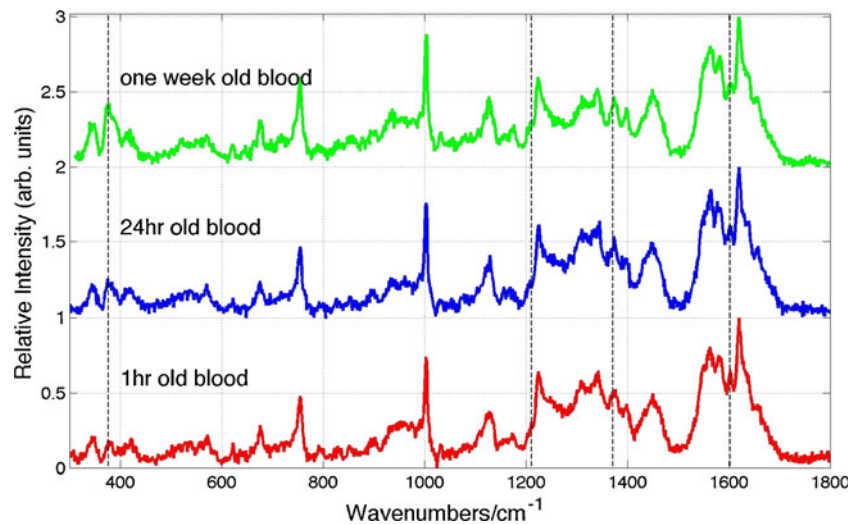
## Discussion

The results described illustrate that the Raman signature of whole human blood is a function of the incident laser power above a threshold fluence (greater than approximately  $2 \times 10^3\text{ W/cm}^2$ ), as well as the age of the blood both before and after it has dried. These factors need to be considered before Raman spectroscopy can be applied to its maximum effect as a blood identification method for forensic applications. In previous studies exploring the use of Raman spectroscopy for identification of body fluids, the effects of high laser power and blood aging could be seen in the published Raman spectra of dried whole human blood. For example, in the Raman spectrum of whole blood excited at  $752.6\text{ nm}$  ( $\times 100$  objective,  $5\text{ mW}$ ) reported by De Wael et al. [8], vibrational bands at 1, 248, 971, 747, and  $666\text{ cm}^{-1}$  clearly indicate that the previously published spectrum has large contributions from heme aggregates (Figs. 1 and 2, Table 1). This does not seem surprising given the excitation fluence resulting from the tight

**Fig. 5** Comparison of the 785-nm-excitation Raman spectra of oxyhemoglobin (oxyHb) whole blood and methemoglobin (metHb) whole blood (upper panel). These samples were dried for about 1–2 h. The metHb whole blood and oxyHb whole blood Raman difference spectrum is displayed in the lower panel. Each spectrum was normalized by the intensity of the  $1,620\text{-cm}^{-1}$  band prior to subtraction. Prominent bands that show changes in intensity on the formation of metHb from oxyHb ( $377$ ,  $419$ ,  $570$ ,  $1,211$ ,  $1,372$ , and  $1,602\text{ cm}^{-1}$ ) are indicated by vertical lines



**Fig. 6** Raman spectrum of whole dried blood excited at 785 nm (1.9 mW) as a function of storage time at 8 °C. Band changes at 377, 1,211, 1,372, and 1,603  $\text{cm}^{-1}$  are indicated



focus of the  $\times 100$  objective used in that study. The 377- $\text{cm}^{-1}$  band, characteristic of auto-oxidation products, such as metHb, exhibits relatively low intensity in this published spectrum [8], indicating that such naturally occurring denaturation products are not extensive in the dried blood sample interrogated.

Lednev and coworkers [9–15] have pioneered the use of Raman spectroscopy for identification of body fluids in forensic science. On the basis of a principal component analysis, it was concluded that each Raman spectrum of dried whole blood excited at 785 nm can be composed of variable amounts of predominantly three components: Hb, fibrin, and a fluorescence component [12, 15]. These three components captured 99 % of the variance. The fibrin component is primarily distinguished by bands at approximately 970, 1,248, and 1,342  $\text{cm}^{-1}$  and a very broad feature with a width of approximately 100  $\text{cm}^{-1}$  peaking at 1,575  $\text{cm}^{-1}$ . These spectroscopic features are all evident in the high-power spectrum of dried whole blood as clearly seen in Figs. 1 and 2. As previously reported and discussed above, the bands at 1,248 and 970  $\text{cm}^{-1}$  have been identified as heme aggregation marker bands resulting from thermally induced or high-laser-power-induced denaturation [21, 25]. Thus, the Raman scattering resulting from excitation of fibrin at 785 nm is not the origin of this component of the dried blood spectrum identified by principal component analysis, but instead is due to a heme aggregate photodegradation product as described above. Furthermore the 785-nm fluorescence, the third component identified in the previous whole blood study [12], is an additional signature of this heme aggregation product as shown in Fig. 1 (inset). It was shown that the Raman spectra of dried whole blood from all donors could be fit with variable amounts of these three components. The Raman contribution from the laser-induced heme aggregates is nonlinearly dependent on the incident 785-nm-laser fluence and thus one might anticipate a considerable variability in

these three signal contributions to the observed spectrum owing to the inhomogeneity of the local heating effect. Such a large range of signal contribution variability was reported in this earlier work [12].

Furthermore, the variable size of the 377- $\text{cm}^{-1}$  band, shown above to be a marker for metHb in whole blood, relative to that due to phenylalanine at 1,003  $\text{cm}^{-1}$  or other vibrational bands not affected by the auto-oxidation, is evident in the spectra of dried whole blood in reports discussing the use of Raman spectroscopy for forensic identification purposes [8, 10, 12]. This observation indicates that an additional source of heterogeneity in these blood spectra is due to the distribution of the ages of the blood samples [19].

## Conclusion

The goal of this report was to indicate that although Raman spectroscopy has a promising future as a valuable method for rapid, inexpensive, and on-site confirmatory identification of human bloodstains, care must be exercised in the implementation of this optical approach. The need for high-quality (i.e., good signal-to-noise ratio) Raman signatures for reliable detection and identification in forensic applications is understood. However, photoinduced denaturation, even in the nominally nonelectronically resonant NIR region of Hb, can readily appear at relatively low fluences (10<sup>4</sup> W/cm<sup>2</sup> or lower) at 785 nm in dried whole blood and result in Raman signals due to heme aggregation. Both of these effects can contribute to spectral inhomogeneity of the Raman signatures used for identification of blood via Raman spectroscopy and to potential limits imposed by the concurrent large fluorescence background. Low laser power eliminates these extraneous components and the signal-to-noise ratio must be compensated by maximized sensitivity in detection. Furthermore, despite the large variety of particles and

molecular components that constitute the complex mixture that is dried whole blood, the Raman spectrum of bloodstains excited at 785 nm is exclusively due to the vibrational components from only a single protein, Hb. The previously reported fibrin component [12] is just the vibrational Raman signature due to the heme aggregation product. With sufficient time on the order of weeks, dried blood exposed to the ambient environment was found to convert to this same denaturation product even in the absence of excessive temperatures or high laser powers, at least for the ambient conditions to which this dried blood sample was exposed.

Apart from signal heterogeneity introduced by laser-induced (thermally induced) heme denaturation effects, additional variable whole blood Raman contributions due to the in vitro aging process involving oxyHb should be accounted for in forensic identification of blood by Raman-based methods. The auto-oxidation of oxyHb to metHb when the blood leaves the human body provides an additional source of spectral heterogeneity which needs to be considered when using 785-nm Raman spectroscopy for identification of body fluids. The metHb spectrum of whole blood is identical to that of metHb RBCs, but exhibits some characteristic vibrational changes from that of whole blood containing oxyHb. Although, this natural blood/heme aging process is an additional challenge to quantitative Raman identification schemes, it is also an opportunity to expand the use of this methodology for forensic applications. The upside of this complication of a time-dependent signal contribution for confirmatory identification of bloodstains is that this in vitro biochemical activity could provide a means for determining the age of blood at crime scenes [17].

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