Characterization of Storage-Induced Red Blood Cell Hemolysis Using Raman Spectroscopy

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ABSTRACT

Background: The therapeutic efficacy and safety of stored red blood cells (RBCs) relies on minimal in-bag hemolysis. The accuracy of current methods of measuring hemolysis can suffer as a result of specimen collection and processing artefacts.

Objective: To test whether Raman spectroscopy could be used to assess hemolysis.

Methods: RBCs were stored for as long as 42 days. Raman spectra of RBCs were measured before and after washing, and hemolysis was measured in supernatant by visible spectroscopy.

Results: Raman spectra indicated increased concentrations of oxyhemoglobin (oxyHb) and methemoglobin (methHb), and decreased

membrane fluidity with storage age. Changes in oxyHb and methHb were associated with the intraerythrocytic and extracellular fractions, respectively. Hemolysis increased in a storage age-dependent manner. Changes in Raman bands reflective of oxyHb, methHb, and RBC membranes correlated with hemolysis; the most statistically significant change was an increased intensity of methHb and decreased membrane fluidity.

Conclusions: These data suggest that Raman spectroscopy may offer a new label-free modality to assess RBC hemolysis during cold storage.

Keywords: storage lesion, hemoglobin, heme, erythrocyte, blood bank, Raman spectroscopy

Transfusion of stored red blood cells (RBCs) is used to treat various patient populations. RBCs are approved for cold storage in preservation solutions for as long as 42 days in the United States. During this time, several morphologic, biochemical, and metabolic changes occur, known collectively as the *storage lesion*. These changes include generation of smaller, denser, and more fragile RBCs, as well as the formation of microvesicles and hemolysis with release of free hemoglobin (Hb) and heme into the supernatant.¹⁻⁶

Abbreviations

RBCs, red blood cells; Hb, hemoglobin; UAB, University of Alabama at Birmingham; IRB, Institutional Review Board; PBS, phosphate buffer saline; Hct, hematocrit; oxyHb, oxyhemoglobin; methHb, methemoglobin; CFH, cell-free heme; deoxyHb, deoxyhemoglobin; He:Ne, helium-neon; NA, numerical aperture; WiRE, Windows-based Raman Environment; RM-ANOVA, repeated measures analysis of variance; FDA, United States Food and Drug Administration; 2,3-DPG, 2,3-diphosphoglycerate; ATP, adenosine triphosphate

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Current concepts suggest that older RBC units may have poorer therapeutic efficacy and the potential for toxicity, especially in patients with trauma and hemorrhage who require massive transfusion.⁷⁻¹⁰ Moreover, significant heterogeneity exists amongst stored RBCs from different donors, such that storage age, by itself, is a relatively poor surrogate for efficacy and safety of RBCs.^{11,12} Thus, improving diagnostic modalities to assess the quality of a given unit of stored RBCs remains a key goal in transfusion medicine and could improve the safety of the RBC supply, inventory management, transfusion protocols, and patient outcomes.

Current methods to evaluate stored RBC quality require specimen collection from a bag or associated segment(s). Further, potentially laborious processing and labelling can introduce artifacts. For example, centrifugation to pellet RBCs may promote hemolysis, yield poor sensitivity, or involve significant time and expense (for instance, metabolic assessments).^{13,14} Also, changes in RBCs during storage, as assessed by testing cells from segments attached to a unit, do not necessarily correlate, at least quantitatively, with changes that occur inside the unit bag.^{1,2} Thus,

noninvasive and sensitive techniques are needed to assess RBCs during storage.

Raman spectroscopy is a label-free, noninvasive spectroscopic method based on the principle of inelastic scattering of monochromatic radiation.¹⁵ Any given band in a Raman spectrum corresponds to a vibrational frequency that is sensitive to the structure, conformation, and environment of the molecule. Raman spectroscopy is uniquely able to provide diagnostic information regarding a variety of biomolecular and biomedical processes.^{16,17} A resonance enhancement to the Raman effect can occur when the photon energy of the laser excitation wavelength is closely matched to the energy of a molecular electronic transition.¹⁵ In these cases, vibrations associated with ligands at the chromophoric active site can be enhanced selectively, with resonance contributions to the Raman intensity as high as 10^5 to 10^6 .¹⁸ Heme proteins, which were some of the earliest experimental examples used to illustrate the resonance Raman effect, have been extensively studied.¹⁹

Over the years, Raman has established its applicability for biomedical research applications, including the study of RBCs.^{18,20,21} For example, Raman has been used for screening single erythrocytes for malaria infection, discerning changes in Hb ligation, and oxidation and conformation state in RBCs from patients with sickle-cell disease and diabetes.^{19,22,23} Recently, storage time-dependent changes in Hb oxygenation were shown to track with morphological changes in the RBC structure and lactate accumulation.^{24–26} In the work reported herein, we studied whether the resonance Raman spectra of Hb-associated vibrational modes in stored RBCs could be used to evaluate storage-dependent hemolysis. Hemolysis is a parameter associated with RBC quality and safety. To date, this process requires specimen handling and centrifugation of cells, which could introduce artefacts, including increased hemolysis.

Materials and Methods

Human-Stored RBC Collection and Processing

Three segments of leukoreduced RBCs in Adsol-1 (attached to individual RBC units, from 6 distinct donors) were collected at a storage age of 7 days from the University of Alabama at Birmingham (UAB) blood bank. All protocols were approved by the UAB Institutional Review Board (IRB). Also, paired

segments were stored until 21 days or 35 days at 4°C in the dark. At each time point, RBCs were collected (400–500 μ L into a 2-mL Eppendorf tube) and centrifuged at 1500 *g* for 10 minutes at 4°C; hemolysis was assessed in the supernatants. Pelleted RBCs were then washed twice and suspended in 1 mL of phosphate buffer saline (PBS; pH, 7.4) to attain a final concentration of approximately 10^7 to 10^8 cells per mL, or hematocrit of 0.1% to 1.0%. This suspension was placed into an aluminum cell chamber with a glass coverslip base and into the specimen chamber of the Raman spectrometer for spectral acquisition. For clarity, data from these measurements are referred to as being from washed RBCs. In addition, 10 drops (each 20 μ L) of stored RBCs (60% hematocrit [Hct]) were collected directly from the segment without washing and casted on aluminum foil sequentially, also for spectral acquisition. For clarity, these measurements are referred to as being from RBCs (60% Hct).

Hemolysis Measurement

Stored RBC supernatants (10–50 μ L) from 7-day or 35-day units were diluted into PBS, and visible absorbance spectra were acquired between 450 nm and 700 nm on a UV-Vis spectrophotometer (Beckman-Coulter Inc) at 1-nm intervals. Concentrations of oxyhemoglobin (oxyHb), methemoglobin (metHb), and cell-free heme (CFH) were determined by spectral deconvolution, as recently described,²⁷ using standard spectra for deoxyhemoglobin (deoxyHb), oxyHb, metHb, and CFH prepared in PBS at pH 7.4 and 6.8. The pH of RBCs stored for 7 days or 35 days are 7.4 and 6.8, respectively. Therefore, standard spectra collected at pH 7.4 and 6.8 were used for deconvolution analyses.

CFH Preparation

Whole blood was collected by venipuncture from healthy volunteers per UAB IRB-approved protocols. CFH was purified from RBCs and the catalase was removed, as previously described.²⁸ All Hb was stored at –80°C in the carbon-monoxide ligated form and was converted to oxyHb immediately before use. MetHb was synthesized using potassium ferricyanide.²⁸ Cell-free oxyHb and metHb were measured by Raman spectroscopy to confirm assignments of specific Raman peaks from spectra of stored RBCs.

Raman Spectroscopy

Raman spectra were recorded using a confocal InVia Raman microspectrometer (Renishaw plc). A 633-nm helium-neon (He:Ne) laser was used as the excitation source.

The system was calibrated to the 520.5-cm⁻¹ line of Si using an internal Si reference before acquiring the spectra from the specimen.

For stored RBCs (60% Hct), the excitation laser beam (~0.5 mW) was focused onto the drop of blood from the specimen using a 50x objective (long working distance, numerical aperture NA = 0.5). Spectra (1800–200 cm⁻¹) were acquired for 3s; a total of 5 scans were accumulated and co-added for each measurement. Three spectra were recorded, each from different spatial positions on each drop of blood taken from each specimen, and averaged. A total of 10 drops were measured, resulting in 10 spectra that were averaged to obtain a single spectrum per donor.

For washed RBC measurements, spectra were recorded at a laser power of approximately 2 mW using a 60x water-immersion objective (NA = 1.00) in confocal mode. Spectra were acquired for 5s; a total of 3 scans were collected and co-added for each measurement. For any individual washed RBC, spectra were recorded from 3 different positions and averaged to yield a representative spectrum from a single cell. Forty individual cells were analyzed per donor, per storage time.

The different settings for spectrum acquisition for RBCs (60% Hct) vs washed RBCs, outlined earlier herein, were optimized in pilot studies to ensure adequate signal-to-noise ratio while minimizing background fluorescence and photodamage. Specifically, the relatively high concentration of Hb in RBCs (60% Hct) in the observed focal plane can lead to high background fluorescence signals, and high absorption can cause photodamage. To limit this occurrence, we used a low laser power setting (~0.5 mW) and a low NA objective (50x long working distance). For preparation of washed single RBC measurements, we used confocal mode to collect spectra with a z-axis resolution of approximately 2 micrometers (the thickness of each RBC is ~2.0–2.5 micrometers). This approach limits additional background signals from PBS and the coverslip. The confocal mode is more effective with a high NA objective; therefore, we used a 60x water immersion objective to collect spectra of single RBCs.

Also, because focal volume is smaller with confocal mode, a higher power setting (~2 mW) was used to increase the signal-to-noise ratio. The range of power used (0.5–2.0 mW) is similar to that used in previous studies and did not induce any observable changes in cell morphology, which indicates

that no photodamage occurred. The different settings for spectrum acquisition for RBC (60% Hct) vs washed single RBC precludes direct comparison of the intensities from Raman spectra of each preparation. Data are plotted to demonstrate relative changes in Raman intensities as a function of storage.

Raman Data Analysis and Processing

We accomplished spectral acquisition and accumulation using Renishaw Windows-based Raman Environment (WiRE) software, version 4.2 (Renishaw plc). Spurious data spikes originating from cosmic rays were removed using the WiRE software. All the spectra at a time point from a donor were averaged to calculate a mean spectrum using Origin 2016 data analysis and graphing software (OriginLab Corporation). The mean spectra were baseline corrected, vector normalized, and analyzed using numerous band-intensities ratios.

Statistical Analysis

We performed statistical analyses using GraphPad Prism (GraphPad Software). Storage time-dependent changes were analyzed by 1-way repeated measures analysis of variance (RM-ANOVA) with Tukey post-test. We compared and analyzed the differences between washed and unwashed specimens using 2-way RM-ANOVA with Sidak multiple comparison post-testing. For testing of correlations, data normality was first determined by the D'Agostino and Pearson omnibus normality test and then by Pearson or Spearman correlations performed accordingly. *Replicates* refer to averaged single spectrum per RBC donor.

Results

Raman Spectra of Stored RBCs

RBCs (60% Hct) were collected from paired segments stored for 7 days, 21 days, and 35 days. Raman spectra were acquired from drops of RBCs to collect spectra that were representative of the entire specimen, including intact RBCs and hemolyzed extracellular components. **Figure 1A** shows representative Raman spectra of RBC (60% Hct) drops at each storage age. Numerous bands are observed in these spectra which, based on precedents in the literature, can be assigned

Figure 1

Representative Raman spectra of stored red blood cells (RBCs) (60% hematocrit [Hct]) and purified hemoglobin (Hb). **A**, Raman spectra RBC (60% Hct): representative averaged spectra from RBC, from a single donor, stored for 7 days, 21 days, or 35 days. **B**, Raman spectra of cell-free ferrous oxygenated Hb (oxyHb) or ferric methemoglobin (metHb) in phosphate-buffered saline (PBS), pH 7.4. Inset represents zoomed portion of spectrum. Dashed lines indicate selective wavenumbers at which clear differences were observed between oxyHb and metHb. **C**, Raman spectra of washed single RBCs at different storage times. Bands indicated by dotted line are spectral features characteristic for oxyHb or metHb.

to distinct vibrational modes of Hb (Table 1).^{19,22,23,26,29,30} We noted differences in the spectra of RBCs at different storage ages. To interpret these spectral changes, we measured the Raman spectra of cell-free human ferrous oxyHb and ferric metHb (Figure 1B). The dashed lines in the figure indicate selected wavenumbers where clear differences were observed between oxyHb and metHb. Previous studies^{19,30} have shown that these particular Raman wavenumbers directly correlate with the oxygenation state of Hb.

The intensities of the Raman vibrations corresponding to oxyHb or metHb are plotted in Figure 2. Figures 2A–2D plot the intensities of bands associated with oxyHb. These occur at 569 cm^{-1} (Figure 2A), 1225 cm^{-1} (Figure 2B), 1565 cm^{-1}

(Figure 2C), and 1638 cm^{-1} (Figure 2D). In this Figure and subsequent Figures, the time-dependent intensities of these bands were normalized to the intensity of the 1366 cm^{-1} peak because the Raman intensity at this wavenumber is independent of differences between oxyHb or metHb using 633-nm excitation.³⁰ Each of the diagnostic oxyHb Raman bands were significantly increased in intensity from RBCs stored for 21 days compared with 7 days and remained at this level at 35 days, except for the 1225 cm^{-1} peak.

Figures 2E and 2F illustrate the intensity changes that occur as a function of storage time in the Raman bands mainly associated with metHb. These metHb-associated vibrational modes have been identified at 506 cm^{-1} (Figure 2E), 1214 cm^{-1} (Figure 2F), 1516 cm^{-1} (Figure 2G), and

Table 1. Observed Bands and Their Assignments Using Previously Published Literature

Raman Shift (cm ⁻¹)	Spectral Assignments
1651	Amide I proteins ^{31,34}
1638	$\nu(\text{C}=\text{C})$ asymmetric stretch (ν_{10}) ^{26,30}
1620	$\nu(\text{C}=\text{C})$ of vinyl groups ^{30,43}
1604–1610	$\nu(\text{C}=\text{C})$ asymmetric stretch (ν_{19}) ^{29,30,43}
1584	$\nu(\text{C}=\text{C})$ asymmetric stretch (ν_{37}) ^{22,30}
1565	$\nu(\text{C}=\text{C})$ stretch (ν_2) ^{30,45,47}
1544	$\nu(\text{C}=\text{C})$ stretch (ν_{11}) ^{29,30}
1516	$\nu(\text{C}=\text{C})$ stretch (ν_{38}) ^{29,30,43}
1447	$\delta(\text{CH}_2/\text{CH}_3)$ ^{34,46}
1427	$\nu(\text{C}=\text{C})$ symmetric stretch (ν_{28}) ^{30,31}
1395	N(pyrrole quarter-ring) stretch (ν_{20}) ^{30,31}
1357	N(pyrrole half-ring) symmetric stretch (ν_4) ^{29,30}
1336	N(pyrrole half-ring) symmetric stretch (ν_{41}) ^{29,30}
1305	$\delta(\text{C}=\text{H})$ asymmetric in plane deformation (ν_{21}) ^{30,31}
1225	$\delta(\text{C}=\text{H})$ in plane deformation (ν_{13}) ^{26,30}
1214	$\delta(\text{C}=\text{H})$ in plane deformation (ν_{23}) ^{26,30}
1172	$\nu(\text{pyrrole half-ring})$ asymmetric stretch (ν_{30}) ^{30,31}
1132	C-C stretch (trans) lipids ^{33,34}
1115–1125	$\nu(\text{pyrrole half-ring})$ asymmetric stretch (ν_{22}) ^{29,30}
1077–1091	$\nu(\text{C}=\text{C})$ asymmetric stretch (ν_{23}), C-C stretch (gauche) lipids, PO ₂ -symmetric stretch ^{33,34}
1064	C-C stretch (trans) lipids ^{33,34}
997	$\nu(\text{C}=\text{C})$ asymmetric stretch (ν_{45}) and phenylalanine ^{29,34}
977	$\delta(\text{pyrrole deformation})$ asymmetric in plane deformation (ν_{46}) and/or $\gamma(\text{C}=\text{H}_2)$ symmetric out-of-plane deformation ^{29,30}
961	C-C stretch in unordered protein ^{34,44}
830	$\gamma(\text{C}=\text{H})$ out of plane deformation (γ_{10}), tyrosine ^{34,44}
850	Tyrosine ^{34,44}
792	$\nu(\text{pyrrole breathing})$ stretch (ν_6) ^{30,46}
755	$\nu(\text{pyrrole breathing})$ stretch (ν_{15}) ^{29,46}
721	C-N stretch lipids ^{33,34}
676	$\delta(\text{pyrrole deformation})$ symmetric in plane deformation (ν_7) ^{30,46}
569	Fe-O ₂ stretch ⁴⁶
506	$\gamma(\text{pyrrole swiveling})$ out of plane deformation (γ_{12}) ²⁹

1610 cm⁻¹ (Figure 2H) (Table 1). The intensity of each of these Raman bands also increased significantly in RBCs stored for 21 days compared with 7 days. Also, the bands at 1214 cm⁻¹ and 1516 cm⁻¹ show additional significant intensity increases at 35 days compared with 21 days.

Raman Spectra of Stored RBCs after Washing

Stored RBCs were washed to remove extracellular, hemolyzed components. Raman spectra of individual washed RBCs were obtained using high-resolution confocal microscopy with a 60x water-immersion objective, so that the spectra reflected only the intracellular environment. We

analyzed the resultant spectra similarly to the stored RBCs (60% Hct) drops, as described earlier herein. Figure 1C shows the Raman spectra of washed RBCs, and Figure 3 plots the Raman peak intensities at the characteristic wavenumbers of oxyHb and metHb. Figures 3A–3D show that intensities at wavenumbers indicative of oxyHb increased at 21 days relative to 7 days. The intensities of the 1225 cm⁻¹ (Figure 3B) and 1565 cm⁻¹ (Figure 3C) peaks also showed significant increments when 35-day spectra were compared with 7-day spectra. For metHb, the 1516 cm⁻¹ mode (Figure 3G) showed no statistically significant intensity differences with storage age. However, Figures 3E, 3F and 3H show statistically significant intensity increments at 21 days relative to 7 days for washed RBC metHb vibrations at 506 cm⁻¹ (Figure 3E), 1214 cm⁻¹ (Figure 3F), and 1610 cm⁻¹ (Figure 3H). For all these modes, no further significant intensity increase was observed at 35 days relative to 21 days.

Raman spectra also report on other, nonheme-derived, biochemical, and molecular features of Hb and the RBC. Figures 4A and 4B show that the intensity at 850 cm⁻¹ relative to 830 cm⁻¹ increases significantly in stored RBCs (60% Hct; part A) and washed RBCs (part B) over time in storage. This intensity-band ratio is a sensitive marker for the state of tyrosine in Hb; an increase in this ratio indicates greater exposure of tyrosine residues to solvent in R-state Hb.^{31,32} Further, intensity at 961 cm⁻¹ (Figure 4C), assigned to a C-C stretching mode Figure 4D, also increased. This band shows significant changes at 21 days compared with 7 days in stored RBCs (60% Hct) (Figure 4C), but not in washed RBCs (Figure 4D).

Raman band ratios also can be used to characterize the C-C stretching vibrations in all-trans hydrocarbon chains. In particular, the skeletal optical modes in the 1000 to 1150 cm⁻¹ range have been extensively studied to characterize acyl chain conformations in lipid membrane model systems, including RBCs.^{33,34} Figure 4E uses the 1064 cm⁻¹ to 721 cm⁻¹ ratio as an indicator of membrane fluidity. The 721 cm⁻¹ Raman band is attributed to the C-N stretching vibration in lipid head groups and is used as an internal standard because it is insensitive to conformational changes. An increase in the 1064 cm⁻¹ to 721 cm⁻¹ ratio is indicative of a more rigid membrane with increased number of trans isomers in the acyl chains. Figures 4G and 4H show that the associated trans C-C marker ratio at 1132 cm⁻¹ (Figure 4G) to 721 cm⁻¹ also increases with storage age, acting jointly with the 1064 cm⁻¹ (Figure 4H) band.

Figure 2

Relative intensity changes of stored red blood cell (RBC) solutions (60% hematocrit [Hct]) at storage day 7, day 21, and day 35; Raman intensity of oxygenated hemoglobin (oxyHb) and methemoglobin (methHb) bands in stored RBC (60% Hct). Each data point represents a value from averaged spectra from single stored RBC (60% Hct) preparation from a single donor. Indicated *P* values were determined by 1-way repeated measures analysis of variance (RM-ANOVA) with Tukey post-test at levels of *P* < 0.05 (indicated by *), *P* < 0.01 (**), or *P* < 0.001 (***). **A**, I_{569}/I_{1366} ; oxyHb. **B**, I_{1225}/I_{1366} ; oxyHb. **C**, I_{1565}/I_{1366} ; oxyHb. **D**, I_{1638}/I_{1366} ; oxyHb. **E**, I_{506}/I_{1366} ; methHb. **F**, I_{1214}/I_{1366} ; methHb. **G**, I_{1516}/I_{1366} ; methHb. **H**, I_{1610}/I_{1366} ; methHb. These are the relative intensities as measured by Raman.

However, this region is also influenced by some contribution from the Hb vibrational modes.

Comparison of Stored RBC (60% Hct) Suspensions with Washed Stored RBCs

Figure 5 shows a paired analysis of the intensity at selected wavenumbers, after calculating the fold change in intensity relative to 7-day spectra. Fold changes were calculated by taking the ratio of the 21-day or 35-day intensities over the original 7-day intensity value. Data are plotted for stored RBC (60% Hct) drops and washed RBCs. Significance between stored RBCs (60% Hct) before and after washing is shown in **Figure 5**. Three of the 4 Raman bands attributed to oxyHb showed significant fold increases of approximately 20% at 21 days relative to 7 days (**Figures 5A–5D**) for stored RBCs (60% Hct), compared with washed RBCs. Differences in the intensity changes between stored RBCs (60% Hct) vs stored RBCs after washing were more pronounced in the Raman peaks associated with methHb (**Figures 5E–5H**), with fold increases observed of approximately 80%. Also, compared with the oxyHb peaks, the methHb bands continued to

exhibit statistically significant increases in intensity at 35 days in stored RBC suspensions (60% Hct) relative to washed stored RBCs.

Figures 5I–5L show fold changes in intensity of bands at 850 cm^{-1} (**Figure 5I**), 961 cm^{-1} (**Figure 5J**), 1064 cm^{-1} (**Figure 5K**), and 1132 cm^{-1} (**Figure 5L**). We observed no differences in 850 cm^{-1} and 1132 cm^{-1} bands between stored RBC solutions (60% Hct) and washed RBCs, whereas increases in 961 cm^{-1} and 1064 cm^{-1} of between 2- and 4-fold were evident at 21 days and 35 days in nonwashed compared with washed RBCs.

Storage-Dependent RBC Hemolysis

We performed biochemical measurements of the extent of hemolysis in the segments attached to each unit as a function of storage age, in parallel with the Raman spectroscopic measurements described earlier herein. Total heme increased from mean (SD) $53.6\text{ (}3.1\text{)}\text{ }\mu\text{M}$ at 7 days to $77.4\text{ (}3.7\text{)}\text{ }\mu\text{M}$ at 35 days ($n = 6$), with oxyHb comprising the majority (70%–80%) of the extracellular heme.

Figure 3

Relative intensity changes of washed red blood cells (RBCs) at storage day 7, day 21, and day 35; Raman intensity of oxygenated hemoglobin (oxyHb) and methemoglobin (methHb) single RBCs after washing to remove extracellular components. Bands from Panels A–D show relative intensity of characteristic oxyHb Raman bands. Panels E–H show relative intensity of characteristic methHb Raman bands. Each data point represents a value from averaged spectra from a washed RBC preparation from a single donor. Indicated *P* values were determined by 1-way repeated measures analysis of variance (RM-ANOVA) with Tukey post-test at levels of *P* < .05 (*), *P* < .01 (* *), or *P* < .001 (***). **A**, I_{569}/I_{1366} ; oxyHb. **B**, I_{1225}/I_{1366} ; oxyHb. **C**, I_{1565}/I_{1366} ; oxyHb. **D**, I_{1638}/I_{1366} ; oxyHb. **E**, I_{506}/I_{1366} ; methHb. **F**, I_{1214}/I_{1366} ; methHb. **G**, I_{1516}/I_{1366} ; methHb. **H**, I_{1610}/I_{1366} ; methHb.

We investigated whether the intensity changes observed in the Raman spectra with storage time (Figures 2–4) correlated with hemolysis. Figure 6 shows the correlations between individual Raman intensity ratios of stored RBC suspensions (60% Hct) and total heme concentration in the stored RBC supernatants. Figures 6A–6D plot the intensity correlations from bands corresponding to oxyHb, and Figures 6E–6H plot the correlations from methHb bands. Where significant, the solid lines show linear regressions, and the dotted lines indicate the 95% confidence bands in the data. Correlation coefficients and *P* values for each of the intensity ratios are indicated on the individual panels.

The data for oxyHb bands (Figures 6A–6D) show correlations between the Raman intensity ratios and total heme for several bands, including the Fe–O₂ vibration at 569 cm⁻¹ (Figure 6A) and the C_αC_m stretch at 1638 cm⁻¹ (Figure 6B), both of which are indicators of oxygenation in RBCs. However, 2 other Raman marker bands for oxyHb, namely, 1225 cm⁻¹ (Figure 6C) and 1565 cm⁻¹ (Figure 6D), did not show significant correlations with supernatant total heme concentration, although closeness to significance is noted. This finding

contrasted with the characteristic methHb bands at 506 cm⁻¹ (Figure 6E), 1214 cm⁻¹ (Figure 6F), 1516 cm⁻¹ (Figure 6G), and 1610 cm⁻¹ (Figure 6H), which all showed a positive association, with correlation coefficients being greater compared with oxyHb bands. Similarly, we observed positive, significant correlations with the 850 cm⁻¹ (Figure 6I), 961 cm⁻¹ (Figure 6J), and 1064 cm⁻¹ (Figure 6K) bands, consistent with increased membrane rigidity (Figure 6L).

The data presented in Figure 6 were reanalyzed to reflect the amount of change (ie, fold changes) on the abscissa and ordinate axes, as opposed to the original data.

Figure 7 shows the correlations between fold changes in Raman peak intensities vs the fold changes in hemolysis (total heme) relative to 7-day RBCs. When plotted in this manner, significant correlations at statistically significant levels (all *P* < .05) were observed for all Raman intensity ratios studied, with the exception of the 850 cm⁻¹ to 830 cm⁻¹ ratio.

Figure 4

Relative intensity changes of stored red blood cell (RBC) solutions (60% hematocrit [Hct]) before and after washing at 850 cm^{-1} , 961 cm^{-1} , 1064 cm^{-1} , and 1132 cm^{-1} . Panels **A**, **C**, **E**, and **G** show relative intensity of selected bands in RBC suspensions (60% Hct). Panels **B**, **D**, **F**, and **H** show relative intensity of selected bands in RBCs after washing. Indicated *P* values were determined by 1-way repeated measures analysis of variance (RM-ANOVA) with Tukey post-test at levels *P* < .05 (*) or *P* < .01 (**). **A**, I_{850}/I_{830} ; in suspensions. **B**, I_{850}/I_{830} ; after washing. **C**, I_{961}/I_{1366} ; in suspensions. **D**, I_{961}/I_{1366} ; after washing. **E**, I_{1064}/I_{721} ; in suspensions. **F**, I_{1064}/I_{721} ; after washing. **G**, I_{1132}/I_{721} ; in suspensions. **H**, I_{1132}/I_{721} ; after washing.

Figure 5

Comparison of Raman spectral changes between red blood cells (RBCs) (60% hematocrit [Hct]) and washed RBCs; changes in Raman intensity of oxygenated hemoglobin (oxyHb), methemoglobin (metHb), and membrane fluidity bands during storage. Intensity at bands for oxyHb (panels A–D), metHb (panels E–H), and membrane fluidity (panels I–L) at 21 days and 35 days were normalized to day-7 values and plotted as fold changes. Data shown are mean (SD) ($n = 6$). Indicated P values compare washed versus unwashed RBCs (60% Hct) and were determined by 2-way repeated measures analysis of variance (RM-ANOVA) with Sidak multiple comparison post-test at levels $P < .05$ (*), $P < .01$ (**). ■ indicates RBCs (60% Hct); ●, washed RBCs. A, I_{1225}/I_{1366} ; for oxyHb. B, I_{1565}/I_{1366} ; for oxyHb. C, I_{1368}/I_{1366} ; for oxyHb. D, I_{506}/I_{1366} ; for oxyHb. E, I_{1214}/I_{1366} ; for metHb. F, I_{1516}/I_{1366} ; for metHb. G, I_{1610}/I_{1366} ; for metHb. H, I_{850}/I_{830} ; for metHb. I, I_{961}/I_{1366} ; for membrane fluidity. J, I_{1064}/I_{721} ; for membrane fluidity. K, I_{1132}/I_{721} ; for membrane fluidity. L, I_{569}/I_{1366} ; for oxyHb.

Discussion

Transfusion of RBCs may be a lifesaving component of blood-based therapeutics in modern medicine. Currently, RBCs are stored at 4°C in solutions that have been designed to allow a shelf life of 42 days. The United States Food and Drug Administration (FDA) criteria for blood storage are hemolysis below 1% and demonstration that greater than 75% of RBCs transfused are recovered within 24 hours of transfusion. Several metabolic changes occur during cold storage of RBCs that lead to lactate accumulation, as well as loss of endogenous antioxidants and membrane flexibility, 2,3-diphosphoglycerate (2,3-DPG), and adenosine triphosphate (ATP).³⁵ All of these changes lead to increased oxygen affinity, cell fragility, and hemolysis, collectively referred to as the *storage lesion*.

This information has been understood for many years. However, recent interest in this area has stemmed from the potential for adverse effects associated with transfusion of stored RBCs, especially with products of hemolysis, which exacerbate inflammation, oxidative stress, microcirculatory dysfunction, and infection in critically ill and severely injured patients who have undergone trauma.^{3,9,10,36–40} Increased understanding of the potential implications of the storage lesion further underscores the need to assess hemolysis before transfusion.

Current methods to assess hemolysis are relatively straightforward, requiring collection of RBCs from the bag or attached segments, the latter approach minimizing potential loss of sterility. However, hemolysis assessment in paired bag-segment specimens has shown that free Hb is higher and free heme is lower in segments compared with bags.^{1,2}

Figure 6

Association between hemolysis and Raman peak intensities from stored red blood cell (RBC) solutions (60% hematocrit [Hct]). Raman intensities from peaks corresponding to oxygenated hemoglobin (oxyHb) (Panels **A–D**), methemoglobin (metHb) (Panels **E–H**), and membrane fluidity (Panels **I–L**) were plotted against total heme concentrations in supernatants. Lines show fitting by linear regression with 95% confidence bands. Correlation coefficients (Pearson) and *P* values are indicated on each panel. **A**, I_{1125}/I_{1366} ; oxyHb. **B**, I_{1565}/I_{1366} ; oxyHb. **C**, I_{1638}/I_{1366} ; oxyHb. **D**, I_{506}/I_{1366} ; oxyHb. **E**, I_{1214}/I_{1366} ; metHb. **F**, I_{1516}/I_{1366} ; metHb. **G**, I_{1610}/I_{1366} ; metHb. **H**, I_{961}/I_{1366} ; metHb. **I**, I_{1064}/I_{721} ; membrane fluidity. **J**, I_{1132}/I_{721} ; membrane fluidity. **K**, I_{569}/I_{1366} ; membrane fluidity. **L**, I_{1225}/I_{1366} ; membrane fluidity.

Thus, segment specimen collection may not accurately reflect the degree of hemolysis in the actual product being transfused into the patient, although the total heme equivalents (free Hb + free heme) are similar.^{1,2} Moreover, current assessment of hemolysis requires centrifugation of the RBC specimen from either source to pellet the cells and to allow measurement of the ratio of Hb to heme in the supernatant. Differences in centrifugation parameters (eg, *g* force, time, temperature, and volume of specimen) are known to affect the degree of hemolysis, partly due to the increased susceptibility of stored RBCs to mechanical disruption.^{41,42} Thus, noninvasive methods that do not require bag or segment specimen collection, and thus could accurately determine the degree of hemolysis, are highly desirable.

For decades, Raman spectroscopy has been applied to the study of Hb and its derivatives and, increasingly, to RBCs as well.^{19,22–26,39} Studies of this type have been performed predominately on model compounds or ex-vivo specimens. The work reported herein is the first demonstration, to our

knowledge, of the use of noninvasive resonance Raman spectroscopy using 633-nm excitation to identify detailed oxyHb and metHb diagnostic markers under clinically relevant RBC storage conditions. Potentially, the results of this research can not only form the basis of a detailed mechanistic understanding of in-bag RBC hemolysis but also could provide an opportunity to develop a clinical tool for noninvasive analysis of time-dependent in-situ storage lesions of RBCs in blood-bag units.

Our data indicate that as a function of storage age, changes in the intensity of bands at specific wavenumbers indicative of increased oxyHb were observed. For example, there was an increase in band intensities at 1565 cm^{-1} (ν_2) due to low spin Fe and at 1638 cm^{-1} (ν_{10}) due to planar porphyrin ring. Because oxygen was not limited in these studies and all measurements were performed in air-saturated buffers, these data suggest an increase in the intrinsic oxygen affinity of Hb, or R-state Hb. This conclusion confirms recently

Figure 7

Association between fold change in hemolysis and in Raman peak intensities. Fold changes (relative to day 7) in Raman intensities from peaks corresponding to oxygenated hemoglobin (oxyHb) (panels **A–D**), methemoglobin (metHb) (Panels **E–H**), and membrane fluidity (Panels **I–L**) were plotted against fold change (relative to day 7) in total heme concentrations in supernatants. Lines show fitting by linear regression with 95% confidence intervals. Correlation coefficients (Pearson for panels **A, B, F, G, I–L** and Spearman for panels **C, D, E, H**) and *P* values are indicated on each panel. **A**, I_{1565}/I_{1366} ; oxyHb. **B**, I_{1638}/I_{1366} ; oxyHb. **C**, I_{1214}/I_{1366} ; oxyHb. **D**, I_{1516}/I_{1366} ; oxyHb. **E**, I_{1516}/I_{1366} ; metHb. **F**, I_{1610}/I_{1366} ; metHb. **G**, I_{850}/I_{830} ; protein conformation. **H**, I_{961}/I_{1366} ; protein conformation. **I**, I_{961}/I_{1366} ; protein conformation. **J**, I_{1064}/I_{721} ; membrane fluidity. **K**, I_{1132}/I_{721} ; membrane fluidity. **L**, I_{1132}/I_{721} ; membrane fluidity.

published data²⁶ and also is consistent with loss of 2,3-diphosphoglyceric acid (2,3 DPG) during storage.

In addition, we observed an increase in the relative intensities of bands indicative of metHb.^{29,30,43} These changes were supported by comparison to cell-free metHb standards. We note that deoxyHb, also a high spin state Hb that could lead to misinterpretation of spectra, was minimized by using air-saturated buffers. The finding that metHb increases in a storage dependent manner likely reflects increased oxidative stress and/or loss of reducing equivalents and metHb reductase activity.

It should be mentioned that visible absorbance spectroscopy, typically used to measure metHb, indicates that metHb is a relatively minor Hb oxidation state compared with oxyHb (ferrous Hb). The relative intensity analysis presented herein is qualitative and/or semiquantitative in

nature; nevertheless, the differences are more sensitive compared with absorption. Also, the ability to distinguish between oxyHb and metHb in RBCs provides a unique, direct spectroscopic approach to assess how oxidation of Hb changes during cold storage. Analysis of the storage-dependent spectra indicates that oxygen affinity increases relatively early, with maximum changes observed by 21 days, whereas metHb continues to accumulate throughout storage.

We also noted that the variance in intensities increased as a function of storage time. For example, in **Figure 3**, the mean (SEM) (*n* = 6) coefficients of variance more than doubled from day 7 to day 35, from 4.7 (0.5) to 12.7 (3.0). Similar increases in variance as a function of storage are observed with other storage lesion end points, including hemolysis or nitric-oxide scavenging kinetics.³⁹ This finding most likely reflects variance associated with differences in the susceptibility of RBCs from

different donors to storage lesion-dependent damage; it suggests that Raman spectra can discern between RBCs that are more or less likely to undergo storage-dependent changes.

We compared the spectra of stored RBCs with or without washing, reasoning that comparison between the 2 may yield qualitative insights into intracellular Hb vs Hb released after hemolysis. The absolute Raman intensities at the same wavenumber of washed vs unwashed RBCs (60% Hct) cannot be compared because of different specimen-collection conditions, settings for spectrum acquisition (eg, laser power), and objectives used. However, storage-dependent trends were similar for oxyHb with either RBC preparation, suggesting that changes in oxygen affinity reflect the intracellular environment. Despite this finding, we observed no significant changes in methHb when RBCs were washed. Because washing removes hemolyzed components, spectra from washed RBC preparations indicate changes that occur exclusively inside or associated with the RBC. This finding suggests that changes in Raman wavenumbers indicative of methHb represent hemolysis in the supernatant.

Data collected by visible spectroscopy showed that average methHb levels increased during storage (0.42 to 4.6 μM , with median levels at day 7 and day 35, respectively). However, the magnitude of these changes in methHb is relatively low compared with oxyHb (38.3 to 63.8 μM , with median levels at day 7 and day 35, respectively) (data not shown). However, our Raman data clearly show continued increases in methHb during storage. This finding underscores the potential for Raman spectroscopy to detect changes in RBC during storage that may not be the highest in magnitude but could still be sensitive indicators of the storage lesion and have clinical implications. Taken altogether, we conclude that assessing the intensities of wavenumbers representing oxyHb in stored RBCs (60% Hct) provides insights into the environment inside the cells, whereas the methHb wavenumbers provide information on hemolysis.

Raman spectral changes also indicated structural changes in globin and the RBC that influence the solubility and oxidative potential of Hb, as well as membrane fluidity. In particular, the skeletal vibrational modes in the 1000 to 1150 cm^{-1} range have been studied extensively to characterize acyl chain conformations in lipid membrane model systems, including RBCs.^{33,34} Increases in intensities of bands at 1064 and 1132 cm^{-1} , assigned to the C-C stretch of all-trans configuration in lipids, relative to the 721 cm^{-1} band of lipids, is consistent with formation of more rigid RBCs during storage. The latter could be postulated to be due to more Hb being bound

to the membrane secondary to oxidative denaturation of the protein. Moreover, increases in 850 cm^{-1} relative to 830 cm^{-1} may reflect structural changes in the globin polypeptide chain of globin with storage time. Also, denaturing of the globin peptide chains was indicated by an increase in the band at 961 cm^{-1} , characteristic of unordered peptides.^{34,44}

Finally, we performed correlation analyses to determine whether the absolute or fold changes in Raman intensities correlated with hemolysis, as measured using more traditional absorbance-based approaches. Some general features of this analysis are intensities at all selected wavenumbers being positively correlated with hemolysis, either significantly or close to significantly; correlations being stronger when fold changes were compared; and correlations being stronger for changes indicative of methHb, denaturation (961 cm^{-1}), or membrane fluidity (1064 cm^{-1}), relative to oxyHb.

Coupled with the emergence of technologies allowing use of hand-held Raman spectroscopy-based devices and the ability to collect spectra through plastic,²⁴ our data suggest that following the storage lesion through Raman spectra testing has the potential to report accurately on the degree of hemolysis in real time. Once clinical studies have determined how hemolysis affects patient outcomes, this technology could be used in blood banks to decide which units are safe to be transfused into the bodies of patients. **LM**

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