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Resonance Raman and UV-Visible Microscopy Reveals that Conditioning Red Blood Cells with Repeated Doses of Sodium Dithionite Increases Haemoglobin Oxygen Uptake

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oxygenated (oxyHb)

deoxygenated Hb (deoxyHb)

resonance Raman Spectroscopy

UV-Vis spectrometry

microfluidics

laser tweezer

Here we report that successive additions of fresh dithionite to a suspension of red blood cells (RBCs) increase the capacity of the cells to uptake oxygen. This effect was not observed when the RBCs were similarly preconditioned using gaseous N_2 to induce short episodes of hypoxia. The effect of successive sodium dithionite and N_2 gas additions on a population of functional erythrocytes was monitored using Raman confocal microscopy, with 514^^nm excitation, and UV–visible microscopy. The results indicate that successive additions of sodium dithionite in a suspension of red blood cells leads to an increase in both the rate and the capacity of the RBCs to uptake oxygen. The sodium dithionite did not cause haemoglobin from lysed RBCs to uptake more oxygen after successive additions and hence this effect was only observed in functional intact RBCs. Experiments performed with polarised Raman spectroscopy suggest that sodium dithionite increases the disorder of Hb in the RBC facilitating oxygen diffusion.

Using a combination of laser tweezer Raman microscopy, UV/Vis microscopy and conventional Raman polarised microscopy we show that successive conditioning of erythrocytes with sodium dithionite increases the uptake and overall concentration of oxygen within the red blood cell. Experiments performed using polarised Raman spectroscopy suggest that sodium dithionite increases the disorder of hemoglobin in the RBC thus facilitating oxygen diffusion within the hemoglobin matrix.

Introduction

The mechanism of oxygen uptake and transport in the red blood cell is fundamental to respiration. It has been long known that haemoglobin in the red blood cell behaves differently to haemoglobin (Hb) in solution. Early studies demonstrated that sodium dithionite anions ($S_2O_4^{2<M->}$) could deoxygenate haemoglobin (Hb) [1]. Hartridge and Roughton demonstrated that the $Na_2S_2O_4$ did not react directly with the HbO_2, but simply removed the oxygen from the solution as fast as it was liberated, and that the direct and quantitative conversion of HbO_2 was a simple first-order dissociation.[1] Since then $S_2O_4^{2<M->}$ addition has become a commonly used procedure to deoxygenate Hb in red blood cell (RBCs)[2–7]. Besides $S_2O_4^{2<M->}$, another commonly used method to obtain deoxygenated RBCs is to bubble dry nitrogen (N_2) gas through a physiological solution containing RBCs[8–10]. An early study using UV-Visible spectroscopy provided evidence of side reactions occur when haemoglobin solutions were exposed to $Na_2S_2O_4$.[11] These experiments revealed that an unstable derivative of Hb is also formed under conditions which favour the formation of an oxidation product of $Na_2S_2O_4$, and is slowly reconverted, in part, into Hb in the presence of excess of $Na_2S_2O_4$. These reactions were shown to accompany the conversion of HbO_2 and methaemoglobin into Hb by $Na_2S_2O_4$ in dilute solution.[11]

Raman microspectroscopy has been widely used to probe the dynamics of intact RBCs[12]. This technique allows real time measurement of living RBCs under changing environmental conditions[13]. Raman spectra provide information about the composition of biological materials including lipids [14], proteins and carbohydrates [15,^16]. However, in the case of RBCs, if the excitation

laser energy is close to the Hb absorption bands in the visible range (i.^e. Q_v, Q_0 and Soret bands) and consequesntly the RBC Raman spectrum is dominated by the haem group contributions due to resonance Raman[17]. Near infrared[18] and visible excitation wavelengths have been used for studying conformational changes[8] of Hb, as well as ordering[17] and spin[19] alterations. RBCs can be also studied using Raman Spectroscopy coupled with microfluidic system [20,^21] or acoustic levitation [22] and, in the past years, new advances have been made in optical tweezers, which allow the manipulation of RBCs in an optical field[23]. Here we apply Raman spectroscopy coupled with optical tweezers in combination UV/visible microscopy to demonstrate that oxygen uptake in RBCs increases after successive additions of $S_2O_4^{2<M->}$ when compared to analogous experiments performed by the addition of N_2.

Results And Discussion

In the first experiment we used a microfluidic device (Figure^^1<figr1>-Top) and laser tweezers to move red blood cells from oxygenated to deoxygenated media. The Raman spectra of a single RBC after moving it from oxygenated to deoxygenated media, **(a)** $\rightarrow$ **(b)**, and then back into the oxygenated media, **(b)** $\rightarrow$ **(c)** is shown in the bottom panel of Figure^^1<xfigr1>. When moving the cell from the oxygenated to deoxygenated buffer there is a decrease in the ν_4 (Hb^{III}-$O_2^{<M->}$), ν_3 (Hb^{III}-$O_2^{<M->}$) and ν_{10} (Hb^{III}-$O_2^{<M->}$) bands at 1377, 1505 (weak) and 1640^^$cm^{<M->1}$. When moving the cell from oxygenated to deoxygenated buffer there is an increase in the ν_4 (Hb^{II}), ν_3 (Hb^{II}) and ν_{10} (Hb^{II}) bands at 1358, 1473 and 1607^^$cm^{<M->1}$, respectively (Table of band assignments is shown in SI4).

Similar changes are observed irrespective of whether the media has been deoxygenated by N_2 bubbling (left) or by the addition of $S_2O_4^{2<M->}$ (right) in **1^b**, however, when the cells are returned to the oxygenated media in **(1^c)**, the response of a single RBC is dependent on the method of deoxygenation. For example, the Raman spectrum of RBCs in the N_2-deoxygenated media (**c** left) is comparable to the initial spectrum in **(1^a)** due to the reversibility of the binding of O_2 to Hb. In contrast, spectra of RBCs from $S_2O_4^{2<M->}$-deoxygenated media (**1^c** right) have an intensity ratio for

the Hb^{III}-$O_2^{<M->}$/ Hb^{II} bands (ν_4) following re-oxygenation that exceeds the intensity ratio of the same bands for the RBC in the initial oxygenated media.

A similar but independent experiment was performed using classical confocal Raman microscopy. In order to compare the effects of deoxygenation with $S_2O_4^{2<M->}$ and N_2 gas treatment over time, Principal Component Analysis (PCA) was performed over the entire series of spectra for repeated cycles of oxygenation and deoxygenation. Figure^^2<figr2> shows the results of PCA for the first score as a function of the time, and the first loading vector. The first principal component (PC) explains more than 90^% of the variance of the dataset in all cases, and was due to the change of the oxidation state of the Hb in the RBCs. This change is supported by the loadings vectors (Panels **2^a** and **2^b**), which show similar changes to those in the spectra and include a positive band at 1640^^$cm^{<M->1}$ (ν_{10}) associated with oxyHb and a negative band at 1607^^$cm^{<M->1}$ (ν_{19}) characteristic of deoxyHb. Panel **2^c** shows the score values corresponding to 40 Raman spectra recorded from individual RBCs in saline solution and following 3 successive additions of $S_2O_4^{2<M->}$. The first dose of $S_2O_4^{2<M->}$ results in a final $S_2O_4^{2<M->}$ concentration of 0.5^^mM. The RBCs were allowed to equilibrate with the atmosphere before each of the second and the third dose of $S_2O_4^{2<M->}$ were added which resulted in 1.0 and 1.5^^mM final concentrations, respectively. Each addition of $S_2O_4^{2<M->}$ is marked by a green asterisk in Figure^^2<xfigr2>. At the start of the experiment the score value is highly positive indicating the majority of Hb is oxygenated. After the addition of the $S_2O_4^{2<M->}$, the oxyHb is quickly converted to deoxyHb, which gives rise to negative scores.

Following each addition of $S_2O_4^{2<M->}$, the oxygenated state of the RBC is gradually recovered (see Figure^^2**c**<xfigr2>), however, there is a clear difference observed in the reoxygenation kinetics in each successive deoxygenation step. After the 1st addition of $S_2O_4^{2<M->}$, it took approximately 5 minutes for the complete conversion of deoxyHb to oxyHb; the time decreased to 4 minutes for the 2nd addition of $S_2O_4^{2<M->}$ and 1.5 minutes for the 3rd addition.

The experiment was repeated using nitrogen gas to deoxygenate the cells as opposed to $S_2O_4^{2<M->}$. The scores plots for this experiment are shown in figure **2^d** and are markedly different compared to those from deoxygenating the cells with $S_2O_4^{2<M->}$ (Figure^^2**a**<xfigr2>). In this case, the

reoxygenation recovery rate is consistent in each successive addition of N_2 gas. This can be readily observed when one compares the full-width-half-maximum of the minima in the score values corresponding to deoxygenated states (c.f. Figures **2^c** and **2^d**). In Figure^^2**c**<xfigr2>, these minima peaks become narrower after each successive addition of $S_2O_4^{2<M->}$, whereas in Figure^^2**d**<xfigr2>, the width of the minima peaks remains relatively unchanged. This suggests some kind of modification is occurring to Hb within the RBC in response to the $S_2O_4^{2<M->}$, which increases the rate of oxygen uptake by Hb.

Complementary experiments were performed using UV-Vis micro-spectroscopy, which enabled minimal invasive monitoring of individual RBCs with improved time resolution. A dataset of spectra from single RBCs exposed to successive oxygenation and deoxygenation cycles was investigated with PCA. Results indicate that the variation in the oxidation state is explained by the 1st principal component, which captured more than 90^% of the explained variance using both $S_2O_4^{2<M->}$ and N_2 gas (see SI3). Figure^^3**a**<figr3> and **3^b** show the loading vectors of the first component for the $S_2O_4^{2<M->}$ and the N_2 experiments, respectively. Both vectors are almost identical, showing a sharp band at 435^^nm with a negative shoulder around 400^^nm indicative of the Soret band shifting from 420 to 430^^nm when oxyHb is converted to deoxyHb. Similarly, the two Q bands at 540 and 570^^nm of oxyHb have negative values whereas the Q band at 550^^nm of deoxyHb has a positive value. The direction of the bands shows that in this case negative and positive values of the first principal component are associated with oxyHb and deoxyHb, respectively.

Figure^^3**c**<xfigr3> shows scores plots after three additions of $S_2O_4^{2<M->}$ (0.5^^mM) over time. At the start, the individual RBC is in the oxyHb state, and after the addition of $S_2O_4^{2<M->}$, highlighted by the *, the oxyHb is quickly transformed to deoxyHb. Similar to the Raman kinetic experiments with $S_2O_4^{2<M->}$, the time it takes to return from deoxyHb to oxyHb decreases from 15 minutes after the 1st addition, to 10 minutes after the 2nd addition and 5 minutes after the 3rd addition. There are quantitative differences in the recovery times determined by Raman and UV-Vis, but this is because the measurements are made on cells from different volunteers; however, the trend in decreasing recovery time is apparent in both datasets. The scores plot depicted in

Figure^^3**d**<xfigr3> show that, once again, the recovery rate did not vary in successive deoxygenation-reoxygenation cycles when using saturated N_2 gas. Therefore the conclusions drawn from the resonance Raman spectroscopy results were confirmed by UV-Vis spectroscopy. Identical UV-Vis experiments were performed on isolated Hb solutions obtained from lysed RBCs (see section E of the SM). In this case, the Hb did not show any change in the kinetics after successive additions of $S_2O_4^{2<M->}$ and the results were comparable to successive deoxygenation cycles using N_2 gas.

Many models have been proposed to explain the diffusion and convection of Hb in the erythrocyte. Klug et al[24,^25]reported that an O_2 gradient can produce a driving force if a carrier like Hb is present that binds O_2 at the high O_2 concentration side and releases it on the low side. Zander and Schmid-Shöbein [26,^27]reported that intracellular convective oxygen transport induced by red blood cell deformation is of greater significance than oxygen diffusion and that its relative role increases with the duration of deoxygenation conditions. Vandergraph and Olsen [28,^29]reported several factors important in O_2 diffusion when developing erythrocyte models. These included cell size and intracellular Hb concentration, morphology, organic phosphate concentration shift, changes in intracellular pH, and the thickness of an **‘’**unstirred sublayer**’’** adjacent to the cell. Bouwer et^^al. [30,^31]determined diffusion co-efficients for a range of Hb concentrations through thin liquid layers. They surmised that Hb diffusion stops if Hb molecules are **‘**just touching**’** and O_2 diffusion stops if the molecules are **‘**closely packed**’.** Bouwer et^^al.[30,^31]calculated the corresponding concentration based on each scenario; namely, 43 and 106^^g/l, respectively. These value were remarkably similar to the reported experimental values of 46 and 100 [31] . Polarised Raman microscopy experiments by our group have shown that the Hb is highly ordered in the red blood cell[17]

We define order as the alignment of haem molecules within the highly packed Hb environment of the cell. This order can change when the quaternary structure is modified and the Hb molecules acquire more degrees of freedom. Given this ordering in the RBC and the lack of change in our experiments using isolated Hb, we suggest that the effect of successive oxygenation and deoxygenation cycles using $S_2O_4^{2<M->}$ is to disrupt the Hb ordering in the cell, facilitating oxygen diffusion (mediated by Hb) through the erythrocyte, which must increase the rate constant for

oxygen uptake and at the same time increase the capacity of red blood cells to carry oxygen (i.^e. the equilibrium O_2-binding constant).

To investigate this hypothesis, deoxygenation-reoxygenation experiments were repeated using polarised Raman spectroscopy. Polarized Raman spectroscopy has been long used to facilitate band assignments and to investigate the intrinsic order of chemical systems. The depolarization ratio is defined as $\rho = \frac{I_\parallel}{I_\perp}$ where $I_\parallel$ is the intensity of the parallel component of scattered light and $I_\perp$ is the intensity of the perpendicular component. In general, totally symmetric modes of A_{1g} symmetry, assuming the D_{4h} point group, have $\rho < 0.5$, while non-totally symmetric modes of B_{1g}, B_{2g}, A_{2g} symmetry have $\rho > 0.5$. Parallel and perpendicular Raman spectra were obtained from untreated RBCs in the oxygenated state and after addition of $S_2O_4^{2<M->}$ followed by reoxygenation with atmospheric air. Figure^^4<figr4> compares the spectra obtained for oxygenated RBCs before and after $S_2O_4^{2<M->}$ deoxygenation to a subsequent spectrum obtained after reoxygenation; in these examples,the spectra are normalised to the band at 755^^cm$^{<M->1}$. Parallel and perpendicular polarised spectra of RBCs show dramatic differences in the intensity of the bands as previously reported [17]

The depolarisation ratio was calculated based on the integrated area of the band in the perpendicular spectrum divided by the integrated area of the same band in the parallel spectrum. The bands assigned to ν_{10}, ν_{19}, ν_{37}, ν_{11}, ν_{21} and ν_{41} are more intense in the perpendicular than in the parallel polarised Raman spectra and hence these modes are generally depolarised. These bands, correspond to $\nu(C_\alpha C_m)_{asym}$, $\nu(C_\alpha C_m)_{asym}$, $\nu(C_\alpha C_m)_{asym}$, $\nu(C_\beta C_\beta)$, $\delta(C_mH)$ and ν(pyr quarter-ring)$_{sym}$ modes, respectively, and are associated with mainly non-totally symmetric modes. On the other hand, ν_4, ν(C<C=>C) and ν_7 bands show a higher intensity in the parallel polarised spectra than in the perpendicular spectra and are assigned to polarised totally symmetric modes ν(pyr half-ring)$_{sym}$, ν(C<C=>C) and δ(pyr deform)$_{sym}$.

Spectra of the oxygenated RBCs before and after the addition of $S_2O_4^{2<M->}$ are very similar. An increase in the depolarization ratio for totally symmetric modes would indicate an increase in disorder of Hb molecules within the cell. However, for the totally symmetric mode ν_7, the

depolarisation ratio increases after the addition of $S_2O_4^{2<M->}$ followed by reoxygenation. This can also be observed in other totally symmetric modes such as the ν (Fe<C->O), ν_4 (pyr half-ring)$_{sym}$, ν_{30} (pyr half-ring)$_{sym}$ and ν_6 ν (pyr breathing) (See SI5 and SI6). The depolarisation ratio increased from 0.22 to 0.31, from 0.44 to 0.52 and from 0.13 to 0.22 for the ν_7, ν_4 and ν_6 bands, respectively. In the case of the ν (Fe<C->O) and ν_{30} bands, the depolarisation ratio was impossible to calculate because the bands were not detected in the perpendicular spectra; however, the band in the parallel spectra decreased in both cases. This increase in the depolarisation ratio after the addition of $S_2O_4^{2<M->}$ indicates an increase in the disorder of haem molecules. The increase in disordered Hb may facilitate oxygen diffusion throughout the cell, which could explain the increase in oxygen recovery of the RBCs exposed to $S_2O_4^{2<M->}$.

Here we show that deoxygenation with dithionite causes changes in the oxygen uptake of RBCs when compared to deoxygenation with N_2 gas. The results show that subsequent deoxygenation/oxygenation cycles produce changes in the oxygen-uptake kinetics of RBCs. An increase in oxygen uptake of a single RBC was observed after conditioning the cell in reduced dithionite buffer. Raman and UV-Vis microspectroscopy showed that the oxygen uptake and reoxygenation kinetics increased as the cells went through successive deoxygenation cycles with $S_2O_4^{2<M->}$. These effects were not observed in isolated Hb, nor were they observed to occur when nitrogen gas was used to deoxygenate cells. Studies performed using polarised Raman microscopy indicate that the $S_2O_4^{2}$ disrupts the highly concentrated environment of Hb in the red blood cell facilitating oxygen diffusion. Our results show that treatment with $S_2O_4^{2<M->}$ decreases the time taken for RBCs to become fully oxygenated and increases the oxygen capacity of the red blood cell under atmospheric conditions. Such results may have important implications in increasing the capacity of red blood cells to uptake and deliver oxygen to patients who require blood transfusions and improving oxygen capacity for athletes and altitude climbers.

Supporting Information Summary

Further details of the experimental and results are available in the supporting information document, which include the following sections: (A) Optical manipulation of red-blood cells across a stable boundary between co-flowing streams of oxygenated and deoxygenated media, (B) Preliminary experiments using the optical trap, (C) Raman Microspectroscopy of single red-blood cells adhered to glass, or gold-coated, surfaces, (D) UV-Visible spectroscopy, (E) Studies on isolated Hb, (F) Polarized Raman Microscopy and (G) Chemicals.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure^^1 Top **-** A schematic diagram showing the translation of a single RBC across a laminar flow boundary between oxygenated and deoxygenated media in a microfluidic device. Bottom - Raman spectra of a single RBC isolated in an optical trap within the fluidic device. **(a)** A spectrum of a RBC recorded in oxygenated media. **(b)** A second spectrum was recorded after approximately 60^^s, following the translation of the RBC into deoxygenated media. **(c)** A final spectrum was recorded, after approximately 60^^s, following the translation of the RBC back into the oxygenated media. The panel of spectra on the left were recorded using deoxygenated media created by bubbling N_2 gas, while the panel of spectra on the right were recorded using deoxygenated media created by addition of dithionite, $S_2O_4^{2<M->}$ (0.5^^mM)..

Figure^^2 The first PC obtained from a PCA performed over the Raman spectra obtained after multiple oxygenation additions. Panels a) and b) Loadings for the experiments performed using sodium dithionite ($Na_2S_2O_4^{2<M->}$) (0.5^^mM) and N_2, respectively. Panels c) and d) Scores of the first PC for the experiments performed using $S_2O_4^{2<M->}$ (0.5^^mM) and N_2 respectively. * marks the addition of $S_2O_4^{2<M->}$, total of 3 additions within the 40 measurements. Black line represents the moving mean calculated using a 3 points window.

Figure^^3 The first PC obtained from a PCA performed over the UV-Vis spectra obtained after multiple oxygenation additions. Panels a) and b) Loadings for the experiments performed using $S_2O_4^{2<M->}$ (0.5^^mM) and N_2, respectively. Panels c) and d) Scores of the first PC for the experiments performed using $S_2O_4^{2<M->}$ (0.5^^mM) and N_2 respectively. * marks the addition of $S_2O_4^{2<M->}$. Black line represents the moving mean calculated using a 3 points window..

Figure^^4 Perpendicular and parallel polarised Raman spectra of oxygenated RBCs before the addition of $S_2O_4^{2<M->}$ and reoxygenation with atmospheric air. Each spectrum is the average of the spectra recorded of 7 to 9 RBCs..