Spectroscopic analysis of myoglobin and cytochrome c dynamics in isolated cardiomyocytes during hypoxia and reoxygenation

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ABSTRACT:

Raman microspectroscopy was applied to monitor the intracellular redox state of myoglobin and cytochrome c from isolated adult rat cardiomyocytes during hypoxia and reoxygenation. The nitrite reductase activity of myoglobin leads to the production of nitric oxide in cells under hypoxic conditions, which is linked to the inhibition of mitochondrial respiration. In this work, the subsequent reoxygenation of cells after hypoxia is shown to lead to increased levels of oxygen-bound myoglobin relative to the initial levels observed under normoxic conditions. Increased levels of reduced cytochrome c in ex vivo cells are also observed during hypoxia and reoxygenation by Raman microspectroscopy. The cellular response to reoxygenation differed dramatically depending on the method used in the preceding step to create hypoxic conditions in the cell suspension, where a chemical agent, sodium dithionite, leads to reduction of cytochromes in addition to removal of dissolved oxygen, and bubbling-N₂ gas leads to displacement of dissolved oxygen only. These results have an impact on the assessment of experimental simulations of hypoxia in cells. The spectroscopic technique employed in this work will be used in the future as an analytical method to monitor the effects of varying levels of oxygen and nutrients supplied to cardiomyocytes during either the preconditioning of cells or the reperfusion of ischaemic tissue.

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1. Introduction

The human heart beats 2.5 – 3 billion times in the average lifetime of 70-80 years\(^1\) due to the continuous and repetitive contraction of muscle cells called ventricular cardiomyocytes. The intracellular haemoprotein, myoglobin, plays a crucial role in providing the energy to maintain the heartbeat through oxygen storage and transportation. Cytochrome \(c\) is part of the mitochondrial electron transport chain where the oxygen molecule is the final electron acceptor that produces adenosine triphosphate (ATP) for cardiac muscle contraction. During and immediately after a myocardial infarction (heart attack), approximately 1 billion cardiomyocytes in the left ventricle undergo rapid cell death leading to cardiac muscle damage and loss of function. There are 1.8 million deaths each year in the European Union caused by coronary artery disease.\(^2\) The current best therapeutic treatment is timely reperfusion of ischaemic tissue using thrombolysis or primary percutaneous coronary intervention to reperfuse the cardiac muscle with oxygenated blood. Although reperfusion of the ischaemic cardiac muscle is essential to salvage function, there is a paradoxical trade-off as the sudden reoxygenation causes ‘reperfusion injury’ which results in yet further death of cardiomyocytes.\(^3\) The cellular processes that underlie reperfusion injury are not fully understood. In this paper, Raman spectroscopy is used to monitor cellular changes in isolated cardiomyocytes during experimental simulations that mimic the low to high transitions in oxygen levels in the reperfusion of ischaemic cardiac tissue.

Laser excitation within the Soret or Q absorption peaks leads to the enhancement of the vibrational Raman transitions of the protoporphyrin IX ligand in haem, and the measured Raman wavenumbers are a sensitive probe of the state of the central-metal ion and the protein environment of the ligand. For instance, the centre wavenumber of the Raman band corresponding to the pyrrole half-ring symmetric stretch, \(\nu_4\), is strongly influenced by the extent of \(\pi\)-backbonding between the metal \(d\) orbitals and the porphyrin \(\pi^*\) orbital.\(^4\)\(^,\)\(^5\) This in turn is determined by a combination of the electron density on the metal ion\(^6\) (\(\nu_4\) is also known as the oxidation state marker band) and the nature of any bond formed by the metal ion to additional ligands.\(^7\) In the ferrous (deoxygenated) state of myoglobin, the haem contains a 5-coordinate high spin metal ion in which the 5\(^{\text{th}}\) coordinate position is occupied by the imidazole group of a proximal histidine and the 6\(^{\text{th}}\) coordinate position is vacant. Because of the significant \(\pi\)-backbonding to the porphyrin ring, \(\nu_4\) has a relatively low wavenumber (~1355 cm\(^{-1}\)). When an oxygen molecule binds reversibly to the 6\(^{\text{th}}\) coordinate position of the ferrous metal ion in myoglobin, a complex is formed with a bent Fe-O-O geometry. Charge transfer in the oxygenated complex means that the central metal ion possesses ferric character in a low spin
electronic state, i.e. $\text{Fe}^\text{II}-\text{O}_2 \leftrightarrow \text{Fe}^\text{III}-\text{O}_2^\text{2-}$. Because of the reduction in $\pi$-backbonding from the ferric metal ion to the porphyrin ligand, $\nu_4$ has a correspondingly higher wavenumber value ($\sim 1370 \text{ cm}^{-1}$) in oxygenated myoglobin. A similar difference in the electronic structure of the haem complex, and wavenumber position of $\nu_4$, is found between deoxygenated and oxygenated haemoglobin, and the reduced and oxidised states of cytochrome c.\textsuperscript{6}

There have been numerous studies in which the oxygenation level of haemoglobin has been monitored in isolated, \textit{ex vivo}, red blood cells by Raman spectroscopy. Detailed assignments of the Raman wavenumber values for the porphyrin modes in cellular haemoglobin have been made at various resonant and non-resonant excitation wavelengths.\textsuperscript{8,9} The effect of transitions from relaxed-to-tense states of the protein\textsuperscript{10}, haem aggregation and protein denaturing\textsuperscript{11} has been characterised, and other studies performed under conditions of dehydration and fixation\textsuperscript{12}. Raman spectroscopy has also been shown to be capable of monitoring the \textit{in vivo} oxygenation levels in tissue.\textsuperscript{13,14}

There are only a few examples of the use of Raman spectroscopy to monitor isolated, \textit{ex vivo}, cardiomyocytes. Inya-Agha et al. have studied dynamic changes during stimulation and cell remodelling using an excitation wavelength that is non-resonant with the haem group.\textsuperscript{15} The authors observed vibrational bands that could be assigned to amino acids and amide linkages in proteins. Ogawa et al. performed resonance Raman spectroscopy on cardiomyocytes using an excitation wavelength coincident with Q peak of the protoporphyrin IX ligand.\textsuperscript{16} In this case, the resolved Raman bands in the spectrum were sparse and attributed to cytochromes. Brazhe et al. also used the same resonant excitation wavelength to record spectra from single cardiomyocytes; which they observed to be dominated by the haem bands from reduced cytochromes $b$ ($\nu_5$ and $\nu_{37}$), $c$ and $c_1$ ($\nu_{15}$ and $\nu_{37}$) and oxygenated myoglobin ($\nu_{17}$ and $\nu_{36}$).\textsuperscript{17} Appropriate ratios calculated for the peak intensities of these bands enabled a semi-quantitative estimation of the amount of reduced cytochromes $b$, $c$ and $c_1$ in different parts of the cell, and the relative proportions of reduced cytochromes were monitored under conditions of oxidative stress. The same authors have performed a similar study in a perfused heart, and observed the changing ratio between oxygenated and deoxygenated myoglobin, along with redox state changes in mitochondrial cytochromes, for \textit{in vivo} cardiomyocytes under conditions of simulated ischaemia.\textsuperscript{18} Resonance Raman spectroscopy was used by Berezhna et al. to monitor conformational changes in cytochrome $c$, within the mitochondria of bovine heart, on the release from the membrane to the mitochondrial matrix. They observed a subtle change in the $\nu_4$ wavenumber of $2 \text{ cm}^{-1}$ using an excitation wavelength resonant with the Soret peak.\textsuperscript{19}

In the present work, the Raman spectra of \textit{ex vivo} cardiomyocytes have been recorded using a excitation wavelength that lies approximately at the minimum between the Soret and Q
absorption bands of the haem group. This excitation wavelength has been found to provide the most sensitive means to observe cellular mechanisms involving both myoglobins and cytochromes during hypoxia and reoxygenation, and this is the first reported example of Raman microspectroscopy of cardiomyocytes at this excitation wavelength. In this work, we have used Raman at 488 nm excitation to analyse the dynamics of reoxygenation of *ex vivo* cardiomyocytes. The results from this study show that Raman microspectroscopy can be used to characterise different cellular responses to reoxygenation of hypoxic cells. As the methods were suitable to study hypoxia, they are likely to be suitable to study ischaemia and, potentially, the method could distinguish between separate populations of cells that recover function, and suffer reperfusion injury. This may also be a suitable analytical tool to monitor the effects of ischaemic preconditioning in cardiac tissue where cells are subjected to a brief periodic sequence of ischaemia (or hypoxia) and reoxygenation (or normoxia) in order to provide protection against reperfusion injury. The results described in this paper also contrast the cellular responses to different experimental methods used to simulate ischaemia, or hypoxia, in the laboratory.

2. Experimental Methods

2.1 The isolation of single ventricular cardiomyocytes

Adult male Wistar rats (250-300 g) were killed by cervical dislocation and single ventricular myocytes isolated by enzymatic dissociation, as described previously. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). The work was done in cooperation with other research programmes, and animals were not used for the sole purpose of the experiments described here. Briefly, the heart was rapidly excised and perfused retrogradely through the aorta for 6 minutes using a Langendorff apparatus with nominally Ca\(^{2+}\)-free Tyrode solution titrated to pH 7.4 with NaOH. A constant flow rate of 10 ml/min was used at 37°C, and the solution was bubbled with 95% O\(_2\) / 5% CO\(_2\). For digestion, the heart was perfused over 7 to 10 minutes with Ca\(^{2+}\)-free Tyrode solution containing type I collagenase (0.4 units/ml), protease type XIV (2.7 units/ml) and 0.05% bovine serum albumin. This was followed by perfusion for 3 minutes with Tyrode solution (containing 2 mM CaCl\(_2\)). Isolation of single myocytes was achieved by gentle agitation in a shaking water bath at 37°C. This typically yielded 70 – 90% quiescent, viable, rod shaped myocytes. Following isolation, myocytes were stored in Tyrode solution at room temperature (approximately 21 °C) at a density of about 0.4 million myocytes/ml and were used for experimentation within 10 hours.
2.2 Cell perfusion and bright-field imaging

The cardiomyocytes were observed by bright-field microscopy whilst perfused with Tyrode solution, which is approximately isotonic with interstitial fluid. The perfusion chamber had a rhombus geometry containing a diagonal length of 30 mm and a width of 15 mm, and was custom machined in Perspex. The lower surface of the chamber was sealed with a #1.5 cover glass. The flow rate was maintained at approximately 4.5 ml/min using a peristaltic pump. Cardiomyocytes settle down on the lower glass surface of the chamber and were not usually displaced by the perfusing solution. All the spectral measurements described in the following section were repeated using different isolated cardiomyocytes that did not undergo hyperconstriction or rigor at the conclusion of the experiment (the details are given in the appropriate figure captions).

Hyperoxic conditions were simulated by bubbling 100% O₂ gas through Tyrode solution prior to its use in the dilution of a suspension of cardiomyocytes. The diluted cell suspensions were stored under 1 atmosphere of pure oxygen gas for 1 hour, and then the spectral measurements on single cardiomyocytes were performed immediately. Hypoxic conditions were simulated by either pre-treatment of Tyrode solution by bubbling 100% N₂ gas (30 minutes), or by the addition of sodium dithionite at a concentration of 8 mM. Hypoxic solutions were prepared just prior to an experiment and transferred immediately to the reservoir in the perfusion apparatus. In the former example, 100% N₂ gas was bubbled continuously through the reservoir to maintain the low level of dissolved oxygen. In the latter example, the strongly reducing dithionite anion, S₂O₄²⁻, reacts with dissolved O₂. The weak acid properties of aqueous S₂O₄²⁻ are buffered by HEPES in Tyrode solution maintaining pH at 7.4. The dithionite solutions retain the ability to remove dissolved oxygen for approximately 30 minutes before the decomposition of the anion leads to the restoration of normoxic conditions. Dithionite ions are expected to reduce all ferric heams to the ferrous state. Reoxygenation of cells was achieved through the pre-treatment of a separate volume of Tyrode solution by bubbling 100% O₂ gas for 30 minutes. This solution was then used to replace the hypoxic Tyrode solution in the reservoir of the perfusion apparatus. 100% O₂ gas was continuously bubbled through the reservoir to maintain high levels of dissolved oxygen during this stage of the experiment.

Both bright-field images, with 100× magnification, and Raman spectra, with a spatial resolution less than 1.0 µm, were recorded from single ventricular cardiomyocytes on a homebuilt inverted microscope. The system employs an oil-immersion, infinity-corrected, objective lens (Nikon,
A white light LED source (400-800 nm, Thorlabs), with a condenser lens, was used to illuminate the cells and images of the blue component of the transmitted light (400-450 nm) were recorded on a charge-coupled device (Basler, piA640-210gm). Raman spectra were recorded by overfilling the back aperture of the objective lens with the 488 nm-beam of a collimated continuous-wave laser. Dichroic mirrors (Chroma) were used to couple the laser light into the imaging optical path and extract the Raman scattered light collected by the objective lens. Spatial filtering of the Raman scattered light was accomplished by focusing, using a 160 mm achromatic lens (Comar), onto a 150 µm pinhole (Comar) in the confocal image plane. The spatially-filtered light was re-collimated with a second achromatic lens of 160 mm, and then focused by a 50 mm achromatic lens onto the 100 µm-width entrance slits of a spectrograph with a 0.500 m imaging triple grating monochromator (Acton Research Corporation, Spectra Pro 2500i; 1800 lines/mm grating; 500 nm blaze wavelength). The detector was a -80 °C cooled, back-illuminated, charge-coupled device (Princeton Instruments, Pixis 100B). Spectral data points were measured in increments of 0.018 nm. A neon mercury lamp was employed for the wavelength calibration of the spectrograph. Unless stated otherwise, the spectra in this paper were recorded with a laser power of approximately 0.9 mW incident on the cells for an acquisition time of 30 s. The laser was shuttered at all times except during the acquisition of a Raman spectrum. Fluorescence background was subtracted from Raman spectra using a polynomial-fitting algorithm in Origin 9.1 software (OriginLab Corp.). Raw spectral data were not subjected to interpolation, curve smoothing or cosmic-ray subtraction algorithms.

Raman spectra have also been recorded for oxygenated, deoxygenated and met-myoglobin, and ferro- and ferri-cytochrome c in solution. Both myoglobin (Sigma Aldrich M1882, ≥ 90 %) and ferric cytochrome c (Sigma Aldrich 30396, ≥ 90 %) from equine heart were used without further purification. A 4 mM stock solution of myoglobin was prepared in 50 mM sodium phosphate buffer adjusted to pH 7.4 with NaOH. Addition of sodium dithionite to the stock solution under 100% nitrogen gas for 5 minutes produced a solution of deoxymyoglobin. Oxymyoglobin solution was prepared by bubbling a steady flow of 100% O2 gas at 5 mL/min. through the deoxymyoglobin solution for 30 minutes. Addition of potassium ferricyanide to the stock solution of myoglobin produced a solution of the oxidised protein, metmyoglobin. A 3 mM stock solution of ferric cytochrome c was prepared in 50 mM sodium phosphate buffer adjusted to pH 7.4 with NaOH. Addition of sodium dithionite to the ferric cytochrome c stock gave a solution of ferrous cytochrome c. The procedures used in this work for preparing myoglobin and cytochrome c solutions follows those described in Ref. 22.
3. Results

3.1 Raman spectroscopy of an isolated cardiomyocyte using a 488 nm excitation laser

Healthy isolated, adult rat, ventricular cardiomyocytes are rod shaped approximately 100 µm in length and 20 µm in width, and contract in response to electrical-field stimulation (Fig. 1A-C). Cardiac muscle cells contain large quantities of the oxygen storage protein, myoglobin, and mitochondrial cytochromes.

Relative intensities of Raman bands for haemoproteins depend on the wavelength of the excitation laser. Symmetric vibrational modes of the porphyrin ligand are enhanced to a greater degree using excitation wavelengths within the Soret absorption peak, whilst asymmetric modes are enhanced to a greater degree using excitation wavelengths within the Q absorption peak. In the work described here, a laser wavelength of 488 nm was employed. This wavelength is located approximately at the absorption minimum between the Soret and Q peaks of iron protoporphyrin IX but still leads to an enhancement of symmetric vibrational modes. The use of this wavelength to record a Raman spectrum of a cardiomyocyte has not been reported before. A shorter wavelength of 405 nm, which is resonant with Soret peak, was found to result in rapid damage to the cells. The objectives of the present work were not served by the use of wavelengths resonant with the Q peak, as the $\nu_4$ band is relatively weak in recorded spectra and distinguishing the Raman bands for myoglobin and cytochromes is challenging.

The Raman spectrum of an isolated ventricular cardiomyocyte in Tyrode solution is shown in the top panel of Fig. 1D. Assignment of the vibrational bands is assisted by comparison to the spectra of pure proteins recorded in aqueous solution. These are shown, in the lower panels of Fig. 1D, for deoxygenated myoglobin, Mb$^{II}$, oxygenated myoglobin, Mb$^{II}$-O$_2$, metmyoglobin, Mb$^{III}$, reduced cytochrome c, Cyt$^{II}$, and oxidised cytochrome c, Cyt$^{III}$. The high wavenumber region, 1350-1650 cm$^{-1}$ is dominated by the vibrational modes of the porphyrin ligand. The aforementioned symmetric $\nu_4$ band lies within 1350 - 1380 cm$^{-1}$ for all haemoproteins, and is sensitive to the oxidation state of the metal centre. Asymmetrical vibrational modes of the porphyrin ligand are observed between 1540 and 1650 cm$^{-1}$ and the position of bands reflect the electronic-spin state of the metal ion. Both Mb$^{II}$ (ferrous, d$^6$) and Mb$^{III}$ (ferric, d$^5$) contain metal ions in the high spin state and display a similar pattern of asymmetric vibrational bands for the porphyrin ligand; $\nu_{11}$ (1545 cm$^{-1}$ shoulder), $\nu_2$ (1565 cm$^{-1}$) and $\nu_{10}$ (1608 cm$^{-1}$). In Mb$^{III}$, $\nu_{10}$ is obscured by a strong $\nu(C_a=C_b)$ band at 1615 cm$^{-1}$.

The 6-coordinate Mb$^{II}$-O$_2$ contains a ferric-like metal ion in a low spin state. In this case, the 1540-1650 cm$^{-1}$ spectral region displays a different intensity pattern of vibrational bands
dominated by the overlapping $\nu_2/\nu_{19}$ bands (1587 cm$^{-1}$) and the $\nu_{10}$ band (1642 cm$^{-1}$). For Mb$\II$-O$_2$, the $\nu_2$ and $\nu_{10}$ bands are blue shifted compared to Mb$\II$, which is due to the different core size (the non-bonded N-N distance) of the porphyrin ligand. The C$_\alpha$-C$_m$ bonds are strengthened on O$_2$ binding as a result of the core size decrease due to the change in the metal centre from a high spin ferrous state to a low spin ferric-like state. The metal can be accommodated in the N$_4$ pocket of the porphyrin in Mb$\II$-O$_2$, whilst the larger metal ion in Mb$\II$ must be located above the N$_4$ pocket of the porphyrin leading to the expansion of the core diameter, the doming of the porphyrin ligand and the weakening of the in-plane $\nu_2$ and $\nu_{10}$ skeletal modes.$^{23}$

The presence of a $\nu_4$ band centred at 1356 cm$^{-1}$ in the spectrum of both the cardiomyocyte and deoxymyoglobin, Mb$\II$, would suggest that the dominant protein observed is myoglobin, and the dominant state of myoglobin is deoxygenated, in isolated ventricular myocytes. Other vibrational bands observed for Mb$\II$ in the cardiomyocyte spectrum are $\nu_{13}$ (1212 cm$^{-1}$), $\nu_3$ (1473 cm$^{-1}$), $\nu_{11}$, $\nu_2$ and $\nu_{10}$. Nevertheless, the intensity of the $\nu_2/\nu_{19}$ and $\nu_{10}$ bands due to Mb$\II$-O$_2$ at 1587 cm$^{-1}$ and 1642 cm$^{-1}$, respectively, and the clear shoulder on the red edge of $\nu_4$ indicates that Mb$\II$-O$_2$ is still present in the cardiomyocyte.

Cytochrome $c$ is an abundant cytochrome found in the inner membrane of mitochondria and will be considered as a representative example of the different cytochromes ($a$, $b$, $c$) present in cardiomyocytes. An imidazole group (from a histidine residue) and a thiol ether group (from a methionine residue) are bound to the 5$^{th}$ and 6$^{th}$ coordinate positions, respectively, of the metal centre in the haem protein. The different environment of the haem group in cytochrome $c$, compared with myoglobin, has an effect on the vibrational wavenumber values of the porphyrin modes and on the relative intensities of Raman bands. This makes it possible to distinguish the vibrational bands that belong to cytochrome $c$ and myoglobin in spectra from live cells. In fact, there does not appear to be a significant contribution from cytochrome $c$ in the Raman spectrum of the ex vivo cardiomyocyte in Fig 1D. The $\nu_4$ band in the solution spectra of ferrocytochrome $c$, Cyt$\II$, and ferricytochrome $c$, Cyt$\III$, are found at the higher wavenumber values of 1363 cm$^{-1}$ and 1374 cm$^{-1}$, respectively, compared with the centre wavenumber of 1356 cm$^{-1}$ measured in the cardiomyocyte spectrum. A comparison of the position and relative intensities of the bands between 1540 and 1650 cm$^{-1}$ is also evidence that cytochrome $c$ is not making a substantial contribution to the measured Raman spectrum for the cardiomyocyte. The high spin complex, Cyt$\III$, has a $\nu_{11}$ band at 1548 cm$^{-1}$ and overlapping bands for $\nu_{19}$, $\nu_2$ and $\nu_{37}$ centred at 1585 cm$^{-1}$. These are similar wavenumber values to the bands observed for the cardiomyocyte; however, the strong $\nu_{10}$ band for Cyt$\II$ measured at 1622 cm$^{-1}$ in solution is not observed in the cardiomyocyte. The $\nu_{11}$, $\nu_2$ and $\nu_{10}$ modes for the low spin complex, Cyt$\III$, have similar band centres at 1563 cm$^{-1}$, 1587 cm$^{-1}$ and 1638 cm$^{-1}$, respectively, and relative intensities to those
observed in the solution spectrum for Mb\textsuperscript{II}-O\textsubscript{2}, and it is likely that these peaks observed in the cardiomyocyte spectrum contain a contribution from Cyt\textsuperscript{III}. Other bands observed in the cytochrome solution spectra are $\nu_29$ at 1404 cm\textsuperscript{-1} (Cyt\textsuperscript{II}) or 1412 cm\textsuperscript{-1} (Cyt\textsuperscript{III}), and $\nu_3$ at 1494 cm\textsuperscript{-1} (Cyt\textsuperscript{II}) or 1505 cm\textsuperscript{-1} (Cyt\textsuperscript{III}). The assignment of the spectra in Fig. 1D is consistent with that reported by Spiro and co-workers.\textsuperscript{4,5,6,23} The Raman spectrum for Cyt\textsuperscript{III} has an overall lower intensity than for Cyt\textsuperscript{II} in measurements that use an excitation wavelength resonant with the Q absorption peak.\textsuperscript{24} In the present work, an excitation wavelength of 488 nm has been used to record Raman spectra, which is located between the Soret and Q absorption peaks of the porphyrin ligand. The different excitation wavelength has a significant effect on relative intensities of vibrational bands for haemoproteins. Although the intensity of bands in the spin marker region, 1540-1650 cm\textsuperscript{-1}, is lower in Cyt\textsuperscript{III}, a similar intensity for the $\nu_4$ band of Cyt\textsuperscript{II} and Cyt\textsuperscript{III} is observed (see Fig. 1). Identical concentrations of reduced and oxidised cytochrome c solutions were used to obtain the data in Fig. 1, and the exact ratio of the peak intensities of the $\nu_4$ band for Cyt\textsuperscript{III} relative to Cyt\textsuperscript{II} is 0.8-0.9.

The spectrum for the cardiomyocyte in Fig. 1D contrasts with the resonance Raman spectra of a cardiomyocyte reported by Brazhe et al.\textsuperscript{17}, in which the vibrational bands for reduced cytochromes were dominant. Once again, in this earlier study, an excitation wavelength of 532 nm resonant with the Q absorption peak was used. The excitation wavelength of 532 nm leads to a stronger enhancement of the vibrational bands between 1540 and 1650 cm\textsuperscript{-1}. This is also the region where the Raman scattering by reduced cytochrome c is strong (see Fig. 1) and this leads to its dominance in the spectrum of a cardiomyocyte in Ref. 17. In contrast, the different excitation wavelength used in the present study (488 nm) results in a stronger enhancement of the $\nu_4$ band, compared with the vibration bands between 1540 and 1650 cm\textsuperscript{-1}, and the $\nu_4$ band has a similar intensity in myoglobin and cytochrome c solution spectra. Consequently, the overall contribution of reduced cytochrome c to the cardiomyocyte spectrum is not as significant in Fig. 1D. Myoglobin is a cytosolic protein and expected to be distributed fairly homogeneously throughout a cardiomyocyte in comparison to cytochromes that are found mainly in mitochondrial membranes. This was reflected by the varying spectral intensities, measured in Ref. 17, of the cytochrome bands at different positions in the cell. In the present work, the measured Raman spectrum, dominated by bands assigned to myoglobin, was found to be approximately the same for confocal measurements made at different positions inside a single cardiomyocyte.
3.2 Hyperoxia in an isolated cardiomyocyte

Blood flow to the heart is an important factor in maintaining high levels of oxygen to ventricular cardiomyocytes. The cells used in the present study were not energetically compromised despite isolation from the heart, and could maintain contractile behaviour in response to electrical stimuli within the time frame of these experiments. A number of control measurements have been made in order to ensure that the high levels of MbII are not due to laser-induced photodissociation of MbII-O2. The spectrum in Fig. 1D was taken on an isolated cardiomyocyte using a 488 nm-laser power of 0.9 mW for 30 s. We have also recorded a single spectrum from a cardiomyocyte with a shorter exposure time of 1 s. Although the intensity of ν₄ was close to the noise level in this spectrum, the band position was still identified as centred at 1356 cm⁻¹. The effects of long term exposure to 488 nm radiation was also examined by recording a sequence of 20 spectra (0.9 mW, 30 s) from an isolated cardiomyocyte at 5 minute intervals. In this case, the first and last spectra showed similar wavenumber positions for the aforementioned vibrational bands and photoinduced damage of the cardiomyocyte could not be detected.

Hyperoxic conditions were simulated to investigate whether or not the level of MbII-O2 in an isolated cardiomyocyte could be increased. The Raman spectrum shown in Fig. 2B was recorded following pre-treatment of cardiomyocytes to a saturated oxygen environment for 1 hour (as described in the Experimental Methods). The appearance of a stronger shoulder on the ν₄ band at 1372 cm⁻¹, and the emergence of ν₂/ν₁₀ and ν₁₀ bands shifted to higher wavenumber values, is indicative of higher levels of MbII-O2 in the ex vivo cardiomyocytes.

3.3 Hypoxia and reoxygenation in an isolated cardiomyocyte

The results in this section demonstrate that Raman spectroscopy can provide information on dynamic changes in the redox state, spin state and oxygen binding of haem proteins in isolated cardiomyocytes. Data was obtained by monitoring cells during perfusion with two different hypoxic Tyrode solutions.

The response of an isolated cardiomyocyte to perfusion of a hypoxic Tyrode solution, in which dissolved O₂ has been displaced by N₂, is illustrated in Fig. 3. All the spectra shown in this figure were recorded on the same cardiomyocyte. After 40 minutes of hypoxia (Fig. 3B), there is no discernable change in the ν₄ band which remains centred at 1356 cm⁻¹ indicating that the ferrous oxidation state remains dominant for haemoproteins in the cardiomyocyte. There is a change in the vibrational bands in the region 1540 - 1650 cm⁻¹. The spectral changes indicate that there is an increasing contribution in the cardiomyocyte from a ferrous haemoprotein in a low spin state. In particular, the intensity increases for bands at 1587 cm⁻¹ and 1622 cm⁻¹. The
latter wavenumber is the position of $\nu_{10}$ for reduced cytochrome $c$, Cyt$^{\mathrm{II}}$, and is more clearly observed in the difference spectrum (right-hand side of Fig. 3) in which the initial measurement made in normal Tyrode solution has been subtracted from the measurement taken after 12 minutes of hypoxia. The appearance of the $1622\ \text{cm}^{-1}$ band is still difficult to distinguish in both raw and difference spectra due to the congestion of vibrational bands in this spectral region. The intensity of the bands for Cyt$^{\mathrm{II}}$ observed during the period of hypoxia is weak in comparison to the corresponding bands for Mb$^{\mathrm{II}}$, and the $\nu_4$ band for Cyt$^{\mathrm{II}}$ (at $1363\ \text{cm}^{-1}$) cannot be separated from the intense $\nu_4$ band for Mb$^{\mathrm{II}}$ (at $1356\ \text{cm}^{-1}$). The difference spectrum in Fig. 3 does show that there is an increase in the intensity at $\sim1360$ relative to $\sim1370\ \text{cm}^{-1}$ which indicates that there is some reduction of ferric to ferrous haemoprotein but it is impossible to distinguish between the loss of Mb$^{\mathrm{II}}$-$\text{O}_2$ ($\leftrightarrow$ Mb$^{\mathrm{III}}$-$\text{O}_2$•$^-$) or Cyt$^{\mathrm{III}}$ due to similar band positions. After 15 minutes of reoxygenation (Fig. 3C), there is an increase in the high wavenumber $\nu_4$ band at $1372\ \text{cm}^{-1}$ (with a concomitant decrease in the low wavenumber $\nu_4$ band at $1356\ \text{cm}^{-1}$) and an increase in both the $\nu_2/\nu_{19}$ and $\nu_{10}$ bands indicating the formation of significant amounts of Mb$^{\mathrm{II}}$-$\text{O}_2$.

A different result was obtained from an experiment in which the period of hypoxia was induced by a Tyrode solution containing 8 mM sodium dithionite, instead of a Tyrode solution pre-treated by bubbling N$_2$ gas. The results of this experiment are shown for a single cardiomyocyte in Fig. 4. In this example, the perfusion of the hypoxic solution for 30 minutes leads to the disappearance of the small peak observed at $1642\ \text{cm}^{-1}$ due to the $\nu_{10}$ band for Mb$^{\mathrm{II}}$-$\text{O}_2$ (Fig. 4B). Unlike the previous example shown in Fig. 3, there is no evidence of an increase in reduced cytochrome $c$ during the period of hypoxia in the experiment; neither in the spectrum shown in Fig. 4B, or a difference spectrum calculated by a baseline subtraction using the spectrum in Fig. 4A (not shown). A more significant change in the spectrum is observed following the subsequent reoxygenation of the cardiomyocyte by perfusion of an O$_2$-saturated Tyrode solution for a further 10 minutes (Fig. 4C). At this stage, separate $\nu_4$ bands are resolved at $1356\ (\text{Mb}^{\mathrm{II}})$, $1363\ (\text{Cyt}^{\mathrm{II}})$ and $1372\ \text{cm}^{-1}$ (Mb$^{\mathrm{II}}$-$\text{O}_2$) accompanied by an increase in the $\nu_{11}$ band at $1548\ \text{cm}^{-1}$ (Cyt$^{\mathrm{II}}$), $\nu_2/\nu_3/\nu_{19}$ bands at $1587\ \text{cm}^{-1}$ (Mb$^{\mathrm{II}}$-$\text{O}_2$ and Cyt$^{\mathrm{II}}$), and $\nu_{10}$ bands at $1622\ (\text{Cyt}^{\mathrm{II}})$ and $1642\ \text{cm}^{-1}$ (Mb$^{\mathrm{II}}$-$\text{O}_2$). The relative intensities indicate a large increase in reduced cytochrome $c$ and a smaller, yet significant, increase in oxygenated myoglobin during the reoxygenation stage of the experiment. The increase in Mb$^{\mathrm{II}}$-$\text{O}_2$ will be at the expense of Mb$^{\mathrm{II}}$ in the cytosol of the cardiomyocyte; however, the explanation for the appearance of strong Cyt$^{\mathrm{II}}$ Raman bands during reoxygenation of the cardiomyocyte is not straightforward, as the appearance of the new Cyt$^{\mathrm{II}}$ bands is not at the expense of existing Cyt$^{\mathrm{III}}$ bands (see Discussion).
The final spectra shown in Fig. 5 were obtained for the perfusion of a hypoxic Tyrode solution containing a higher concentration of sodium dithionite (16 mM). The hypoxic solution was perfused for a total time of 55 minutes. After 3 minutes of hypoxia, there are changes in the vibrational bands observed in the Raman spectrum. Although the peak centres are difficult to deconvolve, there is an increase in the $\nu_4$ band at 1363 cm$^{-1}$ and a significant increase in the $\nu_2/\nu_3/\nu_{19}$ bands at 1587 cm$^{-1}$. This observation is consistent with the previous examples in Fig. 3 and 4, in which it is postulated that there is accumulation of reduced cytochrome $c$.

Decomposition of $\text{S}_2\text{O}_4^{2-}$ ions is significant after 30 minutes, and the levels of dissolved oxygen correspond to normoxic conditions towards the end of the experiment. This leads to reoxygenation of the cardiomyocyte. The dominant $\nu_4$ band is now centred at 1372 cm$^{-1}$ in the spectra measured after 40 and 55 minutes, which suggests that the final amount of Mb$^{\text{II}}$-O$_2$ in the reoxygenated cell (Fig. 5B; top spectrum) exceeds the quantity present under the initial conditions (Fig. 5A). The increase in the $\nu_4$ band at 1372 cm$^{-1}$ is also accompanied by a large increase in the intensity of the $\nu_{10}$ band for Mb$^{\text{II}}$-O$_2$ at 1642 cm$^{-1}$.

4. Discussion

The suitability of Raman spectroscopy to monitor cellular responses to hypoxia and reoxygenation in cardiomyocytes is shown in Figs. 3, 4 and 5. The results also highlight the different response to hypoxia induced via the displacement of O$_2$ by bubbling N$_2$ gas compared to hypoxia induced via the addition of a dithionite salt. Both methods have been accepted widely as suitable conditions to simulate ischaemia and hypoxia in ex vivo and in vivo cells.

There are varying accounts in the literature of the extent of oxygen-bound myoglobin in cardiac tissue, and reported values range from approximately 50% to > 90% depending on the method used and region of myocardium tested. Although these references are reporting on in vivo experiments, the dominance of deoxygenated myoglobin in the ex vivo cardiomyocytes under the initial conditions of normoxia is surprising. The amount of oxygenated myoglobin in cells increases marginally after extensive treatment in an environment of saturated oxygen (Fig. 2B). If the cells were pretreated by hypoxia, then an increase in the pool of oxygen-bound myoglobin in cells was obtained following a relatively short period of reoxygenation that was comparable to that observed under hyperoxic conditions in a saturated-O$_2$ environment; i.e. the relative intensities of the $\nu_4$ bands at 1372/1356 cm$^{-1}$ in the final spectrum of Fig. 3 and 4 is similar to that observed in Fig. 2.
During hypoxia in N₂-bubbled solutions, there is evidence of an increase in reduced cytochrome. Although this is postulated to be cytochrome c, the resolution and intensity of spectral features does not enable the distinction between cytochrome c and other cytochromes. There is a more dramatic increase in CytII observed during the reoxygenation of cells following a period of hypoxia induced by a dithionite salt solution, and, in this instance, the increase is beyond that observed at any time in the experiment with N₂-bubbling. This is expected because the chemical dithionite will reduce the entire pool of ferric haemoproteins in the isolated cardiomyocyte; however, the increased intensity of the $\nu_4$ band for CytII at 1363 cm$^{-1}$ in high oxygen Tyrode after hypoxia (Fig. 4C), cannot be reconciled with a decrease in intensity of a corresponding $\nu_4$ band for oxidised cytochrome c, CytIII in the initial spectrum in normal Tyrode (Fig. 4A). The $\nu_4$ band for CytIII should appear at 1374 cm$^{-1}$, and the absence of a signal for CytIII in Fig. 4A of similar strength to CytII in Fig. 4C is surprising because a similar intensity of the $\nu_4$ bands for CytII and CytIII was observed in solution spectra (see Fig. 1D). It should also be noted that the intensity of the $\nu_4$ band for CytII increases more substantially during the reoxygenation of cells (Fig. 4C), rather than during the actual period of application of the dithionite solution (Fig. 4B). The origin of the small quantity of CytII observed during N₂-bubbling in Fig. 3B and the absence of a Raman signal for oxidised CytIII in Fig. 4A that is comparable to reduced CytII in Fig. 4C is not clear. A possible explanation for the appearance of cytochrome c in the Raman spectra reported in Figs. 4C is that there is a change in the total amount of cytochromes located in the beam waist of the Raman laser. This could be due to the redistribution of cytochromes inside the cell during hypoxia. Dynamic changes in the distribution of cytochrome c have been observed before within HeLa cells during cytokinesis. Another possible explanation is that, due to the different environments, the Raman cross-sections for CytII and CytIII in solution (illustrated by the intensities in reference spectra shown at the bottom of Fig. 1D) are not the same as the Raman cross-section CytII and CytIII in ex vivo cardiomyocytes. Champion et al. have reported that the vibrational Raman spectrum of cytochrome c is altered in solution, and in intact and swollen mitochondria. Furthermore, cardiolipin interacts with cytochrome c in the inner mitochondrial membrane and induces conformation changes in the haemoprotein that include the cleavage of the iron-methionine bond. Reduction of cytochrome c by sodium dithionite or hypoxia in vivo would prevent electron transport and would lead to ATP depletion and eventually cell and tissue death. Oxidised cytochrome c scavenges ROS and an increase in ferrous cytochrome c, via chemical reduction with dithionite, could lead to increased production of ROS during subsequent reoxygenation. The marginal accumulation of ferrous cytochrome c observed in Fig. 3B following the bubbling of N₂ gas might be expected to lead to a much smaller increase in ROS on subsequent reoxygenation. There is evidence that the redox state of cytochrome c in the
cytosol provides a means to regulate apoptosis, but it has also been postulated that the oxidised form is necessary to activate caspases, whilst the reduced form does not initiate programmed cell death.30

Conclusion

Raman microspectroscopy has the capability to monitor both intracellular myoglobin, and cytochrome c during hypoxia and reoxygenation in ex vivo cardiomyocytes. The experimental simulations in this report mimic the low and high oxygen levels experienced by cells during ischaemic preconditioning or reperfusion treatments. The data also highlights the considerably different cellular responses to two common conditions used in the laboratory to simulate hypoxia, with much larger quantities of reduced cytochrome c measured during reoxygenation after dithionite-induced hypoxia, and much weaker effects observed in hypoxia induced by N2 bubbling. The data also illustrates that the detection of oxidised cytochrome c with a Raman excitation wavelength of 488 nm is not possible in control untreated isolated adult rat cardiomyocytes, despite the detection of large quantities of reduced cytochrome c during hypoxia and reoxygenation, and the similar signal strengths observed for oxidised and reduced cytochromes in solution.

Acknowledgements

This work was supported by an Equipment Grant (RG100303), awarded to AJH and NMS, and an International Exchanges Grant (IE121029), awarded to AJH and BRW, from the Royal Society. AA would like to thank the government of Saudi Arabia and King Abdulaziz University for support through award of a PhD scholarship. BRW is funded by an Australian Research Council Future Fellowship Grant (DP110104821).

Data Accessibility

The datasets supporting this article have been uploaded to the Dryad Repository, DOI: doi:10.5061/dryad.qm96r.
Figure captions

**Figure 1:** Frames from a sequence of images taken of a single ventricular cardiomyocyte in (A.) an initial resting state, (B.) an intermediate contracted state and (C.) a final resting state. The contraction occurred spontaneously, without electrical stimulation. Image size is approximately 40 by 60 µm. (D.) Raman spectrum of a single cardiomyocyte in Tyrode solution, and solution spectra of deoxygenated myoglobin, MbII, oxygenated myoglobin, MbII-O2, metmyoglobin, MbIII, reduced cytochrome c, CytII, and oxidised cytochrome c, CytIII. Full details of the spectral assignment are given in the main text. The observed Raman bands at 1000 and 1660 cm⁻¹ are due to the amino acid, phenylalanine (Phe), and the amide group. The experimental measurement was replicated for 30 different cells isolated from 15 different hearts; the example in (A.) for a single cell is representative of all these spectra.

**Figure 2:** Raman spectra of a single cardiomyocyte recorded (A.) during perfusion of normal Tyrode solution, and (B.) following incubation in an oxygen saturated Tyrode solution. The data shown was recorded for two different cells isolated from the same heart. In both examples, the experimental measurement was replicated for 12 different cells isolated from 3 different hearts; the examples shown are representative of all these spectra.

**Figure 3:** Raman spectra of a single cardiomyocyte recorded (A.) during perfusion of normal Tyrode solution, (B.) following perfusion of a hypoxic Tyrode solution (deoxygenated by N₂ gas) for 5, 12, 20, 30 and 40 minutes, and (C.) following reperfusion of oxygenated Tyrode solution for 5, 10 and 15 minutes (or 45, 50 and 55 minutes from the start of the experiment). Difference spectrum for the data recorded after 12 minutes in (B.), obtained by subtracting the spectrum in (A.), is shown on the right-hand side. All data shown was recorded from the same cell but is representative of replicate measurements for 12 different cells isolated from 4 different hearts.

**Figure 4:** Raman spectra of a single cardiomyocyte recorded (A.) during perfusion of normal Tyrode solution, (B.) following perfusion of a hypoxic Tyrode solution (deoxygenated by dithionite salt, S₂O₄⁻²) for 30 minutes, and (C.) following reperfusion of oxygenated Tyrode solution for 10 minutes (or 40 minutes from the start of the experiment). The ν₄ band is shown in the left-hand panel, and the ν₁₁, ν₂, ν₁⁰, ν₃⁷ & ν₁₀ bands in the right-hand panel, at each interval of time. All data shown was recorded from the same cell but is representative of replicate measurements for 12 different cells isolated from 4 different hearts.

**Figure 5:** Raman spectra of a single cardiomyocyte recorded (A.) during perfusion of normal Tyrode solution, and (B.) following perfusion of a hypoxic Tyrode solution (deoxygenated by dithionite, S₂O₄⁻², salt) for 3, 40, and 55 minutes. The ν₄ band is shown in the left-hand panel,
and the $\nu_{11}$, $\nu_{2}$, $\nu_{19}$, $\nu_{37}$ & $\nu_{10}$ bands in the right-hand panel, at each interval of time. All data shown was recorded from the same cell but is representative of replicate measurements for 14 different cells isolated from 5 different hearts.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5


