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Resonance Raman spectroscopy can detect structural changes in haemozoin (malaria pigment) following incubation with chloroquine in infected erythrocytes

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Abstract **Resonance Raman spectroscopy was applied to monitor the effects of chloroquine (CQ) treatment on cultures of *Plasmodium falciparum* trophozoites. A number of bands assigned to A_{1g} and B_{1g} modes characteristic of the haemozoin aggregate are reduced in intensity in the CQ-treated cells, however, no bands from the CQ are observed. The intensity changes are attributed to intermolecular drug binding of the CQ in a sandwich type complex between ferriprotoporphyrin IX (FePPIX) dimer units. It is postulated that the CQ binds via π–π interactions between adjacent and orientated porphyrins thereby disrupting the haemozoin aggregate and reducing excitonic interactions between adjacent haems. The results show the potential of Raman microscopy as a screening tool for FePPIX:drug interactions in live cells.**
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1. Introduction

Malaria is still considered to be one of the most deadly diseases in the world, affecting over 500 million people and causing over 1 million deaths per year [1]. The parasite, *Plasmodium falciparum*, invades the body via the bite of the female *Anopheles* mosquito and undergoes the vertebrate host stages of its lifecycle. During the intraerythrocytic stage haemoglobin is degraded inside the digestive vacuole of the parasite into toxic free haem and denatured globin [2,3]. The process of detoxification involves the conversion of iron-protoporphyrin IX (FePPIX) to haemozoin, also known as malaria pigment, which is an insoluble compound located in the food vacuole of the parasite. Haemozoin is spectroscopically identical to its synthetic analogue β-haematin [4,5], which is known to be an array of dimers linked through reciprocal iron-carboxylate bonds to one of the propionate side chains of an adjacent Fe(III)PPIX moiety [6]. Quinoline drugs such as chloroquine, quinine and amodiaquine are thought to inhibit the FePPIX crystallisation by binding to monomeric or dimeric Fe(III)PPIX, resulting in the build up of toxic free FePPIX which can kill the parasite [7–10]. de Villiers et al. [10] propose a model in which the spontaneously formed aqueous Fe(III)PPIX dimer non-covalently interacts with the unligated faces of two five-coordinate H_2O/HO^-Fe(III)PPIX molecules, with the axial H_2O/HO^- ligands pointing outwards. Three models for drug–FePPIX interactions have been proposed. The first involves inhibition of the growth of the haemozoin crystal by chiral specific binding of the drug to the corrugated surface of haemozoin through the vinyl and methyl groups located on the Fe(III)PPIX surface [11]. In the second model drug binding occurs via a sandwich complex to the face of the crystal through π–π interactions [12]. In the third model quinoline drugs are thought to form π–π interactions with haematin μ-oxo dimers ($[Fe(III)PPIX]_2O$) [13]. This is thought to decrease the availability of monomeric haematin (H_2O/OH^-Fe(III)PPIX) for incorporation into haemozoin by shifting the equilibrium towards the μ-oxo dimer [14]. It should be noted however that the existence of a μ-oxo dimer form of haematin under the conditions of the digestive vacuole has been questioned [10].

While several studies have applied spectroscopic methods to investigate Fe(III)PPIX:drug interactions in solution [13,15] and the solid state [16] hitherto no spectroscopic study has investigated the effect of drug treatment on single *P. falciparum*-infected red blood cells. Using polarisation-resolved resonance Raman spectroscopy Frosch et al. [15] observed small wavenumber shifts when CQ was mixed with haematin in solution, which further supports a non-covalent Fe(III)PPIX:drug complex. Frosch et al. [17] recently reported UV resonance Raman spectra of chloroquine under physiological conditions. In this work mode assignment was performed by comparison with Density Functional Theory (DFT) calculations which suggested that the protonation states of chloroquine greatly influence the molecular geometry, vibrational modes and molecular orbitals important for π–π interactions with haemozoin [17]. Near IR Surface Enhanced Raman Spectroscopy (NIR-SERS) measurements [18] of a CQ–FePPIX complex were compared with data for haematin and CQ alone. The CQ–FePPIX complex indicated an interaction between the quinoline ring of the drug and the porphyrin ring [18].

In this work, we combine Raman microscopy using 782 nm excitation, with Principal Components Analysis (PCA) to investigate the effect of CQ treatment on the physical organisation of haemozoin within the digestive vacuole of *P. falciparum* trophozoites. We present evidence that CQ has an effect on the molecular environment of haemozoin within a red blood cell and provide the first spectroscopic evidence of CQ interfering

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with haemozoin formation, which paves the way to monitor such FePPIX:drug interactions in situ.

2. Experimental

2.1. Cell culture

P. falciparum (a CQ sensitive strain, D10) was maintained in continuous culture using human erythrocytes obtained from the Red Cross Blood Bank, Melbourne [19]. Parasitised erythrocytes were cultured in complete culture medium (CCM) consisting of RPMI 1640 (GIBCO BRL), 25 mM hydroxypiperazine-*N*′-2-ethane sulfonic acid (HEPES, Sigma, pH 7.4), 2 g/l sodium bicarbonate (AnalR) and 4 mM Glutamax (Invitrogen). This was supplemented with 0.16% glucose (AnalR), 0.21 mM hypoxanthine (Sigma), 22 μg/ml gentamycin (Sigma) and 4% human serum and 0.25% Albumax I (GIBCO-BRL). The cultures were kept at 37 °C in a humidified atmosphere of 5% CO_2, 1% O_2 and 90% N_2. Synchronised cultures (~5% parasitemia) of late ring stage parasites were treated for 20 h with 100 nM CQ. This concentration is sufficient to inhibit growth of the parasites by approximately 80% as monitored by uptake of [^{3}H]-hypoxanthine (data not shown). Infected RBCs (10 μl) were suspended in 10 ml of 0.9% saline solution and were placed in an 80 mm diameter glass aluminium sputter coated Petri dish; this was over-coated with poly-L-lysine to adhere the blood cells to the base of the dish. The experiment was repeated three times.

2.2. Raman microscopy

Raman spectra of control and CQ-treated *P. falciparum*-infected erythrocytes were recorded as described previously [20,21]. When the power is kept low and measurements are recorded in growth media in a temperature regulated environment, live infected red blood cells show no visible damage to the membrane or photo-decomposition of the haemoglobin. The Renishaw system 2000 spectrometer used was equipped with a 782 nm diode laser with ~18 mW raw output power and ~3–4 mW power at the sample, a BH2-UMA Olympus optical microscope and a Zeiss water immersion 60× objective. The instrument was calibrated daily using the 520.5 cm^{-1} band of a silicon wafer. Raman spectra were recorded by focusing the laser directly on the haemozoin pigment within the live infected red blood cells. For both the control and the CQ incubated cells spectra were recorded from 30 different cells. Raman spectra were recorded in the spectral range 1800–200 cm^{-1} using a single 10 s exposure at 10% power. All spectra were normalised to the ν_{10} $\nu(C_\alpha C_m)_{asym}$ B_{1g} vibrational mode. Spectra of haematin, haematin μ-oxo dimer and chloroquine were derived by averaging 30 spectra taken at various different positions on several crystals for each compound using a normal 50× objective.

2.3. Data analysis

PCA is a fundamental technique used in multivariate data analysis and involves the decomposition of data into noise and structural components. The dimensionality of the data set is reduced by plotting the objects onto principal components (PC's) with each consecutive component orthogonally positioned with respect to the previous PC. Each PC gives rise to a percentage of variance in the data set, with the explained variance decreasing as the PC increases. A plot of the scores along each PC (scores plot) allows differences and similarities to be easily recognised. Each score represents a single spectrum and is plotted as a point on a two-dimensional axis and the position of each point is dependent on the individual variance contribution of the spectrum to each PC [16]. The loadings plot is used in conjunction with the scores plot to identify the variables that induce the most variability in the data set. In this work, we applied PCA to ascertain biological variability and investigate the uptake of drugs in a population of *P. falciparum* trophozoites. Two different methodologies were used to minimize baseline affects. Firstly, an Extended Multiplicative Scatter Correction (EMSC) was applied to all data to remove physical affects, primarily baseline variation between spectra of each data set. Secondly, a second derivative with a 13 point Savitzky–Golay smoothing function was applied.

2.4. Materials and synthesis

The μ-oxo dimer form of haematin was prepared by dissolving haematin (Sigma–Aldrich) in aqueous base (0.1 M NaOH) and 10% (v/v) pyridine to produce 15.6 mM haematin μ-oxo dimer solution [10,22]. The solution was characterised by its characteristic UV–VIS spectroscopy [10,22]. The 15.6 mM haematin μ-oxo dimer solution was dried onto aluminium sputter coated slide and the characteristic Raman band (ν(Fe–O_{sym})) at 413 cm^{-1} was observed in the Raman spectrum [23–26]. The solution was also dried onto a Golden Gate™ Diamond attenuated total reflectance ATR accessory on a Bruker Equinox FTIR spectrometer and spectra recorded at a resolution of 4 cm^{-1}. The band at ~880 cm^{-1} assigned to the IR ν_{asym} (Fe–O–Fe) absorbance confirms the presence of the μ-oxo dimer (data not shown). Chloroquine diphosphate was purchased from Sigma–Aldrich.

3. Results and discussion

The haemozoin crystal is readily located as a dark pigment granule in brightfield images of live infected red blood cells (Fig. 1, inset). The spectrum from the region of the haemozoin is compared with spectra of haematin and the haematin μ-oxo-dimer in Fig. 1. For comparative purpose the spectra are normalised to the band designated ν_{10}(1623 cm^{-1}). The mode notation is based on that proposed by Abe et al. [27] for the resonance Raman spectra of nickel octa-ethyl porphyrin; where under the D_{4h} symmetry point group the modes are designated ν_1–ν_9 for A_{1g}, ν_{10}–ν_{18} for B_{1g}, ν_{19}–ν_{26} for A_{2g}, ν_{27}–ν_{35} for B_{2g}, and ν_{36}–ν_{55} for E_u modes. The A_{1g} modes are totally symmetric while the B_{1g}, A_{2g}, B_{2g} modes are non-totally symmetric. The E_u modes are IR and Raman active. The symmetry of ferric high spin haems such as haemozoin, haematin and β-hameatin is ideally C_{4v} because the Fe atom is translocated out of the porphyrin plane but the D_{4h} notation is often adopted by convention. The γ symbol is used to designate out-of-plane modes of which there are a number of categories which include (1) out-of-plane wagging modes, (2) tilting, and (3) internal folding of the pyrrole rings. The strong bands in the haemozoin spectrum include the in-plane asymmetric stretch of the porphyrin ring ν_{10}($\nu(C_\alpha C_m)_{asym}$) at 1623 cm^{-1} and the in-plane stretch of the vinyl group ν_{11}($\nu(C_\beta C_\beta)$) at approximately 1552 cm^{-1}. Bands at 1570, 1376, 1238 and 972 $cm^{-1}$are assigned to ν_2($\nu(C_\beta C_\beta)$), ν_4(ν(pyrrole half-ring)$_{sym}$), ν_{42}($\delta(C_mH)$), and ν_{46}(δ(pyrrole deform)$_{asym}$), respectively, and are listed in Table 1. Bands present at 796 and 753 cm^{-1} are assigned to

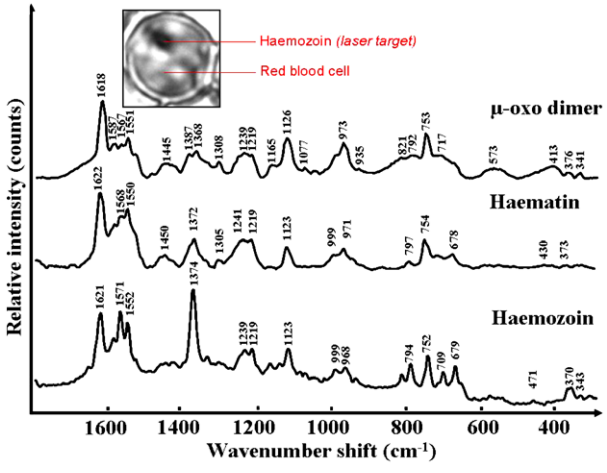

Fig. 1. Resonance Raman spectra of haemozoin in live red blood cells, and of dried samples of haematin and haematin μ-oxo dimer using a 782 nm excitation wavelength. Inset: Brightfield and resonance Raman image of a *P. falciparum*-infected red blood cell. The spot represents the region from which the Raman spectrum was collected.

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