Quantitative Determination of Functional Thiol Groups on Intact Cell Surfaces by Resonance Raman Spectroscopy

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The sensitivity and selectivity of resonance Raman spectroscopy, combined with electronic spectroscopy, has been used to develop a method to quantify the membrane thiol population *in situ* in viable erythrocytes. This technique is based on the thiol–disulfide reaction of Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid). It has the advantage that continuous monitoring of lysis is simple and a correction can be made for any interference resulting from lysis. In addition, the extent of reaction can be expressed as a ratio of the reagent signal, providing an internal calibrant.

Keywords: *Membrane thiol group; Raman spectroscopy*

Introduction

Oxidative stress has been implicated in various disease processes, in particular in the pathogenesis of microvascular complications in patients with diabetes mellitus.[1] Among the most vulnerable targets of oxidants present in the plasma are the exofacial thiol groups on the erythrocyte membrane. The thiol population is concentrated at a few functional sites, mainly on the hexose (60%) and anion (20%) transport proteins[2] and may play a role in the proper functioning of these proteins. Thus, there is a need for methods that can probe membrane thiol concentrations *in situ* in the viable cell.

Previously, electronic spectroscopy was used to detect the product of the reaction of Ellman's reagent, 5,5′-dithio-bis-2-nitrobenzoic acid (ESSE), with membrane-bound thiols.[3] This reaction is illustrated below:

$$\text{MEMBRANE-SH} + \text{ESSE} \rightarrow \text{MEMBRANE-SSE} + \text{ESH}$$
$$\text{ESH} \rightarrow \text{ES}^- + \text{H}^+ \text{ (at pH 7.4)}$$

The anion produced, ES^-, has an absorption band in the visible region at 412 nm. This method, however, is vulnerable to very small degrees of cell lysis. The exofacial thiol concentration on the cell membrane is low and any lysis causes serious interference from the intense Soret band of hemoglobin (Hb) (408–420 nm). This masks the absorbance resulting from ES^-, which is produced by exchange with the membrane thiol population. In addition, the erythrocyte cytosol contains large amounts of glutathione (GSH), which if released by lysis or by diffusion through the membrane, will react with ESSE to produce more anion. The over-all effect is that even a very small amount of cell lysis can provide the majority of the absorbance. This error is clearly not acceptable and consequently there is a need for a more precise, and sensitive method.

This paper reports a method using the same reaction as above, with detection of the anion by resonance Raman scattering. Electronic spectroscopy is used to provide an estimation of lysis for quantitative correction. Methods of analysis based on resonance Raman spectroscopy have the advantage that molecularly specific information is obtained from the sharp vibrational structure and the sensitivity and selectivity allows the detection of very low concentrations. The main advantage of this method is that the sharp vibrational bands allow the identification of separate signals from ES^-, ESSE and Hb. Thus the extent of the reaction can be observed from the ES^- signal and the concentration of ESSE used as an internal calibrant.

Using the intensity of the Hb peak, the extent of lysis can be quickly monitored and, if too high, the sample discarded. However, for very low levels of lysis, it is more precise to calculate the extent of lysis from electronic spectroscopy, since in the Raman spectrum, the Hb band is located on the shoulder of the ESSE band. In addition to the Soret band, the electronic spectrum of Hb has two smaller peaks at 542 and 577 nm. By correlating the absorbance maximum of the 542 nm band in the electronic spectrum of the cell lysate with the ratio of the heights of the Raman scattering peaks of ES^- and ESSE from the Raman lysate spectrum, a correction factor can be found. The correction is based on the assumption that the ratio of Hb to intracellular thiol is constant. The correction factor is multiplied by the absorption at 542 nm in the electronic spectrum from the supernatant from the reaction of whole cells with ESSE, to estimate the proportion of the ES^- Raman scattering intensity due to lysis in the Raman spectrum of the supernatant from the reaction of whole cells with ESSE. This allows the ES^- intensity due to membrane thiols to be calculated.

Experimental

Materials

Ellman's reagent and glutathione were obtained from Sigma (St. Louis, MO, USA), and NaCl and $NaH_2PO_4 \cdot 2H_2O$ were from Fisons (Loughborough, UK).

Raman Spectroscopy

Raman spectra were recorded from samples held in a 1 cm fluorescence cell. The instrument used was an Anaspec (London, UK) modified Cary 81 double spectrometer equipped with a cooled Thorn EMI (UK) 9658R photomultiplier and appropriate photoncounting equipment. A 90° scattering geometry and 5 cm^{-1} slits were used. The samples were irradiated with 100 mW of 457.9 nm radiation from a Model 2020 Spectra-Physics (San Jose, CA, USA) argon ion laser. All spectra were recorded between 1200 and 1450 cm^{-1}.

Solutions

All solutions were made up in phosphate-buffered saline (PBS) (0.125 mol l^{-1} NaH_2PO_4, 0.154 mol l^{-1} NaCl, pH 7.4).

Calibration Graph

Aliquots (2–100 μl) of a 0.2 mmol l^{-1} stock solution of glutathione in PBS were added to 3 ml of 0.5 mmol l^{-1} ESSE and the volume made up to 3.5 ml with PBS. The Raman spectrum of each sample was recorded after 30 min.

Blood Preparation

Freshly drawn blood from a healthy volunteer was collected in a heparinized tube and the analysis carried out immediately. The blood was centrifuged at 2000 rpm for 10 min and the plasma and white cell fractions removed. The packed erythrocytes were washed three times with PBS at 2000 rpm for 5 min.

Hemolysis Correction

To compensate for any intracellular thiol released as a result of cell lysis, a correction had to be applied. Lysates were prepared by adding 50 μl of packed erythrocytes to 2 ml of distilled water and leaving for 1 h. A 60 μl volume of the supernatant was incubated with 3 ml of 0.5 mmol l^{-1} ESSE for 30 min and the Raman scattering and electronic spectra recorded.

Determination of s_r Values

A 0.5 ml volume of packed cells was added to 4.5 ml PBS in a lithium heparin tube. The heparin helps to prevent lysis. The sample was immediately centrifuged at 2000 rpm for 5 min and the PBS discarded. A 3 ml volume of 0.5 mmol l^{-1} ESSE was gently mixed with the erythrocyte pellet. After 30 min, the solution was again centrifuged (2000 rpm for 5 min) and the pale yellow supernatant was removed for analysis. This was repeated six times using aliquots from the same blood sample. Raman and electronic spectra were recorded for each sample.

Release of Intracellular Thiols

A 3 ml volume of PBS was added to 3 ml aliquots of washed erythrocytes. After 30, 60 and 90 min, the supernatant was removed from one of the samples by centrifugation. A 2 ml volume of the supernatant was added to 2 ml of 1 mmol l^{-1} ESSE and after 30 min, the spectrum of each sample was recorded. Lysates were prepared as for the hemolysis correction and the Raman and electronic spectra recorded, to enable compensation for any lysis which may have resulted in thiol in the supernatant.

Results and Discussion

Although Raman spectrometers are usually used as single-beam instruments, modern lasers are relatively stable and consequently quantitation is practical. In this study, a quadrant mounted-shutter arrangement is used, in which the sample in a 1 cm fluorescence cuvette is placed in a slide, so that accurate repositioning can be achieved and the cone of scattered light can exit the sample compartment unimpeded. This method has been used before[4] and the results of this study and the previous one both indicate that effective quantitation is achieved.

To achieve resonance, the frequency of the incident laser light should be chosen to coincide with an absorption band of the resonant species. However for quantitative analysis, the stability and reproducibility of the source is critical. For that reason, the 457.9 nm line of the argon laser was preferred to a line closer to the absorption band maxima of ESSE and ES^-, which would require a tuneable source. A different laser (krypton) would be an alternative, but the line chosen is within the absorption band of ES^- and provides sufficiently increased Raman scattering for effective quantitative analysis.

Raman Spectrum of ESSE

The concentration of thiol groups present in the lysates from erythrocytes has been determined previously by resonance Raman scattering using ESSE.[4] Analysis of the spectrum of ESSE (Fig. 1) shows that the most intense peak is due to a stretching mode of the NO_2 group at approximately 1345 cm^{-1}. The frequency of this mode is sensitive to the charge on the Ellman's species. Thus, the symmetric stretch of the NO_2 group in ESSE is present at 1345 cm^{-1} and in ES^-, at 1325 cm^{-1}. The difference is sufficiently large for the two signals to be distinguished, although there is a significant overlap. The scattering obtained with 457.9 nm excitation is close to resonance for ES^- (λ_{max} 412 nm). For ESSE, the scattering is pre-resonant (λ_{max} 325 nm) and weaker. The ESSE signal is around 35 times less sensitive than that of the anion. ESSE is usually present in excess so that the less sensitive signal from the ESSE molecule can be used as an internal calibration of the system and all other peak intensities can be measured relative to the intensity of the ESSE peak.

At low concentrations, such as would be expected following reaction at the cell surface, the small signal from ES^- is best estimated by determining the size of the peak by subtraction from the top of the ESSE peak rather than the baseline. This method is preferred because of the varying baseline in the Raman spectra, which introduces an error each time it is used to estimate the peak heights. By using the ESSE peak, the baseline is only a factor once, in establishing the intensity of the ESSE signal.

In principle, for the quantitation of thiol groups on erythrocyte membranes, a solution of ESSE at a concentration that produces approximately equally intense Raman scattering from ESSE and the anion ES^-, would provide the best internal calibrant for quantitation. However, consideration of the chemistry led to the choice of a different ratio.

The peak of ESSE at 1345 cm^{-1} has shoulders on either side at about 1325 and 1375 cm^{-1} (Fig. 2). These must be accounted for, since they are close in energy to the peaks due to ES^- and Hb, respectively. In addition, the lower the

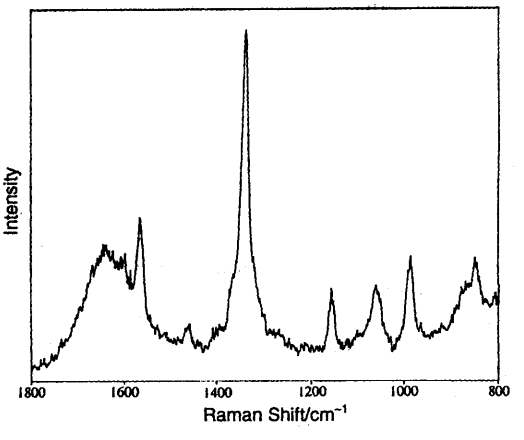

Fig. 1 Raman scattering from a 0.5 mmol l^{-1} solution of ESSE in PBS.

concentration of ESSE, the greater the intensity of the shoulder at about 1325 cm^{-1}. This is thought to be due to the hydroxide-promoted decomposition of ESSE[5] as shown below.

$$ESSE + 2HO^- \rightarrow \tfrac{3}{2} ES^- + \tfrac{1}{2} ESO_2^- + H_2O$$

The small amount of ES^- produced causes an increase in the intensity of the shoulder. Thus, if the concentration of ESSE is too low, a significant concentration of anion is produced and if the concentration is too high, ESSE dominates the spectrum and makes quantitation difficult. The most effective solution is to choose the lowest possible concentration of ESSE, such that anion formation is limited to a value to which a correction could easily be applied. This optimum concentration was found to be 0.5 mmol l^{-1}. At this concentration the scattering intensity of the shoulder on the low energy side at 1325 cm^{-1} was found to remain constant relative to the scattering from the main ESSE band at 1345 cm^{-1}.

Calibration Graph

A calibration graph of the reaction of GSH and ESSE was constructed (Fig. 3). The plot of ES^-/ESSE against the number of moles of thiol added, gave a correlation coefficient of −0.9 and a standard deviation of 0.09. Thus, in principle, the ratio of the intensities of ES^-/ESSE can be used to estimate the number of thiol groups which are present on the membrane. However the handling of the cells and addition of ESSE may cause some lysis. This releases GSH and Hb to contribute to the reactive thiol population. With careful procedures this can be minimized, but to accurately assess the number of thiol groups on the membrane, a correction must be applied.

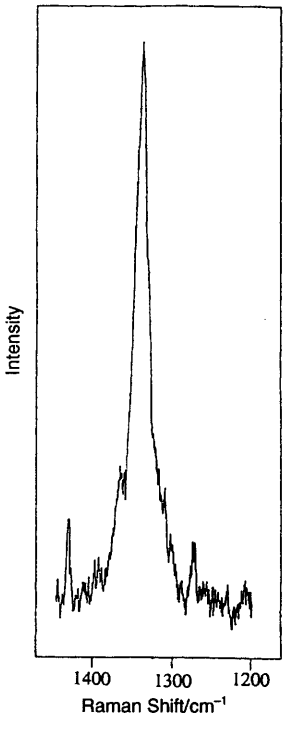

Fig. 2 The NO_2 stretching mode of ESSE at approximately 1345 cm^{-1}.

Lysis Correction

One key advantage of the Raman method over existing methods is that the signal due to the ν_4 mode of Hb at 1375 cm^{-1} is distinct from that of ESSE and anion peaks so that approximate estimation of the extent of lysis can be made by inspection. During these experiments, lysis was kept to a minimum and careful treatment of the cells was vital. Any distinct lysis led to the termination of the experiment. Without such a precaution, determination of membrane thiol groups by spectrophotometry is prone to error. A correction can be applied to account for the anion produced as a result of the reaction of ESSE with intracellular thiol, mainly GSH, which is released into the supernatant upon cell lysis. Since this correction is based on the assumption that the ratio of Hb to intracellular thiol in red cell lysate is constant, only a relatively small correction can be made. The Raman spectrum from lysate, [Fig. 4(*b*)], clearly identifies the presence of Hb and of ES^- due to intracellular thiol.

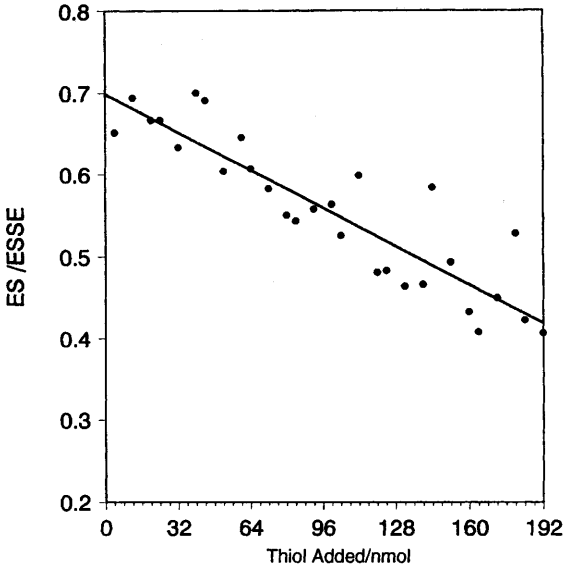

Fig. 3 Calibration graph of the ES^-/ESSE ratio, as measured from the Raman spectra, against the number of moles of thiol added to 0.5 mmol l^{-1} ESSE (total volume, 3.5 ml). [Note the ES^- intensity is measured as the difference from the top of the ESSE peak to the top of the ES^- peak, to reduce the baseline error. Hence the slope of the graph (see text)].

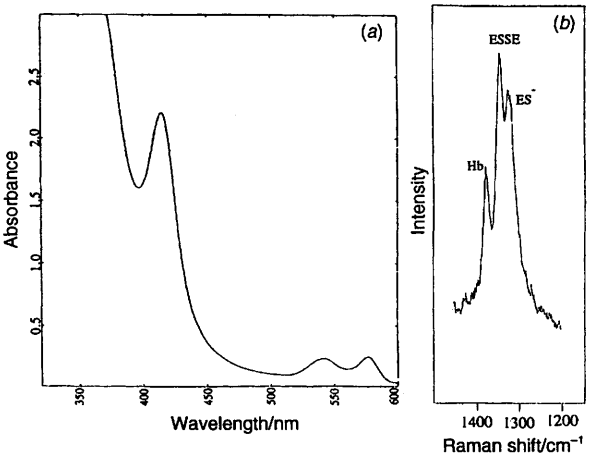

Fig. 4 (*a*) Electronic and (*b*) Raman spectra from the supernatant of the reaction of cell lysate with 0.5 mmol l^{-1} ESSE (3 ml).

However, because of the error introduced by a correction to the Hb peak for the shoulder at 1375 cm^{-1} in the Raman spectrum of ESSE, the amount of Hb was estimated separately using the 542 nm band in the electronic spectrum of the lysate, [Fig. 4(*a*)]. This value was then correlated with the ratio of the scattering intensities of ES^- and ESSE in the Raman spectrum of the lysate [Fig. 4(*b*)]. $(ES^-/ESSE)/Hb_{542\ nm}$ from six lysates from the same blood sample, gave a correction value of 7.107 ± 0.446 (s_r 6.3%).

The $(ES^-/ESSE)/Hb_{542\ nm}$ ratio, (7.107 for this particular sample), can be used to correct for the small ES^- scattering intensity which is due to lysis in the Raman spectrum of the supernatant, following centrifugation of the whole cell suspension after reaction with ESSE. The correction ratio (7.107) is multiplied with the absorbance at 542 nm, in the electronic spectrum of the supernatant of ESSE-treated whole cells [Fig. 5(*a*)]. The resulting value is then multiplied by the ESSE intensity in the Raman spectrum of the supernatant from ESSE-treated whole cells [Fig. 5(*b*)] to give ES^- due to lysis. The ES^- intensity due to lysis is then subtracted from the total ES^- intensity in the Raman spectrum of the ESSE-treated whole cells, leaving the ES^- signal due to membrane thiols.

The supernatant from the incubation of ESSE with a suspension of intact erythrocytes was analysed six times using both Raman scattering and electronic spectroscopies (Fig. 5). The electronic spectrum [Fig. 5(*a*)] was used to correct for lysis as described above. The number of moles of thiol was found from the calibration graph using the relative intensity, $(ES^-_{corr})/ESSE$, from the Raman spectrum [Fig. 5(*b*)]. The replicate analysis gave an $(ES^-_{corr})/ESSE$ ratio of 0.576 ± 0.007 (s_r 1.2%). From the calibration graph, the number of thiol groups present on the membrane of 0.5 ml of erythrocytes was found to be 85 nmol.

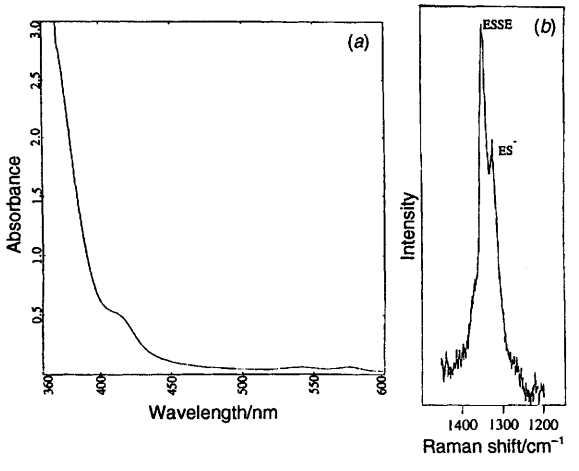

Fig. 5 (*a*) Electronic and (*b*) Raman spectra from the supernatant of the reaction of 0.5 mmol l^{-1} ESSE (3 ml) with 0.5 ml of packed erythrocytes.

Release of Intracellular Thiols

It has been claimed that GSH can leak from red cells without lysis. To determine whether appreciable amounts of GSH were released from the cell without lysis, the supernatants from erythrocytes suspended in PBS for 30, 60 and 90 min, were reacted with ESSE. The Raman spectra showed no ES^- intensity which could not be attributed to lysis, as estimated by the ES^-/ESSE-to-Hb peak ratio. This experiment was repeated four times with the same result in each case. Hence it can be concluded that appreciable amounts of GSH are not transported across the erythrocyte membrane within the timescale of the experiment.

Conclusion

The sensitivity and selectivity of resonance Raman spectroscopy combined with electronic spectroscopy has enabled the determination of the concentration of thiol groups present on the erythrocyte membrane. This method is applicable *in situ* with viable cells and allows a correction for intracellular thiols released by cell lysis. Most of the problems with this method arise from the thiol reagent, ESSE, in that at low concentrations the scattering intensity particularly of the shoulders on the main peak at 1345 cm^{-1} can vary. The assessment of membrane thiols *in situ* in a manner which permits rapid analysis is a useful method for studies of oxidative stress and for studies of sugar and anion transport. Modern advances in Raman spectroscopy such as the use of charge coupled device detectors and curve fitting should greatly enhance the signal-to-noise ratio and allow better resolution of the signals, providing a powerful method for the measurement of membrane thiols.

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