Electroanalytical adaptation of the classical Ellman’s Assay: Determination of total thiols in incubation media from a biological sample (excised articular cartilage)

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The total thiols in a medium containing authentic biological sample (articular cartilage) has been determined electrochemically. The detection strategy utilises the electrochemical adaptation of the Ellman’s reaction process via the electrochemically initiated reaction of thiols with diethyl-p-phenylenediamine. The strategy has been examined over a biologically significant pH range and the behaviour observed rationalised. The detection of thiols within the biological sample is shown to proceed with micromolar levels of thiol determined within the sample.

The detection of thiol containing amino acids has recently come to the fore due to their importance in biological processes1. As a consequence they are used as biomarkers for a wide range of diseases; an increased level of homocysteine is indicative of cardiovascular disease2, atherosclerosis3, and is associated with common pregnancy complications4, where as high concentrations of glutathione can signify a patient is suffering from AIDS-related dementia, Alzheimer’s disease or Parkinson’s disease5,6. Therefore the physiological importance of thiols is well recognised within the medical and analytical communities.

A large number of comprehensive reviews on the analytical assays for thiol detection have been published7,8, and signify that the majority of the preferred determination processes are based on chromatographic separation before detection. However, these protocols often require the matrix pretreatment followed by post-column sample derivatisation. Therefore there is a pressing need for the development of a protocol which produces fast and sensitive responses with minimal sample pretreatment.

Electroanalytical techniques are well represented in the literature9 and could provide a means of producing such a protocol. These have focused on the direct oxidation of thiols (RSH) at bare solid substrates10-16 and the possibility of suitable redox indicators and electrocatalysts capable of improving both sensitivity and selectivity of the electrode response (for example metal complexes17, metallo-phthalocyanines18,19, bromine reduction20,21 and enzymes22,23).

A more recent development in the electroanalytical determination of thiols species has been the electrochemically initiated derivatisation of phenylene-diamine species by thiols (see Scheme 1)24. The reaction procedure has been shown to be generic in nature and as such an electrochemical adaptation of the classical Ellman’s test25 has been developed (Scheme 2). This involves the reaction of the thiol species and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), producing two equivalents of TBNA and one equivalent of the oxidised thiols species (Scheme 2). The newly generated TBNA species can subsequently attack the electrochemically oxidised DEPD species thereby forming the electrochemically

Scheme 1—The proposed detection pathway

Scheme 2—Ellman’s reaction
active TBNA-DEPD adduct which provides the analytical signal. This methodology has been shown to work for a wide range of thiol species each producing the same analytical signal and therefore allows the determination of the total thiol (R-SH) concentration in the sample of interest.

In the paper outlined this previously reported technique has been used to determine the total thiol content in a incubation medium from a biological sample. The results reveal experimental adjustments to those reported previously which have improved the sensitivity and allowed the determination of thiols within the matrix.

Materials and Methods

All reagents were of the highest grade available and used without further purification. All solutions and subsequent dilutions were prepared daily using deionised water from an Elgastat (Elga, UK) UHQ grade water system with a resistivity of not less than 18 MΩ cm and were refrigerated when not in use. Electrochemical measurements were conducted using an Autolab computer controlled potentiostat (Eco-Chemie, Utrecht, Netherlands) with a standard three electrode configuration and a typical cell volume of 5 cm³ maintained at a temperature of 20°C ± 2°C. Glassy carbon (GC, 3 mm diameter, BAS Technicol, UK) served as the working electrode with spiral wound platinum wire as the counter electrode and a saturated calomel (Radiometer, Copenhagen) reference electrode. The GC electrodes were polished between experiments with diamond pastes of decreasing particle size (6 μm to 1 μm, Kemet, UK).

Bovine articular cartilage preparation

The bovine articular cartilage was excised on the day of analysis from the metacarpal-phalangeal joints of 2-3 feet and washed several times with 0.15 M NaCl. This was followed by a further three washes with a Salts and Glucose medium (see Appendix). The cartilage was then divided evenly into two samples, with approximately 15 mL of Salts and Glucose added to each sample. The anoxic media was bubbled with nitrogen through a cannula for 5 min before the bottle was sealed. These samples were then incubated on a rotary Hybaid at 37°C from 20 to 45.5 h (Table 1).

After the incubation period the samples were analysed immediately by means of a standard addition experiments (see below) in order to determine the concentration of thiol species present.

General assay protocol for the determination of total thiols in the sample

For electrochemical experiments, 2.5 mL of the biological sample was placed into the electrochemical cell. To this solution aliquots of DEPD (25 μM) and DTNB (25 μM) were added. This solution was then degassed thoroughly with nitrogen prior to commencing the electrochemical measurements. Once the electrochemical experiments were being conducted a nitrogen stream was passed across the solution to maintain an inert (oxygen free) atmosphere.

Linear sweep voltammetry (starting potential +0.5 V, pre-conditioning time 2 s, end potential -0.2 V, scan rate 50 mV s⁻¹) was utilised throughout the experiments with a standard addition protocol being utilised to determine the concentration of thiol species. In order to overcome cumulative electrode fouling by the species within the sample the electrode was polished after each experiment with the voltammetric response being recorded three times.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incubation conditions</th>
<th>Incubation period, h</th>
<th>Dry weight of cartilage, g</th>
<th>pH of incubation medium at end of experiment</th>
<th>Concentration of total thiols in incubation medium at end, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>7.0</td>
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<tr>
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<td>1.09</td>
<td>7.3</td>
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<tr>
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<td>Anoxic</td>
<td>45.5</td>
<td>2.53</td>
<td>5.9</td>
<td>11.0</td>
</tr>
</tbody>
</table>

*The bottle containing the cartilage was stood in boiling water for 20 minutes. The cartilage was then cooled and washed several times in 0.15 M NaCl before anoxic incubation began.
between each addition in order to ensure reproducibility.

Results and Discussion

The reaction procedure has been examined in the incubation solution and over a range of biologically relevant pH.

Total thiols (Ellman's assay)

Initial studies focused on the examination of the reaction process in the incubation buffer (see appendix) at pH 7.4. The corresponding linear sweep voltammograms detailing the reduction of DEPD (25 μM) and DTNB (25 μM) in the incubation buffer to increasing additions of glutathione (5 μM) are detailed in Fig. 1. In the absence of the glutathione a single reduction peak is observed at +0.075 V (vs. SCE), which can be attributed to the two-electron reduction of the oxidised DEPD species as detailed in Scheme 1. Upon the introduction of the thiol species (glutathione) to the solution a new reduction wave at ~0.025 V is observed. This can be assigned to the reduction of the oxidised TBNA-DEPD adduct as depicted in Scheme 2.

Analysis of this new reduction wave by means of a standard addition plot (inserted in Fig. 1) revealed a linear range from 5 to 20 μM. In order to show that the limit of detection could be further decreased an analogous experiment was undertaken: 1 μM glutathione additions were added to the solution. It is seen that on addition of the glutathione to the sample solution a new reduction wave was observed at ~0.015 V consistent with that detailed in Scheme 1. Analysis of these sets of data for the 1 μM and 5 μM standard addition plots revealed a linear range from 1 to 20 μM and a corresponding limit of detection of 0.51 μM, which is an enhancement with that reported previously.

In order to study the reaction process further the effect of pH on the analytical signal was examined. This is required as the used incubation medium pH varies between the samples (Table 1) and therefore in order to minimise sample pre-treatment (adjusting the pH of the solution) it would be convenient for the reaction process to work over biologically relevant range of pH (5-7.5). The voltammetric response was examined over a pH range (5 to 7) each solution containing 50 μM DEPD and 50 μM DTNB. The corresponding wave shapes obtained were analogous to those observed at pH 7.5 (Fig. 1); in the absence of any thiols species only a single reduction wave was observed corresponding to the reduction of the oxidised DEPD species, and on the addition of a thiol (cysteine, 5 to 25 μM) to the solution a new reduction wave was observed at potentials lower than that for the reduction of the DEPD species which is attributed to the reduction of the oxidised DEPD-TBNA species.

Analysis of the voltammetric waves by means of plots of peak current for the reduction attributed to the reduction of the DEPD-TBNA species against concentration are detailed in Fig. 2. These results reveal that as the pH decreases the sensitivity of the electrochemical Ellman's reaction also decreases. This behaviour is confirmed by the analysis of the analytical parameters obtained for each pH (Table 2). This data reveals that as the pH is decreased the limit of detection increases consistent with a decrease in the sensitivity. This decrease in sensitivity can be explained by a knowledge of the pKₐ of TBNA which is 4.9. It can be rationalised that as pH tends towards

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**Fig. 1**—Linear sweep voltammograms (scan rate = 50 mVs⁻¹) detailing the response of 25 μM concentration of DEPD and DTNB in Salts and Glucose Medium to increasing additions of 5 μM glutathione at glassy carbon electrode. Insert: A plot of peak current (at ~0.150V vs SCE) against concentration for a solution containing the 25 μM DEPD and 25 μM DTNB and increasing concentrations of glutathione.

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**Fig. 2**—The plots of peak height against concentration of cysteine for a solution containing the 50 μM DEPD and 50 μM DTNB in various pH buffers: (●) pH 7 phosphate buffer, (▲) pH 6 phosphate buffer, (●) pH 5 acetate buffer.
this value the sulphur moiety of the TBNA species will become protonated and as such that the reaction with DEPD will be inhibited.

**Analysis of the medium in which cartilage had been incubated**

The assay procedure for the used incubation medium has been described previously with a standard addition protocol being developed in order to determine the quantity of reduced thiol present.

The initial voltammetric response was recorded in the used incubation medium before the addition of the DEPD and DTNB species, to ensure there was no electroactive species in the potential range studied. The corresponding linear sweep voltammogram (not shown) indeed revealed no appreciable voltammetric activity in the potential range and therefore allows the total reduced thiol to be determined using the proposed methodology.

After this preliminary study the relevant concentrations (25 µM) of DEPD and DTNB was added to the solution. The corresponding reductive linear sweep voltammogram for the response of a glassy carbon electrode placed in the used incubation medium in the presence of DEPD and DTNB are detailed in Fig. 3. These reveal two reductive waves at +0.135 V and +0.020 V corresponding to the reduction of the oxidised DEPD and DEPD-TBNA adducts.

In order to check the reproducibility of the initial voltammetric signal shown in Fig. 3, several repetitive scans were recorded. However, it was observed that upon repetitive scanning the clarity of the voltammetric signal was decreased. This diminishing signal can be tentatively attributed to the fouling of the electrode by species within the biological sample. In order to overcome this problem of electrode fouling the electrode was polished between each voltammetric scan, and the voltammetric signal was recorded three times between each addition in order to verify the stability and reproducibility of the electrode response.

Also detailed in Fig. 3 is the effect of increasing the concentration of glutathione in the sample solution. Upon the addition of glutathione (1 to 5 µM) to the sample the reductive wave at +0.020 V increases in magnitude consistent with the results detailed above and therefore confirms that this reduction wave is due to the reduction of the DEPD-TBNA species. The corresponding standard addition plot for the sample is inserted in Fig. 3. The results reveal a linear response to glutathione additions, with the results showing that 9.3 µM is present in the biological articular cartilage.

Further analysis of other biological articular cartilage samples were also undertaken in order to examine the applicability of the detection procedures for biological procedures. The resulting concentrations of total thiols determined in a range of media, which had undergone various preparation conditions are summarised in Table 1. These results show how the concentrations of total thiol varies between each sample and may be related to the age or history of the animal which is being examined.

It can also be seen from Table 1 the effect of boiling the cartilage before anoxic incubation in order to kill the cells. No thiols were detected in the solution from this treatment, showing the dependence of thiol production on living cells.

**Conclusions**

The above report illustrates the practical application of a previously developed electrochemical detection protocol for the determination of total thiol species produced by biological cartilage. The reaction
was examined over a range of biologically relevant pH (5-7.5) and it was found that as the pH was decreased the sensitivity was also found to decrease whilst the limit of detection increased.

Furthermore the detection methodology has been utilised for incubation medium from a real biological sample with problems caused by surface fouling overcome by the use of repetitive polishing. The fact that thiols were detected in the sub-micromolar level in the medium shows the detection process works and encourages the adaptation of such protocols into single shot screen printed electrodes which would obviate the need for repetitive polishing in the analytical experiment.

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Appendix
Components of “Salts and Glucose” Medium
Potassium chloride 400 mg/L
Magnesium sulphate, anhydrous 98 mg/L
Sodium chloride 5900 mg/L
HEPES 5958 mg/L
Sodium dihydrogen orthophosphate, anhydrous 100 mg/L
Calcium chloride, dehydrate 265 mg/L
Glucose 1000 mg/L

Adjusted with sodium hydroxide to pH 7.4 in air.

References