

Molecular anchors—mimicking metabolic processes in thiol analysis

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The interaction between various novel naphthoquinones and glutathione was explored using a variety of electrochemical techniques. An adamantane derivatised quinone was found to adsorb strongly to carbon surfaces providing a robust film possessing a distinct and consistent voltammetric profile which is markedly different from solution based species. The anodic peak process was found to respond to increasing additions of glutathione and the analytical merits have been assessed. In contrast to conventional thiol–quinone electrochemistry, the detection pathway involves the solubilisation and removal of the hydrophobic quinone derivative through conjugation with glutathione. The peak potential (+0.2 V vs. Ag/AgCl) is such that an unambiguous response to glutathione can be achieved in the presence of a significant excess (1 mM) of common physiological components.

Introduction

Glutathione is a sulfur containing tripeptide and is one of the prime protective agents within physiological systems. It facilitates detoxification through a number of mechanistic pathways ranging from direct and enzyme mediated electron transfer through to metal complexation and more elaborate conjugation processes.^{1–3} The latter is best exemplified through its role in paracetamol metabolism whereby the reactive quinone imine intermediates resulting from metabolism of the analgesic are speedily removed through Michael addition by the glutathione present within the liver.³ The toxicity associated with overdose is related to the effective depletion of the glutathione reserves. The hydrophilic nature of the peptide also facilitates the excretion of conjugated metabolites through increasing the solubility of hitherto hydrophobic moieties.^{1–3} The present communication has sought to explore and exploit the native metabolic action outlined above as a means of quantifying thiols using a novel electrochemical approach.

Quinone–thiol interactions have been investigated as both a spectroscopic and electrochemical diagnostic tool for the quantification of reduced thiol species.^{4–7} Naphthoquinone, in particular, has been shown to exhibit higher selectivity than most benzoquinone derivatives as the former is largely unaffected by the presence of ascorbate—a prime interferent in electroanalytical investigations.⁷ The basic assay is highlighted in Fig. 1A where cyclic voltammograms detail the typical responses obtained when glutathione is added to a solution containing naphthoquinone. The magnitude of the reduction peak decreases within increasing concentration of the thiol and

can be attributed to the reaction scheme highlighted in Fig. 1B. The naphthoquinone (I) is subject to a 1,4-Michael addition by the nucleophilic thiol species (II) resulting in the generation of the corresponding reduced conjugate (III). As such, when the cathodic scan commences after each addition there is progressively less oxidised naphthoquinone (I) available within the bulk of the solution and hence the magnitude of the reductive electrode process diminishes accordingly. It is worthwhile noting that the oxidation process does not increase but rather broadens out. This is in line with the emergence of the new redox couple associated with the conjugate (III) rather than the simple redox cycling of the native naphthoquinone.

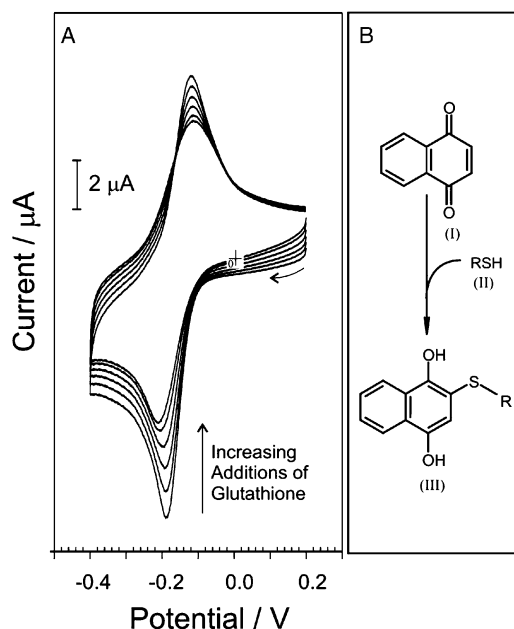


Fig. 1 (A) Response of naphthoquinone (1 mM, pH 7) to increasing additions of glutathione (25 μM). (B) Reaction scheme.

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Table 1 Naphthoquinone derivatives investigated

R	
	(IV)
	(V)
	σ (VI)
	m (VII)
	p (VIII)
	(IX)
	(X)

The key parameter in most assays of this type is that both indicator and target analyte are present within the solution.^{4–7} In this instance, however, we have sought to explore the potential for localising the naphthoquinone indicator directly at the electrode surface. This would have the advantage of generating an unambiguous signal which would be modified in the presence of thiol.

Our strategy was to develop a number of bifunctional molecular assemblies through tethering naphthoquinone derivatives to an appropriate pendant group. The former serves as the thiol selective hook with the latter providing the hydrophobic anchor through which the assembly could reversibly adhere to the electrode substrate. The principal species investigated are shown in Table 1.

Results and discussion

There was considerable variation in the response characteristics for the various derivatives, with IV through to VIII all producing an initial voltammetric behaviour analogous to that of the naphthoquinone (I). There was, however, no appreciable change in the profile with IV and V upon the addition of glutathione and this can be attributed to the electron donating properties inherent to the hydroxy and methoxy functional groups. The increase in electron density across the double bond effectively reduces the susceptibility to nucleophilic attack. The converse is true with the remaining derivatives—all of which show marked reactivity towards glutathione. The response of the *p*-tosyl derivative (VIII) is highlighted in Fig. 2 where the emergence of a new redox peak process associated with the reduction of the conjugate can be clearly observed at -0.2 V.

Voltammograms detailing the electrode response to the solution based acyloxy (1 mM, pH 7) derivative are detailed in Fig. 3. The profile is substantially more complex than that observed with the tosyl systems and is attributed to two factors. Hydrolysis of the ester bond is relatively rapid, resulting in the emergence of peaks (-0.4 V and -0.29 V) associated with the parent 2-hydroxy-1,4-naphthoquinone (IV). The identity of the latter was confirmed by adding an aliquot of a standard solution of IV to the cell (dotted line, Fig. 3).

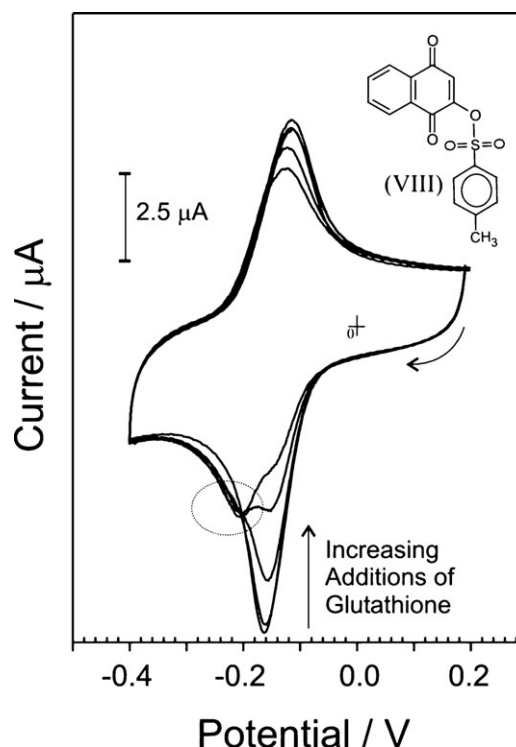


Fig. 2 Response of VIII (1 mM, pH 7) to increasing glutathione concentration (25 μ M) and the emergence of a conjugate redox process (dotted circle).

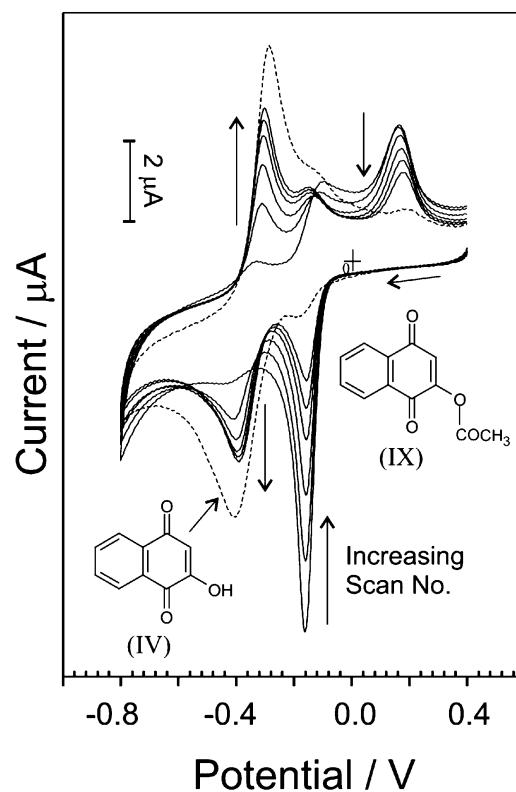


Fig. 3 Repetitive cyclic voltammograms of IX (1 mM, pH 7) highlighting the hydrolysis of the ester linkage. Cyclic voltammogram of IV is included for comparison.

The second factor is that the peak separation between the reduction (-0.18 V) and associated oxidation ($+0.19$ V) process for the ester derivative is 370 mV which is significantly greater than that found for the previous investigations (I \rightarrow VIII). The peak separations observed with the 2-hydroxy (IV), 2-methoxy and *p*-tosyl naphthoquinone (VIII) were 110, 95, 92 mV respectively and, while larger than that expected of a 2 electron system, are nevertheless consistent with the quasi reversible behaviour of the simple NQ derivatives at carbon substrates. The separation is a consequence of both the ester functionality and the increased adsorption of the derivative at the electrode surface. This is clear from the Gaussian shaped profile observed in the ester peaks ($+0.19$ V) which contrast those of the previous examples where diffusion is still an important contributing factor.

The derivatives investigated thus far have provided some valuable insights into the nature of the tether that needs to bind the quinone to the substrate anchor but none are sufficiently hydrophobic in themselves to serve as our detection indicator. The proposed detection technique requires a quinone derivative that is effectively insoluble within aqueous solution, hence the inclusion of the hydrophobic adamantane pendant group (X) within Table 1.

The adamantane substituent was purposefully attached to a 2-hydroxy-1,4-naphthoquinone core through ester linkages (X) rather than an ether linkage. While the latter would be inherently more stable to hydrolysis, the preliminary investigations of V implied that there would be little reaction between the available ring position and thiol targets whereas the electron withdrawing properties of the ester functionality would retain the native quinone reactivity towards thiol moieties. It was hoped that the greater steric bulk of the adamantane pendant would impede hydrolysis.

Derivative X was found to be essentially insoluble in aqueous buffer (pH 7) but could be anchored to the electrode substrate through either solvent casting or dip coating (from either an acetone solution of the ester or aqueous suspension). Cyclic voltammograms detailing the response of an electrode modified with X in fresh buffer (pH 7) are detailed in Fig. 4A. The profile obtained is again distinct from that obtained with the simple solution based naphthoquinone (Fig. 1) and is characterised by the sharp reduction and oxidation peak processes expected for adsorbed redox species. The peak separation (410 mV) is again substantially greater than that expected for such a couple and mirrors the response obtained with the simpler, methyl ester (Fig. 3). The major advantage is that in this instance there is no hydrolysis—confirmed by the absence of any 2-hydroxy-1,4-naphthoquinone peaks (IV).

The quinone peaks were found to be stable in the buffer solution and did not diminish in magnitude, thus confirming the anchor like properties of the derivative. Cyclic voltammograms detailing the response of the modified electrode to increasing glutathione concentration (25 μ M) are shown in Fig. 4B. The introduction of the thiol serves to solubilise the adsorbed quinone and effectively lifts the anchor into the bulk of the solution. This is shown by the marked decreases in the heights of both the oxidation and reduction peaks associated with the adsorbed quinone–adamantane assembly and is

analogous to the behaviour observed with the simple naphthoquinone solution detailed in Fig. 1A.

The emergence of a second oxidation process (-0.12 V) in the cyclic voltammograms detailed in Fig. 4B is attributed to the resulting glutathione–quinone conjugate. This exhibits the broad diffusion limited profile expected of a solution based system and clearly contrasts the sharp adsorption profile obtained in the absence of the thiol. Square wave voltammograms detailing the response of the oxidation peak processes to increasing glutathione are shown in the inset in Fig. 4C.

The solubilising effect of the glutathione conjugate as the principal mode of action at the electrode surface was confirmed by removing the electrode from the test solution (after holding at $+0.4$ V for 30 s) and then re-scanning the voltammogram in fresh buffer solution. In the absence of glutathione this could be done repeatedly with only a minimal decrease (less than 1%) in the quinone peak heights. In the case where the glutathione (25 μ M) is allowed to react with the quinone assembly, the subsequent fresh buffer test resulted in the complete removal of the quinone signature. Derivative X was further modified with a methyl group substituted into the free quinone ring position (derivative XI) to preclude reaction with glutathione. The entire process was repeated—with the new indicator deposited onto the electrode from an acetone solution and placed in fresh buffer. The voltammetric profile (not shown) was analogous to that for X and, as in the previous case, the electrode could be repeatedly removed and replaced in fresh buffer without accruing any significant loss in signal intensity. In this case, however, there was no change in the profile upon the introduction of glutathione. This confirmed the necessity of having the free proton as the thiol selective hook and that the process is not a simple redox interchange.

The reduction of quinones within well buffered aqueous media ordinarily will tend to follow a 2 electron/2 proton

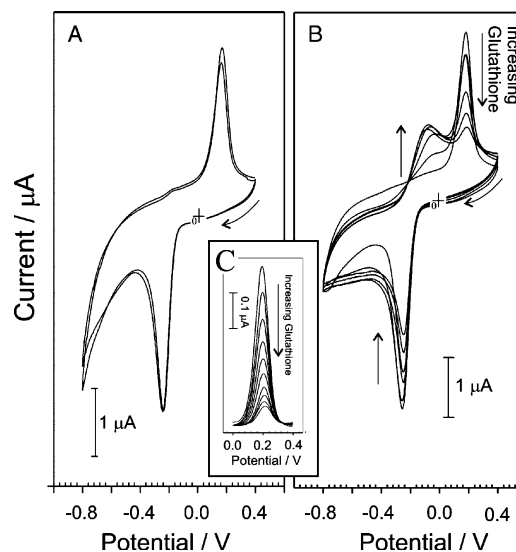


Fig. 4 (A) Voltammograms detailing the response of adsorbed X in fresh pH 7 buffer. (B) Influence of glutathione (25 μ M additions) on the cyclic voltammetric behaviour of adsorbed IV in pH 7 buffer. (C) Square wave voltammograms detailing the removal of adsorbed X with increasing glutathione concentration.

route—whereby the initial reduction to the anion is rapidly followed by protonation leading to the production of the hydroquinone analogue through an ECEC process. In aprotic or poorly buffered medium, then, it could be expected that sequential 1 electron conversions to the semiquinone anion and lastly to the dianion would be observed.^{8–12} Interestingly, the oxidation of hydroquinones within such systems tends to exhibit electrochemically irreversible behaviour as a consequence of the localised lowering of pH. The voltammograms detailed in Fig. 4A show a number of similarities with the latter and may arise as a consequence of the film structure on the electrode surface. This may be attributed to a combination of the hydrophobic nature of the film and the possible exclusion of the quinone moieties from the bulk solution. This would mean that the redox species experience an environment akin to that of the poorly buffered systems and hence explain the deviation of the profile from conventional reversible systems normally expected in buffered aqueous media. Close inspection of the voltammetric profile detailed in Fig. 4A reveals the presence of a small anodic process at -0.05 V. The intensity of this process was found to increase markedly relative to the existing, and hitherto principal, anodic process ($+0.2$ V) when the scan rate was increased (Fig. 5). This can be attributed to the re-oxidation of the anionic form of the semiquinone prior to protonation. This is now rate limiting as indicated by the scan rate dependence. At slow scan rates there is sufficient time for the protonation of the anionic sites within the film before scan reversal. The increased scan rate effectively captures the transition between semiquinone anion and quinone with the oxidation of the hydroquinone formed in competition by the ECEC process appearing as the anodic peak at higher potentials ($+0.2$ V). A similar process is observed with the acyloxy (IX) derivative shown in Fig. 3.

Upon reducing the quinone to the hydroquinone form the derivative becomes momentarily more soluble as a consequence of the conversion from quinone to anion to the

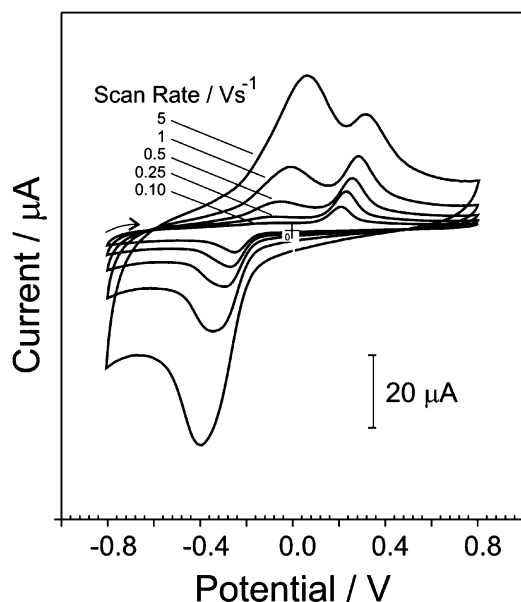


Fig. 5 Influence of scan rate on the voltammetric response of adsorbed X in fresh pH 7 buffer.

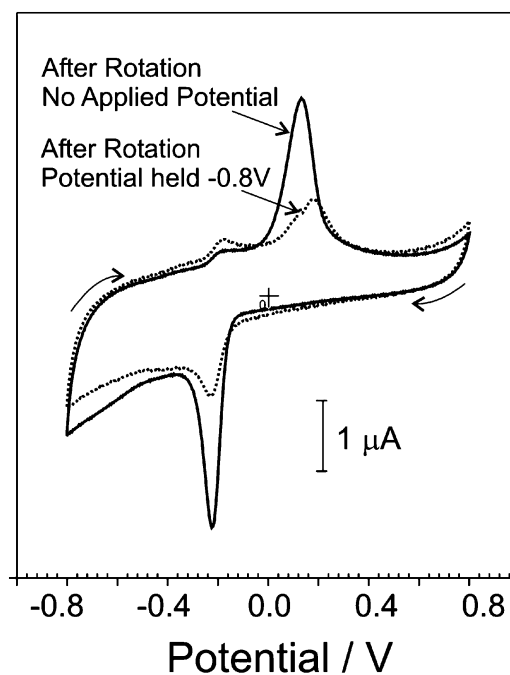


Fig. 6 Cyclic voltammograms detailing the response obtained after rotation (3000 rpm, 2 min) at open circuit (solid line) and at -0.8 V (dotted line).

protonated form. As such it is possible that in a convective system significant losses of material may occur. In order to test this hypothesis a rotating disk experiment was conducted. The rationale behind the experiment is that if there is a significant difference in the solubility of the components as a consequence of the reduction process then the rotation should remove the material from the electrode before it can reassemble within the layer. The derivative was adsorbed onto a 3 mm glassy carbon disk electrode and placed within fresh pH 7 buffer. The electrode was rotated repeatedly at 3000 rpm for two minute periods until a stable voltammogram was obtained. This was done under open circuit and was designed to effectively remove loosely bound material. The cyclic voltammogram obtained is shown in Fig. 6 and was found to be stable, indicating that the oxidised form is strongly adsorbed to the electrode surface. The electrode was again rotated at 3000 rpm but in this instance the electrode potential was held at -0.8 V—the potential being applied after commencing the rotation. The voltammograms show that upon rotation at -0.8 V substantial losses of material for the adsorbed layer occurred. It was not, however, possible to completely remove the quinone layer.

Analytical characterisation

Essentially identical responses were obtained to glutathione, cysteine and homocysteine and they were linear up to $250 \mu\text{M}$ of thiol (peak current/A = -0.00128 ; [glutathione/mol L⁻¹] -6.6×10^{-9} ; $N = 5$; $R^2 = 0.992$) with a detection limit in the order of $5 \mu\text{M}$ (based on $3s_b$). The narrow linear range is due to the film coverage as the signal is based on a decreasing peak intensity. Increasing the adsorption time had little effect on increasing the height of the oxidation peak but it is susceptible

to variation in electrode area. This will obviously influence the inter-electrode reproducibility (>10%) within the present system given the *ad hoc* nature of the preparation.

The selectivity of the approach was assessed in terms of the influence of common functional groups that could have nucleophilic character and hence could act in much the same way as the thiol targets. There was no response to lysine (up to 1 mM) but this could be expected as the alkyl amino will be predominantly protonated at physiological pH. The response to histidine was also assessed as the imidazole substituent possesses amine functionality that has a pK_a of 5 and hence nucleophile potential.^{13–15} There was essentially no response to the amino acid (1 mM). In this case the lack of response can be attributed to the resonance within the imidazole ring which under the conditions of the experiment did not present an effective challenge to the film. It is likely, however, that reaction would occur over a longer timescale.¹³ The influence of ascorbate, paracetamol and urate was also assessed. Only the former presented problems for the interpretation of the voltammetric profile as the others are oxidised at a potential significantly more positive than the quinone oxidation peak. Square wave voltammograms detailing the response of the film to increasing additions of ascorbate (200 μ M) are shown in Fig. 7.

The peak separation between the quinone and ascorbate is close but resolution is still possible. It is also important to note that the concentration of ascorbate in the test solution is significantly higher than those expected within normal physiological fluid (typically 100–200 μ M¹⁴) and hence the pre-

sence of the antioxidant should not compromise thiol detection. There is another feature of the film and the proposed detection method which could be of analytical value and is significantly different from conventional electrochemical thiol measurements. Should there be a case where the voltammetric peak of the quinone oxidation is obscured by the presence of another electroactive component, matrix exchange could be employed to counter the lack of initial resolution—providing the interfering substance does not chemically interact with the film. It can be seen from Fig. 7 (dotted line) that the peak integrity is retained upon removing the electrode from the solution and placing it in fresh buffer. It could be anticipated that upon exposing the electrode to the test solution any thiols present would react and induce the removal of the film constituents. Upon removal of the electrode to fresh solution the concentration of thiol could be unambiguously quantified from the change in the film peak height. This is admittedly more clumsy than a direct analysis. Nevertheless, the chief concept of thiol solubilisation as a diagnostic tool has been shown to be, in principle, viable with a sensitive signal that is within a dynamic range potentially suitable for a host of biomedical applications.

Conclusions

A range of novel naphthoquinones have been investigated with a view to assessing the molecular architecture needed to induce their speedy and electrochemically quantifiable reaction with reduced thiols. The potential applicability of a wholly new electroanalytical strategy for the determination of glutathione, cysteine and homocysteine has been evaluated and two distinct approaches outlined. The system has been shown to be selective for RSH functionality with little interference from other physiological agents. A strategy has been developed that outlines a potential route for further exploitation and a molecular framework highlighting the key structural and electronic properties required for future development has been highlighted. While the detection rationale is not suitable for *in vivo* applications, there is increasing interest in the use of thiols as biomarkers for oxidative stress processes in a wide range of health care contexts and it is possible that the approach advocated here may be of value within *in vitro* based decentralised testing contexts.⁷ As the detection pathway involves the cumulative removal of the indicating species, any potential sensor would necessarily be a single shot device. Nevertheless, there is increasing interest in the analysis of antioxidant thiols in a host of diverse biochemical processes such that their role can be fully understood and disease pathways elucidated.^{16–20} This present communication offers the possibility of adding another diagnostic tool through which these can be investigated.

Experimental

Methods and materials

Electrochemical measurements were conducted using a μ Autolab type III computer controlled potentiostat (Eco-Chemie, Utrecht, The Netherlands) using a three electrode

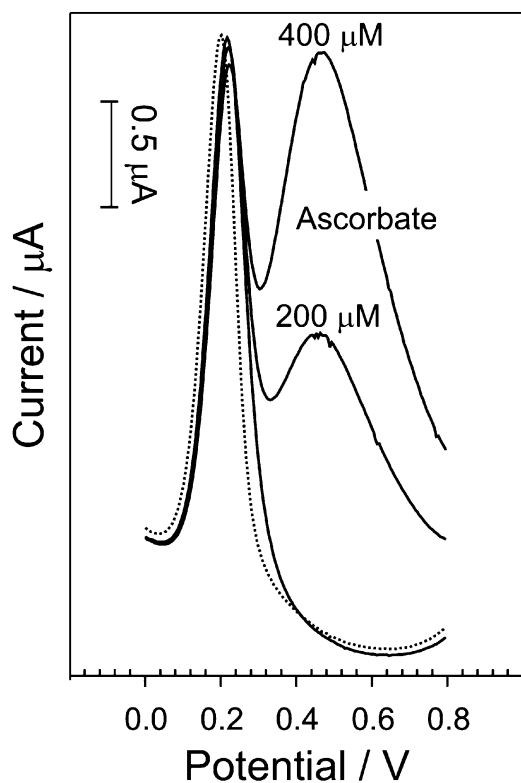


Fig. 7 Square wave voltammograms detailing the response obtained in the presence of ascorbate. The response of the same film after replacing the solution with fresh buffer is also shown (dotted line).

configuration consisting of a glassy carbon working electrode (3 mm diameter, BAS Technicol, UK), a platinum wire counter electrode and a 3 M NaCl Ag | AgCl half cell reference electrode (BAS Technicol, UK). All measurements were done under nitrogen. NMR spectra were measured on a JEOL ECX 400 MHz spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). Mass spectra were recorded at the EPSRC Mass Spectrometry Centre at Swansea University. Microanalysis was carried out using a Leeman CE 440 automatic elemental analyser. Flash chromatography was performed on 40–63 silica gel (Merck). All chemicals and solvents were bought from either Acros Organics or Sigma Aldrich and used without further purification. Quinone derivatives IV and V were obtained from Sigma.

Syntheses

General procedure. To a stirred solution of the appropriate starting quinone (typically 1 g) and a two fold excess of acid chloride (toluenesulfonyl chloride, acetyl chloride or adamantane-1-carbonyl chloride) in acetone (160 ml) was added anhydrous potassium carbonate (1.58 g). The reaction was stirred overnight at room temperature. The red reaction mixture was poured into dichloromethane (50 ml) and washed with distilled water (3 × 100 ml). The organic layer was isolated, dried with sodium sulfate and evaporated under reduced pressure. The crude product obtained was purified by column chromatography using silica gel as adsorbent and chloroform as eluent.

Derivative VI: 1,4-Naphthalenedione, 2-[[[(2-methylphenyl)sulfonyl]oxy]. Yield 1.15 g (27%) of 1,4-naphthalenedione, 2-[[[(2-methylphenyl)sulfonyl]oxy] (as the second fraction), as a bright yellow solid.

¹H NMR (d-CDCl₃) δ 2.81 (s, 3 H), 6.94 (s, 1 H), 7.34–7.38 (t, 1 H), 7.43–7.45 (d, 1 H), 7.57–7.60 (t, 1 H), 7.74–7.77 (m, 2 H), 7.98–8.00 (d, 1 H), 8.06–8.08 (m, 2 H). ¹³C NMR (d-CDCl₃) δ 20.48, 125.22, 126.31, 126.49, 126.88, 130.18, 130.61, 131.50, 132.95, 133.90, 134.14, 134.55, 134.90, 139.51, 151.25, 178.11, 184.10. CIMS (*m/z*) 346.1 [M + NH₄]⁺. Anal. Calcd for C₁₇H₁₂O₅S: C, 62.19; H, 3.68. Found: C, 62.34; H, 3.37%.

Derivative VII: 1,4-Naphthalenedione, 2-[[[(3-methylphenyl)sulfonyl]oxy]. Yield 0.51 g (27%) of 1,4-naphthalenedione, 2-[[[(3-methylphenyl)sulfonyl]oxy] (as the second fraction) as a bright yellow solid.

¹H NMR (d-CDCl₃) δ 2.47 (s, 3 H), 6.91 (s, 1 H), 7.47–7.54 (m, 2 H), 7.75–7.77 (m, 2 H), 7.83 (brs, 1 H), 7.85 (brs, 1 H), 8.07–8.09 (m, 2 H). ¹³C NMR (d-CDCl₃) δ 21.31, 125.60, 125.68, 126.52, 126.94, 128.67, 129.28, 130.69, 131.59, 134.18, 134.55, 135.13, 135.82, 139.94, 151.38, 178.15, 184.16. Accurate LCMS (*m/z*) 346.074 [M + NH₄]⁺. Anal. Calcd for C₁₇H₁₂O₅S: C, 62.19; H, 3.68. Found: C, 62.05; H, 3.40%.

Derivative VIII: 1,4-Naphthalenedione, 2-[[[(4-methylphenyl)sulfonyl]oxy]. Yield 0.45 g (23.8%) of 1,4-naphthalenedione, 2-[[[(4-methylphenyl)sulfonyl]oxy] (as the second fraction), as a bright yellow solid.

¹H NMR (d-CDCl₃) δ 2.47 (s, 3 H), 6.91 (s, 1 H), 7.36–7.40 (d, 2 H), 7.75–7.77 (m, 2 H), 8.06–8.08 (m, 2 H). ¹³C NMR (d-CDCl₃) δ 21.93, 125.82, 126.63, 127.07, 128.64, 130.06,

130.72, 131.72, 132.36, 134.30, 134.66, 146.52, 151.54, 184.13, 184.31.

CIMS (*m/z*) 346.1 [M + NH₄]⁺. Anal. Calcd for C₁₇H₁₂O₅S: C, 62.19; H, 3.68. Found: C, 61.80; H, 3.68%.

Derivative IX: 2-Acetoxy-1,4-naphthoquinone. Yield 0.21 g (84.6%) of 2-acetoxy-1,4-naphthoquinone (as the first fraction), as a bright yellow solid.

¹H NMR (d-CDCl₃) δ 2.39 (s, 3 H), 6.75 (s, 1 H), 7.76–7.77 (m, 2 H), 8.08–8.10 (m, 2 H). ¹³C NMR (d-CDCl₃) δ 20.67, 126.13, 126.60, 127.06, 131.30, 134.50, 134.10, 134.50, 154.42, 167.83, 178.70, 184.64. EIMS (*m/z*) 216. Anal. Calcd for C₁₇H₁₂O₅S: C, 66.67; H, 3.73. Found: C, 66.48; H, 4.00%.

Derivative X: 2-(Tricyclo[3.3.1.1^{3,7}]decane-1-carboxylic acid), 1,4-naphthoquinone ester. Yield 0.61 g (63.2%) of 2-(tricyclo[3.3.1.1^{3,7}]decane-1-carboxylic acid), 1,4-naphthoquinone ester (as the second fraction), as a bright yellow solid.

¹H NMR (d-CDCl₃) δ 1.68 (brs, 6 H), 1.84 (brs, 3 H), 2.00 (brs, 6 H), 6.63 (s, 1 H), 7.65–7.68 (m, 2 H), 7.97–7.99 (m, 2 H). ¹³C NMR (d-CDCl₃) δ 27.86, 36.38, 38.73, 126.03, 126.50, 126.90, 131.14, 132.01, 134.00, 134.38, 155.22, 173.39, 174.82, 178.67, 184.54. Accurate LCMS (*m/z*) 354.170 [M + NH₄]⁺. Anal. Calcd for C₂₁H₂₀O₄: C, 74.90; H, 5.99. Found: C, 74.75; H, 5.98%.

2-Hydroxy-3-methyl-1,4-naphthoquinone. This was synthesised from 2-hydroxy-1,4-naphthoquinone, hydrogen peroxide and sulfuric acid using the procedure recorded by Kaushik.¹⁵

Derivative XI: 3-Methyl-2-(tricyclo[3.3.1.1^{3,7}]decane-1-carboxylic acid), 1,4-naphthoquinone ester. Yield 0.26 g (67.9%) of 3-methyl-2-(tricyclo[3.3.1.1^{3,7}]decane-1-carboxylic acid), 1,4-naphthoquinone ester (as the second fraction), as a bright yellow solid.

¹H NMR (d-CDCl₃) δ 1.78 (brs, 6 H), 1.94 (brs, 3 H), 2.07 (s, 3 H), 2.11–2.13 (brs, 6 H), 7.72–7.73 (m, 2 H), 8.06–8.08 (d, 1 H), 8.08–8.10 (d, 1 H). ¹³C NMR (d-CDCl₃) δ 9.70, 27.80, 36.33, 38.73, 41.34, 126.49, 126.61, 130.91, 132.06, 133.74, 133.95, 135.93, 151.48, 174.62, 178.01, 184.77. EIMS (*m/z*) 351 (M⁺). Accurate CI + (ammonia) (*m/z*) 368.185 [M + NH₄]⁺. Anal. Calcd for C₂₁H₂₀O₄: C, 75.41; H, 6.33. Found: C, 75.31; H, 6.21%.

Acknowledgements

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