Unravelling the Occurrence of Mediator-Blood Protein Interactions *via* the Redox Catalysis of the Physiological Gasotransmitter Hydrogen Sulphide

Haydn J. Ward,[1] Rhys A. Ward,[2] Nathan S. Lawrence,[2] Jay D. Wadhawan[*1,2]

[1]*Department of Chemistry, The University of Hull,*
Cottingham Road, Kingston-upon-Hull HU6 7RX, United Kingdom.

[2]*Department of Chemical Engineering, The University of Hull,*
Cottingham Road, Kingston-upon-Hull HU6 7RX, United Kingdom.

To be communicated to *ChemistrySelect*

[*]Corresponding author
E.mail: j.wadhawan@hull.ac.uk (JDW)

Abstract

Ferrocenemethanol is employed as an aqueous, homogeneous redox mediator for hydrogen sulphide. The reaction is seen to follow an EC' mechanism over the range $6 \leq pH \leq 9$, with bisulphide reacting four times more rapidly than hydrogen sulphide. In the presence of 10 vol.% hæmolysed blood, greater concentrations of sulphide (as H_2S or as HS^-) are required to achieve the same degree of redox catalysis, compared with the absence of the blood proteins, at both pH 7 and pH 9. It is suggested that this phenomenon derives from a ferrocenemethanol/blood protein interaction, which is first titrated by the sulphide species. This can give rise to a titration-based electroanalytical assay for sulphide in blood, whilst being important for blood-based electrochemical bioassays involving hydrophilic ferrocene derivatives.

Key words: hydrogen sulphide, redox catalysis, ferrocene derivatives, electroanalysis, protein interactions, bioelectrochemistry.

Although recognised as a toxic gas, hydrogen sulphide is currently considered to be a potentially significant physiological signalling molecule (gasotransmitter).[1],[2] Its aqueous solubility (*ca.* 4 g L^{-1} at 20 °C)[3] and acidity constant (pK_{a1} = 6.88 at 25 °C)[4] indicate that it can be found in blood plasma (pH 7.4), predominantly as HS^-, at low concentrations between 20-40 μM, with some reports approaching 300 μM.[2] Although the detection of H_2S or HS^- within blood plasma is challenging, owing to its binding with blood such as plasma proteins,[5] it is nevertheless important for understanding the roles it plays in biology and therapeutics.

A variety of electrochemical strategies for monitoring H_2S have been considered and reviewed.[6-9] One of these is a redox catalytic pathway (EC' mechanism), wherein a mediator (RFc) is oxidised heterogeneously, and this catalyses the oxidation of sulphide in aqueous solution (Scheme 1).[10-12]

$$RFc - e^- \rightleftharpoons RFc^+$$

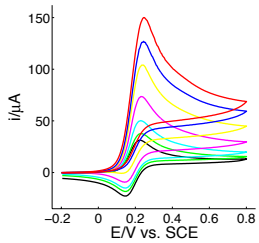
$$RFc^+ + S_{TOT} \xrightarrow{k_{eff}} RFc + S_{TOT}^{+\bullet} \left\{ \begin{array}{c} RFc^+ + H_2S \xrightarrow{k_{H_2S}} RFc + HS^\bullet + H^+ \\ RFc^+ + HS^- \xrightarrow{k_{HS^-}} RFc + HS^\bullet \end{array} \right\}$$

$$HS^\bullet \xrightarrow{fast} \frac{1}{2} HSSH \rightarrow \rightarrow \text{Products}$$

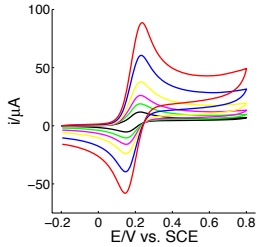
***Scheme 1**: One-electron EC' mechanism for the competing oxidation of bisulphide and hydrogen sulphide using a ferrocenemethanol mediator (RFc). Note that S_{TOT} refers to the total sulphidic species present (bisulphide and hydrogen sulphide), not elemental sulphur.*

Figure 1a illustrates the resulting voltammetric waveshape change, using ferrocenemethanol (RFc) as the mediator for bisulphide oxidation in aqueous solution at pH 9. In the absence of H_2S/HS^-, this mediator undergoes a one-electron oxidation that is effectively electrochemically reversible (peak potentials are independent of scan rate and pH; peak-to-peak separation at 0.1 V s^{-1} is 81 ± 2 mV, independent of pH; data not shown) at glassy carbon electrodes in aqueous solution, and is under diffusion control (peak oxidative currents are directly proportional to the square root of the scan rate; data not shown), with a diffusion coefficient derived from the (reversible) Randles-Ševčík equation as 5.6 ± 1.0 x 10^{-6} cm^2 s^{-1}, independent of pH in the range 6.0 ≤ pH ≤ 9.0, in agreement with literature values.[13],[14] Increasing additions of sulphide cause the peak oxidation current to rise and the reverse peak to diminish in size (Figure 1a). In the pH range tested, the extent of peak current increase with sulphide addition is, as expected, greatest at pH 9 and weakest at pH 6, with, in all cases, the shape of the voltammograms

(a)



(b)(i)



(b)(ii)

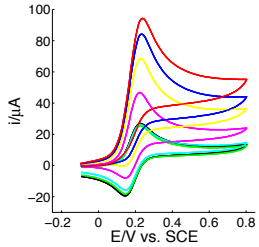


Figure 1

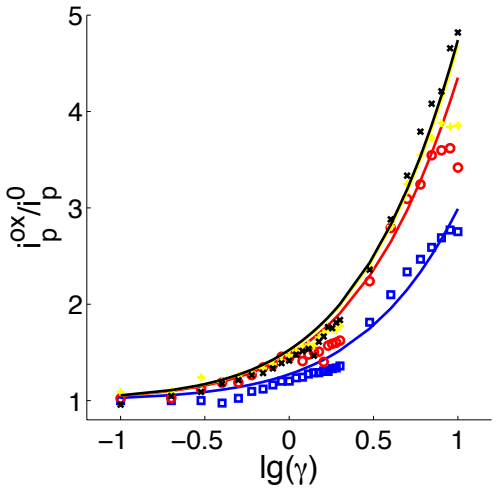
Cyclic voltammograms (i-E curve) of 2.0 mM ferrocenemethanol in aqueous solution containing 0.1 M H_3BO_3 and 0.1 M KCl at pH 9.0 (a), pH 7.0 (unbuffered, bii), and at pH 9.0 with 10 vol.% laked horse blood present (bi), at a scan rate of 0.1 V s^{-1} (a, bii) or variable scan rates (bi), in the presence (a, bii) or absence (bi) of sulphide. In (a) and (bii), the lines correspond to sulphide concentrations of 0 (black), 1.0 (green), 3.0 (blue), 6.0 (magenta), 10 (yellow), 14 (blue) and 20 (red) mM; in (bi), the lines correspond to scan rates of 0.02 (black), 0.05 (green), 0.1 (magenta), 0.2 (yellow), 0.5 (blue) and 1.0 (red) V s^{-1}.

resembling those of the the "general kinetics" (KG) EC' zone.[15],[16] Accordingly, the estimation of the pH-dependent rate constant for the homogeneously catalysed reaction can only be undertaken *via* recourse to numerical simulation.

We make the assumptions that (1) all of the heterogeneous electron transfer kinetics are fast (electrochemically reversible); (2) all diffusion coefficients are identical (this is reasonable for the dilute solutions used); (3) the direct, heterogeneous oxidation of the sulphidic species does not take place in the potential range considered; (4) the bimolecular reaction between the oxidised mediator and the sulphidic species is rate-limiting, so that the oxidised sulphidic species is at steady-state, enabling the homogeneous bimolecular electron transfer reaction to be considered chemically irreversible; and (5) the outer-sphere, bimolecular, electron transfer reaction is very much faster than competing nucleophilic reactions such as (i) the reaction between $FcCH_2OH$ and HS^-/H_2S, and (ii) the reaction between chloride ions and the electrogenerated ferricenium derivative – reactions that are known to be slow at room temperature.[17],[18] In the calculated voltammograms, since the reaction between the ferricenium species and bisulphide competes with that for H_2S, an effective rate constant, k_{eff}, was employed for a total sulphur species concentration, $\left[S_{TOT}\right]=\left[H_2S\right]+\left[HS^-\right]$, such that it depends on pH: $k_{eff}=\frac{k_{H_2S}\left[H^+\right]+k_{HS^-}K_{a_1}}{K_{a_1}+\left[H^+\right]}$. Thus, comparison of theoretical working curves with experimental peak currents (Figure 2a) indicates $k_{H_2S}=375\pm42\ M^{-1}s^{-1}$ and $k_{HS^-}=2230\pm116\ M^{-1}s^{-1}$, in agreement with earlier estimates for similar compounds,[11],[12] and confirming the greater reactivity of deprotonated over protonated sulphide species.[19]

When hæmolysed (laked) horse blood is present (at 10 vol.%), the cyclic voltammograms of ferrocenemethanol appear to be almost identical to those in its absence (Figure 1bi), including, electrochemical reversibility and similar diffusion coefficient ($5.6 \pm 0.2 \times 10^{-6}$ cm^2 s^{-1}). The lack of observed catalysis in the voltammograms suggests, based on the kinetic zone diagram,[16] assuming similar reaction kinetics, that any sulphydryl species (thiol or sulphidic species) already present in the blood, are of, at most, concentrations of 140 μM in the electrochemical cell. Accounting for the dilution, this upper limit correlates well typical values observed in horse blood plasma.[20] Thus, we infer that even though such addition sulphydryl thiols may be present in the blood electrolyte used, their concentration is too low for them to impact on the voltammetry. Likewise, even though reaction

(a)



(b)

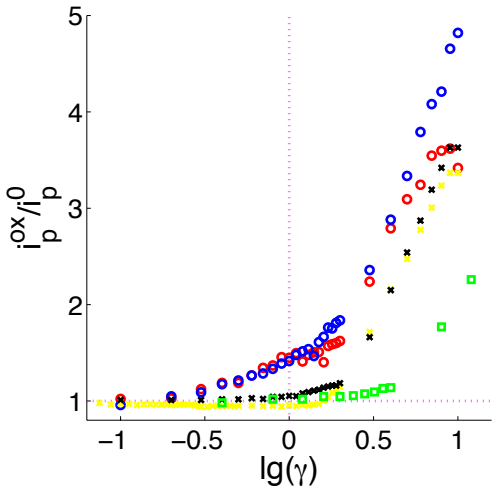


Figure 2

(a) Variation of experimental (symbols) ratios of peak oxidative current, observed at 0.1 V s^{-1}, of 2.0 mM ferrocenemethanol in the presence (i_p^{Ox}) and absence (i_p^0) of sulphide with the excess ratio, $\gamma = \frac{[sulphide]}{[ferrocenemethanol]}$ for experiments undertaken in aqueous solution containing 0.1 M H_3BO_3 and 0.1 M KCl at pH 9.0 (black crosses), pH 8.0 (yellow pluses), pH 7.0 (red circles) or pH 6.0 (blue squares). The solid lines illustrate the comparison of experimental data with ratios predicted theoretically under the EC' mechanism as a function of the excess ratio, with k_{eff} = 591 (blue), 1429 (red), 2099 (yellow) or 2216 (black) M^{-1} s^{-1}.

(b) Comparison of the effect of 10 vol.% laked horse blood on the observation of redox catalysis at 0.1 V s^{-1} with 2.0 mM ferrocenemethanol at pH 9.0 (black crosses in the presence of blood, vs. blue circles in the absence of blood); pH 7.0 (yellow crosses in the presence of blood, vs. red circles in the absence of blood); and 0.5 mM ferrocenemethanol at pH 9.0 in the presence of blood (green squares). The dashed, magenta lines correspond to i_p^{Ox}/i_p^0 = 1.0 and γ = 1.0.

kinetics between oxidised ferricenium derivatives and ascorbic acid are faster than the corresponding reaction with sulphidic species ($10^5 – 10^7$ M^{-1} s^{-1}),[21] the kinetic zone diagram indicates that the concentration of ascorbic acid in the electrochemical cell is less than 60 – 90 μM, since no catalysis is observed. This range is actually larger than typical ascorbic acid concentrations in horse blood (*ca.* 50 μM).[20] Hence, from an electrochemical perspective, the diluted blood electrolyte used behaves as though it free from the reduced (or oxidisied) forms of thiols/antioxidants.

However, the homogeneous redox catalysis requires significantly larger amount of sulphide stock solution to be added, at both pH 7 and pH 9, *q.v.* Figure 2b. Whilst this, at first sight, might be thought as due to the presence of blood-based oxidants in the electrolyte solution, this is discounted since catalysis is observed to occur when the sulphidic species has a concentration greater than, or equal to, the ferrocene derivative concentration (*viz.*, $\gamma \geq 1$), at mediator concentrations that vary by a factor of four – see Figure 2b. (Note that all other things remaining constant, the degree of catalysis observed in the EC' mechanism is affected by both the mediator concentration and the analyte excess ratio.) Thus, the relative ratio of sulphidic species-to-mediator is more significant that those of ferrocence-to-blood oxidant, or sulphidic species-to-blood oxidant. This indicates that there is a matrix effect on the ferrocene derivative, rather than a matrix effect on the sulphidic species, and that the sulphidic species has to titrate this first. Accordingly, given earlier literature work that demonstrates kinetic effects of ferrocene/protein interactions through electrochemical immunoassay,[22] we suggest the presence of a blood protein/ferrocenemethanol complex ($FcCH_2O$-BP), which rapidly forms in a pre-equilibrium process and serves to decrease the pH of the blood/electrolyte suspension. This $FcCH_2O$-BP complex, whilst electroactive, must be fast in reacting with the nucleophilic sulphide species to afford a less active blood protein thiol (BP-SH or BP-S^-) as a further pre-equilibrium to the electrode reaction, Scheme 2.

$$FcCH_2OH + BP \rightleftharpoons FcCH_2O - BP + H^+$$

$$FcCH_2O - BP + H_2S \rightleftharpoons BP - SH + FcCH_2OH$$

$$FcCH_2O - BP + HS^- \rightleftharpoons BP - S^- - BP + FcCH_2OH$$

Scheme 2: *Proposed reaction pre-equilibria for the formation of a mediator/blood proteins (BP) complex, and its nucleophic reaction with sulphide.*

In summary, the presence of blood proteins may encourage association with an hydrophilic mediator with nucleophilic moieties. This can elicit an unusual change in the reaction mechanism, which, for substrates that also can behave as nucleophiles, such as sulphides, requires a larger concentration present prior to their electroanalytical detection. This might provide for a potential titration-based electrochemical determination of sulphide species in blood-based matrices, in which a ferrocene-model blood solution of known ferrocene concentration is titrated through standard addition aliquots of a blood solution containing sulphide at unknown concentration; the transition from the "no catalysis" to the KG zone, using a threshold value for i_p^{Ox}/i_p^0 as a guide, would enable the unknown sulphide concentration to be determined. This approach holds the conventional advantages of voltammetric waveshape sensing systems over fixed-point amperometric, or reference drifting potentiometric methods.

Given the ubiquity of ferrocene derivatives for monitoring blood analytes,[23],[24] the further understanding this type of phenomenon might become more relevant to overcome sensor performance issues during the tightening of international monitoring standards.[25],[26]

Experimental Methods

All chemical reagents utilised in this work were of the highest available grade, and were purchased from Sigma-Aldrich, Strem (ferrocenemethanol), or Oxoid, Ltd. (laked horse blood), with standard, departmental ethical assessments undertaken for the experiments undertaken. Deionised water was taken from an Elgastat system with a resistivity of at least 18 MΩ cm. All experiments were performed at room temperature (23 ± 2 ^{o}C) and pressure, inside a fume cupboard.

Cyclic voltammetry was performed in a 100 mL five-necked glass cell, using a computer-controlled potentiostat (Autolab PGSTAT 30, Eco Chemie), at a 3.0 mm (diameter) glassy carbon working electrode, with a saturated calomel (SCE) reference electrode and graphite rod counter electrode. The aqueous electrolyte solution, chosen to be 0.1 M boric acid with 0.1 M potassium chloride, was buffered at pH 9.0, or kept unbuffered but at pH 6.0, 7.0 or 8.0, by adjusting the bulk solution pH using either aqueous NaOH or HCl. In the case of experiments in the presence of blood, the aqueous electrolyte comprised 10 vol.% laked horse blood, 90 vol.% 0.1 M boric acid with 0.1 M KCl, with the solution being kept at either pH 9.0 or

pH 7.0. These blood systems were actually fine, heterogeneous suspensions, so required a small degree of agitation to afford an apparent homogenisation of the solution prior to experimentation. These electrolytes were freshly prepared as stock solutions, and were mixed with ferrocenemethanol mediator (in the electrochemical cell, 50 mL) using a magnetic stirrer, or used to form a sulphide stock solution (by dissolving the equivalent of 0.1 M Na_2S). Aqueous solutions were rigorously degassed with argon (BOC) prior to electrochemical experimentation, during which period, the working electrode was polished using a MECAPOL P230 lapping machine (Presi), using a clean and wet, napped polishing cloth impregnated with 0.3 μm alumina slurry, for *ca.* five minutes, so as to overcome historical electrode fouling issues. In the case of experiments with blood, polishing was undertaken on a polishing cloth separate to any other experiment, so as to reduce the risk of biological contamination in the laboratory; apparatus were sterilised using hot water. In order to investigate redox catalysis, aliquots of the stock sulphide electrolyte were added to the electrolyte solution, with thorough mixing of the electrolyte before undertaking voltammetric measurements.

Theoretical voltammograms corresponding to the EC' mechanism were computed through numerical solution of the relevant reaction-diffusion equations,[15] subject to the relevant boundary conditions and assumptions described, using a finite difference method to linearize the bimolecular electron transfer rate constant,[27] and a pentadiagonal matrix algorithm to solve coupled equations.[27] Programs, written and compiled in gfortran, were executed on a MacBook Air laptop running with a 1.3 GHz Intel Core i5 processor, with 4 GB of DDR3 RAM at 1600 MHz speed. Single voltammograms were computed within ten minutes. This time intensive computation occurred in order to achieve the required convergence of the concentration profiles, using a closely spaced finite difference grid comprising 500000 spatial nodes and 2000 temporal nodes. At a fixed scan rate of 0.1 V s^{-1}, and fixed mediator concentration of 2.0 mM, these simulations enabled working curves to be obtained for variable excess factors (γ) in the range $0 \leq \gamma \leq 10.25$, for five effective bimolecular rate constants in the range $1 \leq \lg(k_{eff}/M^{-1}\ s^{-1}) \leq 5$. Cubic spline interpolation of these working curves,[28] enabled the variation of the peak current ratio with excess factor to be identified for more accurate fitting with experimental data. The latter was undertaken through identification of the least squares fit.[28]

Acknowledgements

We thank The University of Hull for funding this work. It is a pleasure to acknowledge useful discussions on the physiological significance of hydrogen sulphide with Dr. Jonathan Knaggs, Dr. Laura Sadofsky and Professor Alyn Morice (all Hull-York Medical School). We are grateful to two anonymous reviewers who have helped improve the manuscript and its presentation.

Conflicts of Interest

There are no conflicts to declare.

Dedication

This work is dedicated to Professor Marcin Opallo, on the occasion of his 65th birthday.

References

[1] D. J. Lefer, *Proc. Nat. Acad. Sci.*, 2007, **104**, 1709.
[2] K. R. Olsen, *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, 2011, **301**, R297.
[3] J. J. Carroll, A. E. Mather, *Geochim. Cosmochim. Acta*, 1989, **53**, 1163.
[4] J. J. Horn, T. McCreedy, J. D. Wadhawan, *Anal. Methods*, 2010, **2**, 1346.
[5] N. L. Whitfield, E. L. Kreimer, F. C. Verdial, N. Skovgaard, K. R. Olsen, *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, 2008, **294**, R1930.
[6] N. S. Lawrence, J. Davis, R. G. Compton, *Talanta*, 2000, **52**, 771.
[7] T. Xu, N. Scafe, L.-P. Xu, S. Zhou, K. A. Al-Ghanem, S. Mahboob, B. Fugetsu, X. Zhang, *Analyst*, 2016, **141**, 1185.
[8] R. Karunya, K. S. Jayaprakash, R. Gaikwad, P. Sajeesh, K. Ramshad, K. M. Muraleedharan, M. Dixit, P. R. Thangaraj, A. K. Sen, *Sci. Reports*, 2019, **9**, 3258.
[9] K. Manibalan, V. Mani, P.-C. Chang, C.-H. Huang, S.-T. Huang, K. Marchlewicz, S. Neethirajan, *Biosens. Bioelectron.*, 2017, **96**, 233.
[10] N. S. Lawrence, M. Thompson, C. Prado, L. Jiang, T. G. J. Jones, R. G. Compton, *Electroanalysis*, 2002, **14**, 499.
[11] N. S. Lawrence, G. J. Tustin, M. Faulkner, T. G. J. Jones, *Electrochim. Acta*, 2006, **52**, 499.
[12] C. E. Banks, A. S. Yashina, G. J. Tustin, V. G. H. Lafitte, T. G. J. Jones, N. S. Lawrence, *Electroanalysis*, 2007, **19**, 2518.
[13] R. Wilson, A. P. F. Turner, *Biosens. Bioelectron.*, 1992, **7**, 165.
[14] R. A. Ward, A. Charlton, K. J. Welham, P. Baker, S. H. Zein, J. Tomkinson, D. I. Richards, S. M. Kelly, N. S. Lawrence, J. D. Wadhawan, *Electrochem. Commun.*, 2021, **124**, 106942.
[15] C. P. Andrieux, C. Blocman, J.-M. Dumas-Bouchiat, F. M'Halla, J.-M. Savéant, *J. Electroanal. Chem.*, 1980, **113**, 19.
[16] J.-M. Savéant, K. Su, *J. Electroanal. Chem.*, 1984, **171**, 341.
[17] C. Combs, H. I. S. Ashmore, A. F. Bridges, C. Swanson, W. D. Stephens, *J. Org. Chem.*, 1969, **34**, 1511.
[18] R. Prins, A. R. Korswagen, A. G. T. G. Kortbeck, *J. Organomet. Chem.*, 1972, **39**, 335.
[19] M. Eigen, K. Kustin, *J. Am. Chem. Soc.*, 1960, **82**, 5952.
[20] D. J. Marlin, K. Fenn, N. Smith, C. D. Deaton, C. A. Roberts, P. A. Harris, C. Dunster, F. J. Kelly, *J. Nutrition*, 2002, **132**, 1622S
[21] M. H. Pournaghi-Azar, R. Ojani, *Talanta*, 1995, **42**, 1839.
[22] L. I. Partington, S. L. Atkin, E. S. Kilpatrick, S. H. Morris, M. Piper, N. S. Lawrence, J. D. Wadhawan, *J. Electroanal. Chem.*, 2018, **819**, 533.
[23] A. E. G. Cass, G. Davis, G. D. Françis, H. A. O. Hill, W. J. Aston, I. J. Higgins, E. V. Plotskin, L. D. L. Scott, A. P. F. Turner, *Anal. Chem.*, 1984, **56**, 667.
[24] See, for example, P. Ugo, P. Marafini, M. Meneghello, *Bioanalytical Chemistry: From Biomolecular Recognition to Nanobiosensing*, De Gruyter, Berlin, 2021.
[25] B. H. Ginsberg, *J. Diabetes Sci. Technol.*, 2009, **3**, 903.

[26] G. Freckmann, C. Schmid, A. Baumstark, M. Rutschmann, C. Haug, L. Heineman, *J. Diabetes Sci. Technol.*, 2015, **9**, 885.
[27] R. G. Compton, E. Laborda, K. R. Ward, *Understanding Voltammetry: Simulation of Electrode Processes*, Imperial College Press, London, 2014.
[28] See, for example, W. H. Press, S. A. Teukolsky, W. T. Vetterling, B. P. Flannery, *Numerical Recipes in C: The Art of Scientific Computing*, 2nd edn., Cambridge University Press, Cambridge, 1992.

Graphical Abstract