# **RSC Advances**



View Article Online





Cite this: RSC Adv., 2015, 5, 89003

# Conversion of nitrous oxide to nitrogen by cobaltsubstituted myoglobin†

Trevor D. Rapson, \*a Soeren Warneke, a Mustafa M. Musameh, b Helen Dacres, a Ben C. T. Macdonald<sup>a</sup> and Stephen C. Trowell<sup>a</sup>

Developing technology to decrease greenhouse gas emissions is one of the greatest challenges we face in the  $21^{st}$  century. Nitrous oxide (N<sub>2</sub>O) is an important greenhouse gas, which is estimated to contribute 6% of the overall global warming effect. Herein we report the use of cobalt substituted heme proteins to reduce N<sub>2</sub>O to nitrogen (N<sub>2</sub>). This catalysis was electrochemically driven using methyl viologen or benzyl viologen as electron transfer partners for cobalt myoglobin. Using bulk electrolysis we demonstrated the production of  $^{15}N_2$  from  $^{15}N_2$ . This catalysis, however, was noted to be poor, most likely due to oxidative damage to the protein scaffold.

Received 29th July 2015 Accepted 13th October 2015

DOI: 10.1039/c5ra15036a

www.rsc.org/advances

### Introduction

Nitrous oxide has a global warming potential approximately 300 times greater than carbon dioxide over a 100 years time horizon. In the stratosphere, N2O is oxidized to NO and NO2, which catalyze the destruction of ozone.1,2 Unlike other greenhouse gases such as carbon dioxide, N2O is largely produced by natural systems, predominantly as an intermediate in the denitrification pathway.3 Over the last century, there has been a 20% increase in atmospheric N2O concentration, attributed to the use of nitrogen-based fertilizers, intensive pastoral farming and waste-water treatment. It is estimated that up to 10% of anthropogenic N<sub>2</sub>O emissions are from the biological treatment of sewage, manure and industrial effluents.4 Such N2O emissions from sewage works, are a feasible target for point source remediation.

Metalloproteins that can catalyze the reduction of N<sub>2</sub>O to N<sub>2</sub> have been investigated for their potential to help reduce nitrous oxide emissions.<sup>5</sup> However, nitrous oxide binds very poorly to metal ions making it difficult to activate it towards reduction. In the face of this constraint, nature has evolved a class of copper dependent N2O reductases (N2OR) that catalyze the final step of denitrification.5 N2OR are soluble enzymes, located in the bacterial periplasm. One promising approach currently being explored is the use of N2OR produced in genetically modified crops to 'scrub' N<sub>2</sub>O emissions from soils. Naturally occurring N<sub>2</sub>OR enzymes are, however, inactivated by exposure to O<sub>2</sub> and the activity of N<sub>2</sub>OR is also sensitive to pH, which makes them unsuitable, without further modification, for bioremediation.<sup>7</sup>

In addition to copper N<sub>2</sub>O reductases, N<sub>2</sub>O has been reported to react with a number of other transition metal complexes,8 in particular the cobalt corrins found in cobalamin (Vit B12 - ESI Fig. 1†). Nitrous oxide has been shown to inhibit the activity of cobalamin dependent methionine synthase.9 The mechanism of inhibition has been proposed to involve a reaction between Co<sup>I</sup> and N<sub>2</sub>O. The cobalamin cofactor alone, when reduced with sodium borohydride to Co<sup>I</sup>, reacts with N<sub>2</sub>O to produce N<sub>2</sub> and Co<sup>II</sup>. 10,11 To date, neither methionine synthase nor cobalamin have been used in N2O remediation, largely because of their oxygen sensitivity. In order to develop an alternative strategy for mitigating N2O, we sought to develop a N2OR that is not inhibited by oxygen. Other desirable characteristics are that the protein needs to be readily produced allowing large-scale production making the process commercially viable.

Heme proteins are attractive scaffolds for engineering, as their gas binding properties can be 'tuned' through modifying the amino acid residues surrounding the heme cofactor. In particular the binding of oxygen to the heme center is stabilized by electrostatic interactions between the polar Fe-O bond and distal pocket residues such as histidine or tyrosine.12,13 If this interaction is removed through site directed mutagenesis, the affinity of the iron heme center for oxygen dramatically decreases.13,14 This could be used to prevent O2 from binding to the heme centre.15 Furthermore, the native heme group can readily be removed and the protein can be reconstituted with an artificial porphyrin or other macrocycle. For example, iron protoporphyrin IX can be substituted with copper, cobalt or manganese protoporphyrin IX16 or cobalt corrins.17 More recently it has become possible to express heme proteins with artificial metalloporphyrins.18

Given the N2O reactivity of cobalt metal centers within corrin rings (e.g. cobalamin), we decided to use cobalt macrocycles to make cobalt substituted heme proteins, to determine if they

<sup>&</sup>lt;sup>a</sup>CSIRO, Black Mountain, ACT, 2601, Australia. E-mail: trevor.rapson@csiro.au <sup>b</sup>CSIRO Clayton, VIC, 3168, Australia

<sup>†</sup> Electronic supplementary information (ESI) available. 10.1039/c5ra15036a

**RSC Advances** 

have N<sub>2</sub>OR activity. Cobalt protoporphyrin IX (where the iron in heme b has been substituted for a cobalt - see ESI - Fig. 1†), which is commercially available, was used as the cobalt macrocycle. We chose myoglobin as our initial protein scaffold, as this protein is known to be amenable to heme substitution.

### **Experimental section**

#### 2.1 Chemicals

All chemicals were purchased from Sigma Aldrich and used as received unless stated otherwise. Cobalt protoporphryin IX was purchased from Frontier Scientific and unlabelled nitrous oxide from Coregas. 15N-N2O (100%) was purchased from Sigma Aldrich, US branch.

### 2.2 Preparation of cobalt myoglobin (CoMb)

The heme group was removed from horse heart myoglobin (FeMb) using the acid/butanone method described previously to generate apoMb.16,19 Reconstitution of apoMb with cobalt protoporphyrin following the method described by Hoffman and Petering<sup>20</sup> with a modification that sodium dithionite was not used, preparing cobalt myoglobin as Co<sup>III</sup>Mb as developed by Hambright and Lemelle.21 Briefly apoMb was incubated with a two times excess of cobalt protoporphyrin (CoPPIX) dissolved in a minimal amount of pyridine at 4 °C overnight. Unbound CoPPIX was removed using a PD10 column from GE Healthcare eluting CoMb with of 50 mM phosphate (pH 7.4). CoMb was then concentrated to the required concentration using spin dialysis.22

#### 2.3 Cyclic voltammetry

Cyclic voltammetry was carried out using a BAS Epsilon potentiostat with a C3 cell stand. A three-electrode system was employed comprising a glassy carbon electrode, Pt wire counter and Ag/AgCl reference electrode. Anaerobic conditions were established through purging with argon. During experiements an argon blanket was used to maintain anaerobic conditions. Nitrous oxide was added from a saturated solution (prepared by exhaustive purging with N2O). Buffer solutions of 50 mM phosphate (pH 7.4) and 50 mM Tris (pH 7.5) were used with either methyl viologen or benzyl viologen (at varying concentrations 5 µM to 50 µM) as the mediator. The use of 2-hydroxy-1,4-napthoquinone as a mediator was tested however no catalysis was observed.

For cyclic voltammetry, the enzyme electrodes were prepared firstly by polishing the glassy carbon electrode according to the manufacturers instructions (BASi). Secondly 10 µL of a 100 µM protein solution was dried at 4 °C and finally covered with a presoaked dialysis membrane (MW cut-off 3.5 kDa), fastened to the electrode using a rubber O-ring. All potentials are quoted vs. Normal Hydrogen Electrode (NHE), calculated as Ag/AgCl + 196 mV.

#### 2.4 Bulk electrolysis

Bulk electrolysis was carried out using a homemade air-tight electrochemical cell. A carbon cloth electrode (AVCar HCB

 $1071 - 2 \times 5$  cm) was employed as the working electrode with a Ag/AgCl electrode immersed in the analyte solution while a Pt wire counter electrode was separated from the main solution by a CoralPor<sup>TM</sup> porous frit. The electrochemical solution was  $\sim$ 80 mL of 50 mM phosphate buffer with CoMb (15  $\mu$ M) and methyl viologen (2 mM). To remove atmospheric nitrogen the electrochemical solution was thoroughly purged with helium with stirring for 90 minutes. A mixture of 9 mL <sup>14</sup>N-N<sub>2</sub>O (100%) and 6 mL <sup>15</sup>N-N<sub>2</sub>O (100%) was added into the headspace of the electrochemical cell and the electrochemical solution was stirred for 90 minutes to ensure that equilibrium of N<sub>2</sub>O between solution and headspace was reached.

Controlled potential was applied to the carbon cloth electrode (-600 mV vs. NHE) using a BAS Epsilon potentiostat. 1 mL samples of the headspace were removed using an airtight syringe and immediately placed into a sample tube filled with helium. To avoid a negative pressure within the bulk electrochemical cell, 1 mL of helium gas was immediately injected following sampling. The concentration of <sup>14</sup>N-N<sub>2</sub>, <sup>15</sup>N-N<sub>2</sub>, <sup>14</sup>N-N<sub>2</sub>O and <sup>15</sup>N-N<sub>2</sub>O was determined at the UC Davis Isotope Analysis facility (full details in ESI†). Due to the expense of <sup>15</sup>N-N<sub>2</sub>O this experiment could only be carried out once.

#### 3. Results and discussion

Co<sup>III</sup>Mb prepared was characterized using UV-Visible spectroscopy and was similar to that reported by Hambright and Lemelle (Fig. 1,  $\lambda_{\text{max}}$  425 nm).<sup>21,23</sup> The Co<sup>II</sup>Mb form could be readily obtained with the addition of sodium dithionite (Fig. 1,  $\lambda_{\text{max}}$  406 nm).

The nitrous oxide reductase activity of CoMb was subsequently tested using mediated catalytic voltammetry. Methyl viologen (-456 mV vs. normal hydrogen electrode - NHE) was chosen as the mediator, as it is typically used in N<sub>2</sub>OR activity assays,24 and to generate CoI in cobalamin dependent methionine synthase.9 The enzyme electrodes were prepared by immobilizing CoMb under a dialysis membrane covering a glassy carbon electrode as described by Bernhardt and coworkers.25 Experiments were initially conducted under anaerobic conditions using an argon blanket. Nitrous oxide was

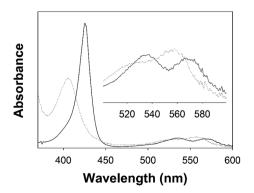


Fig. 1 The UV-Vis spectrum of Co<sup>III</sup>Mb (solid line) as prepared by reconstitution and Co<sup>II</sup>Mb (dotted line) obtained by the reduction of Co<sup>III</sup>Mb with sodium dithionite.

Paper

C C MV BV

Fig. 2 Catalytic conversion of  $N_2O$  to  $N_2$ . The voltammetric response before (dashed line) and after the addition of  $N_2O$  is shown (solid line) for: (A) cobalt myoglobin (CoMb) mediated by methyl viologen (MV - 40  $\mu$ M). (B) CoMb mediated by benzyl viologen (BV - 40  $\mu$ M) and (C) Iron myoglobin (FeMb) with benzyl viologen (BV - 40  $\mu$ M, scan rate = 5 mV s<sup>-1</sup>). The arrow indicates the increase in current with the addition of  $N_2O$ .

Potential (mV vs. NHE)

added to the electrochemical cell by purging the solution with anaerobic  $N_2O$  gas to yield a saturated  $N_2O$  solution ( $\sim$ 25 mM).

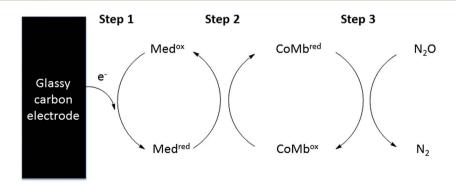
Addition of  $N_2O$  to the electrochemical cell resulted in significant amplification of the reductive current and the waveform changed from a peak-shaped transient voltammogram to a sigmoidal steady-state voltammogram (Fig. 2A). A catalytic response from CoMb was also observed when an alternative mediator, benzyl viologen ( $-370 \text{ mV } \nu s$ . NHE) was used. In this case the catalytic current was only 60% of that obtained using methyl viologen (Fig. 2B). A higher potential mediator 2-hydroxy-1,4-napthoquionone ( $-137 \text{ mV } \nu s$ . NHE) was tested, however no catalytic currents were observed (data not shown).

The pronounced catalytic current observed for CoMb was in contrast to the absence of catalysis with native FeMb (Fig. 2C). Furthermore, no changes were noted when  $N_2O$  was added to an electrochemical cell with only methyl viologen or benzyl viologen and no CoMb or with the protein alone without a mediator (ESI Fig. 2†).

The results observed for CoMb can be interpreted as shown in Scheme 1. Methyl viologen is reduced at the electrode through heterogeneous electron transfer (Step 1). CoMb in turn is reduced by methyl viologen to  $Co^I$  (Step 2) which is able to reduce  $N_2O$  to  $N_2$  (Step 3). The amplified catalytic current noted with the addition of  $N_2O$ , is due to the rapid reduction of methyl viologen driving the enzymatic conversion of  $N_2O$  to  $N_2$ .

The voltammetric sweep rate is a powerful variable to probe the mechanisms of electrochemical processes. For both methyl viologen and benzyl viologen mediated experiments, the scan rate was varied between 5 mV s<sup>-1</sup> and 500 mV s<sup>-1</sup>. It was clearly observed, for both methyl viologen and benzyl viologen that, as the scan rate increased, the heterogeneous reduction and oxidation of the mediator became too fast for homogenous electron transfer between the mediator and CoMb. At low scan rates (5 mV s<sup>-1</sup>) sigmoidal catalytic responses were noted, as the scan rate was increased the voltammograms became more peak shaped (Fig. 3). This result provided strong evidence that an enzymatic response due to the reduction of N<sub>2</sub>O by CoMb was being observed at slow scan rates (<20 mV s<sup>-1</sup>).

The N<sub>2</sub>O concentration dependence of the catalytic reaction was investigated by incrementally adding aliquots from a mediator solution saturated with N2O (25 mM) to the electrochemical cell. There was an increase in the catalytic current with increasing N<sub>2</sub>O concentrations (Fig. 4), however the catalytic current did not appear to follow standard Michaelis-Menten kinetics. The plot of catalytic current vs. N2O concentration could be fitted to a sigmoidal curve (ESI Fig. 3†). The origin of this unusual concentration dependence is unclear. There are a number of reports which indicate that N<sub>2</sub>O binds to several non-iron sites within heme proteins such as myoglobin and hemoglobin.26,27 It is possible that binding of N2O to such non-heme sites gives rise to an allosteric effect on the N2O reductase activity of CoMb. Alternatively, the enzyme electrode was prepared by immobilizing CoMb under a dialysis membrane. This immobilization was required to obtain catalytic currents with N2O, but the use of a membrane could



Scheme 1 The proposed pathway of electrochemical enzymatic catalysis whereby the mediator (either methyl viologen or benzyl viologen) supplies electrons to cobalt myoglobin (CoMb).

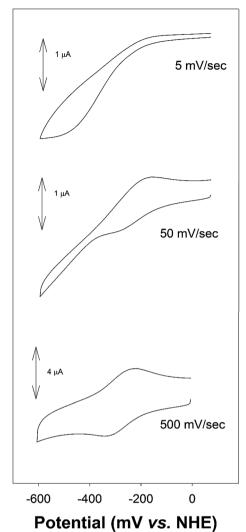


Fig. 3 The effect of scan rate on the catalytic voltammogram obtained for CoMb in the presence of N<sub>2</sub>O ( $\sim$ 25 mM) with benzyl viologen as a mediator (40  $\mu$ M).

interfere with the diffusion of  $N_2O$  from the solution to the enzyme electrode.

In order to confirm that CoMb reduces N2O to N2 we characterized the reaction products from a bulk electrolysis of isotopically labelled N<sub>2</sub>O (approximately 34% <sup>15</sup>N-N<sub>2</sub>O) carried out in a helium atmosphere. A potential of -600 mV vs. NHE was applied to the carbon-cloth working electrode. Samples from the headspace were removed at different times and were analyzed for concentrations of <sup>15</sup>N-N<sub>2</sub> and N<sub>2</sub>. Over the course of the experiment, there was an increase in the levels of total N<sub>2</sub> (11.3 to 19.6  $\mu$ mol - Fig. 5) and  $^{15}$ N-N<sub>2</sub> (41 to 72 nmol - ESI Table 1†). There was also a doubling in the ratio of <sup>15</sup>N-N<sub>2</sub> (a product of the enzymatic reaction) to <sup>14</sup>N-N<sub>2</sub> (ESI Table 1†) from  $1.57 \times 10^{-5}$  to  $3.42 \times 10^{-5}$ . The atom percentage of  $^{15}$ N-N<sub>2</sub> however was low ( $\sim$ 0.36%) indicating that there was a high level of contamination with atmospheric nitrogen. This contamination unfortunately was unavoidable due to the experimental constraints in using syringes to sample the headspace of the

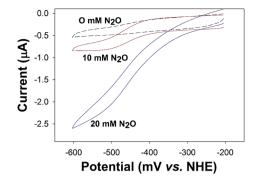


Fig. 4 The effect of  $N_2O$  concentration on the voltammetric response from CoMb. Methyl viologen (40  $\mu$ M) was used as the mediator with a scan rate of 5 mV s<sup>-1</sup>.  $N_2O$  concentration was varied by adding aliquots of a mediator solution saturated with  $N_2O$ .

bulk electrolysis cell rather than using a sampling loop attached directly to a mass spectrometer.

The pronounced catalytic currents observed upon the addition of  $N_2O$  when CoMb is immobilized on a glassy carbon electrode indicate that through a simple iron to cobalt substitution, myoglobin can be turned into a  $N_2O$ -reductase. No catalytic currents were noted when FeMb was used rather than CoMb or in the presence of the mediators alone, confirming that CoMb is the active center for the reduction of  $N_2O$  and the mediator.

The current electrochemical setup does not allow the oxygen sensitivity of CoMb to be fully examined, as the redox properties of the low potential mediators used are adversely affected by the presence of oxygen. In order to investigate this further, we are exploring options to achieve direct electron transfer between the cobalt metal center and an electrode, using options such as protein film voltammetry<sup>28,29</sup> or site-specific covalent attachment to gold electrodes.<sup>30</sup> There have been some reports of direct electron transfer to CoMb on pyrolytic graphite electrodes<sup>31,32</sup> however these experiments employed surfactant films, which have been shown to cause the release of heme from myoglobin.<sup>33</sup>

It is important to note that, unlike naturally occurring  $N_2OR$ , CoMb is not inactivated by oxygen. Before enzyme assays can be performed with naturally occurring  $N_2OR$ , an activation step

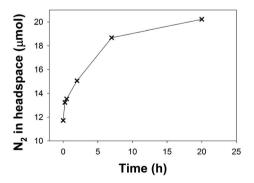


Fig. 5 Reaction product analysis from the headspace of the bulk electrolysis of CoMb in the presence of  $^{15}N$ -labelled  $N_2O$ . A constant potential of -600 mV vs. NHE was applied to a carbon cloth electrode with methyl viologen (2 mM) as mediator.

Paper

needs to be carried out whereby the enzyme is reduced using high concentrations of methyl viologen (3 mM) and dithionite (1.5 mM) for 3 h.24 Here, no pre-treatment was required for CoMb; the protein was prepared and used as Co<sup>III</sup>Mb. The reactive Co<sup>I</sup> state required to reduce N<sub>2</sub>O was generated electrochemically during cyclic voltammetry.

There have been reports previously of heme proteins, such as cytochrome P450 and myoglobin, reducing N2O.34,35 These examples required the production of Fe<sup>I</sup> to reduce N<sub>2</sub>O and therefore catalysis was only noted at large over-potentials  $\sim$  -850 mV vs. NHE. Using CoMb, the potential required for catalysis is -450 mV, 400 mV higher, which avoids non-specific reduction processes.

While cyclic voltammetry suggested that CoMb is able to reduce N2O, bulk electrolysis was carried out to confirm the production of  $N_2$  from the enzymatic reaction. To overcome contamination with atmospheric N<sub>2</sub>, <sup>15</sup>N labelled nitrous oxide was used with a 40% 15N enrichment. Through the course of bulk electrolysis both an increase in 15N-N2 (Fig. 5) and a doubling in the ratio of  $^{15}N-N_2$  to  $^{14}N-N_2$  (Table 1†). This result shows that CoMb is able to convert N2O to N2.

There are two possible explanations for the relatively low amount of 15N-N2 measured in the headspace in relation to <sup>14</sup>N-N<sub>2</sub>. The first is that CoMb selectively reduces <sup>14</sup>N<sub>2</sub>O over <sup>15</sup>N<sub>2</sub>O. Given that in our experiment a mix of <sup>14</sup>N<sub>2</sub>O (60%) and  $^{15}N_2O$  (40%), the larger increase in  $^{14}N_2$  (~8 µmol, Table 1†) noted could be due to the enzymatic conversion of 14N2O to <sup>14</sup>N<sub>2</sub>. The second and more likely explanation is that while CoMb is able to reduce N2O to N2, the enzyme is not very efficient. In the case of cobalamin dependent methionine synthase, Drummond and Matthews observed that the reduction of N<sub>2</sub>O by cobalamin produces a potent oxidant, which damages the protein structure proximal to the cobalamin cofactor. 9,36 They propose that N2O undergoes a single electron transfer with cobalamin resulting in a hydroxyl radical. It is likely that in the case of CoMb a similar one-electron reduction of N2O takes places which damages CoMb preventing further catalysis. We plan to continue to investigate if indeed oxidative damage is occurring to CoMb during the conversion of N2O to N2 and will investigate using alternative heme protein scaffolds which might be more resistant to oxidative damage.37,38

### Conclusion

Here we demonstrate that the use of cobalt-substituted myoglobin represents a new option for the development of N<sub>2</sub>O mitigation strategies, based on reducing N<sub>2</sub>O to N<sub>2</sub>. To continue this research both the effect of oxygen on the N2O activity of CoMb and whether CoMb is indeed oxidatively damaged during catalysis needs to be explored further.

## Acknowledgements

We thank Dr Yen Troung for preparing the carbon cloth electrodes.

### References

- 1 D. J. Wuebbles, Science, 2009, 326, 56-57.
- 2 T. D. Rapson and H. Dacres, Trends Anal. Chem., 2014, 54,
- 3 J. N. Galloway, J. D. Aber, J. W. Erisman, S. P. Seitzinger, R. W. Howarth, E. B. Cowling and B. J. Cosby, Bioscience, 2003, 53, 341-356.
- 4 J. Desloover, S. E. Vlaeminck, P. Clauwaert, W. Verstraete and N. Boon, Curr. Opin. Biotechnol., 2012, 23, 474-482.
- 5 D. Richardson, H. Felgate, N. Watmough, A. Thomson and E. Baggs, Trends Biotechnol., 2009, 27, 388-397.
- 6 S. Wan, T. L. Ward and I. Altosaar, Trends Biotechnol., 2012, 30, 410-415.
- 7 S. R. Pauleta, S. Dell'Acqua and I. Moura, Coord. Chem. Rev., 2013, 257, 332-349.
- 8 W. B. Tolman, Angew. Chem., Int. Ed. Engl., 2010, 49, 1018-
- 9 J. T. Drummond and R. G. Matthews, Biochemistry, 1994, 33, 3732-3741.
- 10 R. G. S. Banks, R. J. Henderson and M. Pratt, J. Chem. Soc. A, 1968, 2886-2889.
- 11 R. G. S. Banks, R. J. Henderson and J. M. Pratt, Chem. Commun., 1967, 387-388.
- 12 E. M. Boon and M. A. Marletta, J. Inorg. Biochem., 2005, 99,
- 13 J. S. Olson and G. N. Phillips, J. Biol. Inorg. Chem., 1997, 2,
- 14 E. M. Boon and M. A. Marletta, Curr. Opin. Chem. Biol., 2005, 9, 441-446.
- 15 A.-L. Tsai, E. Martin, V. Berka and J. S. Olson, Antioxid. Redox Signaling, 2012, 17, 1246-1263.
- 16 F. Ascoli, M. R. Fanelli and E. Antonini, Methods Enzymol., 1981, 76, 72-87.
- 17 T. Hayashi, Y. Morita, E. Mizohata, K. Oohora, J. Ohbayashi, T. Inoue and Y. Hisaeda, Chem. Commun., 2014, 50, 12560-12563.
- 18 J. J. Woodward, N. I. Martin and M. A. Marletta, Nat. Methods, 2007, 4, 43-45.
- 19 F. W. Teale, Biochim. Biophys. Acta, 1959, 35, 543.
- 20 B. M. Hoffman and D. H. Petering, Proc. Natl. Acad. Sci. U. S. A., 1970, 67, 637-643.
- 21 P. Hambright, S. Lemelle and K. Alston, Inorg. Chim. Acta, 1984, 92, 167-172.
- 22 T. D. Rapson, H. Dacres and S. C. Trowell, RSC Adv., 2014, 4, 10269-10272.
- 23 Z. N. Zahran, L. Chooback, D. M. Copeland, A. H. West and G. B. Richter-Addo, J. Inorg. Biochem., 2008, 102, 216-233.
- 24 S. Dell'acqua, S. R. Pauleta, E. Monzani, A. S. Pereira, L. Casella, J. J. G. Moura and I. Moura, Biochemistry, 2008, 47, 10852-10862.
- 25 P. Kalimuthu, K. Fischer-Schrader, G. Schwarz and P. V. Bernhardt, J. Phys. Chem. B, 2013, 117, 7569-7577.
- 26 A. Dong, P. Huang, X. J. Zhao, V. Sampath and W. S. Caughey, J. Biol. Chem., 1994, 269, 23911-23917.

27 O. Einarsdóttir and W. S. Caughey, J. Biol. Chem., 1988, 263, 9199–9205.

- 28 T. D. Rapson, U. Kappler, G. R. Hanson and P. V Bernhardt, *Biochim. Biophys. Acta*, 2011, **1807**, 108–118.
- 29 P. V. Bernhardt, Aust. J. Chem., 2006, 59, 233.

**RSC Advances** 

- 30 S. Mukherjee, K. Sengupta, M. R. Das, S. S. Jana and A. Dey, *J. Biol. Inorg. Chem.*, 2012, **17**, 1009–1023.
- 31 J. Gao and J. F. Rusling, J. Electroanal. Chem., 1998, 449, 1-4.
- 32 C. Li, K. Nishiyama and I. Taniguchi, *Electrochim. Acta*, 2000, **45**, 2883–2888.
- 33 M. T. de Groot, M. Merkx and M. T. M. Koper, *Electrochem. Commun.*, 2006, **8**, 999–1004.

- 34 C. E. Immoos, J. Chou, M. Bayachou, E. Blair, J. Greaves and P. J. Farmer, *J. Am. Chem. Soc.*, 2004, **126**, 4934–4942.
- 35 M. Bayachou, L. Elkbir and P. J. Farmer, *Inorg. Chem.*, 2000, **39**, 289–293.
- 36 J. T. Drummond and R. G. Matthews, *Biochemistry*, 1994, **33**, 3742–3750.
- 37 T. D. Rapson, J. S. Church, H. E. Trueman, H. Dacres, T. D. Sutherland and S. C. Trowell, *Biosens. Bioelectron.*, 2014, 62, 214–220.
- 38 T. D. Rapson, T. D. Sutherland, J. S. Church, H. E. Trueman, H. Dacres and S. C. Trowell, ACS Biomater. Sci. Eng., 2015, DOI: 10.1021/acsbiomaterials.5b0023.