Protein film-electrochemical EPR spectroscopy as a technique to investigate redox reactions in biomolecules

Kaltum Abdiaziz,[1] Enrico Salvadori,[1,2] Katarzyna P. Sokol,[3] Erwin Reisner,[3] Maxie M. Roessler*,[1,4]

[1]School of Biological and Chemical Sciences and Materials Research Institute, Queen Mary University of London, Mile End Road, London E1 4NS, UK
[2]Department of Chemistry, NIS Centre and INSTM Reference Center, University of Turin, Turin, Via Giuria 7, 10125, Italy
[3]Department of Chemistry, University of Cambridge, Cambridge CB2 1EW, UK
[4]Department of Chemistry, Imperial College London, Molecular Sciences Research Hub, White City Campus, 80 Wood Lane, W12 0BZ
* corresponding author: m.roessler@imperial.ac.uk

Table of Contents

1. Materials 2
2. Mesoporous ITO electrode preparation 2
3. Film-electrochemistry 3
4. Functionalisation of mesoporous ITO electrodes 3
5. Electrochemical impedance spectroscopy 4
6. EPR spectroscopic measurements and analysis 5
7. Sensitivity: determining the concentration of redox-active species 6
8. Supplementary Figures and Tables 7
9. References 15

1. Materials

All materials were purchased form Sigma Aldrich unless stated otherwise: Indium tin oxide (ITO) nanoparticles (<50 nm diameter), fluorine doped tin oxide (FTO) coated glass (7 Ω/sq), methanol, isopropanol, acetone, sodium chloride, potassium chloride, potassium ferricyanide, MilliQ water, deionised water, phosphate-buffered saline (PBS) buffer, acetic acid, ethanol, Cu,Zn Superoxide Dismutase, 4-aminoTEMPO, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 3-phosphonopropionic acid, triethylamine (NEt_3), Thermo Scientific Pierce BCA Protein Assay Kit.

Titanium wire (Ti, 98%, outer diameter (O.D.) 0.320 μm, Advent Research Materials) was used to connect the meso-ITO working electrode to the potentiostat. The titanium wire was coiled only inside the meso-ITO structure. Bare Pt wire (98%, O.D. 0.125 μm, Advent Research Materials) was used as the counter electrode and Ag wire for the pseudo-reference electrode (98%, O.D. 0.125 μm, Advent Research Materials).

2. Mesoporous ITO electrode preparation

Mesoporous ITO (meso-ITO) electrode on flat glass slide (henceforth known as flat meso-ITO electrode) was prepared following a previously published protocol.[1] 3-dimentional (cylindrical) meso-ITO electrode was prepared using borosilicate glass tubes (Stuart SMP10/1) and titanium wire (Ti, 0.320 μm). Glass tube and Ti wire were sonicated in isopropanol and ethanol for 20 mins each and dried at room temperature in air. ITO nanoparticles (52 mg, 20 wt %) were sonicated for 60 mins in an acetic acid: EtOH (57 μL: 200 μL) solution. The resulting ITO mixture (70 μL) was pipetted into the glass tube, with the Ti wire (15 mm in length, the bottom 5 mm of the Ti wire was coiled) before centrifuging at 4000 rpm for 40 mins (1520 g, Universal 320R Hettich zentrifugen). The supernatant was removed and replaced with methanol, and the setup was centrifuged again at 4000 rpm for 40 mins. Excess solvent was removed, and the meso-ITO structure was dried (40 ^{o}C for 3 days; shorter timeframes were found to lead to insufficient drying levels) before annealing in a furnace with the following programme: 1 ^{o}C/min to 300 ^{o}C for 20 mins and then slowly cooled to RT in the furnace chamber (5 ^{o}C/min to RT). The meso-ITO structure was carefully pulled out of the glass tube by gently pulling on the Ti wire, rinsed with ethanol and dried before use.

3. Film-electrochemistry

All electrochemical studies were carried out using EmStat 3+ potentiostat (Palmsens) with PS-Trace software. Protein film electrochemical experiments were carried out using the standard three-electrode configuration consisting of the mesoporous indium tin oxide (cylindrical meso-ITO) electrode as the working electrode, teflon-insulated silver wire (Ag wire) as pseudo-reference electrode and bare platinum wire as counter electrode. All protein-film electrochemical experiments were performed in an anaerobic glovebox (MBraun, < 0.05 ppm O_2) in buffer (PBS and 150 mM NaCl, adjusted to pH 7.0 or 8.0) at room temperature (22-24 °C), except cyclic voltammetry experiments involving flat meso-ITO electrodes which were performed on the bench in argon-degassed buffer. A new working electrode was used for each experiment. For peak current vs scan rate measurements (Fig. 3A in the main manuscript), we note that measurements at higher scan rates were not feasible because the overall conductivity of the circuit is then limiting relative to electron transfer at the surface (i.e. redox-peaks broaden beyond detection). In potentiometric titrations, meso-ITO electrodes were poised at different potentials until a stable current was reached, which occurred in less than 5 mins, before the sample was flash frozen in a dry ice/acetone bath. The meso-ITO structure was found to be unstable to repeated freeze-thaw cycles. A new electrode was therefore assembled for each spectroelectrochemical sample given that measurements had to be conducted on frozen samples. All redox potentials are given against the standard hydrogen electrode (SHE), where $E_{SHE} = E_{Ag\ wire} + 0.222$ V. The reference electrode (pseudo Ag wire) was calibrated using the redox potential of ferricyanide (pH 7.00) against a saturated calomel electrode (SCE).

Functionalisation of mesoporous ITO electrodes

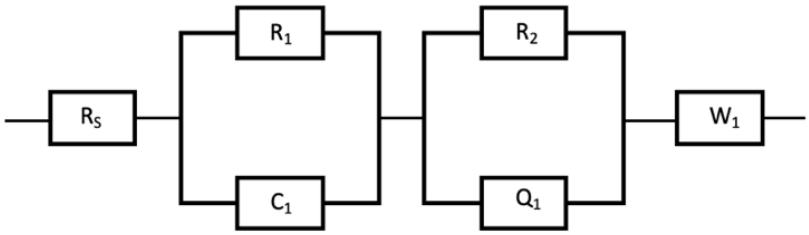
The surface of the meso-ITO electrode was functionalized according to published procedures.[2] The pulled-out meso-ITO 3D electrodes were successively cleaned with isopropanol, acetone and water. The cleaned meso-ITO electrodes were then placed in a solution containing H_2O, H_2O_2 (30%) and NH_4OH (30%) in 5:1:1 ratio at 70 ^oC for 1 hr and then washed with deionised water. 3-phosphonopropionic acid (10 mM) was self-assembled on meso-ITO in an ethanolic solution containing triethylamine (NEt_3, 20 mM) at room

temperature for 24 hrs to give a self-assembled monolayer (SAM). Phosphonic acids are known to have a high affinity to metal oxides.[3,4] The SAM-modified meso-ITO electrodes were rinsed with EtOH and heated at 140 ^{o}C in an argon atmosphere for 24 hrs. The electrode was then placed in NEt_3 (5% v/v) in EtOH for 30 min at room temperature, rinsed with EtOH and finally dried. For SOD, the SAM-modified working electrode was immersed in solution of SOD (1 mM in PBS buffer) for 30 mins at 4 °C to allow sufficient association.

To anchor 4-aminoTEMPO covalently on ITO, the SAM-modified meso-ITO electrode was placed in a deionised water solution of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, 10 mM) and N-hydroxysuccinimide (NHS, 10 mM) for 1 hr and washed with excess deionised water before immersing in a solution of 4-aminoTEMPO (10 mM) for 12 hrs. Any unbound protein or 4-aminoTEMPO molecules were washed out with deionised water before electrochemical measurements.

4. Electrochemical impedance spectroscopy

Impedance spectra were collected using an Ivium potentiostat (CompactStat, Alvatek) against Ag/AgCl reference electrode at an open circuit voltage of 1 KHz down to 1 mHz (AC amplitude, 10 mV; sampling rate, 10 points per decade). Impedance spectra were recorded in a phosphate buffer (100 mM NaCl, 50 mM KCl and 20 mM phosphate, pH 7.4) with 5 mM $K_6[Fe(CN_6)]$. The impedance plots were fitted to an equivalent circuit using an in-built fitting program to determine the values for the resistance in the WE. The circuit used to fit the plots is shown below:



R_S – solution resistance
R_1/ R_2 – resistance
C_1 – capacitance
Q_1 – Q factor
W_1 – mass transport component

5. EPR spectroscopic measurements and analysis

Low-temperature EPR measurements were performed in the Centre for Advanced ESR (CAESR) located in the Department of Chemistry of the University of Oxford, using an EMXmicro X-band CW spectrometer (Bruker BioSpin GmbH, Germany), equipped with a helium flow cryostat (ESR900, Oxford instruments) and a rectangular resonator module with 10 mm sample access (X-band Super High Sensitivity Probehead, ER 4122SHQE). Room-temperature EPR measurements were carried out in SPIN-Lab at Imperial College, using a Bruker Elexsys E580 CW EPR spectrometer operating at X-band frequencies and equipped with rectangular resonator module with 10 mm sample access (X-band Super High Sensitivity Probehead, ER 4122SHQE). EPR measurements were conducted with 2 mW microwave power, 100 kHz modulation frequency and 1 G (for 4-amino TEMPO) or 7 G (for SOD) modulation amplitude.

The spectra of the empty resonator and of samples containing only buffer (including only the glass tube) were found to be identical. For CW measurements the Q value, as reported by the built-in Q indicator in the Xepr programme (typically Q = 1200 $\pm$ 100), was used as a guide to position each sample in the same location in the resonator. All data analysis was carried out using EasySpin.[5]

Spin quantification of the number of 4-aminoTEMPO molecules immobilised on the meso-ITO structure was determined by EPR at room temperature. Whilst all PFE measurements were carried out in water (to mimic the requirements for proteins) the high dielectric constant of water required room temperature EPR measurements to be carried out in acetic acid. Electrochemistry in acetic acid was not possible without very high concentrations of electrolyte to make the solution conductive enough for PFE measurements (at which point room-temperature EPR measurement became impossible due to high conductivity). For room temperature measurements in acetic acid, the PFE-EPR entire cell could be placed into the centre of the resonator, allowing quantification of the number of 4-amino TEMPO molecules in the meso-ITO structure by EPR. To determine the rotational correlation times of 4-amino TEMPO (Figure S4 and Table S3), simulated EPR spectra were fitted to the experimental data. All the parameters, *g* and *A* values were fixed based on typical values for 4-aminoTEMPO in solution (*g* = 2.0075, 2.006, 2.0035; *A* = 21.5, 21.8, 90.8 MHz).[6]

6. Sensitivity: determining the concentration of redox-active species

The number of molecules of SOD and 4-amino TEMPO immobilised on meso-ITO electrode was determined using UV-Vis spectroscopy. The PFE-EPR setup with SOD or 4-amino TEMPO immobilised was sonicated in buffer (pH 2, 50 μL) until the entire meso-ITO structure detached from the titanium wire and completely dispersed in the solution. The suspension was then centrifuged, and the supernatant was recovered. This procedure was repeated until there was no more SOD or 4-amino TEMPO detected. To determine the concentration of SOD in solution, the bicinchoninic acid assay (Thermo Scientific Pierce BCA Protein Assay Kit) was used. 4-amino TEMPO exhibits an absorbance at 426 nm which was used to generate the calibration curve.

7. Supplementary Figures and Tables

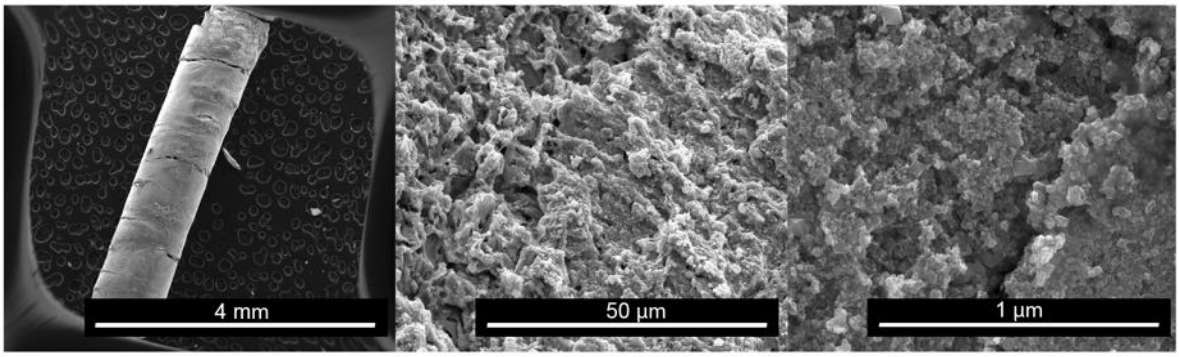


Figure S1. Scanning electron microscopy (SEM) images of PFE-EPR electrode with mesoporous ITO co-assembled using 50 nm ITO nanoparticles.

Table S1. Analysis of impedance data shown in Figure 3B of the main manuscript.

Electrode surface	Solution resistance (Ohms)	Total Resistance (Ohm)	Resistivity (Ohm m)	Conductivity $(Ohm\ m)^{-1}$
Bare ITO	11	37.4	9.9484	0.100
Phosphonic acid	18.9	60.4	16.0664	0.062
4-aminoTEMPO	15.2	109.3	29.0738	0.034

Table S2. Comparison of resistivity and conductivity between different materials.

	Conductor	Semiconductor	Insulator	PFE-EPR setup (meso-ITO)	FTO coated glass slide (Sigma Aldrich)
Resistivity (Ohm m)	10^{-9} to 10^{-4}	10^{-3} to 10^{3}	10^{4} to 10^{17}	9.94	~ 7
Conductivity $(Ohm\ m)^{-1}$	10^{7}	10^{-6} to 10^{4}	10^{-10} to 10^{-20}	0.10	0.14

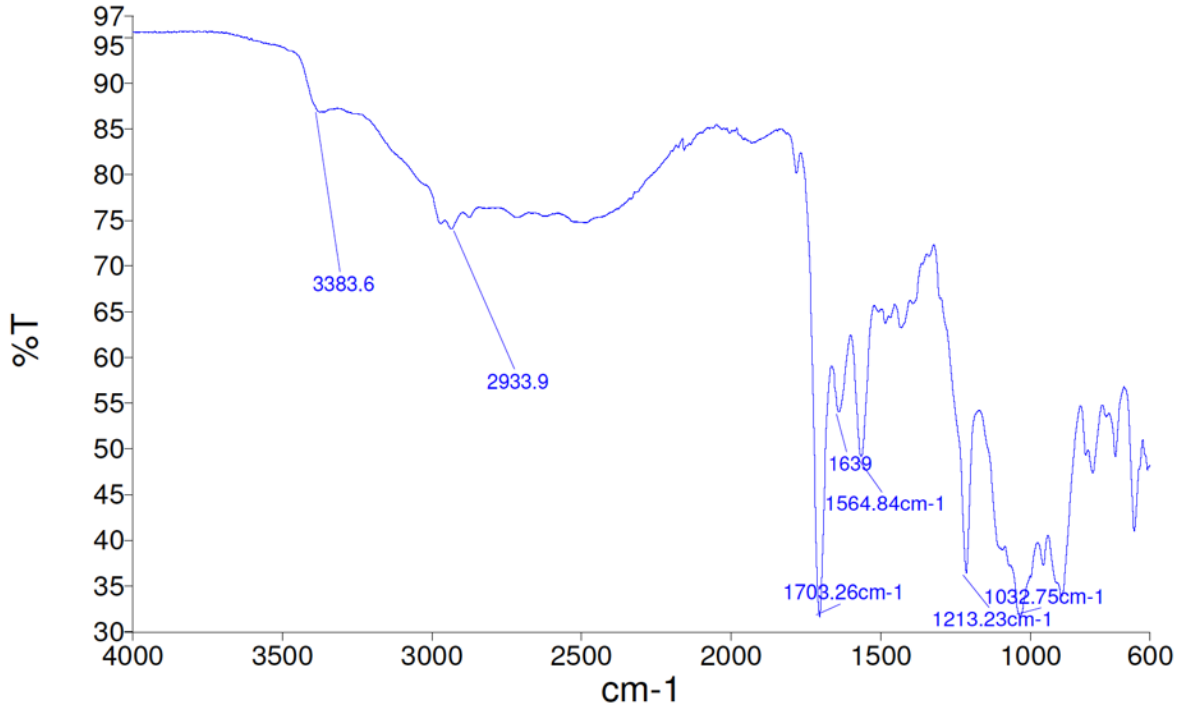


Figure S2. Attenuated total reflection FTIR of the coupling reaction between 3-phosphopropanoic acid and 4-aminoTEMPO with coupling reagents EDC/NHS in water. The distinctive peak at 1703 cm^{-1} corresponds to an amide C=O bond and shows that the coupling reaction was successful.

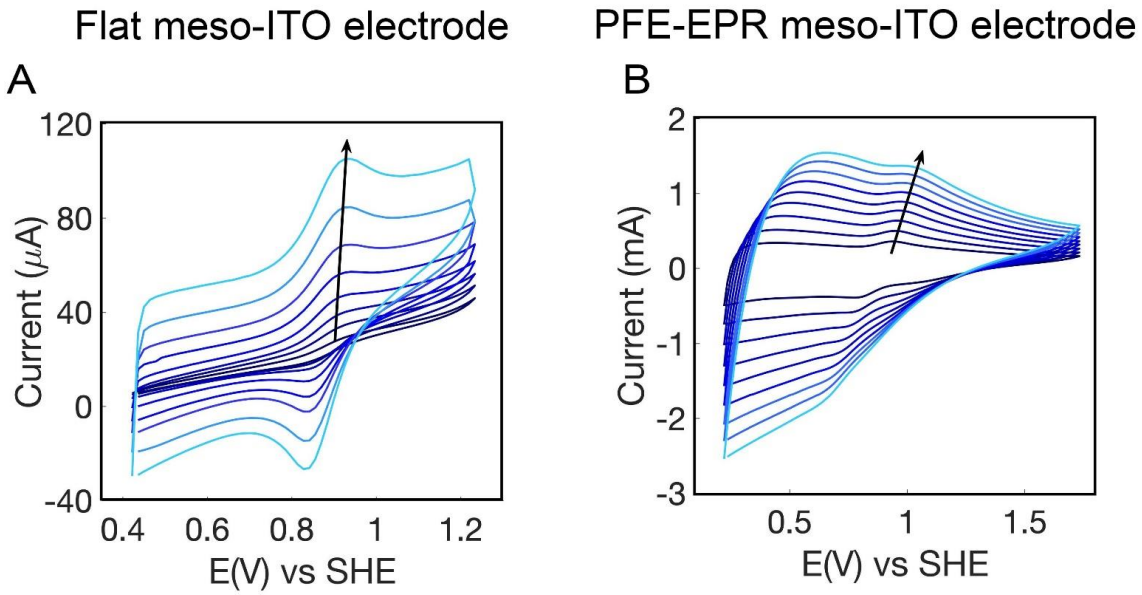


Figure S3. (A) Overlay of CV scans of 4-amino TEMPO immobilised on flat *meso-ITO* electrode at multiple scan rates (20, 40, 60, 100, 125, 150, 175, 200 mV/*s*). (B) CV scans of 4-amino TEMPO immobilised on PFE-EPR – meso-ITO annealed at 300 °C at multiple scan rates (20, 30, 40, 50, 60, 70, 80, 90, 100 mV/s). Protein-film electrochemistry was carried out in PBS, 0.15M NaCl (pH 7.00) at room temperature.

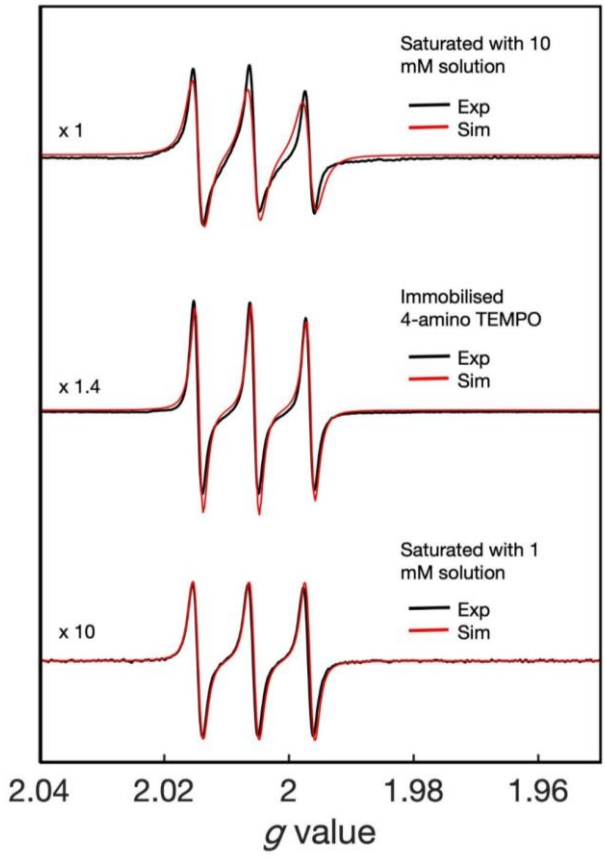


Figure S4. Comparison of the EPR spectra of 4-amino TEMPO immobilised on the PFE-EPR setup and a control experiment in which 4-amino TEMPO was not covalently anchored to meso-ITO, with a 10 mM and 1 mM solution saturating the ITO pores. In the non-immobilised samples, the meso-ITO electrodes were not functionalised with the phosphonate group. Top: non-immobilised 10 mM 4-amino TEMPO in acetic acid; middle: immobilised 4-amino TEMPO; bottom: non-immobilised 1 mM 4-amino TEMPO in acetic acid. The distortion visible in the EPR spectrum at 10 mM (top) is attributed to hindered tumbling of the molecules within the pores at such high concentration[7] and is not apparent the 1 mM solution (bottom spectrum). Distortion is minimal in the surface-anchored 4-amino TEMPO (middle spectrum) which however offers nearly an order of magnitude better sensitivity compared to the 1 mM saturated solution. See Table S3 for the corresponding rotational correlation times determined from simulations (red). CW measurements were carried out at RT, 2 mW power, 100 kHz modulation frequency and 1 G modulation amplitude.

Table S3. Rotational correlation times (τ_{corr}) determined from the EPR spectra of 4-aminoTEMPO shown in Figure S4. The correlation times are in agreement with 4-amino TEMPO immobilised on peptides and large molecules.[6,8]

	τ_{corr} (ns)
MesoITO saturated with 10 mM 4-amino TEMPO	1.36
Immobilised 4-aminoTEMPO	0.116
MesoITO saturated with 1 mM 4-amino TEMPO	0.031

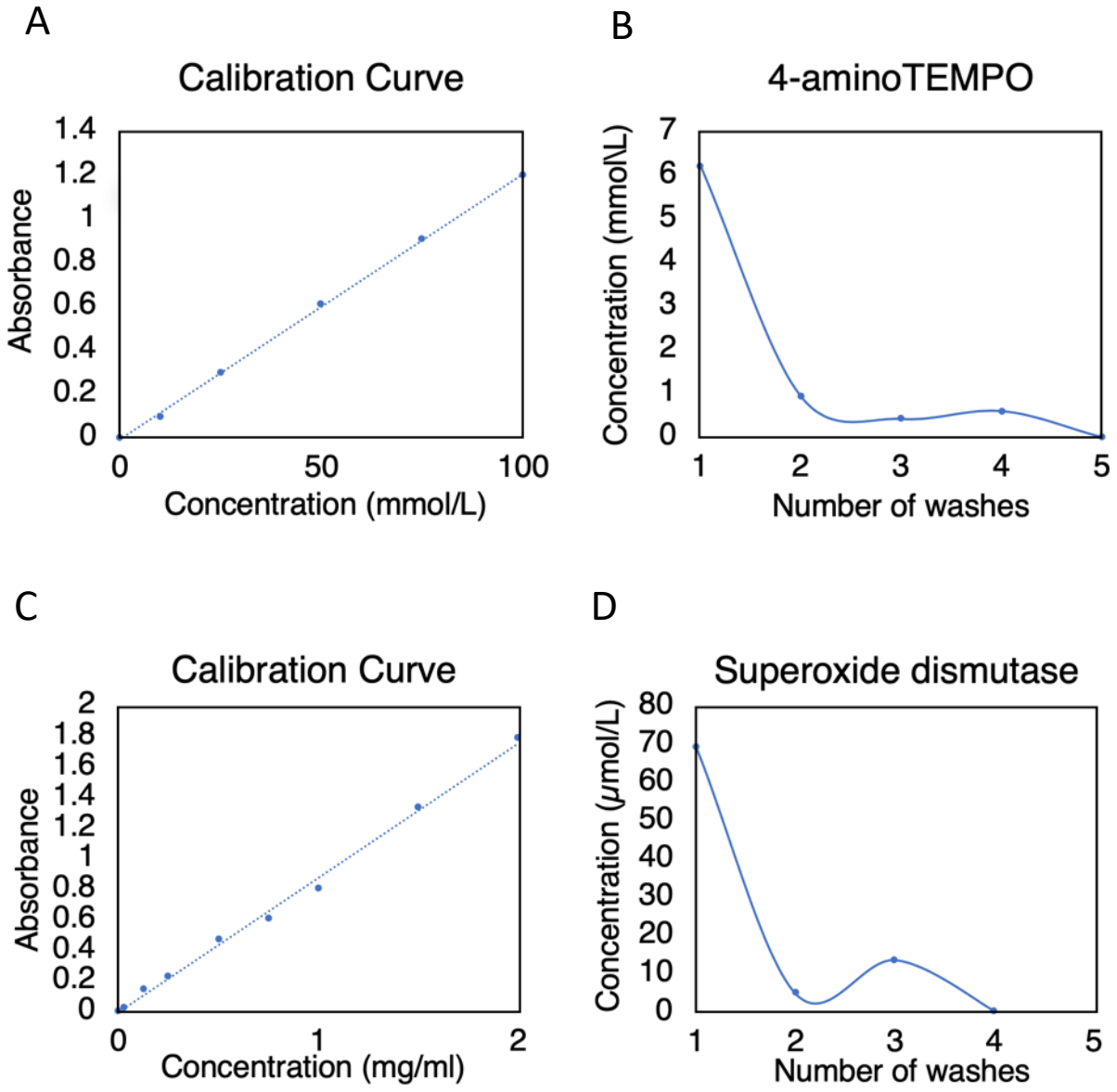


Figure S5. Quantification of surface-bound 4-amino TEMPO and SOD in the PFE-EPR set-up. (A, C) Standard calibration curve of SOD and 4-aminoTEMPO. The calibration curve for 4-amino TEMPO (A) was determined by measuring absorbance at 426 nm. The concentration of SOD (C) in solution was determined by bicinchoninic acid assay (Thermo Scientific Pierce BCA Protein Assay Kit). (B) Concentration of 4-amino TEMPO disassociated from meso-ITO electrode for each successive wash. (D) Concentration of SOD disassociated from meso-ITO electrode for each successive wash. The concentration of redox active species was determined by sonication and disruption of interactions between the working electrode and the SOD or TEMPO (see ESI section 8).

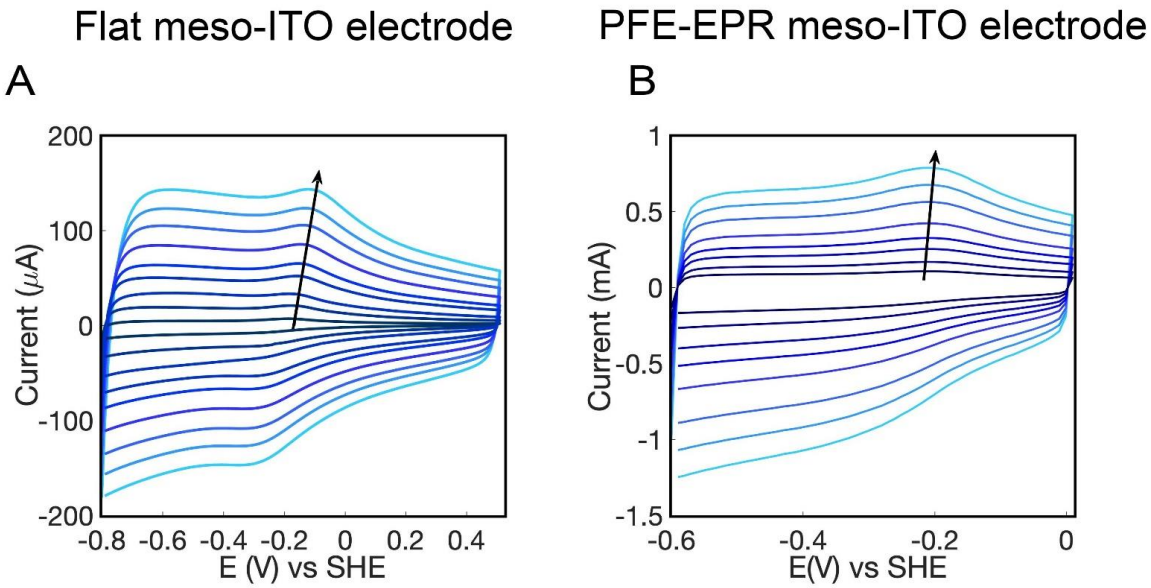


Figure S6. (A) Overlay of CV scans of Superoxide Dismutase (SOD) immobilised on flat meso-ITO electrode at multiple scan rates (20, 40, 60, 100, 125, 150, 175, 200 mV/s). (B) CV scans of SOD immobilised on PFE-EPR – meso-ITO annealed at 300 °C at multiple scan rates (20, 30, 40, 50, 60, 70, 80, 90, 100 mV/s). Protein-film electrochemistry was carried out in PBS, 0.15M NaCl (pH 8.02) at room temperature.

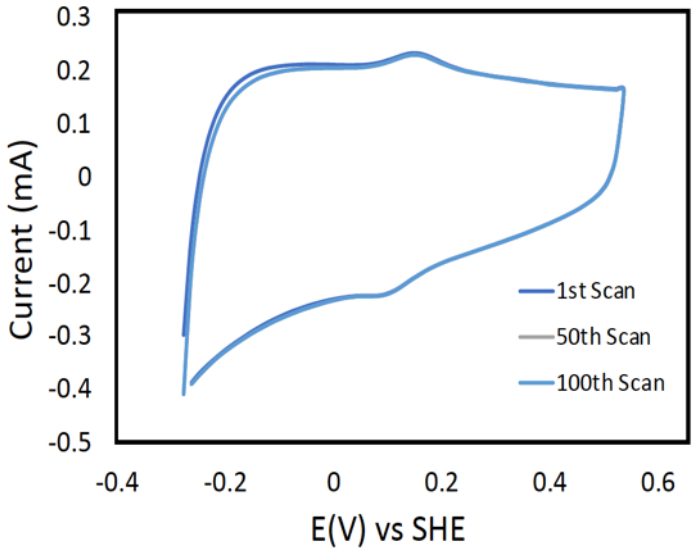


Figure S7. Cyclic voltammogram of Superoxide Dismutase (SOD) in PBS, 0.15M NaCl (pH 8.02) at scan rates of 20 mVs^{-1} after 1, 50 and 100 scans. SOD was immobilised on PFE-EPR setup-mesoITO annealed at 300°C.

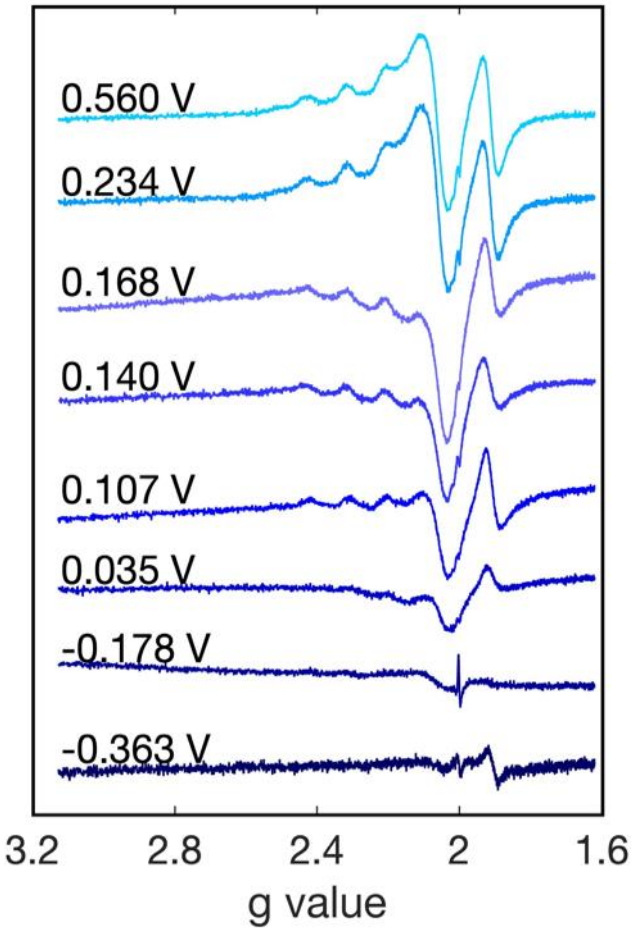


Figure S8. Potentiometric titration of SOD immobilised on PFE-EPR setup. Continuous-wave EPR spectra of surface bound SOD reduced at different potentials vs SHE. Measurements were carried out at 16 K, 2 mW power (non-saturating conditions), 100 kHz modulation frequency and 7 G modulation amplitude.

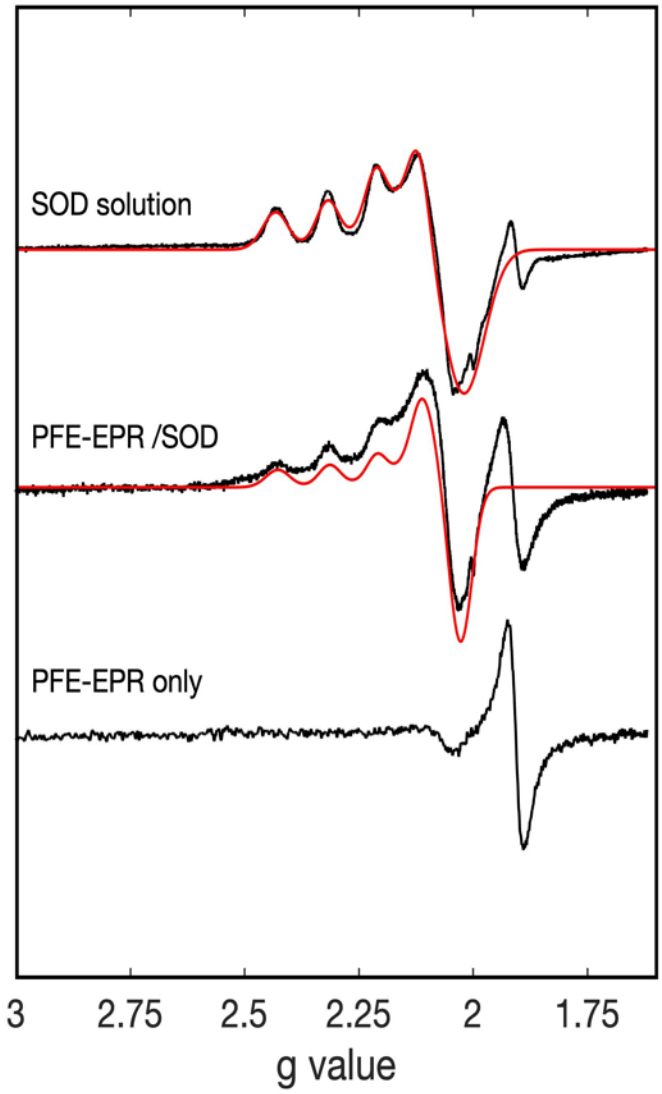


Figure S9. CW spectra of 1 mM SOD in solution (top), immobilised on PFE-EPR setup (middle) and PFE-EPR setup. Continuous-wave EPR spectra of surface bound SOD reduced at different potentials. Measurements were carried out at 16 K, 2 mW power (non-saturating conditions), 100 kHz modulation frequency and 7 G modulation amplitude. The radical signal (g = 1.907) arises from ITO. In order to immobilize SOD onto the mesoITO structure the electrode was immersed in a 1 mM solution of SOD which led to cross-contamination of the SOD solution, hence the radical signal is also present in the SOD solution spectrum.

8. References

1 M. Kato, T. Cardona, A. W. Rutherford and E. Reisner, *J. Am. Chem. Soc.*, 2012, **134**, 8332–8335.

2 M. Kato, T. Cardona, A. W. Rutherford and E. Reisner, *J. Am. Chem. Soc.*, 2013, **135**, 10610–10613.

3 J. Willkomm, K. L. Orchard, A. Reynal, E. Pastor, J. R. Durrant and E. Reisner, *Chem. Soc. Rev*, 2016, **45**, 9.

4 K. E. Dalle, J. Warnan, J. J. Leung, B. Reuillard, I. S. Karmel and E. Reisner, 2019, **119**, 2752–2875.

5 S. Stoll and A. Schweiger, *J. Magn. Reson.*, 2006, **178**, 42–55.

6 H. N. Sultani, H. H. Haeri, D. Hinderberger and B. Westermann, *Org. Biomol. Chem.*, 2016, **14**, 11336–11341.

7 M. M. Haugland, J. E. Lovett and E. A. Anderson, *Chem. Soc. Rev.*, 2018, **47**, 668–680.

8 M. Matthies, K. Glinka, M. Theiling, K. Hideg and H.-J. Steinhoff, *Appl Magn Reson*, 2016, **47**, 627–641.