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Supplementary Information

Electrochemically-generated ferricyanide enables thiol-ene capture of protein-protein binding

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1. Methods

BCA Assay

The BCA assay was prepared in a 96-well plate. Standards of Bovine Serum Albumine (BSA) were prepared at the concentrations 2, 1.5, 1, 0.75, 0.5 and 0.25 mg/mL. 12.5 µL of each solution was applied to the 96-well plate in duplicate. 12.5 µL of each sample was also loaded into the plate in triplicate. A solution of BCA was prepared using BCA (BCA reagent A) and copper (II) sulphate (BCA reagent B) in a proportion on 50:1 and 100 µL of that solution was applied to each well. The plate was incubated at 37 ^{o}C with gentle shaking, for 30 min. The absorbance of each well was analysed at 562 nm, using the Nanodrop. The BSA concentration range was used to draw a calibration curve, that was used to calculate the concentration of each sample.

SDS-PAGE

Labelling samples were analysed on a 12% acrylamide gel (resolving gel: 1.3 mL 1.5 M Tris-Cl pH 6.8, 1.5 mL 40% acrylamide/bis-acrylamide (29:1), 2 mL dH_2O, 50 µL 10% SDS, 50 µL 10% ammonium persulfate (APS), 5 µL Tetramethylethylenediamine (TEMED); stacking gel: 630 µL 0.5 M Tris-Cl pH 6.8, 300 µL acrylamide/bis-acrylamide (29:1), 1.3 mL dH_2O, 25 µL 10% SDS, 25 µL 10% APS, 2.5 µL TEMED). Fisher's EZ-RunTM Pre-Stained Rec Protein Ladder was loaded alongside the samples. Protein separation was performed at 150 V for 80 min in running buffer (25 mM Tris, 190 mM Gly, 1% SDS) and visualised by anti-HA western blotting.

Anti-HA Western Blotting

The transfer sandwich was prepared with a nitrocellulose membrane (GE Healthcare, Illinois USA) soaked in blotting transfer buffer (25 mM Tris, 190 mM Gly, 20% MeOH), filter papers and sponges. Proteins was performed in blotting transfer buffer for 18 h at 15 V. Prior to immunoblotting, the membrane was incubated with blocking buffer (5% skimmed milk powder in PBS-Tween 20 0.1%) for 1 h at room temperature. The primary mouse monoclonal anti-HA antibody (1:2,000 dilution in blocking buffer) (Biolegend, California USA, Cat. Number 901501) was incubated with the membrane for 1 h, at room temperature, with gentle shaking. The membrane was then washed with PBS-Tween 20 0.1% (3 x 4 min) and PBS (2 x 4 min). The secondary antibody (1:4,000 dilution in blocking buffer) (Peroxidase conjugated AffiniPure Goat Anti-Mouse IgG (H+L), Jackson ImmunoResearch, Cambridgeshire UK, Cat. Number 115-035-166) was added to the membrane and incubated for 1 h at room temperature with gentle shaking. The membrane was then washed with PBS-Tween 20 0.1% (4 x 4 min), PBS (3 x 4 min) and dH_2O (1 x 4 min). Chemiluminescence was visualised with Pierce ECL western blotting substrate (Thermofisher, Massachusetts USA). The membrane was imaged in a Chemidoc XRS+ (Biorad, California USA).

OTUB1 expression and purification

BL21 (DE3) cells transfected with a pET28a-LIC vector containing an N-terminal His6 tagged OTUB1 were cultured in LB medium (8 mL), containing kanamycin (100 µg/mL), for 18 h at 37 ^{o}C, 180 rpm. The resulting culture was transferred into fresh LB medium (300 mL), containing kanamycin (100 µg/mL), and grown at 37 ^{o}C at 180 rpm until an OD_{600} of 0.6 to 0.9 was reached. IPTG was then added at a final concentration of 0.4 mM and the bacteria were incubated for 20 h at 18 ^{o}C, 180 rpm. The cells were centrifuged at 6,000 rpm, for 15 min, and the resulting pellet was re-suspended in 25 mL of homogenate buffer (50 mM Tris-Cl pH 7.4, 5 mM $MgCl_2$, 250 mM sucrose) containing PMSF (20 µM). Cell lysis was performed using a sonication tip, for 5 min with a 3-sec pulse. The lysate was centrifuged at 13,000 rpm, for 45 min. Ni NTA agarose resin (Sigma-Aldrich, Missouri, USA) (1.5 mL, 1:1 suspension in 20 % EtOH) was centrifuged at 2,200 rpm for 5 min and the supernatant was discarded. dH_2O (2 x 0.7 mL) was added to the beads which were gently inverted until the resin was fully resuspended. The resulting solution was centrifuged at 2,200 rpm and the supernatant was discarded. Ni wash buffer (1.4 mL, 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole) was added to the resin which was transferred to the clarified supernatant and incubated overnight at 4 ^{o}C with rolling. The resin was centrifuged at 2,200 rpm and the supernatant was discarded. Ni wash buffer (1 mL) was added to the resin which was fully resuspended by gentle inversion and centrifuged at 2,200 rpm for 5 min. This wash step was repeated four times. The supernatant was discarded after each washing step. Ni elution buffer A (4 x 0.7 mL; 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 150 mM imidazole) was added to the resin that was resuspended by gentle inversion. The solution was centrifuged at 2,200 rpm for 5 min and the supernatants from these washes were pooled in clean microcentrifuge tubes. A final wash step was carried out with Ni elution buffer B (1 mL; 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 300 mM imidazole) at 2,200 rpm for 5 min. The supernatant was discarded, and the beads were stored in 20% EtOH. The pooled supernatants were concentrated in a 10 kDa MW cut-off Vivaspin 500 centrifugal concentrators in a centrifuge at 9,000 rpm to a final concentration of 50 µL. Ni wash buffer (450 µL) was added to the tubes and concentrated to 50 µL in a centrifuge at 9,000 rpm. This wash step was repeated, and the solution was resuspended in storage buffer (150 µL, 20 mM Tris-Cl pH 8.0, 1mM DTT, 10% glycerol, 50 mM NaCl). The concentration was measured by nanodrop (12.81 µg/µL in 200 µL).[1, 2]

Expression and purification of HA-$^{1-75}$Ub-MESNa

BL21 (DE3) cells containing a pTYB2 plasmid encoding for a HA-tagged ^{75}Ub fusion protein with an intein domain and chitin-binding domain (HA-$^{1-75}$Ub-intein-CBD) were cultured in LB medium (8 mL), containing ampicillin (100 µg/mL), at 37 °C, 180 rpm, for 18 h. The resulting culture was transferred into fresh LB medium (300 mL), containing ampicillin (100 µg/mL), and grown for an additional 2.5 hours, at 37 °C, 180 rpm, up to an OD_{600} 0.6-0.9. IPTG was then added at a final concentration of 0.4 mM and the bacteria were incubated for 20 h, at 18 °C, 180 rpm. The cell culture was centrifuged at 6,000 rpm, for 15 min, and the resulting pellet was re-suspended in column buffer (50 mM HEPES pH 6.8, 100 mM NaOAc). Cell lysis was performed using a sonication tip, for 5 min with a 3-sec pulse. The lysate mixture was centrifuged for 45 min, at 13,000 rpm. Chitin resin (5 mL) (New England Biolabs) was added to a column and the system was equilibrated using column buffer (50 mL). The supernatant was added and the column was washed using column buffer (50 mL). Column buffer containing MESNa (15 mL, 50 mM) was then run through the column and the system was incubated at 37 °C, for 18 h, with gentle shaking. HA-$^{1-75}$Ub-MESNa was eluted with column buffer (8 mL) and the sample concentrated by centrifugation at 11,000 rpm in 5 kDa MW cut-off Vivaspin 500 centrifugal concentrators (Sartorious, Göttingen Germany), until a volume of 500 µL. HA-$^{1-75}$Ub-MESNa was loaded into a NAP-5 column (GE Healthcare, Illinois USA) and desalted with column buffer (1 mL). Final protein concentration was determined using Nanodrop (3.2 mg/mL, 1 mL).[1-3]

Synthesis of HA-$^{1-75}$Ub-alkene probe 1

A solution of *N*-Hydroxysuccinimide (0.2 M, 45 µL) and Tris-Cl pH 7.5 (100 mM, 10 µL) was added to HA-$^{1-75}$Ub-MESNA in column buffer (3.2 mg/mL, 500 µL) and the mixture was incubated at 37 °C, for 10 min, with gentle shaking. Allylamine (15 µL, 0.2 mmol) was dissolved in a solution of MeCN-dH_2O (1:1, 50 µL) and added to the reaction mixture. The pH was adjusted to 10. The reaction was incubated at 37 °C, for 20 h, with gentle shaking. After this incubation, the reaction mixture was loaded into a NAP-5 column, desalted with column buffer (1 mL) and concentrated by spinning at 11,000 rom in a 5 kDa MW cut-off Vivaspin centrifugal concentrator. The protein concentration was determined using a BCA assay (4.7 mg/mL, 100 µL).[3]

***In vitro* thiol-ene labelling of OTUB1 with HA-$^{1-75}$Ub-alkene probe 1, with potassium ferricyanide**

Probe **1** (2 µg) was incubated with recombinant DUB OTUB1 (1 µg). The final volume was adjusted to 31 µL with homogenate buffer (50 mM Tris-Cl pH 7.4, 5 mM $MgCl_2$, 250 mM sucrose). The mixtures were pre-incubated for 90 min, at 37 °C, with gentle shaking, before the addition of initiators. For DPAP (MW 256.30 g/mol)/MAP (MW 150.17 g/mol) initiation, DPAP (0.5 mM) and MAP (0.5 mM) were added to the system and following degassing, the mixture was irradiated with UV at 254 nm in a Luzchem LZC-4 UV Reactor (14 lamps of 8W, 112W) for 2 min. For redox initiation, variable concentrations and incubation times were attempted for potassium ferricyanide (concentration range 2 µM – 1 mM; incubation times 1 – 90 min, 37 °C with gentle shaking). Upon completion, reducing sample buffer (31 µL) (0.2 M Tris-Cl pH 6.8, 30% glycerol, 0.4% β-mercaptoethanol, 9% SDS, 0.1 % bromophenol blue) was added and the proteins were denatured at 95 °C for 5 min. Labelling was analysed using SDS-PAGE and visualised using silver anti-HA western blotting.

***In vitro* thiol-ene labelling of OTUB1 with HA-$^{1-75}$Ub-alkene probe 1, with electrochemically-generated potassium ferricyanide**

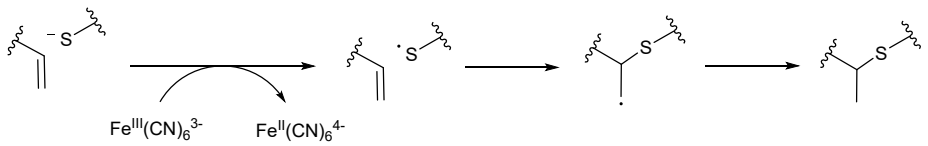
Probe **1** (2 µg) was incubated with recombinant DUB OTUB1 (1 µg). The final volume was adjusted to 31 µL with homogenate buffer (50 mM Tris-Cl pH 7.4, 5 mM $MgCl_2$, 250 mM sucrose). The mixtures were pre-incubated for 90 min, at 37 °C, with gentle shaking, before the addition of initiators. For DPAP/MAP initiation, DPAP (0.5 mM) and MAP (0.5 mM) were added to the system and following degassing, the mixture was irradiated with UV at 254 nm in a Luzchem LZC-4 UV Reactor (14 lamps of 8W, 112W) for 2 min. For redox initiation, different concentrations of potassium ferrocyanide (1 mM and 10 mM) were added and varying amounts of charge were applied to generate concentrations of ferricyanide 2.5 – 500 µM. Incubation times 1 - 90 min were attempted at 37 °C, with gentle shaking. Upon completion, reducing sample buffer (31 µL) (0.2 M Tris-Cl pH 6.8, 30% glycerol, 0.4% β-mercaptoethanol, 9% SDS, 0.1 % bromophenol blue) was added and the proteins were denatured at 95 °C for 5 min. Labelling was analysed using SDS-PAGE and visualised using silver anti-HA western blotting.

2. Electrochemical System Design and Reaction Conditions

The ferro/ferricyanide redox couple is a reversible one-electron transfer reaction and the process can be studied using cyclic voltammetry. Conventionally, in electroanalytical chemistry, a three-electrode setup is used, including a working reference and counter electrode. Figure S2a (black line) depicts the cyclic voltametric response of a glassy carbon working electrode (radius 1.5 mm) in a solution containing 1 mM Fe(II) and 50 mM NaCl, as measured against an Ag/AgCl reference electrode. A clear oxidation wave is observed at about +0.2 V vs Ag/AgCl. The present experimental system involving the use and modification of proteins requires the use of only small sample volumes, ca. 31 µL. In contrast, conventional electroanalytical measurements are often undertaken using mL volumes. Hence, a bespoke cell design needed to be created to allow the synthetic procedure to be accomplished. It was deemed that rather than using a full-three electrode system, a simplified two-electrode system would provide a more optimal design allowing easier miniaturisation of the cell. The use of a two-electrode system is certainly feasible; however, the resulting voltammogram is notably distorted, as shown in Figure S2a (red line) where the counter and reference electrode connections from the potentiostat have been electrically joined and connected to a platinum wire. Under this two-electrode system, in the resulting voltammogram, the peak-to-peak separation has increased to about 0.7 V, reflecting the lack of potentiostatic control. Given that the aim of this work is not focused on the direct analysis of the system's current but is simply using the electrochemical reaction to *in situ* generate the needed reagent (ferricyanide), this voltametric distortion does not create an experimental impediment. The cell design for the protein-based experiments comprised of an inverted glassy carbon electrode (radius 1.5 mm), with a pipette tip as a collar to hold the solution was fabricated, a schematic of this cell design is shown in the main article Figure 1. The use of this inverted electrode design enables voltametric experiments to be performed on 10s of microlitres of solution. The electrochemical cell was completed with the addition of a coiled platinum counter electrode submerged in the analyte solution. Figure S2b depicts two voltametric responses of this cell recorded whilst it contained 31 µL of solution, where either the sample has been diluted using distilled water (dH_2O) or the homogenate buffer has been added, mimicking the solution composition that is used for the protein-based experiments. Both voltametric signals are highly distorted, but it is notable that in both cases the oxidative and reductive peaks for the ferro/ferricyanide redox are still clearly observable despite being shifted to markedly higher potentials. This shift in the voltametric peaks does not in this case reflect a change in the solution phase thermodynamics or kinetics of the process understudy but is simply due to the presence of an uncompensated solution phase resistance arising due to the use of the two-electrode cel design. Although the voltametric peaks are shifted, importantly the total amount of material being oxidised at the working electrode can still be monitored through measurement of the charge passed.

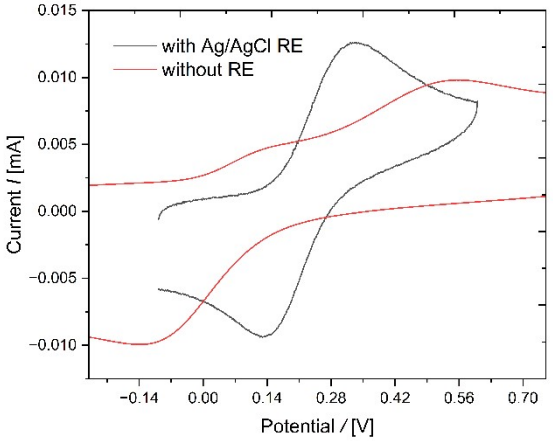
In the protein experiment, the aim is to electrochemically convert the solution phase ferrocyanide to ferricyanide. Table S1 reports the required charge that needed to be passed in order to generate the desired ferricyanide concentration in the 31 µL of protein solution. The active glassy carbon disk electrode has a radius of 1.5 mm and this is embedded in a non-conductive support such that the end of the entire electrode construction has a radius of 3 mm. Consequently, using the inverted cell design shown in the main body of the text when 31 µL of solution is added to the cell, the solution in the absence of the counter electrode will have a depth of approximately 1 mm. At these length scales and due to the fact that the solution is removed from the cell after electrolysis and incubated for 30 min, the solution can be assumed to be relatively homogeneous over the course of the incubation period. To electrochemically generate the required ferricyanide, a slow linear sweep voltammogram was started and the magnitude of the total charge passed monitored. When the desired charge was obtained, the electrochemical reaction was halted and the solution removed from the electrochemical cell. This approach was designed to ensure the required quantity of ferrocyanide was converted to ferricyanide at the working electrode. In this solution, the counter electrode reaction is likely to involve proton reduction at the platinum counter electrode. To ensure that the generation of the desired quantity of ferricyanide could be achieved over the timeframe of a few minutes, for the higher concentrations of ferricyanide (250 µM and 500 µM), a bulk concentration of 10 mM ferrocyanide was used.

3. Supplementary Figure S1: Proposed thiol-ene initiation mechanism with potassium ferricyanide.

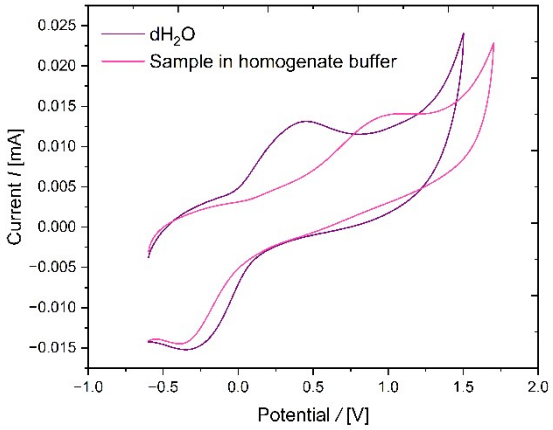


4. **Supplementary Figure S2: Cyclic voltammograms with three electrode and two electrode set-ups, and set-up with dH2O and homogenate buffer, alkene probe and OTUB1.** a) Comparison between cyclic voltammograms using three electrode and two electrode set-ups; b) Comparison between pipette tip set-up with dH_2O and homogenate buffer, alkene probe and DUB enzyme with addition of Fe^{2+} (1 mM) and NaCl (50 mM). The scan rate was 100 and 200 mV/s respectively.

a)



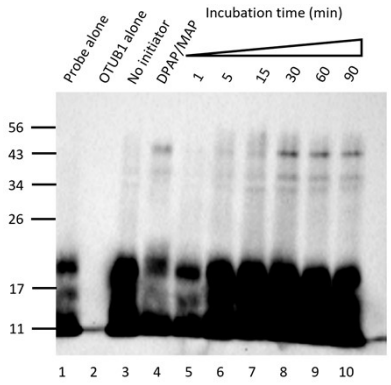
b)



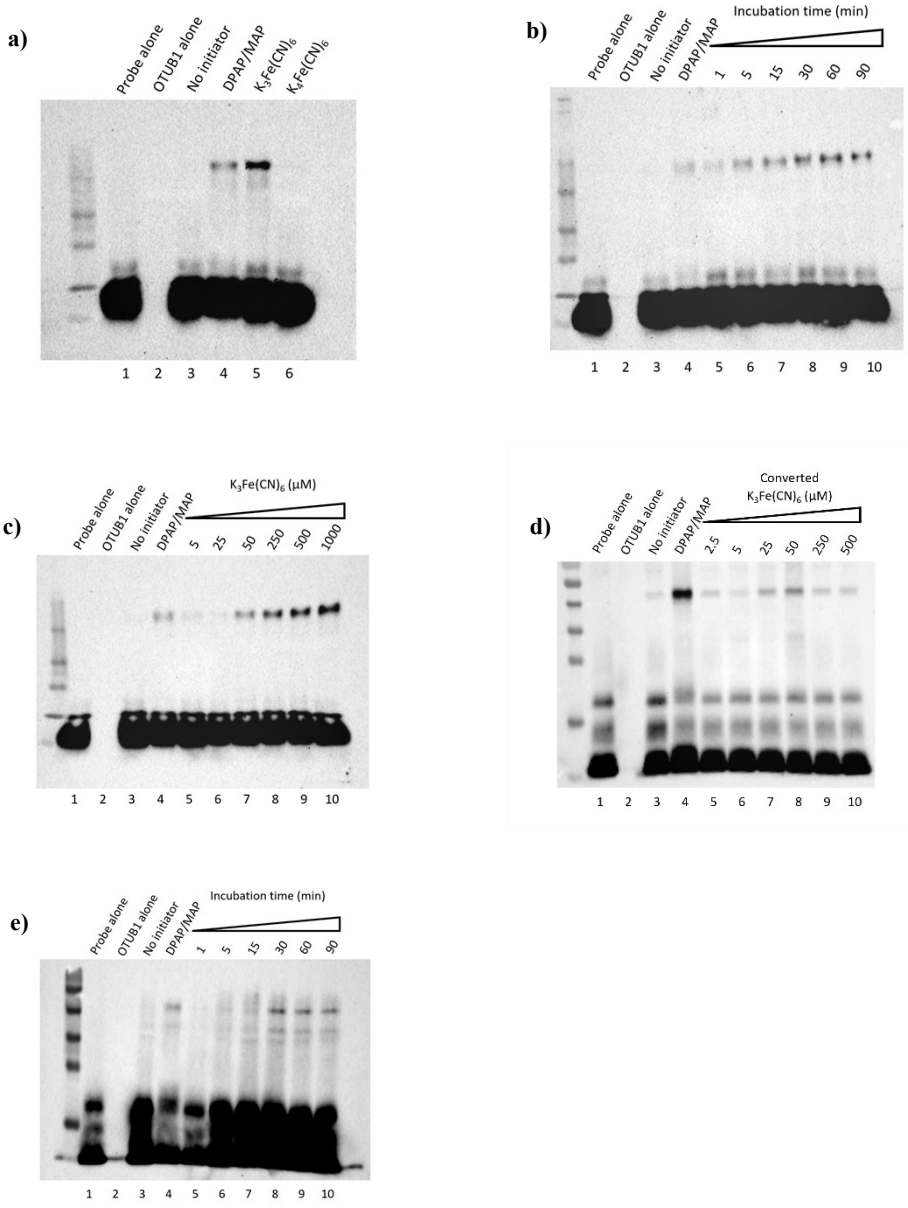
5. **Supplementary Table S1: Conversion of concentration into charge in homogenate buffer, in a volume of 31 µL.** Amount of charge required to convert 1 mM of ferrocyanide into 2.5 – 50 µM of ferricyanide (entries 1-4) and 10 mM of ferrocyanide into 250 – 500 µM of ferricyanide (entries 5-6).

Entry	Concentration (µM)	Charge (µC)
1	2.5	7.5
2	5	15
3	25	75
4	50	150
5	250	750
6	500	1500

6. **Supplementary Figure S3: Incubation time optimisation for the labelling of OTUB1 (1 µg) with probe 1 (2 µg), using electrochemically-generated Fe (III) as chemical initiator.** Assay visualised by anti-HA Western Blotting. Samples were incubated for 90 min at 37°C, prior to initiator addition. DPAP (500 µM) and MAP (500 µM) were added to lane 4, which was degassed and irradiated with UV light (254 nm) for 2 min. Potassium ferrocyanide (10 mM) was added to lanes 5-10 and a charge of 150 µC was applied to the samples to generate a final concentration of ferricyanide of 500 µM. Lanes 5-10 were then incubated for varying periods of time.



7. Supplementary Figure S4. Uncropped Western Blots and SDS-PAGE, with protein ladder, for Figures 2-5 and Supplementary Figure S3.



8. Supplementary references

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