Supporting Information: Structure-Redox Response Correlation in a Few Select Heme Systems Using X-Ray Absorption Spectro-Electrochemistry

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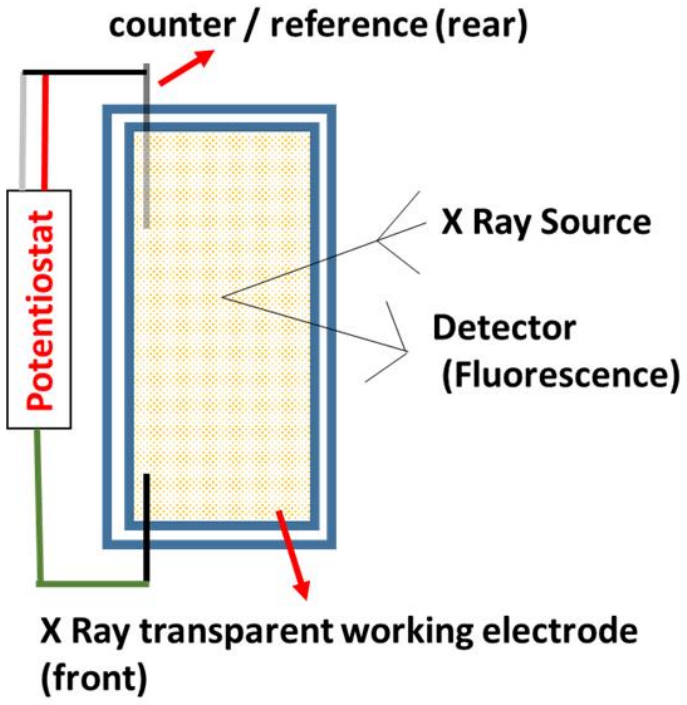
S1. Preparation of samples for XAS measurements:

We prepare solutions of heme proteins in PBS buffer (pH 7.2; made in accordance with the Cold Spring Harbour protocol available online).[1] Protein solutions are 20mg/mL in concentration, to maximize XAS signals. We cannot go to concentrations beyond 20 mg/mL due to agglomeration.

The solutions are stored at 4°C. Hemin is not soluble in PBS buffer directly, and a few drops of NaOH need to be added to ensure formation of solution. The pH of the solution is ~ 7.8. No precipitation of $Fe(OH)_3$ is observed in this condition (validated by UV-visible measurements). All amino acids are added to this solution of hemin before measurement – no significant change in pH is seen on addition of amino acids.

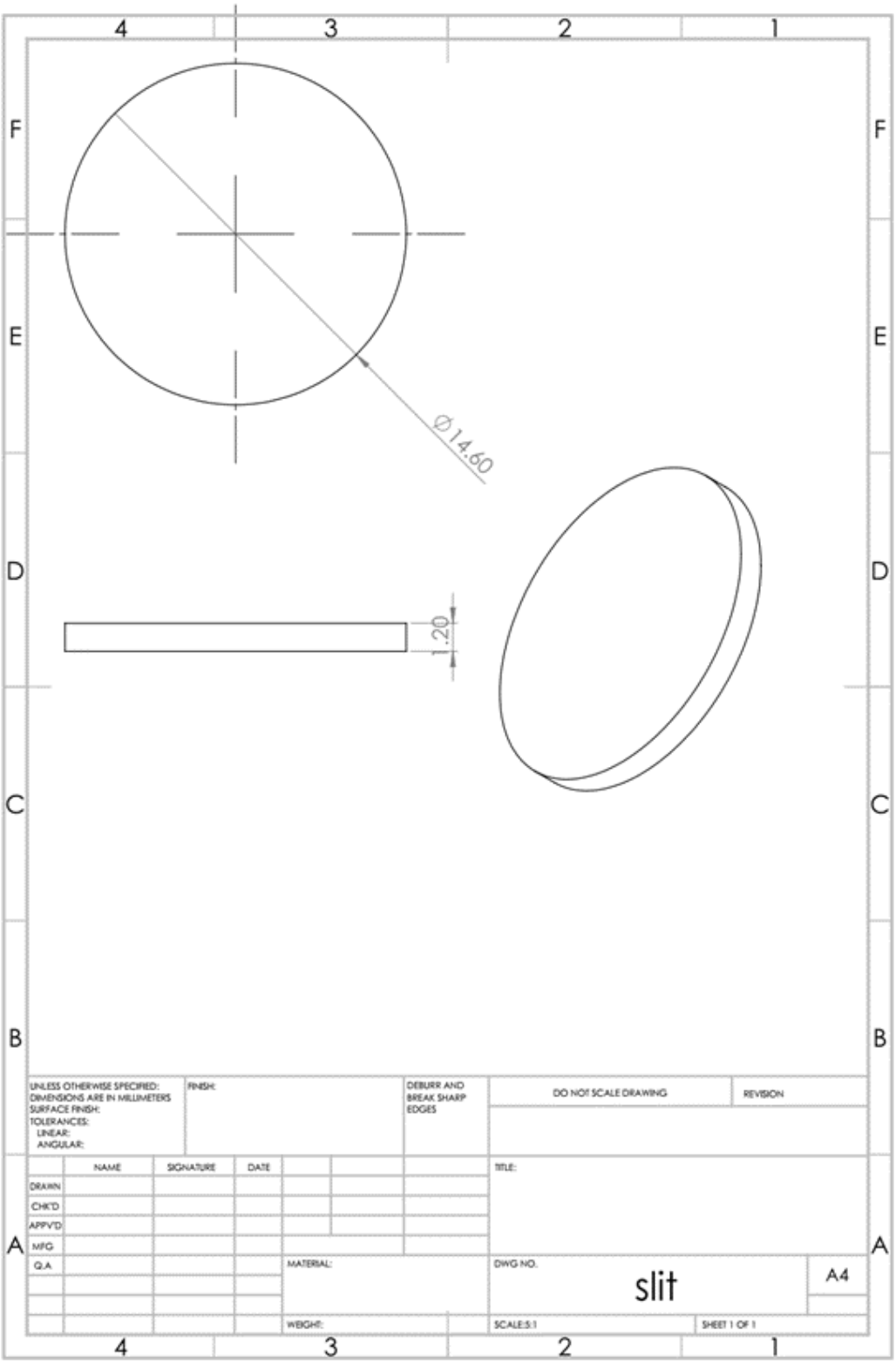
S2. Scheme for XAS data collection and cell design:

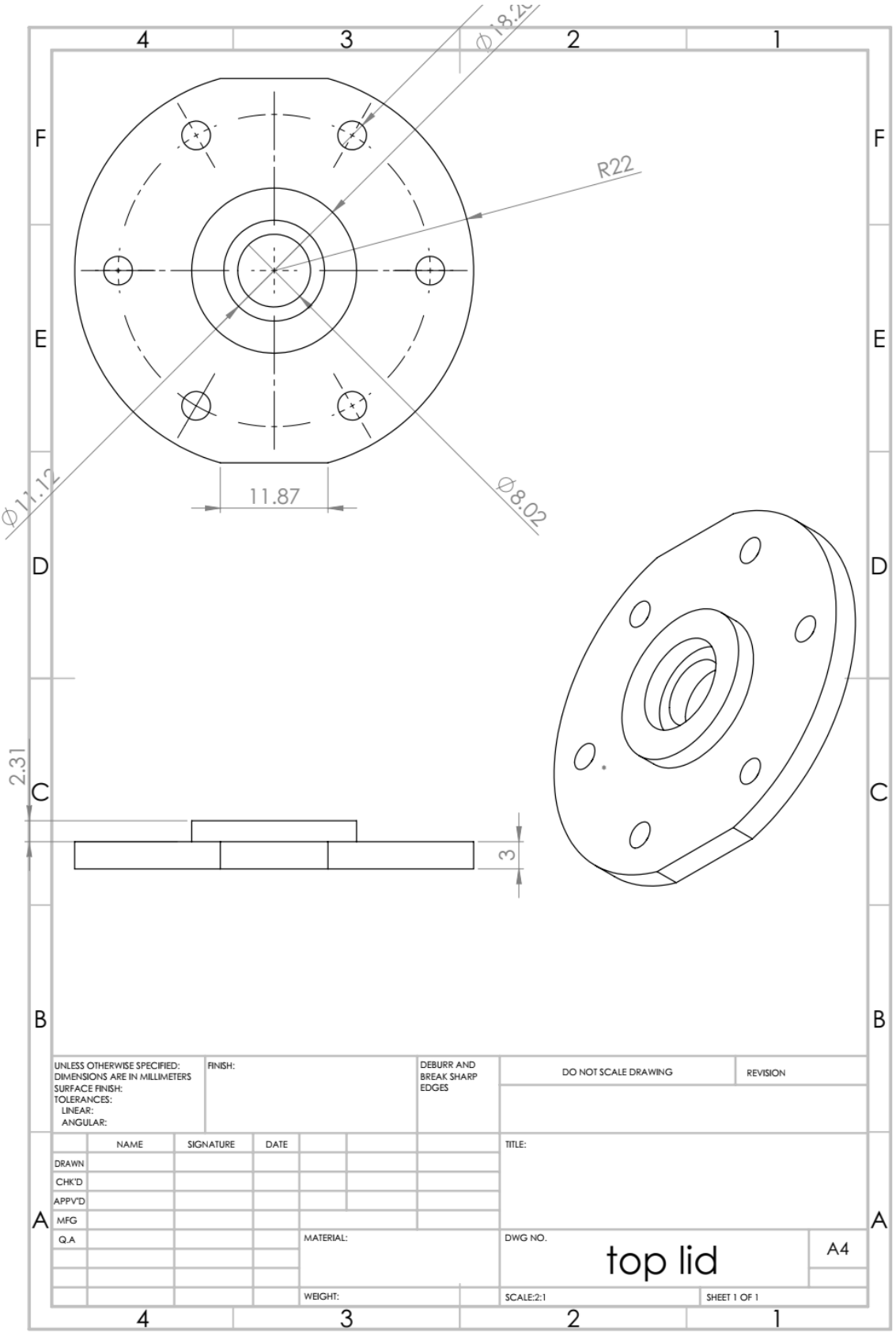
At a maximum solubility of 20mg/mL for the protein, the concentration of iron is too low to yield proper transmission signals in X-ray absorption spectroscopy. All XAS data reported in this paper are collected in fluorescence mode. We use an in-house fabricated cell with an X-ray transparent working electrode facing the X-ray source (further details in **Materials and Methods,** see **S3** for technical drawing). To reduce radiation- induced damage, we keep all measurement as short as possible to yield statistically meaningful signal-noise ratio. Each experimental run ideally last for a few minutes. We collect absorption spectra of each sample after the X-ray exposure – no significant shifts in the bands rule out any radiation damage.



Scheme S1: Mode at which XANES – CV in operando is carried out: the X – ray transparent electrode of the cell faces the beam and data is collected in fluorescence geometry, the counter / reference electrodes lie at the rear face of the cell. The cell is connected to a potentiostat for the electrochemical cycling.

S3. Technical drawing of cell components:

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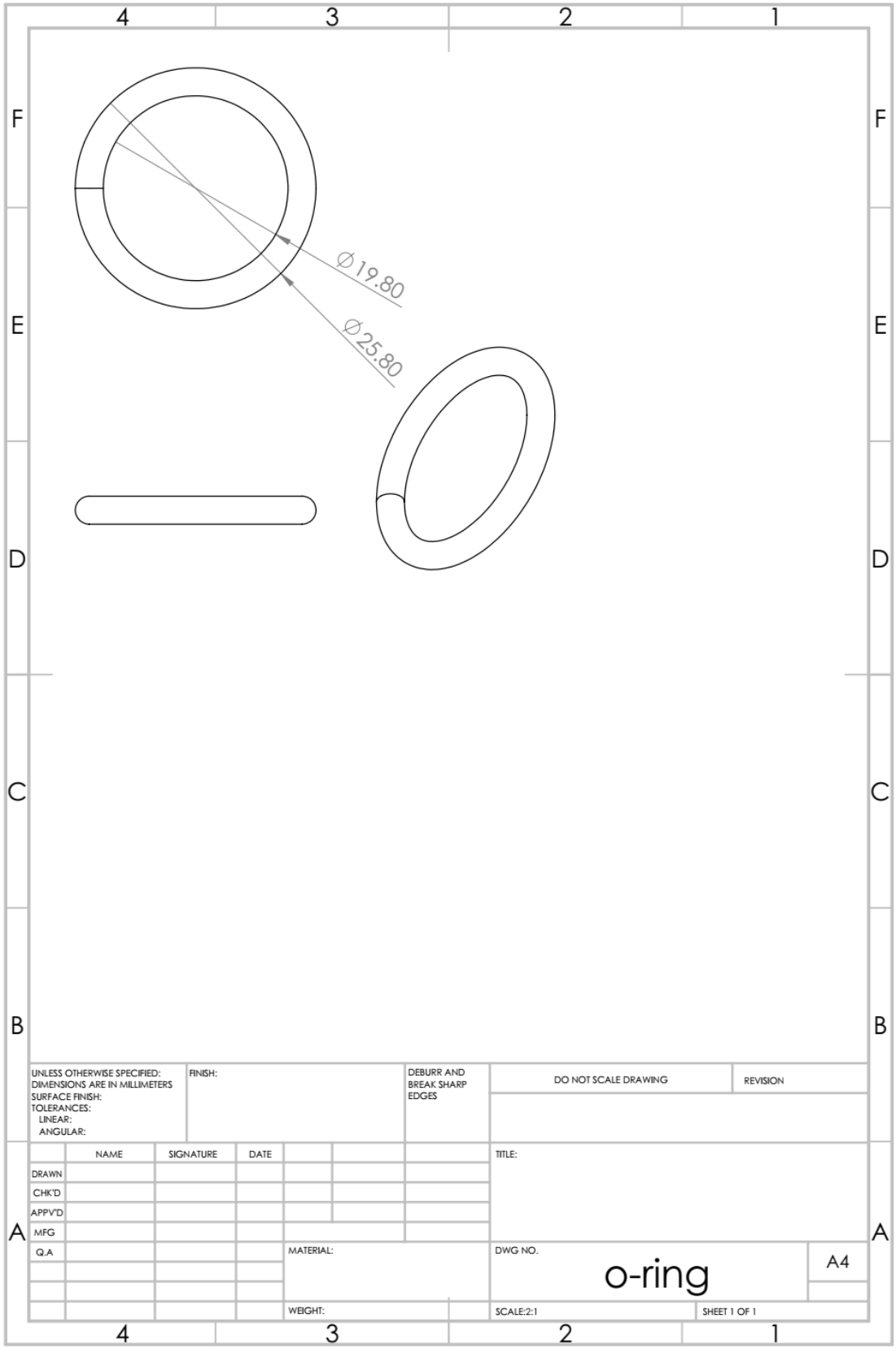
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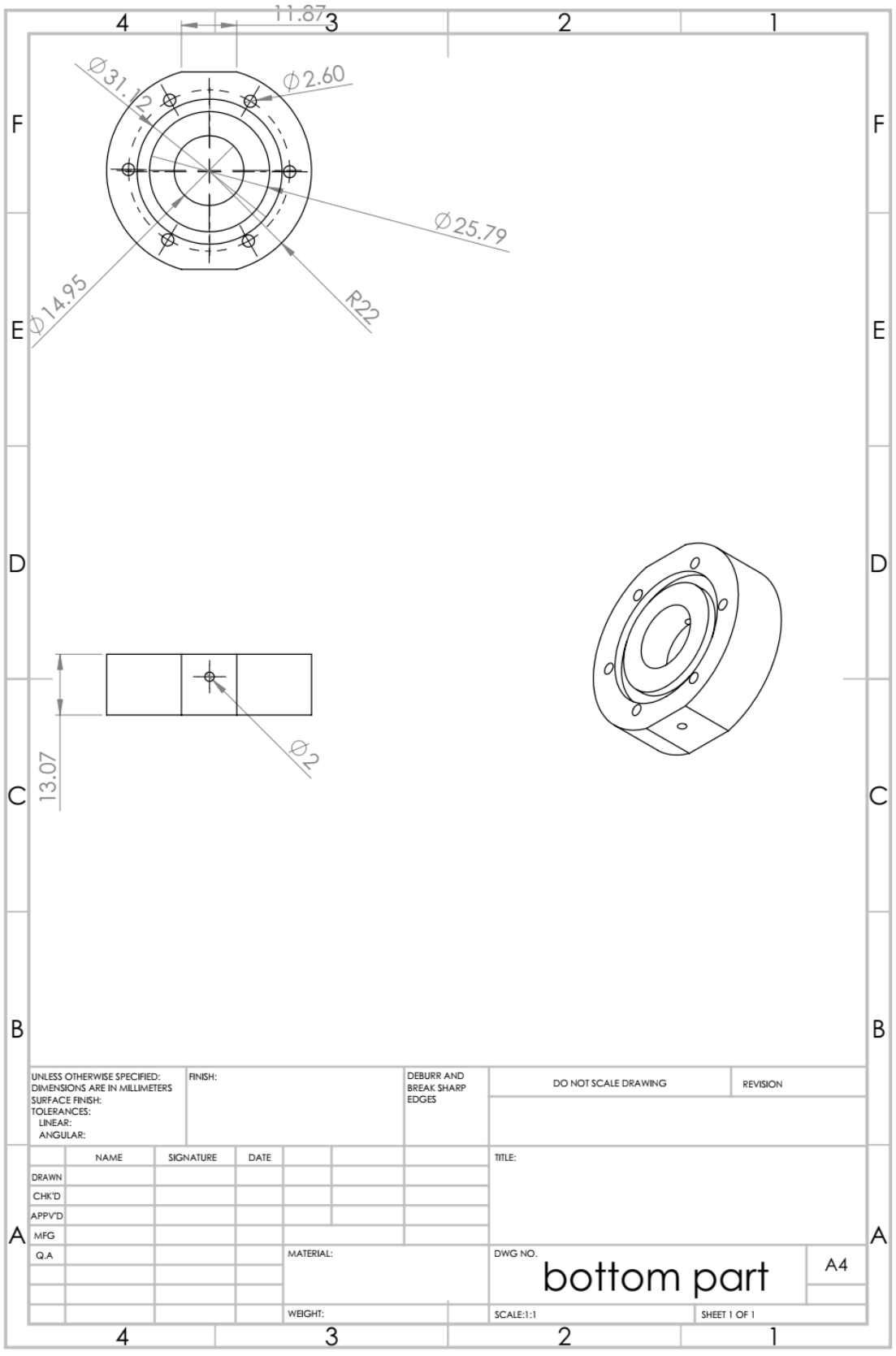

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S4. Reversibility of cyclic voltammograms: conversion efficiencies:

It is important to keep in mind that the extent of spectral changes observed during the in-operando spectro-electrochemistry experiments also depend on the 'conversion efficiencies', i.e. the number / percentage of molecules that shift from one oxidation state to another, during the course of the electrochemical cycling. We try to estimate the 'conversion efficiency' (so to speak) during the voltammetric experiments by integrating the area under the voltammogram peaks under reduction and oxidation cycling and comparing their area ratio:

$$\frac{A(P_{Oxd})}{A(P_{red})}$$

We keep the reduction peak area as the denominator as we start with both compounds in formally +III oxidation state and the reduction step precedes the oxidation step. For hemin, this ratio is found to be approximately 1, indicating close to 100% conversion / cycling efficiency – not unlike that expected for a reversible redox couple. For hemoglobin, however, this ratio is close to 0.3.

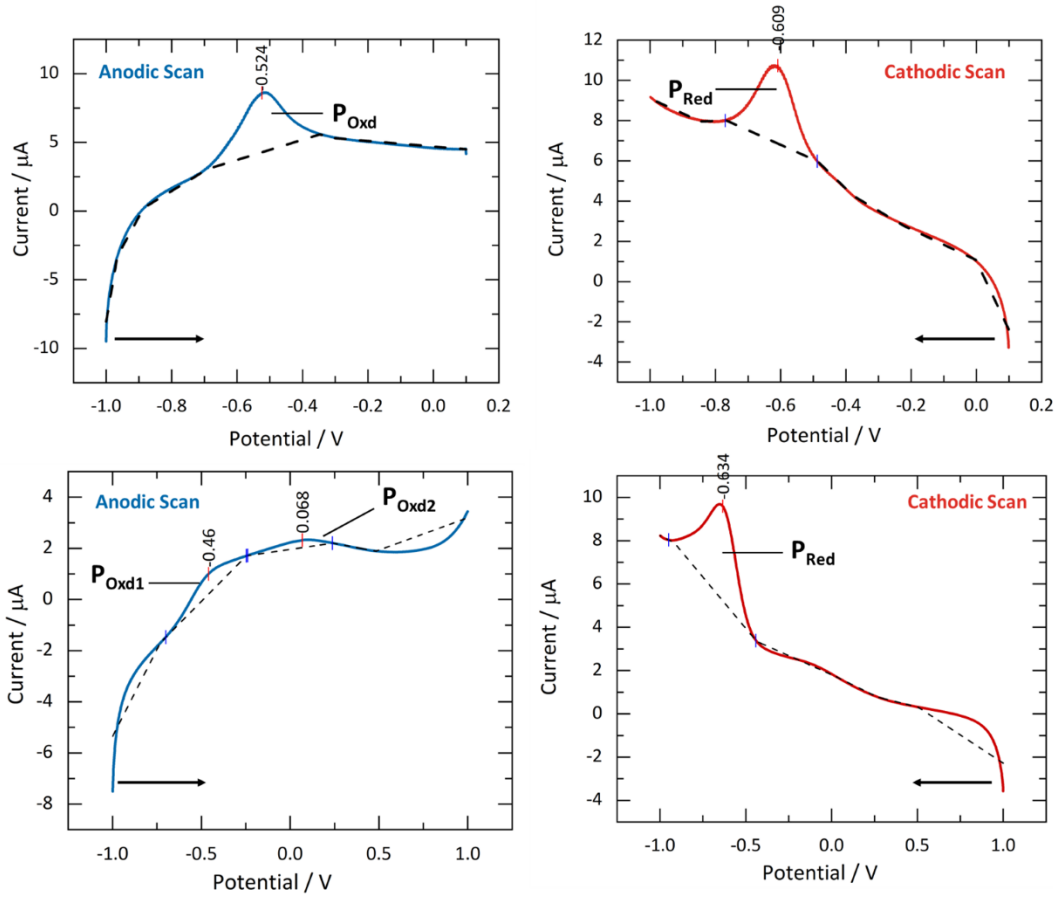

Figure S1: : Anodic sweep (oxidation, left) and cathodic sweep (reduction, right) from cyclic voltammetry of hemin (top panel), and hemoglobin (bottom panel) in solution, as reported in the manuscript, plotted separately with the oxidation and reduction peaks marked. For meaningful integration data, the cathodic current (negative by IUPAC convention, adopted in this manuscript) is shown as positive.

However, we would also like to add here that the conversion efficiencies are 'in-built' in the way we generally describe a voltammetric response of a redox couple. Hemin is reversible implying an almost hundred percent conversion efficiency while hemoglobin is quasi-reversible indicating that the reversibility is lower.

In terms of molecular numbers, we know from literature that the surface monolayer coverage of a heme protein in a similar electrochemistry experiment is 1.89×10^{-11} mol cm^{-2}.[2] With the electrode we have used (surface area 9.49×10^{-1} cm^2), we can expect a surface coverage of 17.94×10^{-12} mol. Out of this approximately 5.38×10^{-12} mol are re-oxidized in the anodic scan following the initial cathodic scan (assuming a 100% reduction in the monolayer for the initial cathodic scan).

S5. UV-Visible spectra of hemoglobin under different reducing conditions:

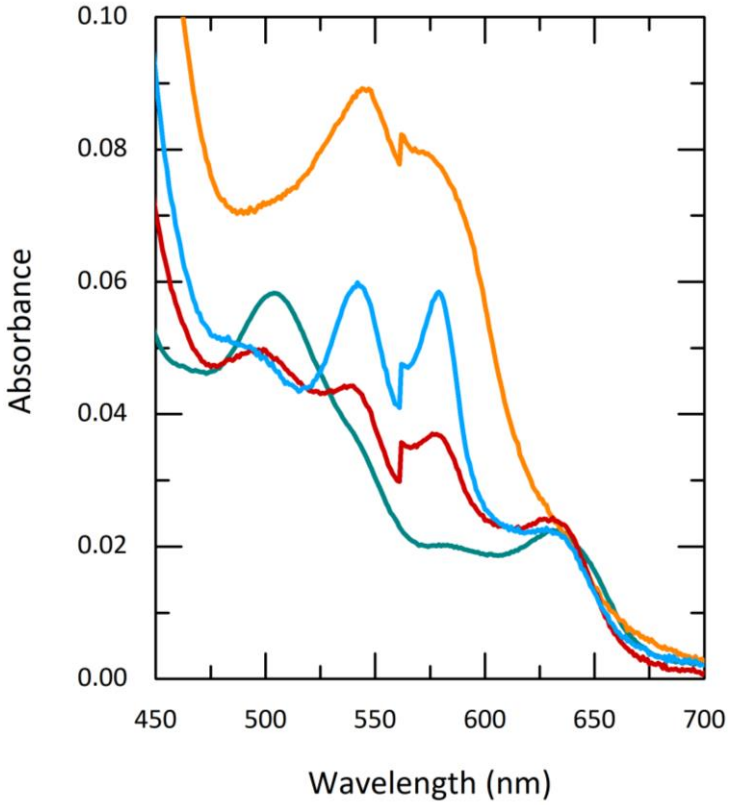

Figure S2: We know from the literature that changes in redox state and coordination environment in/around the iron center in heme is reflected in changes in the UV-Vis spectra, especially in the high wavelength (Q band) region. Here we compare the spectra of *met* protein (grey), and protein reduced with borohydride (orange), ascorbic acid (blue), and ferrocyanide (maroon). Notably, the splitting in the Q band area is affected.

S6. Derivative XANES plot for hemin coordinated to different amino acids in solution:

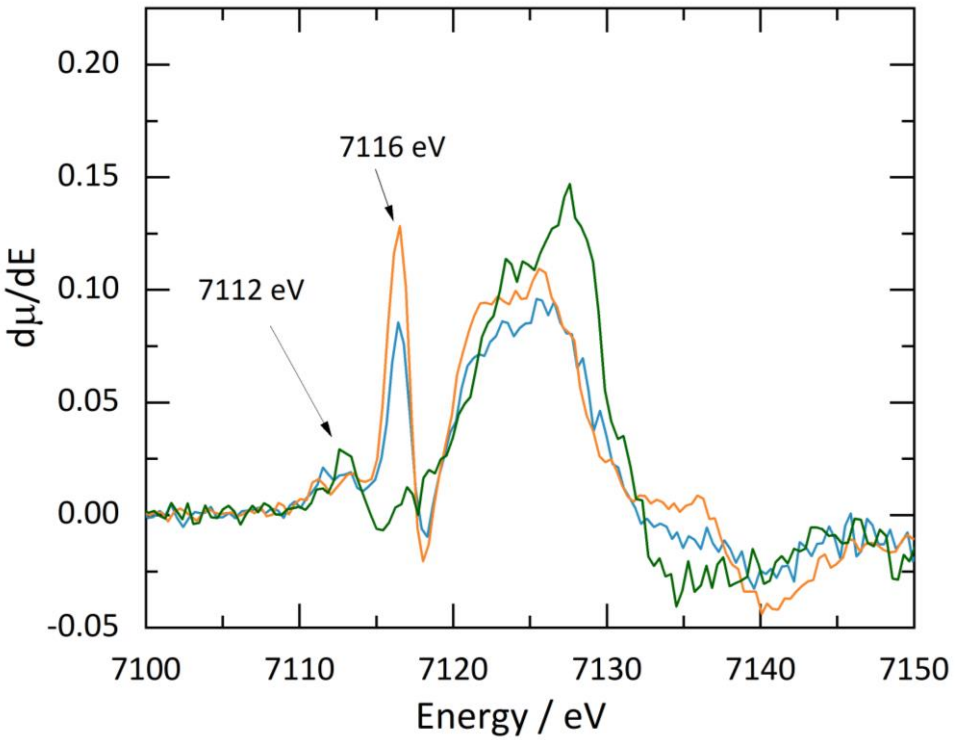

Figure S3: First derivative for absorption with energy, for hemin coordinated with cysteine (green), histidine (blue), and tryptophan (orange). The feature at ~ 7116 eV is more prominent for histidine and tryptophan and is governed by ligands that coordinate to the iron center. We also note that Hemin coordinated to cysteine has a slightly higher rising edge (~ 7127 eV) compared to the histidine and tryptophan analogues ~ 7125 eV)

S7. XANES of Hemin mixed with three coordinating species:

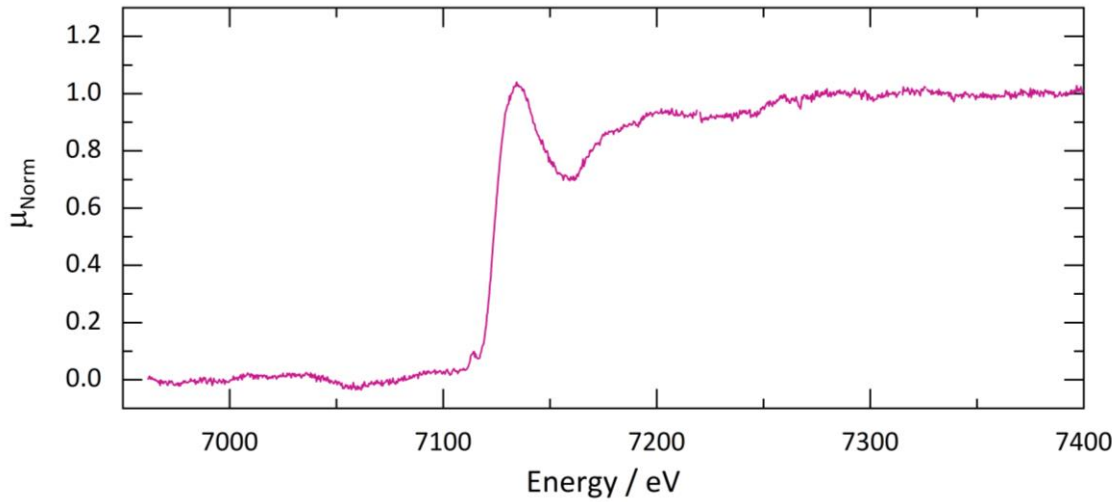

Figure S4: XANES spectrum for hemin freely mixed with all three amino acids (histidine, cysteine, and tryptophan) in solution. The spectrum shows a single pre-edge feature at ~ 7115 eV, qualitatively resembling the XANES of a heme protein.

S8. XANES of myoglobin:

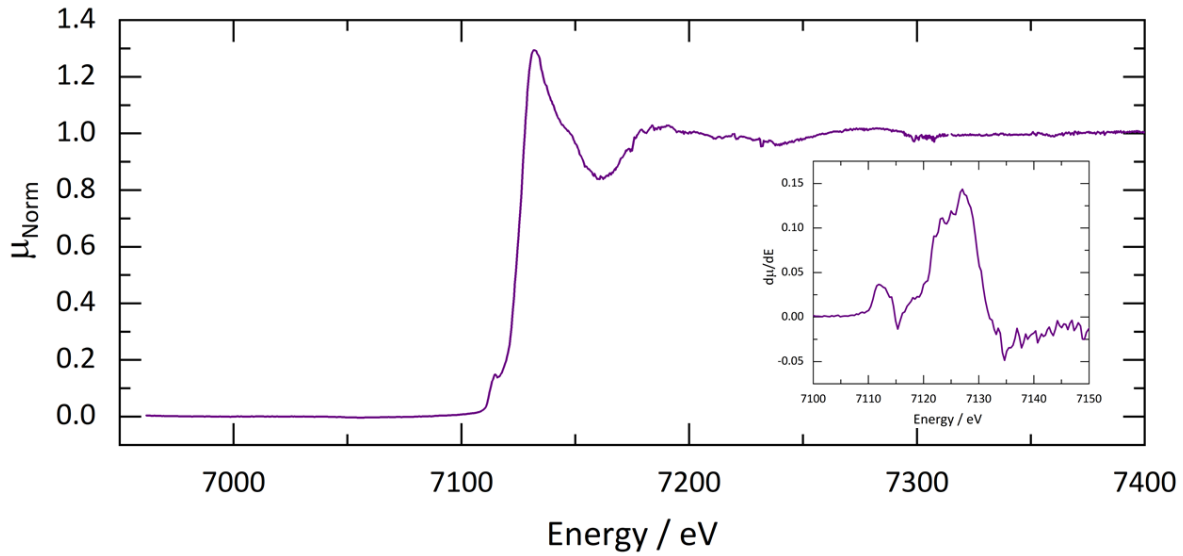

Figure S5: XANES of myoglobin in solution (first derivative plot inset). Note the neat pre-edge feature at ~ 7112 eV. This feature is invariant with haemoglobin, indicating similar coordination environment around the active site.

S9. Theoretical Framework and data analysis:

X – rays, when passing through matter get absorbed according to the Lambert Beer law:[3]

$$I = I_0 e^{-\mu t} \dots\dots\dots (28)$$

Here I is the intensity of the transmitted X – ray, I_0 is the intensity of the incident X – ray, μ is the X – ray absorption coefficient. The X – ray absorption coefficient is mathematically defined as:

$$\mu = \frac{\rho Z^4}{AE^3} \dots\dots\dots (29)$$

Here ρ is the sample density, Z is the atomic number of the sample (for pure elemental sample), A is the atomic mass, and E is the x – ray energy. When X – rays having energy greater than / equal to the energy of the core level electron in an atom, a core level electron is thrown into continuum on interacting with the X – rays (**Figure S6**).

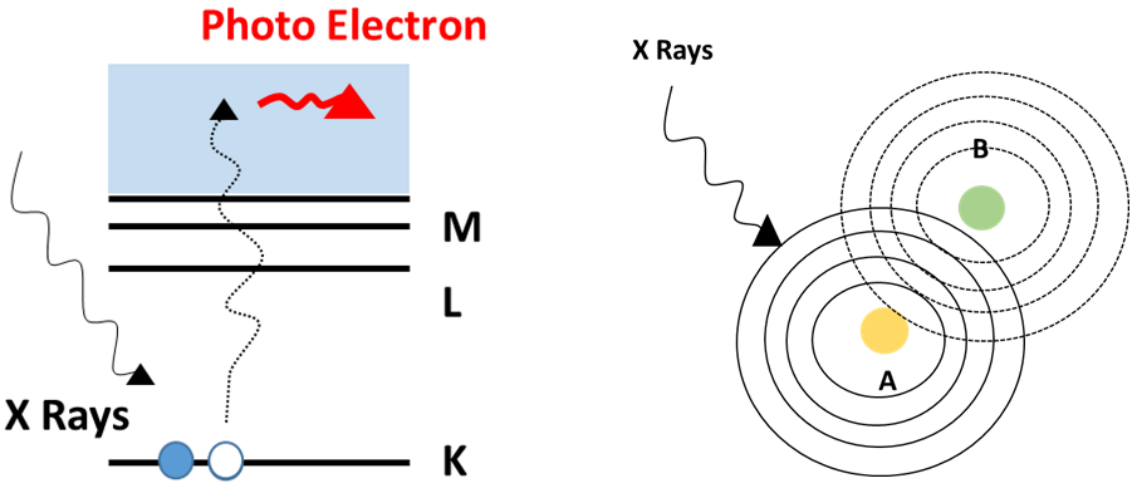

Figure S6: (Left) Phenomena following X – ray absorption, a core electron being promoted to continuum leaving a core hole behind and generating a photo – electron. **(Right)** Phenomenology of generation of EXAFS oscillation, where backscattering of the photo – electron wave from a neighboring atom gives rise to interference leading to oscillations observed.

This gives rise to a sharp edge in the X – ray absorption spectrum. Once the absorption takes place, the atom is in excited state with a core hole and a photoelectron. The excited state may relax through either fluorescence or auger mode. In a typical X – ray absorption experiment, the absorption coefficient is measured as a function of energy.

$$\mu(E) = \log \frac{I_0}{I} \dots\dots\dots (30)$$

The X – ray absorption spectrum as a function of energy is represented in **Figure S7**. The region near the absorption edge is known as the X – ray Absorption Near Edge Structure (XANES), whereas the oscillations away from the edge are known as Extended X – Ray Absorption Fine Structure (EXAFS). A phenomenological explanation for generation of EXAFS is as follows – the photo electron from the atom behaves like a radial wave and spreads across the lattice / solution. This is back – scattered from the neighbouring atom and interference between the radiated and backscattered wave gives rise to the fine structure (**Figure S6**).

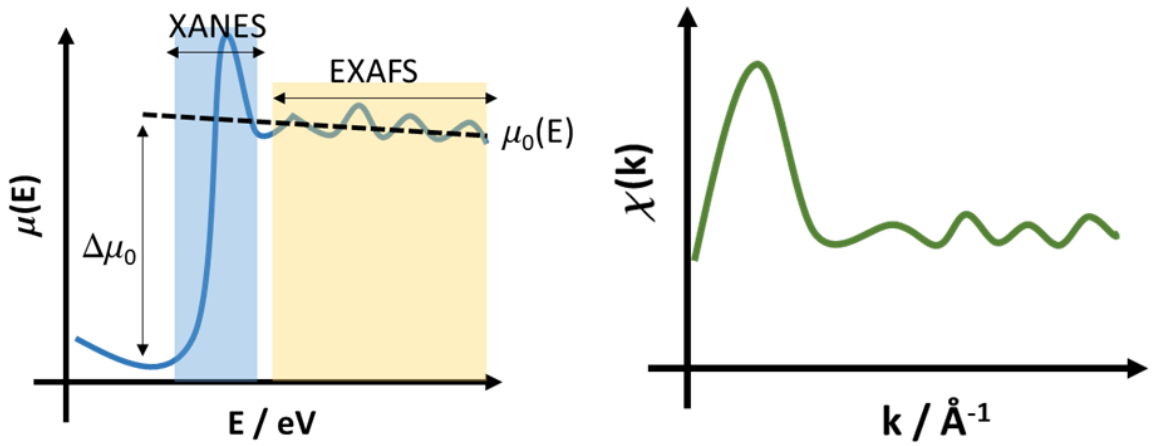

Figure S7: (Left) Typical X – ray absorption spectra, showing the XANES and EXAFS regions. The jump in the absorption at the threshold energy is represented by $\Delta\mu_0$. **(Right)** EXAFS oscillations observed in the wave number domain.

The XANES region of the X – ray absorption spectrum primarily is affected by – oxidation state, spin state, and coordination environment. The EXAFS region of the X – ray absorption spectrum is affected by radial distribution, coordination number, and disorder in the matrix. While a quantitative theory for XANES is not easy, the phenomenon of EXAFS can be modelled using simple mathematics.[4] The EXAFS fine structure can be defined as:

$$\chi(E) = \frac{\mu(E) - \mu_0(E)}{\Delta\mu_0(E)} \dots\dots\dots (31)$$

Here μ_0 (E) is the smooth background function representing the absorption of an isolated atom, and $\Delta\mu_0$ is the measured jump in the absorption at the threshold energy E_0. As EXAFS is phenomenologically understood in terms of the photoelectron wave, it is studied in the wavenumber domain. The wavenumber, k is defined as:

$$k = \sqrt{\frac{2m(E - E_0)}{\hbar^2}} \dots\dots\dots (32)$$

The EXAFS χ(k) in the wavenumber domain is defined as:

$$\chi(k) = \sum_j \frac{N_j f_j(k) e^{-2k^2\sigma_j^2}}{kR_j^2} \sin\left[2kR_j + \delta_j(k)\right] \dots\dots\dots (33)$$

Here, f(k) and δ(k) are the scattering amplitudes and phase shifts of the neighboring atoms respectively, which depend on the atomic number. N is the number of neighboring atom, R is the distance to the neighboring atom, and σ^2 is the disorder in the distance.

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