Elucidating Film Loss and the Role of Hydrogen Bonding of Adsorbed Redox Enzymes by Electrochemical Quartz Crystal Microbalance Analysis

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ABSTRACT

The immobilization of redox enzymes on electrodes enables the efficient and selective electrocatalysis of useful reactions such as the reversible interconversion of protons (H^{+}) to dihydrogen (H₂), and formate to carbon dioxide (CO₂) with hydrogenase (H₂ase) and formate dehydrogenase (FDH), respectively. However, their immobilization on electrodes to produce electroactive protein films for direct electron transfer (DET) at the protein-electrode interface is not well understood and reasons for their activity loss remain vague, limiting their performance to hour timescales. Here, we report the immobilization of a [NiFeSe]-H₂ase and a [W]-FDH from Desulfovibrio vulgaris Hildenborough on a range of charged and neutral self-assembled monolayer (SAM)-modified gold electrodes with varying hydrogen bond (H-bond) donor capabilities. The key factors dominating the activity and stability of the immobilized enzymes are determined using protein film voltammetry (PFV), chronoamperometry (CA) and electrochemical quartz crystal microbalance (E-QCM) analysis. Electrostatic and H-bonding interactions are resolved, with electrostatic interactions responsible for enzyme orientation whilst enzyme desorption is strongly limited when H-bonding is present at the enzyme-electrode interface. Conversely, enzyme stability is drastically reduced in the absence of H-bonding and desorptive enzyme loss is confirmed as the main reason for activity decay by E-QCM during CA. This study provides insights into the possible reasons for reduced activity of immobilized redox enzymes and the role of film loss, in particular H-bonding, in stabilizing bioelectrode performance, promoting avenues for future improvements in bioelectrocatalysis.

INTRODUCTION

Redox enzymes carry out key reactions in biology with unparalleled efficiency at their active sites upon electron exchange with their physiological partners.¹⁻³ Enzymes are often large and complex structures, containing transition-metal active sites buried deep within the protein that must be electronically connected to the outer surface *via* electron transfer centers such as iron-sulfur clusters (FeS).⁴ Charge exchange with the active site is facilitated when the outermost (distal) electron transfer site closely approaches the redox site of the natural redox partner (< 14 Å).⁵

The charge flowing to and from a redox enzyme can be redirected *in vitro* by immobilization of the isolated enzyme to an electrode.⁶ The stable binding of enzymes to electrode surfaces in an electroactive orientation represents a challenge in fundamental and applied bioelectrochemistry, particularly to elucidate enzyme mechanisms and for their use in sensing as well as catalysis.⁷⁻¹² Orientation control of enzymes on surfaces is critical as it allows the distal electron transfer site to closely approach the electrode for direct electron transfer (DET) in the absence of any redox mediators.

The elucidation of the true turnover frequency (TOF) of enzymes immobilized on electrodes is of key interest, however, disparities in these values exist depending on the technique used to quantify the amount of adsorbed enzyme on the electrode. One method is to analyze the nonturnover signals of the redox centers in the protein, which is observed by inhibition of the enzyme to prevent catalysis from occurring.⁷ This provides an estimate of the amount of electroactive enzyme loaded on the electrode surface. TOFs calculated by this method reach and – in some cases – exceed those measured in solution assays, however, the signal intensities are usually very weak due to the low surface coverage of enzyme $(2 - 5 \text{ pmol cm}^{-2})$ and the resulting low density of redox centers, making this technique unreliable for large proteins such as H₂ase and FDH.¹³ On the other hand, the gross loading of enzyme can be measured by gravimetric techniques such as quartz crystal microbalance (QCM) and surface plasmon resonance (SPR), however, TOF values are typically on the order of 10¹ s⁻¹, significantly below those determined by solution assays (10⁴ s⁻¹) and reasons for these observed differences are yet to be understood.¹⁴ Possible explanations include an inaccurate measure of electroactive enzyme loading, a large proportion of enzyme unable to undergo DET due to poor orientation, a considerable amount of immobilized enzyme being denatured and therefore inactive, or a suboptimal environment when the enzyme is immobilized, all of which could be reducing the TOF when compared to freely diffusing in solution.¹⁵⁻¹⁸

The stability of enzymes on an electrode is another consideration, as ideally the correctly orientated enzyme should maintain activity on the electrode over long periods of time. However, enzymes are susceptible to a range of inactivation mechanisms that may occur during catalysis such as desorption, reorientation and protein unfolding.^{19,20} Due to the complexity of enzymes and their attachment to surfaces, it is difficult to pinpoint the exact reasons for activity loss, but they can broadly be grouped into non-desorptive (denaturation and reorientation) and desorptive (loss of electroactive enzyme from the electrode) processes. To limit desorptive activity loss, the covalent coupling of the enzyme to the electrode has been used to prevent enzyme leeching from the electrode.^{10,21,24} However, this is chemically non-trivial and while some reports claimed improvements in long term activity, others have observed reduced operational stability when compared to non-covalently bound enzyme.²⁵

Therefore, to improve the DET performance and stable integration in bioelectrodes, the enzymeelectrode interface must be understood and designed to provide a desirable interaction by taking inspiration from the natural biological enzyme-redox partner interactions.¹⁸ [NiFeSe]-hydrogenase $(H_2ase)^{26}$ and [W]-formate dehydrogenase (FDH)²⁷ from *Desulfovibrio vulgaris* Hildenborough (*Dv*H) are examples of highly efficient redox enzymes that can reversibly interconvert proton $(H^+)/H_2$ and CO₂/HCO₂⁻, respectively.^{28,29} Although they are different enzymes with distinct protein structures and active sites, they both possess a natural dipole moment with a local negative region around the distal FeS cluster. This interacts strongly with a local positive region near the electron accepting heme of the natural redox partner cytochrome $c_3 (cyt-c_3)^{1.2}$ to enable fast and efficient electron transfer *in vivo* (Figure 1a). This can provide inspiration for the design of a chemically modified electrode surface that can orientate the enzyme in the same manner as the cyt-c₃, as mimicking the enzyme's natural environment on an electrode offers the best opportunity to match the high activities achieved *in vivo*.

Electrode materials for enzyme immobilization can range from carbon (graphite, carbon nanotubes, graphene), to metal oxides (indium tin oxide (ITO), titanium dioxide (TiO₂)), and gold (Au).³⁰ Carbon electrodes, such as pyrolytic graphite edge and carbon nanotubes have been successfully used to immobilize redox enzymes for DET, but they contain different aromatic, hydroxyl and carbonyl moieties and are non-planar, complicating the ability to control and study the orientation of a protein.^{10,18,31,32} Metal oxides such as ITO and TiO₂ have also been used for the immobilization of H₂ase and FDH and the resulting protein film exploited in electrocatalysis and solar fuel synthesis.^{9,33-37} However, while these materials are desirable for many applications they possess ill-defined surface terminations, making the factors that limit their activity rates to orders of magnitude below solution assays difficult to determine. It is also challenging to engineer their surfaces to mimic the enzyme's natural redox partner. While ITO and TiO₂ colloids can be chemically modified with alkylphosphonic acids, they are prone to hydrolyze in aqueous solutions, display instabilities (ITO) or a lack of conductivity (TiO₂) at certain potential ranges³⁸ and can

yield disordered self-assembled monolayers (SAMs) due to inefficient packing on the rough metal oxide surface.³⁹

Au is a highly planar noble metal that is stable over a wide redox window and can be easily modified with thiols to form stable, highly-ordered SAMs that have been thoroughly characterized, providing a well-defined, surface tunable model electrode surface on which to immobilize redox enzymes.^{40,41} The SAM can be designed to control enzyme orientation and be exploited to probe the effects of surface termination on enzyme stability (Figure 1b).⁴¹⁻⁴³ Moreover, the use of Au enables spectro-electrochemical approaches, such as surface-enhanced infrared absorption spectroscopy, as well as gravimetric techniques such as QCM.^{14,44} This allows for the *operando* study of the vibrational structure of the protein backbone and the active site of redox enzymes, as well as their adsorption onto surfaces, making this the ideal electrode to understand the enzyme-electrode interface,^{45,46} providing information that can then be transferred to other less well-defined surfaces such as carbon and metal oxides to improve the performance of enzymes for applications such as biofuel cells and photoelectrochemical devices.^{35-37,47,48}



Figure 1. (a) Electrostatic surface potentials (red = negative, blue = positive) across DvH [NiFeSe]-H₂ase, DvH [W]-FDH, and DvH cyt-c₃ (pdb: 5JSH, 6SDR and 2CTH, respectively), containing all redox centers including hemes, FeS clusters, the NiFeSe and W active sites, molybdopterin cofactors and their *in vivo* electrostatic and hydrogen bonding interactions to one another (inset). (b) Oriented immobilization of DvH [NiFeSe]-H₂ase and [W]-FDH on a SAM-

modified Au electrode with hydrogen bonding and non-hydrogen bonding SAMs and the possible non-covalent interactions at the enzyme-electrode interface (inset).

The oriented immobilization of $H_2ase^{22,49-52}$ and FDH^{17,23,53,54} for DET on electrodes has been demonstrated, but the reasons for their activity loss are not fully understood and are commonly described as "film loss" to encompass a varied range of processes speculated to be responsible for observed decreases in current density.^{15,47}

To understand the activity and stability of H₂ase and FDH on electrodes, the exact nature of the binding of physisorbed redox enzymes on modified electrodes needs to be understood as rational design is one of the most promising means to provide the step change in activities necessary to allow enzymes to approach their maximum activities determined in solution.⁵⁵ For example, protein binding to the modified electrodes is often assumed to be mainly governed by electrostatic interactions, yet additional non-covalent interactions such as hydrogen bonding (H-bonding), hydrophobic interactions and van der Waals (vdW) interactions, which can exist *in vivo* with their respective redox partners, are less commonly investigated and their net contribution to bioelectrocatalytic performance is not well known.^{53,56} Moreover, these interactions may be important for the effective immobilization of enzymes on surfaces, and could provide a greater understanding of the nature and contribution of the multiple interactions present that are required to develop systems with better enzyme orientation, activity and stability.

In this work, the non-covalent interactions that govern enzyme orientation, binding, activity, and stability at the enzyme-electrode interface were elucidated for DvH H₂ase and FDH. The influence of electrostatics and H-bonding on enzyme immobilization and orientation were probed on a range of SAM-Au electrodes using protein film voltammetry (PFV), chronoamperometry (CA) and electrochemical quartz crystal microbalance (E-QCM). By using rationally chosen SAMs, strong

evidence for the presence and role of H-bonding interactions on protein stabilization, similar to those thought to exist *in vivo*, was observed at the enzyme-electrode interface for the first time, providing an insight into the approaches needed to improve redox enzyme performance on electrodes to allow them to approach their maximal rates that can be achieved in solution assays.^{25,57}

RESULTS AND DISCUSSION

Electrostatic orientation on SAM-modified Au electrodes

First, SAM-modified Au electrodes were prepared by immersing a gold rotating disk electrode (RDE) (geometric area = 0.0314 cm²) in aqueous solutions of the relevant thiol (10 mM) overnight. Five SAMs were used to represent different charge and H-bonding ability: 2-mercaptoethanol (2-ME°), 3-mercaptopropionate (3-MPA⁻), 2-dimethylammoniumethanethiol (2-DMAET⁺), 2-trimethylammoniumethanethiol (2-TMAET⁺) and 2-ammoniumethanethiol (2-AET⁺) (Figure 2a). At pH 6, according to their respective pK_a (Figure 2a), these thiols generate a surface charge denoted by their superscript.^{21,58-62} To confirm the assembly and net charge of thiol-based SAMs on gold, 2-AET⁺ was functionalized onto gold nanoparticles and the pH-dependent charge confirmed by zeta-potential measurements (Figure S1).

The orientation of H₂ase and FDH immobilized on the SAM-modified electrodes at pH 6 was found to be strongly dependent on the surface charge. Good's buffers at pH 6 for H₂ase and pH 8 for FDH were selected to provide optimal electrolyte conditions for proton reduction²⁶ and formate oxidation²⁷, respectively – the reactions of interest in the subsequent E-QCM experiments. Bubble formation from CO₂ purged buffers in the E-QCM experiments prevented the accurate study of CO₂ reduction with FDH. Electrochemical DET activity for both H^+ reduction and H_2 oxidation for H_2 ase, and formate oxidation for FDH was highest using the positively charged SAMs 2-DMAET⁺, 2-TMAET⁺ and 2-AET⁺, which can be ascribed to the electrostatic attraction to the negatively charged region surrounding the distal FeS cluster in both enzymes (Figure 2b, c).



Figure 2. (a) Thiol-based SAMs used to control the enzyme-orientation and stability on the electrode, their electrostatic and H-bonding properties at pH 6 and their surface pK_a values. ^{*}Denotes the bulk solution pK_a in the case where the surface pK_a value is not available (2-ME°) or a where a pH independent head group indicates no pK_a value is measured (2-TMAET⁺). (b) Activity of H₂ase adsorbed on different SAM-modified gold electrodes taken from the current recorded by PFV at +0.1 V (filled squares) and -0.1 V vs RHE (empty squares). (c) Activity of FDH adsorbed on different SAM-modified gold electrodes taken from the current recorded by PFV at +0.5 V vs RHE (filled triangles). Conditions: MES/KCl (50 mM/50 mM, pH 6), 1 atm H₂ for H₂ase (10 pmol), HEPES/KCl/formate (50 mM/50 mM/20 mM, pH 8) for FDH (40 pmol) activated by incubation with 1,4-dithiothreitol (DTT, 50 mM). v = 5 mV s⁻¹, ω = 2000 rpm, 25 °C.

The electrocatalytic waveshape of FDH immobilized on the three positively charged electrodes $(2-AET^+, 2-DMAET^+, 2-TMAET^+)$ displayed hysteresis under N₂ (Figure S2b), which disappears upon saturation of the electrolyte with CO₂ with a concurrent decrease in activity (Figure S3). This is likely due to the oxidation of formate to CO₂ at positive overpotentials in the N₂ saturated electrolyte, which affects the intrinsic activity of the enzyme on the reverse scan possibly due to saturation of the substrate channels of the enzyme, leading to hysteresis. Similar inhibition of H₂ase was observed in the presence of H₂.¹⁹ To retain sufficient activity and to prevent the use of CO₂ purged buffers which would affect subsequent E-QCM experiments, an N₂ saturated electrolyte was used for all FDH experiments and a potential of +0.5 V vs RHE was chosen for current analysis, which is the point at which hysteresis is at a minimum, allowing for confidence in the currents analyzed.

The redox mediators methyl viologen (MV²⁺, 250 μ M, $E^{0*} = -0.09$ V vs RHE at pH 6) and benzyl viologen (BV²⁺, 250 μ M, $E^{0*} = +0.11$ V vs RHE at pH 8) can be used to estimate the amount of enzyme immobilized on the surface irrespective of orientation *via* mediated electron transfer (MET) as any enzyme not oriented via the distal FeS cluster can undergo MET, resulting in an increase in current density (*j*).^{18,63} Subsequently, the amount of DET current compared to MET current provides a $j_{\text{DET}}/j_{\text{MET}}$ value, which signifies the proportion of enzyme bound to the electrode in a favorable orientation *via* the distal FeS cluster, where j_{MET} includes any contribution from DET. Adding mediators showed little to no net increase over the DET currents for H₂ oxidation by H₂ase or formate oxidation by FDH on the three positively charged electrodes, which suggests near quantitative binding of H₂ase and FDH in the correct orientation for DET as evidenced by a near unity $j_{\text{DET}}/j_{\text{MET}}$ value (Figure 3).

In contrast, a lower *j* was observed for 2-ME°, which indicates non-optimal orientation and only a fraction of the enzyme immobilized in DET orientation (Figure 2b, c). The addition of redox mediators led to an increase in anodic *j* (j_{ox}) for both enzymes on the neutral electrode (Figure 3), although this was less pronounced for H₂ase, as shown by the catalytic waveshape in the representative protein film voltammograms (Figure S2).¹⁸

A similarly low j_{ox} of $3.3 \pm 2 \,\mu$ A cm⁻² and $5.9 \pm 2.7 \,\mu$ A cm⁻² was observed for H₂ase and FDH on 3-MPA⁻, respectively, which can be assigned to the repulsion of the distal FeS region of the enzymes (Figure 2b, c). The poor orientation of the enzymes on 2-ME^o and 3-MPA⁻ was confirmed by the addition of redox mediators, which significantly increased j_{ox} , resulting in a j_{DET}/j_{MET} of 0.35 ± 0.07 and 0.16 ± 0.04 for H₂ase and FDH, respectively on 3-MPA⁻ (Figure 3). An enzyme-free CV in the presence of BV²⁺ displayed a much lower j_{ox} than in the presence of FDH, confirming that the increase in j_{ox} was due to enzymatic MET (Figure S4).



Figure 3. DET to MET current density ratio extracted from the PFV response for H₂ase for H₂ oxidation at +0.1 V vs RHE (squares) and FDH for formate oxidation at +0.5 V vs RHE (triangles) on each SAM-modified electrode. Conditions: H₂ase (10 pmol), MES/KCl (50 mM/50 mM, pH 6), 1 atm H₂, MV²⁺ (250 μ M). FDH (40 pmol), DTT (50 mM), HEPES/KCl/formate (50 mM/50 mM/20 mM, pH 8), BV²⁺ (250 μ M). v = 5 mV s⁻¹, ω = 2000 rpm, 25°C. Error bars represent the standard deviation for a sample size of n = 3.

Stability of the enzyme film by CA

In addition to orientation in an electroactive configuration, the design of electrode surfaces to promote enzyme films with high stability on electrodes is another key feature in bioelectrocatalysis. The electrocatalytic stability of H₂ase on each of the well-orientated positively charged electrodes was therefore assessed by CA at an applied potential (E_{app}) of +0.1 V vs RHE (Figure 4). 2-AET⁺ and 2-DMAET⁺ displayed a similar decrease in electrocatalytic activity, with a loss in current of (46±9.6) % and (52±5.4) % over 2 h, supporting that the film stability was not detrimentally affected by the presence of methyl moieties. On the other hand, 2-TMAET⁺ exhibited

a rapid current decay in the first 20 min and lost (97±6.7) % of its activity over 2 h. This difference may be explained by the differing H-bonding abilities of each SAM on the electrode. 2-AET⁺ and 2-DMAET⁺ are protonated primary and secondary amines and can act as strong H-bond donors,⁶⁴ whereas 2-TMAET⁺ is a quaternary ammonium cation that cannot act as a H-bond donor.⁶⁵



Figure 4. CA of H₂ase for H₂ oxidation adsorbed on 2-AET⁺, 2-DMAET⁺ and 2-TMAET⁺. Conditions: MES/KCl (50 mM/50 mM, pH 6), 1 atm H₂, H₂ase (10 pmol), 25°C, ω = 2000 rpm, E_{app} = +0.1 V vs RHE. Error bars represent the standard deviation for a sample size of n = 3.

The reasons for the loss in electrocatalytic activity for each of the H₂ase-films can be attributed to either desorptive or non-desorptive processes, which cannot be determined by electrochemistry alone. To further understand factors contributing to film loss, gravimetric techniques such as QCM combined with electrochemistry can provide a better understanding of the enzyme-electrode interface.

Investigating electrostatic interactions by E-QCM

QCM can quantify the loading of enzymes on an electrode and, in combination with electrochemical analysis (E-QCM), can be used to probe changes at the enzyme-electrode interface under turnover conditions.⁴⁴ A typical monolayer film of H₂ase on 2-AET⁺-modified gold QCM sensors reached (5.0 ± 0.3) pmol cm⁻², whereas a lower loading was observed for 2-TMAET⁺ ((3.2 ± 0.1) pmol cm⁻²; Figure 5a; Equation S1). These surface coverages are comparable to the monolayer coverage observed previously for H₂ase on planar TiO₂,³⁷ and is comparable to a theoretical monolayer loading of 3 - 10 pmol cm⁻² of H₂ase ($8.5 \text{ nm} \times 7.5 \text{ nm} \times 6.5 \text{ nm}$) depending on the orientation of the enzyme upon immobilization. Therefore, the loading values generated by E-QCM can be used to accurately determine turnover frequencies (TOFs) without the uncertainty of an assumed enzyme loading provided the majority of the immobilized enzymes are orientated for DET. However, limitations arise here as the proportion of loaded enzyme that may be denatured upon immobilization is unknown.

The strength of the enzyme-electrode interaction was probed by washing the H₂ase-adsorbed SAM-modified electrodes with enzyme-free MES/KCl (50 mM/ 50 mM, pH 6) buffer, followed by 3 M KCl to shield electrostatic interactions between the enzyme and electrode and decouple the ratio of electrostatically bound to non-electrostatically bound H₂ase (Figure 5b). A small decrease in adsorbed H₂ase was observed for both 2-AET⁺ and 2-TMAET⁺ when switching from the denser enzyme-containing solution to the enzyme-free buffer signaling removal of physisorbed enzyme bound on top of the underlying monolayer protein film but, surprisingly, no further H₂ase desorption was observed after 3 M KCl on 2-AET⁺ indicating that all enzyme molecules were bound by non-electrostatic interactions. FDH also remained quantitatively bound to 2-AET⁺ after





Figure 5. (a) QCM profile for the immobilization of H_2 as on 2-AET⁺ and 2-TMAET⁺-modified Au sensors. (b) Desorption profiles of H_2 as on the SAM-modified Au sensors after a buffer

exchange followed by exposure to 3 M KCl. (c) PFV responses of H_2 ase on the SAM-modified sensors after buffer exchange and 3 M KCl. Conditions: MES/KCl (50 mM/ 50 mM, pH 6), 1 atm H_2 , H_2 ase (16 nM), flow rate = 0.141 mL min⁻¹, 25 °C. Error bars represent the standard deviation for a sample size of n = 3 across three independent sensors.

This observation is unexpected as the most commonly referenced physisorbed interaction at the enzyme-electrode interface is electrostatic, without reference to other possible interactions.^{56,66} The activity of the remaining H₂ase was confirmed by protein film voltammograms in MES/KCl (50 mM/ 50 mM, pH 6) buffer solution after each ionic solution washing step, where negligible current loss was observed after exposure to 3 M KCl (Figure 5c), with quantitative activity also observed for FDH on 2-AET⁺ after 3 M KCl (Figure S5c). Additionally, no desorption was observed by QCM for H₂ase washed with 3 M KCl on 3-MPA⁻ even though the negative charge of the electrode misorients the enzyme, supporting that the protein is still bound strongly to the electrode by other non-covalent interactions regardless of orientation (Figure S6b). Enzyme-enzyme interactions were also investigated by loading only half of the expected monolayer of H₂ase on 2-AET⁺ followed by the same washing procedure, with no desorption occurring after 3 M KCl (Figure S7). This hints that the enzyme was stable on the surface at sub-monolayer coverages where the possibility of interactions between enzymes may be minimized due to regions of increased inter-enzyme spacing.

This provides strong evidence for distinct non-covalent interactions that separately govern orientation and immobilization at the enzyme-electrode interface. This is also possible for FDH on TiO_2 where 60% of FDH remained adsorbed after exposure to 3 M KCl.⁵⁴ Due to the presence of H-bonding between H₂ase and cyt-c₃.^{67,68} it is possible that H-bonding is the main non-covalent interaction playing a role in immobilization at the H₂ase-2-AET⁺ interface, preventing enzyme

desorption when electrostatic interactions are shielded by the 3M KCl solution. H-bonding for physisorbed enzymes to electrodes has been suggested previously for Cu, Zn superoxide dismutase on cysteine-modified Au electrodes, where H-bonding between -NH₃⁺ of the cysteine zwitterion and -OH of Thr135 was proposed, although no evidence for this interaction was provided.^{69,70}

To validate the hypothesis of H-bonding, H₂ase adsorbed on the 2-TMAET⁺-modified QCM Au sensor was subject to 3 M KCl, and a loss of (38±5) % compared to the initial loading of H₂ase was observed (Figure 5b), whilst FDH-adsorbed on 2-TMAET+ led to a loss of (20±8) % compared to the initial loading (Figure S8b). This could be rationalized by the absence of H-bonding between the enzyme and 2-TMAET⁺, leading to a larger contribution from electrostatic interactions to immobilization, ultimately prompting enzyme desorption at high salt concentrations when these interactions were shielded. Mediator-free protein film voltammograms recorded after the observed desorption confirmed loss of H₂ase (Figure 5c) and FDH (Figure S8c) from the 2-TMAET⁺ sensor, with the current decreasing by a larger proportion than the loading possibly being due to nondesorptive activity loss such as reorientation or active site degradation occurring simultaneously with desorptive activity loss. To confirm that the desorption of protein from 2-TMAET⁺ was not due to a more hydrophobic surface, H₂ase was loaded onto a propanethiol-modified Au sensor, a purely hydrophobic and non-electrostatic surface, whereupon no desorption after 3 M KCl was observed, indicating that hydrophobic interactions between the enzyme and electrode were also stable in the presence of high ionic concentrations (Figure S9).

Due to the previous observation of a near quantitative amount of enzyme orientated for DET on the positively charged electrodes (Figure 3), TOFs of the monolayer enzyme films were extracted from the loading and electrocatalytic data by E-QCM (Table 1) with the assumption that j_{DET}/j_{MET} at -0.1 V vs RHE and +0.5 V vs RHE for H₂ase and FDH, respectively, on 2-AET⁺ and 2-TMAET⁺

is 1 (Figure S10 and Figure S11). The highest apparent TOFs (TOF_{apparent}) were observed on 2-AET+ (21.1±1.8 s⁻¹ for H₂ase and 17.5±0.8 s⁻¹ for FDH) and 2-TMAET+ (17.7±2.9 s⁻¹ for H₂ase and 26.8±1.2 s⁻¹ for FDH) taking into account the gross enzyme loading by QCM. This is much lower than the activities observed by conventional solution assays for H₂ase and FDH (5201 s⁻¹ and 1100 s⁻¹ for H⁺ reduction and formate oxidation, respectively, see Materials section), which was also observed for bilirubin oxidase on SAM-modified Au by electrochemical SPR and E-QCM.^{25,71,72} The reason for this discrepancy is unclear and remains a common challenge in the field of PFV (Table S1).^{17,49} In this work, we have so far been able to rule out poor orientation and enzyme desorption as factors that contribute to the low electrochemical TOFs, and although this work signifies progress towards an understanding of why the electrochemical TOFs are much lower than solution TOFs, further work in the PFV field is necessary to elucidate this. Some reasons for this difference in activity could be (i) protein deconformation upon immobilization with a heterogeneous substrate as opposed to a homogeneous soluble redox partner (redox mediator in *vitro* or cyt-c₃ *in vivo*), (ii) protein crowding on electrodes altering the rate of enzyme reactions, or (iii) electric field induced protein denaturation.^{15,71,73-76} One key question that remains is whether the majority of the loaded enzymes are inactive, or whether the intrinsic activity of each enzyme molecule is lower, and consequently the method used to calculate the TOF is extremely important. From our results, the low TOFs on electrodes conveys the need to better understand and optimize interfacial electron transfer by methods other than solely orientation to realize limiting currents similar to the activities observed in solution assays.

Nevertheless, the electrode activity values herein are similar to DET TOFs observed for various redox enzymes on SAM-Au, metal oxide, functionalized graphite, Ketjen Black and carbon cloth electrodes, thus providing a comparable system with the current state of the art in the PFV field

with which to further analyze the function of non-covalent interactions at the enzyme-electrode interface (Table S1).^{9,71,75,77-79}

H₂ase loaded on 3-MPA⁻ displayed a significantly lower TOF_{apparent} of 1.5±0.1 s⁻¹ by E-QCM, initially indicating that the TOF is limited by electron transfer due to the suboptimal orientation of the enzyme molecules on the electrode. However, the TOF_{apparent} considers only the total enzyme loading (Equation S2). Therefore, to calculate the real TOF (TOF_{actual}) of H₂ase on 3-MPA⁻, the actual amount of electroactive enzyme (electroactive loading) generating catalytic current must be considered as opposed to the total amount of immobilized enzyme (Equation S3). This was realized by the addition of MV²⁺ to the H₂ase-adsorbed 3-MPA⁻ QCM sensor, which led to a drastic increase in the current density (*j*) from (1.27±0.1) μ A cm⁻² to (21.0±1.3) μ A cm⁻², exhibiting a *j*_{DET}/*j*_{MET} of 0.06±0.01 at a total loading of 4.4±0.2 pmol cm⁻² (Figure S6c). Therefore, assuming that the intrinsic enzyme activity is unaffected by orientation, of the total H₂ase loaded on 3-MPA⁻, only (0.26 \pm 0.02) pmol cm⁻² of H₂ase are in direct electronic communication with the 3-MPA⁻ electrode. This results in a TOF_{actual} of 25.4±1.8 s⁻¹, within the same order of magnitude of H₂ase on the positively charged electrodes, and within error of the TOF_{actual} of 25.9±1.6 s⁻¹ for H₂ase on 3-MPA-in the presence of the redox mediator MV²⁺ (Figure S6c), confirming that intrinsic enzyme activity is retained on negative electrodes when compared to positive electrodes, but still much lower than their activity in solution. This analysis emphasizes the importance of knowing both the loading and orientation information to accurately measure an enzyme TOF, something which lacks consideration in the bioelectrocatalysis field.

Enzymatic system ^a	Total Loading (pmol cm ⁻²) ^b	Electroactive Loading (pmol cm ⁻²) ^c	lj _{DET} l (µA cm ⁻²) ^d	<i>j_{MET}</i> (µA cm ^{−2}) ^{<i>e</i>}	jdet/jmet	TOF _{apparent} (s ⁻¹) ^f	$\mathrm{TOF}_{\mathrm{actual}}(\mathrm{s}^{-1})^{\mathrm{g}}$
H ₂ asel2- AET ⁺ I _{DET}	5.1±0.1	5.1±0.1	20.6±1.8	20.6±1.8	1.0	21.1±1.8	21.1±1.8
H ₂ asel2- TMAET ⁺ _{DET}	3.2±0.1	3.2±0.1	10.9±1.4	10.9±1.4	1.0	17.7±2.9	17.7±2.9
FDHl2- AET+l _{DET}	3.7±0.3	3.7±0.3	12.5±0.5	12.5±0.5	1.0	17.5±0.8	17.5±0.8
FDHI2- TMAET ⁺ I _{DET}	2.8±0.2	2.8±0.2	14.4±0.6	14.4±0.6	1.0	26.8±1.2	26.8±1.2
H2asel3- MPA ^{-I} DET	4.4±0.2	0.26±0.02	1.27±0.1	21.0±1.3	0.06±0.01	1.5±0.1	25.4±1.8
H ₂ asel3- MPA ⁻ I _{MET}	4.4±0.2	4.4±0.2	-	21.0±1.3	-	-	25.9±1.6

Table 1. Summary of information obtained from E-QCM analysis for H₂ase and FDH on SAM-modified Au sensors.

^{*a*}The subscripts _{DET} or _{MET} refers to the current density (j_{DET} or j_{MET}) used when calculating the catalytic current *i* used in Equations S2 and S3. ^{*b*}Total loading is calculated using Equation S1 in the Materials and Methods section. ^{*c*}Electroactive loading is calculated by considering j_{DET}/j_{MET} values for each enzymatic system. ^{*d*}Values of $|j_{DET}|$ for H₂ase are obtained from PFV responses at E = -0.1 V vs RHE, while $|j_{DET}|$ for FDH are obtained from PFV responses at E = +0.5 V vs RHE. ^{*e*}Values of $|j_{MET}|$ are obtained by the addition of 250 μ M MV²⁺. ^{*f*}TOF_{apparent} is calculated using Equation S3 in the Experimental section. ^{*g*}TOF_{actual} is calculated by using Equation S3 in the Experimental section, taking into account j_{DET}/j_{MET} , where j_{MET} includes the contribution from DET. All data was acquired from E-QCM experiments.

Deconvoluting desorptive and non-desorptive activity loss with E-QCM

E-QCM allows for monitoring the change in surface coverage *operando* and to probe the different surface conditions leading to either desorptive or non-desorptive activity loss. CA was performed on 2-AET⁺ and 2-TMAET⁺ QCM Au sensors preloaded with 5.0 and 3.2 pmol H₂ase cm⁻², respectively, after which a 2-minute pre-equilibration was applied at a constant potential before beginning the CA. Figure 6a shows the change in enzyme loading (top) and current decay (bottom) during CA. No change in protein loading was observed over 1 h, but the proton reduction

current at -0.1 V vs RHE decays by (65±10) % for the H-bond capable 2-AET⁺, indicating that the mechanism for activity loss was non-desorptive due to the presence of H-bonds between the protein and electrode. It was previously speculated that strong electrostatic interactions may destabilize bilirubin oxidase on 6-mercaptohexanoic acid-modified electrodes,⁷⁵ which could be a possible driver of the observed non-desorptive activity loss for H₂ase and FDH on 2-AET⁺.

Comparing the two H-bonding extremes, the H-bond diminished 2-TMAET⁺ displayed simultaneous enzyme desorption of (14 ± 1.3) % ((0.45 ± 0.04) pmol cm⁻²) of adsorbed H₂ase with a current decay of (86±9) % in the first 20 min, indicating that potential-induced enzyme desorption from the electrode is only possible in the absence of H-bonding (Figure 6a). The same trend was observed for FDH, where negligible enzyme desorption was observed with a current decay of (75±11) % for formate oxidation at +0.4 V vs RHE on 2-AET⁺. Significant FDH desorption of (26±5) % ((0.76±0.16) pmol cm⁻²) was observed with a current decay of (96±6) % for FDH on 2-TMAET⁺ for formate oxidation at +0.4 V vs RHE after 1 h (Figure 6b).

Thus, we provide evidence for the first time that H-bonding stabilizes bioelectrocatalysis at the enzyme-electrode interface for both oxidative and reductive reactions.



Figure 6. E-QCM CA after loading (a) H₂ase and (b) FDH on 2-AET⁺ and 2-TMAET⁺ sensors (lower panel) and their corresponding changes in loading (upper panel) *operando*. The shapes of the loading curves before the vertical dotted line are due to a 2-minute pre-equilibration applied at the E_{app} to prevent large capacitance spikes at the start of the current measurement. The vertical dotted line indicates the start of the CA measurement at the applied potential, E_{app} = -0.1 V vs RHE (H₂ase, H⁺ reduction) and +0.4 V vs RHE (FDH, formate oxidation) after a 2 min electrode equilibration. Conditions: MES/KCl (50 mM/ 50 mM, pH 6), H₂ase (16 nM). HEPES/KCl/formate (50 mM/ 50 mM/ 20 mM, pH 8), FDH (66 nM), DTT (50 mM), flow rate = 0.141 mL min⁻¹, N₂ atmosphere, 25 °C. Error bars represent the standard deviation for a sample size of n = 3.

CONCLUSION

We have confirmed the importance of electrostatic interactions for enzyme orientation and activity, but also provide the first strong evidence for the presence and role of H-bonding in promoting the stability of two model redox enzymes at the electrode interface. Both CA and E-QCM confirmed the presence of H-bonding as a dominant non-covalent interaction that, when removed, resulted in desorption of H₂ase and FDH films from the electrode due to electrostatic interactions alone being insufficient at preventing desorptive activity loss. We find that resolving other non-covalent interactions outside of electrostatics is critical for a full understanding of the enzyme-electrode interface. Furthermore, a distinction between the total amount of enzyme loaded and the amount of electroactive enzyme wired to the electrode was elucidated by E-QCM. When factored into the calculation of the TOF, it was observed that the intrinsic activity rate for any enzyme directly wired to the electrode is unaffected by the charge of the electrode, however, remains significantly lower than the solution activity of the enzyme. By understanding the parallels between the enzyme-redox partner interactions in vivo and the enzyme-electrode interface, we have resolved the surface conditions that lead to either desorptive or non-desorptive processes for activity degradation and have decoupled them unequivocally for the first time. The understanding of the importance of Hbonding for enzyme stability can help tune the rational design of molecular surfaces to enhance bioelectrocatalytic performances and underlines the importance of characterizing the presence and function of interactions at the enzyme-electrode interface for future improvements in bioelectrode stability and activity that can help enzymes immobilized on electrodes achieve the exceptionally high rates seen in solution assays that make them such desirable catalytic systems.

EXPERIMENTAL SECTION

Materials

The following chemicals and materials were obtained from commercial suppliers and used without further purification unless otherwise stated: hydrogen gas (BOC), ethanol (VWR Chemicals), hydrogen peroxide (H_2O_2 , Sigma Aldrich, 33%), sulfuric acid (H_2SO_4 , Sigma Aldrich, 99%), methyl viologen dichloride hydrate (MV²⁺, Sigma Aldrich, 98%), benzyl viologen dichloride hydrate (BV²⁺, Sigma Aldrich, 98%), Parafilm® M (Sigma Aldrich), potassium chloride (KCl, Fisher Chemical), rubber septa (Subaseal), 2-(N-morpholino)ethanesulfonic sodium salt (MES, Sigma Aldrich), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic sodium salt (HEPES, Sigma Aldrich), sodium hydroxide (NaOH, Sigma Aldrich, ≥97%), 2-aminoethanethiol hydrochloride (2-AET, Sigma Aldrich, 98%), 3-mercaptopropionic acid (3-MPA, Sigma Aldrich, ≥99%), 2-(dimethyl)aminoethanethiol hydrochloride (2-DMAET, Sigma Aldrich, 95%), 2mercaptoethanol (2-ME, 99%, Sigma Aldrich), gold(III) chloride trihydrate (HAuCl₄, Sigma Aldrich, ≥99.9%), sodium borohydride (NaBH₄, Sigma Aldrich, ≥98.0%), DL-Dithiothreitol (DTT, Fisher, $\geq 98.0\%$), sodium formate (Sigma Aldrich, $\geq 99.0\%$). Buffer solutions were prepared using water from a Simplicity UV MilliQ system (18.2 MQ cm at 25°C) and consisted of MES (50 mM) and KCl (50 mM) or HEPES (50 mM), KCl (50 mM) and sodium formate (20 mM). Gases $(N_2, N_2 \text{ with } 2\% \text{ CH}_4 \text{ and } H_2)$ were supplied by BOC.

The following compounds were synthesized as reported previously: 2-(Trimethylammonium)ethyl thiol (2-TMAET).⁸⁰ [NiFeSe]-H₂ase and [W]-FDH from *Desulfovibrio vulgaris* Hildenborough was expressed, purified and characterized according to a published method.^{27,81} All purification steps were performed under aerobic conditions at 4 °C. 10 μ M H₂ase stock solutions with an activity of 5201 ± 293 s⁻¹ for H₂ production were stored in a buffer solution (20 mM Tris-HCl, pH 7.6) at -40° C under N₂ atmosphere. 40 μ M FDH stock solutions with an activity of 1100 s⁻¹ for formate oxidation and 320 s⁻¹ for CO₂ reduction were stored in a buffer solution (20 mM Tris-HCl, 10% glycerol, 10 mM NaNO₃, pH 7.6) at -40° C under N₂ atmosphere.

All measurements with H₂ase and FDH were carried out in an anaerobic glovebox (MBraun, N₂ atmosphere, < 0.1 ppm O₂). Potentials for the electrostatic surface contours of enzymes were calculated with the APBS Electrostatics plugin [https://server.poissonboltzmann.org/pdb2pqr] with correction for charges of the FeS clusters, Selenocysteine, Nickel and Tungsten in the active site.⁸² PyMOL (version 2.3.4, Schrodinger, LLC) was used for enzyme visualization.

Physical Methods

¹H and ¹³C NMR spectra were recorded on a Bruker DPX-400 MHz or a Bruker 500 MHz DCH cryoprobe spectrometer at room temperature. Chemical shifts are given in ppm and coupling constants in Hz. Chemical shifts for ¹H NMR spectra are referenced relative to residual protons in the deuterated solvent (D₂O: ¹H = 4.8 ppm, Methanol-d₄ : ¹³C = 49.1 ppm). High resolution-mass spectra (MS) were recorded using a ThermoScientific Orbitrap Classic mass spectrometer.

Gold Substrate Preparation

Two independent gold disk electrodes of 2 mm diameter (Pine Instruments) were cleaned by immersion in *piranha* solution (3:1 concentrated H₂SO₄/33 % H₂O₂) for 5 min (*Caution! Piranha solution is very corrosive and may explode if contained in a closed vessel*), then were gently rinsed with Milli-Q water, polished with 0.05 μ m alumina (Buehler) and were ultrasonicated in H₂O followed by EtOH for 2 min. Finally, the electrodes were electrochemically cleaned by repetitive cycling between -0.3 and +1.2 V (vs Ag/AgCl) in 0.05 M H₂SO₄ at a scan rate of 50 mV s⁻¹ under N₂ until a stable voltammogram was observed (around 15 cycles). The amount of charge under the

gold oxide reduction peak at +0.9 V vs AglAgCl was used by integrating said peak to yield the real electroactive surface area by taking into account the theoretical charge of $390 \pm 10 \ \mu\text{C} \ \text{cm}^{-2}$ for the reduction of a gold oxide monolayer for the two independent electrodes.⁸³ The electrodes were found to have an electroactive surface area (A_{electroactive}) of (0.165±0.1) cm² and (0.162±0.05) cm². The relevant self-assembled monolayers (SAMs) were formed by immersing the Au substrates in a 10 mM aqueous solution of the relevant thiol overnight.

Synthesis of 2-(Trimethylammonium)ethyl thiol (2-TMAET⁺)

The synthesis was performed according to a modified literature procedure.^{84,85} In brief, 2-(bromoethyl)-triethylammonium bromide (5.0 g, 20.2 mmol) was dissolved in 25 mL distilled water. Potassium thioacetate (3.01 g, 26.3 mmol) was added and the stirred solution was heated to 60 °C. After 16 h, the reaction mixture was concentrated under reduced pressure. The product was extracted by stirring the solid in 100 mL (MeOH/CH₂Cl₂, 1:1) at room temperature for 30 min. KBr was removed by filtration over celite. The filtrate was concentrated under reduced pressure and the extraction/filtration were repeated twice to ensure removal of all KBr. The product was collected as a red-white solid (2.77 g, 56.4%), of which 1.5 g was added to hydrochloric acid (HCl, 1 M, 7.5 mL). The reaction mixture was refluxed at 110 °C for 16 h under an inert gas atmosphere. The solvent and volatile by-products were removed in vacuo to yield a white-yellow solid. The product was further purified by dissolution in 0.5 mL MeOH while stirring and continuously heating. At 50 °C, 0.2 mL MeOH was added. At 70 °C, all compound was fully dissolved. The solution was cooled slowly to room temperature and further down to 0 °C with an ice-bath. After the product precipitated, the supernatant was removed with a syringe. To obtain a very pure product, the recrystallization process was repeated while sacrificing the yield. The product was collected as a white hygroscopic solid and dried in vacuo (38.6 mg, 4.0%). ¹H NMR (D₂O, 400

MHz): $\delta = 2.95 (2H, CH_2)$, 3.15 (9H, NMe₃), 3.55 (2H, CH₂) ¹³C NMR (Methanol-d4, 101 MHz) $\delta = 69.56$, 53.64, 17.75. MS m/z: MS calculated for C₅H₁₄NS⁺ 120.08, found 120.12.

Synthesis of 2-aminoethanethiol-capped gold nanoparticles

2-aminoethanethiol-capped gold nanoparticles (2-AETIAuNP) were synthesized using a previously reported method.⁸⁶ In brief, 2-AET (400 μ L, 213 mM) was added to HAuCl₄ (40 mL, 1.42 mM) and was gently stirred for 20 min at room temperature. NaBH₄ (10 μ L, 10 mM) was quickly added, and the mixture stirred vigorously in the dark at room temperature for 10 min to yield a wine-red solution of 2-AET-AuNPs, roughly 40–50 nm in diameter as determined by dynamic light scattering.

Preparation of H₂**ase-modified electrodes**

Enzyme-modified electrodes were prepared in an anaerobic glovebox (MBraun, N₂ atmosphere, <0.1 ppm O₂). DvH-H₂ase (1 µL, 10 µM) was diluted in 4 µL MES (50 mM) with KCl (50 mM) at pH 6 and dropcast onto SAM-modified Au electrodes. The resulting H₂aselSAMlAu electrode was left to dry for 15 min, then gently rinsed with buffer to remove any loosely physisorbed enzyme.

Preparation of FDH-modified electrodes

Enzyme-modified electrodes were prepared in an anaerobic glovebox (MBraun, N₂ atmosphere, <0.1 ppm O₂). *Dv*H-FDH (1 μ L, 40 μ M) was mixed in a 1:1 *v:v* ratio with DTT (50 mM, 1 μ L) in MES/KCl at pH 6 for 15 min. The resulting mixture was then diluted in 3 μ L in MES buffer (50 mM) with KCl (50 mM) at pH 6 and dropcast onto SAM-modified Au electrodes. The resulting FDHISAMIAu electrode was left to dry for 15 min, then gently rinsed with buffer to remove any loosely physisorbed enzyme and excess DTT.

Protein film voltammetry

A gas-tight two compartment cell with a Nafion membrane separating the compartments was equipped with a three-electrode setup, consisting of a AglAgCl (saturated KCl) reference electrode (BASi). Unless otherwise stated, all potentials are quoted with respect to the reversible hydrogen electrode (RHE) using the conversion $E_{\text{RHE}} = E_{\text{AglAgCl}} + 0.197 + (0.059 \times \text{pH}) \text{ V} (25 \text{ °C})$, alongside a Pt wire counter electrode, and a H₂aselSAMIAu or FDHISAMIAu rotating disk working electrode (RDE). An electrolyte solution containing MES (50 mM) and KCl (50 mM) at pH 6 for H_2 as or HEPES (50 mM), KCl (50 mM) and formate (20 mM) at pH 8 for FDH was prepared by dissolving the relevant free acids, their sodium salts, and KCl in MilliQ H_2O . The electrochemical cell was filled with 8 mL of electrolyte, sealed with rubber septa, constantly kept at 25°C, and purged with the relevant gas if required for 15 min before the start of the measurement. All electrochemical experiments were performed with an Ivium CompactStat potentiostat and a Pine Instruments rotating disk electrode rotator and voltammograms were recorded with a scan rate of 5 mV s⁻¹ at a rotation speed (ω) of 2000 rpm. Errors bars are \pm sample standard deviation (s) estimated from at least three experiments across the two independent electrodes. All data processing was performed using Python 3.8.2.

Electrochemical quartz crystal microbalance analysis

E-QCM experiments were conducted with a Biolin Q-Sense Explorer module and a customdesigned QCM electrochemical cell in an anaerobic glovebox (MBraun, N₂ atmosphere, < 0.1 ppm O₂). Typically, a gold-coated quartz chip (0.79 cm²) was cleaned using the same procedure as for the gold working electrode, followed by a 15 min UV-ozone treatment, after which the Au sensor was immersed in a 10 mM aqueous solution of the relevant thiol overnight, and rinsed with ultrapure water (Milli-Q, >18.2 MΩ.cm) prior to use. Prior to measuring, enzyme-free MES buffer solution (50 mM) with KCl (50 mM) at pH 6 was cycled through at 0.141 mL min⁻¹ for 10 min to generate a stable baseline. Following this, an enzyme-containing buffer solution (16 nM H₂ase or 66 nM FDH in MES/KCl (50 mM/50 mM)) was injected into the cell. Enzyme adsorption was quantified by monitoring changes in the resonance frequency of the piezoelectric quartz chip. The frequency was related to the mass through the Equation S1⁸⁷:

$$\Delta f = -\frac{2f_o^2}{A\sqrt{p_q\mu_q}}\Delta m \tag{S1}$$

where f_0 is the resonance frequency of the quartz oscillator, A is the piezoelectrically active crystal area, Δm is the change in mass, p_q is the density of quartz, and μ_q is the shear modulus of quartz. To convert the mass adsorbed to quantity of enzyme, an assumption was made that 25% of the adsorbed mass consisted of water molecules bound to the enzyme, which was 91.68 kDa for H₂ase and 138.3 kDa for FDH in weight.^{26,27}

For *operando* electrochemical analysis, once FDH was fully loaded after 2 h, a 50 mM solution of DTT was injected and flown through the cell for 10 min, after which the flow was stopped, and the solution kept on the FDHISAMIAu chip for a further 20 min. Then, the DTT solution was replaced with HEPES/KCl/formate (50 mM/50 mM/20 mM, pH 8), after which electrochemical analysis was carried out. H₂ase was measured in MES/KCl (50 mM/50 mM, pH 6) as is with no prior activation needed. TOFs were calculated using Equation S2 and S3:

$$TOF_{apparent} = \frac{i}{-nFA\Gamma}$$
(S2)

$$TOF_{actual} = \frac{i}{-nFA\Gamma j_{DET}/j_{MET}}$$
(S3)

where *i* is the catalytic current (negative for reductive processes, positive for oxidative processes by convention, calculated from the product of j_{DET} and the electrode surface area), *n* is the number of electrons involved in the reaction (2 for the reduction of H⁺ to H₂, 2 for the oxidation of HCO₂⁻ to CO₂), *F* is Faraday's constant, *A* is the surface area of the electrode (0.79 cm²), Γ is the coverage of the enzyme, TOF_{apparent} and TOF_{actual} is the enzyme's intrinsic rate constant/turnover frequency, and j_{DET}/j_{MET} is the ratio of direct electron transfer current and mediated electron transfer current.

For the KCl desorption studies, ionic solutions of KCl in MES (50 mM, pH 6) were prepared and injected into the cell for 30–40 mins until no continuous change in frequency was observed. The KCl solution was then replaced by the required buffer solution (MES/KCl (50 mM/ 50 mM, pH 6) for H₂ase or HEPES/KCl/formate (50 mM/50 mM/20 mM, pH 8) for FDH for a further 30-40 mins until the frequency response stabilized, after which the CVs were recorded. The seventh harmonic (f_7) was used in all data analysis. Errors bars are ± sample standard deviation (s) derived from at least three experiments across at least three independent Au sensors. All data processing was performed using Python 3.8.2.

Other instrumentation

Zeta potential and nanoparticle diameter was measured using a Malvern Zetasizer Nano ZS. The sample was dispersed in MES/KCl (pH 6 and 7) and HEPES/KCl (pH 8) solutions (50 mM / 50 mM) and allowed to stand prior to measurements in disposable cuvettes (Malvern). Measurements were conducted as three replicates; average results were quoted using the standard deviation as the error.

ASSOCIATED CONTENT

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Notes

The authors declare no competing financial interest.

Supporting Information.

Zeta potential measurements of 2-AET|AuNP; protein film voltammograms of H₂ase and FDH on SAM-modified Au electrodes; protein film voltammograms of FDH on 2-AET⁺ under N₂ and CO₂; enzyme-free CVs in the presence and absence of BV²⁺; FDH E-QCM on 2-AET⁺; H₂ase E-QCM on 3-MPA⁻; E-QCM of sub-monolayer H₂ase on 2-AET⁺; E-QCM of FDH on 2-TMAET⁺; E-QCM of H₂ase on propanethiol; E-QCM MET protein film voltammograms of H₂ase and FDH on 2-AET⁺ and 2-TMAET⁺; table comparing solution assays activities to electrochemical activities for various bioelectrocatalytic systems.

This material is available free of charge on the ACS Publications website at DOI: xxxxx. Experimental section (Figures S1 – S11, Table S1) (PDF)

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