Understanding the Local Chemical Environment of Bioelectrocatalysis

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Abstract

Bioelectrochemistry employs an array of high-surface area meso and macroporous electrode architectures to increase protein loading and the electrochemical current response. Whilst the local chemical environment has been studied in small molecule and heterogenous electrocatalysis, conditions in enzyme electrochemistry are still commonly established based on bulk solution assays, without appropriate consideration of the non-equilibrium conditions of the confined electrode space. Here, we apply electrochemical and computational techniques to explore the local environment of fuel-producing oxidoreductases within porous electrode architectures. This improved understanding of the local environment enabled simple manipulation of the electrolyte solution, by adjusting the bulk pH and buffer pK_a, to achieve an optimum local pH for maximal activity of the immobilised enzyme. When applied to macroporous inverse opal electrodes, the benefits of higher loading and increased mass transport were employed and, consequently, the electrolyte adjusted to reach \( -8.0 \text{ mA cm}^{-2} \) for the H\(_2\) evolution reaction (HER) and \( -3.6 \text{ mA cm}^{-2} \) for the CO\(_2\) reduction reaction (CO\(_2\)RR), demonstrating an 18-fold improvement on previously reported enzymatic CO\(_2\)RR systems. This research emphasises the critical importance of understanding the confined enzymatic chemical environment, thus expanding the known capabilities of enzyme bioelectrocatalysis. These considerations and insights can be directly applied to both bio(photo)electrochemical fuel and chemical synthesis as well as enzymatic fuel cells to significantly improve the fundamental understanding of the enzyme-electrode interface as well as device performance.
Introduction

Enzymatic electrochemistry has rapidly expanded into research fields that encompass applications across disciplines from fundamental understanding of enzymology to biosensors, biofuel cells and semi-artificial photosynthesis.\(^1\,\,^2\) Hydrogenases (H\(_2\)ases) combine protons and electrons to reversibly produce H\(_2\) at the thermodynamic potential using Fe or NiFe active sites and have therefore been extensively studied as a model system for reversible electrocatalysis.\(^3\,\,^4\) Formate dehydrogenases (FDH) have similarly garnered attention due to their ability to reversibly reduce CO\(_2\) to formate, with high selectivity when immobilised on an electrode.\(^5\,\,^6\,\,^7\) Bioelectrochemistry on thin-film electrodes has long provided mechanistic and analytical insight into enzyme function, as well as establishing bioelectrolysis as a potential method of product synthesis.\(^1\,\,^3\,\,^9\) Porous electrodes enable higher enzyme loading, and hence higher current densities, improving the overall performance by increasing the consumption or production of desired chemicals.\(^10\,\,^11\,\,^12\,\,^13\,\,^14\,\,^15\)

Solution assays provide insight into the influence of bulk pH and ionic strength on enzyme activity and the conclusions have informed the choice of electrolyte in electrochemical studies.\(^16\,\,^17\) However, the high current densities achievable using the latest generation of high surface area electrode scaffolds, usually used as stationary electrodes, presents a significantly different local chemical environment during turnover to that of bulk solution assays. With enzymes operating at a high rate of catalysis, for example D. vulgaris Hildenborough (DvH) [NiFeSe]-H\(_2\)ase has a turnover frequency (TOF) of \(~8300\) s\(^{-1}\) for H\(_2\) evolution,\(^18\) the local proton concentration is likely to change significantly if the enzyme is placed in a porous environment. Therefore, consideration of local concentration gradients, as opposed to the bulk electrolyte solution, appears to be critical to understand and optimise enzymatic activity in such porous electrodes.

The precise conditions of the local chemical environment are decisive for enzyme activity and the local environment must therefore be tailored to the nature of the immobilised enzyme for optimal catalysis. Both the enzymatic H\(_2\) evolution reaction (HER) (eq. 1) and formate
production by the enzymatic CO$_2$ reduction reaction (CO$_2$RR) (eq. 2) involves the net consumption of protons as part of their mechanism, leading to an increased local pH at the electrode surface.$^{19,20}$ Due to their high activity and selectivity, H$_2$ases can provide a simple, illustrative model system of how catalysis causes local pH changes within porous architectures, whilst FDHs can demonstrate the effect for the CO$_2$RR.

\[
2H^+_{(aq)} + 2e^- \rightleftharpoons H_2_{(g)} \quad (1)
\]
\[
CO_2_{(aq)} + 2e^- + H^+_{(aq)} \rightleftharpoons HCOO^-_{(aq)} \quad (2)
\]

The local CO$_2$RR environment has been extensively modelled and conclusions applied to improve the selectivity of heterogeneous,$^{21-25}$ and heterogenised molecular catalysts.$^{26-29}$ Due to competition between HER and CO$_2$RR when using synthetic catalysts, compromises, such as basicity, are often made to minimise the HER even at the cost of CO$_2$RR activity due to the overall benefit of selectivity.$^{30,31}$ The complexity of these interfacial interactions is considerably reduced when considering enzymatic catalysis due to the inherent specificity, selectivity and low overpotential requirements of the protein to drive catalysis.$^{32,33}$ Without the competing HER, the local environment in enzymatic systems can be controlled without compromise to facilitate the CO$_2$RR. Therefore, enzymatic CO$_2$ electroreduction provides a paragon for the development of more ‘ideal’ synthetic molecular catalysts in the future.

Here, the local chemical environment of enzymatic HER and CO$_2$RR systems were studied by bioelectrochemistry and computational methods using a finite element model (FEM). Firstly for the HER, and then for the CO$_2$RR, the change in local pH on mesoporous electrodes were studied bioelectrochemically by the current density obtained in solutions with buffers of different pK$_a$. For this purpose, $DvH$ [NiFeSe] H$_2$ase and $DvH$ W-FDH were chosen as enzymes with high activity for their respective reduction reactions and low product inhibition (Figure S1).$^{16,34}$ The pH-activity solution assays for H$_2$ase and FDH had previously been undertaken, showing an optimum pH of 6 and 7.1 respectively (Figure S2). Independently, a FEM was developed from basic physical principles and enzyme-activity dependence studies
for pH, buffer concentration and substrate concentration. The accuracy of the model was then confirmed by predicting the current density outcome of each experiment. The excellent match between the experimental and simulated results validated the FEM and hence the model could be used to simulate the changes in local pH, and other species concentrations. The model was then used to iterate the optimum conditions for each system and the resulting increase in current density was demonstrated experimentally. Finally, the conclusions were experimentally tested on a different electrode architecture, an inverse opal electrode, to demonstrate the wide-ranging applicability of this study.

Results and Discussion

Methodology and Simulation

To demonstrate the effect of local chemical environment changes in confined electrodes, first a mesoporous indium-tin-oxide (mesoITO) film on fluorine-doped tin oxide-coated glass (synthesised 50 nm ITO particles, film thickness of 8.5 μm, Figures S3-5) was chosen as the electrode. The mesoporous structure could be simulated as a randomly close packed structure and hence did not require too computationally expensive modelling. ITO was chosen as the electrode material due to its conductivity and high affinity for enzyme binding and versatility to prepare porous materials. The enzymes, H₂ase for the HER and FDH for the CO₂RR, were then immobilised onto the electrode by dropcasting. A three-electrode set-up was used in the chosen electrolyte solution to measure the resultant current density and the solution was stirred to maximise mass transport to the electrode. To demonstrate the reproducibility of the findings at least three repeats were measured for each experiment, each with a newly assembled bioelectrode, in a fresh electrolyte solution.

For the electrolyte, two zwitterionic Good’s buffers were selected for this study: 2-(N-morpholino)ethanesulfonic acid (MES) (pKₐ = 6.27) and 3-(N-morpholino)-propanesulfonic acid (MOPS) (pKₐ = 7.18) (Figure 1a,b). These buffers were chosen due to their similar
chemical composition to ensure that any changes in enzyme productivity could be attributed to the different pKₐ as opposed to physical or chemical interactions with the electrode or enzyme. These buffers are also known to not coordinate with the main metals present in enzymes and reference electrodes. The effect of the specific buffer concentration on the enzyme activity was measured for this study and the results presented in the Supporting Information, Enzyme Current Density, and the buffer concentration has been kept below inhibitory levels throughout this study. The electrolyte solution for the CO₂RR also contains CO₂ and its pH-dependent equilibrium with carbonic acid (H₂CO₃), bicarbonate (HCO₃⁻) and carbonate (CO₃²⁻), which has been further discussed in the Supporting Information, Equilibria. To accurately prepare the electrolyte solutions, taking into account the influence of ionic strength, equations to calculate the activity coefficients of all species were applied using the freeware chemical equilibrium model Visual MINTEQ (Supporting Information, Ionic Strength). In initially compared electrolytes, the ionic strength has been kept constant by the addition of KCl. The buffers were prepared by combination of the correct concentration of acid and base buffer species to give the desired bulk pH. The pH at which MES and MOPS have identical buffer capacity is 6.54 and so this was selected as the bulk pH (Figure 1b and Supporting Information, Buffer Capacity).
Figure 1. Analysis of Good’s buffer species MES and MOPS. (a) Chemical structure of MES and MOPS zwitterions. (b) Graph demonstrating the theoretical change in ratio of each buffer species MES (blue) and MOPS (orange) with pH (protonated Good’s buffer shown as dashed line and anion as solid line). The grey dotted vertical line demonstrates the pH at which the buffer capacity of the two buffers is equal.

The FEM was constructed to simulate the effect of local environment changes on enzymatic HER (DvH [NiFeSe] H₂ase) and CO₂RR (DvH W-FDH) in the mesoporous electrode, using COMSOL Multiphysics 5.5. Similar models have been applied to follow the concentration of H₂ as substrate for H₂ oxidation and O₂ inhibition in redox hydrogel films or carbon nanotubes, though these models did not consider the concomitant change in local pH or other electrolyte species.⁴⁶,⁴⁷ The model, and its governing parameters and equations, is described in detail in the Supporting Information, *Finite Element Model*, and is summarised in Figure 2.

The model uses the Nernst-Planck equation to describe the potential distribution within the electrode, alongside the kinetics of the enzyme which is assumed to be homogenously distributed within the porous electrode. Furthermore, known modifiers on enzyme activity such as pH and substrate concentration that are described using common analytical expressions such as Michaelis-Menten kinetics are included where appropriate. Finally, this is combined...
with a mass transport model that incorporates the kinetics and thermodynamics of solution reactions. This approach gives a model that is predictive of electrode currents and performance and, critically, is completely independent of the experimental data. This prediction of experimental currents across a range of conditions and the excellent agreement of the predicted and experimental values provides validation of the model. Furthermore, it is indicative of the absence of additional unconsidered interactions, demonstrating that a more comprehensive analysis than that presented was not required.

Once the model was validated, it has been used to simulate a quantified understanding of the previously inaccessible local environment, demonstrating the time-dependent changes in the local pH and other species concentrations. The predictive nature of this FEM could then allow the solution composition to be iterated for the best experimental system performance and highest simulated current densities, which were then validated experimentally. Once the local environment had been optimised for the enzymatic HER and CO₂RR on mesoporous electrodes, a novel electrode architecture was introduced to demonstrate the applicability of the conclusions on electrolyte composition to a different system. Hierarchical inverse opal (IO) electrodes were chosen due to their macro and mesoporous structure which enables increased enzyme loading compared to purely mesoporous electrodes, as has been previously demonstrated.¹⁰,⁴⁸ A shift to TiO₂ also allowed the application of more negative reduction potentials (~0.8 V vs. SHE) than that being used with mesoITO (~0.6 V vs. SHE) due to the degradation of ITO at more reducing potentials. The IO-TiO₂ electrodes were constructed with co-assembled ~21 nm anatase TiO₂ particles and 750 nm diameter polystyrene spheres and then annealed to give IO macropores with a total film thickness of 40 μm (Figure S6). With these electrodes the principles of local chemical environment adjustment for enzymatic electrochemistry were applied and hence significant improvements in the current density were again achieved.
Figure 2. Graphic illustrating the different parameters which were considered in the FEM. Including enzyme activity dependence, enzyme-electrode kinetics, geometry parameters, solution properties and potential distributions.

Local Chemical Environment of the HER

Electrochemical experiments with DvH [NiFeSe] H$_2$ase (25 pmol) on the mesoITO electrode were undertaken in MES and MOPS buffered electrolyte solution. The 100 mM MES electrolyte has been labelled MES and the 100 mM MOPS electrolyte as MOPS, both with the same buffer capacity at a bulk pH 6.54 (Figure 1b). The mesoITO|H$_2$ase electrode in the MOPS electrolyte solution displayed 60% higher catalytic activity for proton reduction than the MES solution at an applied bias of $-0.6$ V vs. SHE (Figure 3a). The H$_2$ produced was measured by gas chromatography of a sample of the reaction headspace and a Faradaic efficiency (FE) of $(88 \pm 5)\%$ was determined (Figure 3b). The average current density of the repeats, with a new bioelectrode in new electrolyte for each repeat, has been shown as the
solid line with the standard deviation \( N \geq 3 \) as the shaded area/error bars. The reproducibility of the system is demonstrated by the narrow standard deviation of both the current density and the product quantification. Voltammetry of the mesoITO|H\textsubscript{2}ase and mesoITO electrodes are also shown in Figure S7, showing minimal current density for the enzyme-free control electrodes.

As described in *Methodology and Simulation*, the experimental system was also simulated using a FEM and the predicted current density shown as a dotted line over the experimental results (Figure 3a). Assuming a FE of 100%, the predicted H\textsubscript{2} evolution is shown as separate bars in Figure 3b. The simulated current densities were in close agreement to the experimental data for both the MES and MOPS electrolytes, supporting the validity of the model and its description of the enzyme kinetics, activity and solution mass transport. With the FEM matching the experimental conditions and outcomes, the model was then used to simulate the changes in local pH and buffer capacity within the electrode over time (Figure 3c,d), and as a function of distance from the electrode (Figure S8).

Due to the change in local proton concentration as the catalysis occurs, the pH at the electrode instantly increases (Figure 3c). The peak activity of proton reduction by DvH [NiFeSe] H\textsubscript{2}ase in solution assays was shown to be at pH 6, with a reasonably sharp decrease in activity on deviation from the optimum (Figure S2).\textsuperscript{34} Therefore as the local pH increases away from the optimum, a decrease in the rate of catalysis of the enzyme will occur. However, the change in local proton concentration is reduced when using an electrolyte solution with a high buffer capacity at that local pH (Figure 3d). Therefore, we observe a lower local pH change in the electrodes for the MOPS buffer (pK\textsubscript{a} = 7.2) compared with MES (pK\textsubscript{a} = 6.3) at a local pH of >7.5 (\( \Delta \text{pH} = 1.24 \) and 1.76 for MOPS and MES, respectively). Consequently, the enzyme activity is higher with the MOPS electrolyte solution and therefore higher experimental current densities were observed than using MES solution (Figure 3a). This can additionally be observed in the decrease in current density between the first and second scan of the initial voltammetry scans (Figure S9).
Figure 3. Bioelectrochemistry of mesoITO\textit{H}$_2$\textit{ase} in MES (blue), MOPS (orange) and the optimum electrolyte MES$_{\text{opt}}$ (dark blue) and simulations demonstrating the local environment changes. (a) Chronoamperometry (CA) of mesoITO\textit{H}$_2$\textit{ase} with an applied bias of $-0.6$ V vs. SHE. Experimental data shown by solid line with standard deviation as area. Simulated current density shown by dashed line. (b) Experimental \textit{H}$_2$ evolution and FE$_{\text{H}_2}$ from CA after 60 min and simulated \textit{H}$_2$ evolution, assumed 100% FE$_{\text{H}_2}$, shown by lighter, dashed bar. (c) Simulated change in the average pH within the porous electrode during the 60 min CA. (d) Simulated change in the average buffer capacity within the porous electrode during the 60 min CA. Conditions: MES [MES (100 mM), KCl (50 mM), pH 6.54 and ionic strength (125 mM)], MOPS [MOPS (100 mM), KCl (105 mM), pH 6.54 and ionic strength (125 mM)]. MES$_{\text{opt}}$ [MES (240 mM), KCl (175 mM), pH 4.24]. \textit{H}$_2$\textit{ase} (25 pmol), N$_2$ atmosphere, stirred, 25 °C.
The combination of experimentation and computation was therefore able to demonstrate that porous electrodes with confined enzymes exhibit a significant local pH change, thereby affecting the current and product output. Consequently, the bulk pH should not be the optimum pH for the enzyme, but instead should be aligned as to give a local pH at the optimum. It can also be concluded that the pK_a of the electrolyte buffer should be matched to the local pH, not the bulk pH. Qualitatively, for DvH H_2ase HER the optimum pH is 6 (Figure S2), and therefore a lower bulk pH of about 4-5 would likely be appropriate with a buffer pK_a of 6 (for example MES).

Finally, to demonstrate the importance of accounting for the local environment, and to show the improvements that result, the FEM was used to predict the optimum solution conditions for DvH H_2ase H_2 evolution on the mesoITO electrode. By iteration of the solution properties in the FEM, the system could be optimised to give the highest predicted current densities, taking into consideration the contrasting effects of MES concentration on enzyme activity (Supporting Information, Enzyme Current Density) and buffer capacity for an optimised local pH. The optimal solution determined by the FEM was a MES concentration of 240 mM and a bulk pH of 4.24, which predicted a resulting local pH of 5.94, closely matching the optimum (pH 6) for HER with DvH H_2ase, and a simulated current density of 4.4 mA cm^{-2}.

The FEM-suggested optimum conditions (MES_{opt}) were then tested experimentally and gave initial current densities (~4.8 mA cm^{-2}) and H_2 production (50 ± 3 μmol cm^{-2} h^{-1}) six times the original MES electrolyte solution and close to the predicted value from the FEM (Figure 3). The reversible nature of the impact of low pH on enzyme activity was demonstrated by the almost instantaneous return in MES_{opt} to high activity despite the low starting pH of 4.24. However, the optimised system exhibited deterioration of current densities over the hour. The decrease can be attributed to the build-up of hydrogen gas in the pores and on the electrode as demonstrated by the rejuvenation of the activity after the bubbles were removed from the electrode (Figure S10). Whilst the simulation does account for the mass transport effects of bubble formation (Supporting Information, Solution Domain), it does not account for bubble
blocking or other effects. For example, H₂ bubble formation may cause increased film loss, through enzyme displacement, re-orientation and degradation. H₂ also affects the thermodynamic potential according to the Nernst equation, an effect even more pronounced at the low overpotentials applied here. Though usually H₂ is a key inhibitor of H₂ases, DvH [NiFeSe] H₂ase is less susceptible to H₂ inhibition as other H₂ases and is hence an ideal candidate for application in confined architectures.\(^{34,49}\) However, if the local concentration was high enough it is likely that inhibition would still occur. Thus the simulation does not show the same decline in current density over time and predicts a greater amount of H₂ (77.5 \(\mu\text{mol cm}^{-2}\)). Therefore, the optimised electrolyte has effectively reached the highest current densities achievable in this electrode system due to the limitations imposed by the production of H₂, and other accelerated sources of enzyme-film loss.

**Local Chemical Environment of the CO₂RR**

CO₂, the substrate for FDH, forms the CO₂/HCO₃⁻ (pK\(_1\) = 6.34) and HCO₃⁻/CO₃²⁻ (pK\(_2\) = 10.32) buffer equilibria when dissolved in H₂O (Supporting Information, *Equilibria*). However, on the relatively short timescales within the diffusion layer at the electrode, the slow kinetics of CO₂ hydration result in minimal interconversion of CO₂ and HCO₃⁻ in response to the shifting equilibria. Therefore the CO₂ concentration is almost unchanged by differing pH within the diffusion layer.\(^{50}\) As such the buffering effect of CO₂/HCO₃⁻, and similarly the formation of aqueous CO₂ from HCO₃⁻, is limited in its mitigation of the local concentration gradients.\(^{28,51,52}\)

In heterogeneous and heterogenised molecular catalyst systems, the poor buffering of the CO₂/HCO₃⁻ equilibrium benefits the system by enhancing the local pH change, which slows down the competing HER, improving selectivity for the CO₂RR.\(^{22,25}\) However, in enzymatic CO₂RR, the HER is negligible due to high selectivity and low overpotential requirements preventing the HER from occurring directly at the metal oxide electrode. Therefore, increasing the buffer capacity would be beneficial, and so an additional, faster-buffering species can be introduced. Three electrolyte solutions were therefore chosen, consisting firstly of a
‘bicarbonate only’ electrolyte (BC) and then two alternative electrolytes which include an additional 100 mM buffer as follows: BC-MES and BC-MOPS. A pH of 6.54 was again selected as at this pH the buffer capacity of electrolyte BC-MES and BC-MOPS is equal (Figure S11a), and also the CO$_2$ equilibria species concentrations do not change between all the electrolytes (Figure S11b).

From the electrochemical experiments on a mesoITO scaffold with FDH (52 pmol) at an applied bias of −0.6 V vs. SHE, we observe significant activity in the BC-MES and further enhanced current in the BC-MOPS electrolyte solution (Figure 4a). As before, repeats of each solution were made and a narrow standard deviation ($N \geq 3$) demonstrated the reproducibility of the system. The FEM current density prediction was shown to closely match the experimental results, again confirming the accuracy of the model. The formate production was measured by ion chromatography and displayed a FE of $(97 \pm 7)$% (Figure 4b) and negligible average H$_2$ evolution with a FE of $(0.2 \pm 0.2)$%, thus demonstrating the excellent selectivity of FDH. The mesoITO electrode without FDH showed minimal current (Figure S12).

From the FEM evaluation of the local electrode environment, it is apparent that the local pH is significantly reduced at the electrode with electrolyte BC-MES and BC-MOPS compared to BC (Figure 4c). The highest buffer capacity at the electrode (BC-MOPS) again causes the lowest local pH change (Figure 4d). A pH of 7.1 has been shown in solution assays to be the optimum pH of the FDH for CO$_2$ reduction, and therefore increasing the pH beyond this optimum decreases enzyme activity (Figure S2).\(^{16}\) Whilst the increased initial buffer capacity of BC-MES improves upon the BC system, the higher pK$_a$ of MOPS enables a high buffer capacity at the local pH in BC-MOPS, thus minimising the local pH change and increasing activity. Equally, voltammetry of the FDH-modified mesoITO electrode showed a small drop in current density between scan 1 and 2 in electrolyte BC (Figure S13), whereas BC-MES and BC-MOPS showed stable current density between successive scans (Figure S14), indicative of the smaller change in local pH in these solutions. The simulation results showing the pH and buffer capacity as a function of the distance from the surface are shown in Figure S15.
The local change in CO₂ equilibria species concentration over time was also simulated and the results are discussed in the Supporting Information (Figure S16).

Figure 4. Bioelectrochemistry of mesoITO|FDH in electrolyte BC (purple), BC-MES (blue), BC-MOPS (orange) and the optimum electrolyte BC-MOPS_{opt} (brown), and simulation demonstrating the local environment changes. (a) CA of mesoITO|FDH with an applied bias of −0.6 V vs. SHE. Experimental data shown by solid line with error bars as area. Simulated current density shown by dashed line. (b) Experimental formate production and FE_{HCOO} from CA after 60 min and simulated formate production, assumed 100% FE_{HCOO}, shown by lighter, dashed bar. c) Change in the average pH within the electrode during the 60 min CA. d) Change in the average total buffer capacity (solid lines) within the electrode during the 60 min CA. The individual buffer capacity of each Good’s buffer (dashed lines) and HCO₃⁻ (dotted lines) have also been shown. Conditions: BC [NaHCO₃ (71 mM), KCl (138 mM), pH 6.54], BC-MES [MES (100 mM), NaHCO₃ (72 mM), KCl (56 mM), pH 6.54], BC-MOPS [MOPS (100 mM), NaHCO₃ (71 mM) KCl (113 mM), pH 6.54], BC-MOPS_{opt} [MOPS (86 mM), NaHCO₃ (50 mM), KCl (175 mM) and pH 6.39], FDH (52 pmol), CO₂ atmosphere, 25 °C.
The experimental data shows continued functionality of the FDH in electrolyte BC despite the local pH increase to 8.4 at which the solution assay suggests <20% activity. However the CO₂ concentration would have changed dramatically above pH 7.5 in the assay as the addition of 50 mM of NaHCO₃ was used to provide CO₂, rather than saturated CO₂.¹⁶ Therefore, the enzyme may in fact function better at a higher bulk pH than the assay suggests, as supported by the slightly lower predicted current densities from the FEM model in electrolyte BC. The influence of low CO₂ concentration as opposed to the pH effect on the solution assay is further corroborated by the continued oxidation activity of the enzyme at high pH.¹⁶ These results further demonstrate the importance of considering the effect of pH on CO₂ when designing experiments and suggest that solution assays may be more accurate with saturated CO₂.

The FEM was then used to iterate the optimum electrolyte for enzymatic CO₂RR to provide the greatest predicted currents. The resultant conditions consisted of MOPS (86 mM) and NaHCO₃ (50 mM) at a bulk pH of 6.39. Experimentally, this optimum electrolyte (BC-MOPSopt) gave 20% higher current densities (Figure 4). Thus showing again that a high buffer capacity and matching the pKₐ of the additional buffer to the local pH, as opposed to the bulk pH, is critical to stabilising the local pH and hence maximising product formation.

**Local Chemical Environment of Macroporous Electrodes**

The above local environment optimisation conclusions were then applied to hierarchical inverse opal (IO) electrodes to demonstrate their applicability to alternative electrode architectures (Figure S6). The mesoporous scaffold enables high loading of the enzyme whilst the macropores, formed by a polystyrene sphere co-assembly procedure, increase the diffusion of electrolyte species, such as substrates and products, into and out of the electrode. With the IO-TiO₂ electrodes the loading could be doubled from that used with mesoITO and more negative reduction potentials (−0.8 V vs. SHE) were able to be applied. The less-
confined IO structure should also increase the mass transport of protons to the local environment of the enzyme, decreasing the local pH increase caused by catalysis.

First, the IO electrode IO-TiO$_2$|H$_2$ase$_{25}$ was run with the same loading (25 pmol) and at the same applied potential (−0.6 V vs. SHE) with 240 mM MES and a bulk pH of 4.24 to demonstrate the increased mass transport of the macroporous structure (Figure 5). As the local pH increased less than in the confined mesoITO structure, the local pH remained below the optimum and therefore the current density for IO-TiO$_2$ was significantly lower (−2.2 mA cm$^{-2}$) than for mesoITO (−4.8 mA cm$^{-2}$). If the local proton consumption is then increased beyond the mass transport limit by doubling the enzyme loading and applying a more reductive potential (IO-TiO$_2$|H$_2$ase$_{50}$), the local pH again increases as in the mesoporous electrode. Therefore, the current density increased to −8.0 mA cm$^{-2}$, producing 83 μmol cm$^{-2}$ of H$_2$ at a FE of 92% with a H$_2$ase-based turnover number (TON) for H$_2$ of 3.2·10$^5$ and turnover frequency (TOF) of 9 s$^{-1}$. The TON and TOF was calculated from the product quantification and the enzyme loading, and since the number of active enzymes in direct electron transfer with the surface is unknown, the TON and TOF of these systems therefore represent the lower limits of the actual values. Due to the significantly higher current densities obtained for this system, even greater bubble effects are expected, which can be observed in the decrease in current density over the one hour-long experiment.
The stability of CO₂ reduction by the mesoITO|FDH₅₂ electrode, with the same loading (52 pmol), was demonstrated over 10 hours producing 22 μmol cm⁻² h⁻¹ of formate with a FE of 103% (Figure 6). As with H₂ase, the increased mass transport of the macroporous IO structure can be observed by the lower current density of IO-TiO₂|FDH₅₂ at −0.6 V vs. SHE. On increasing the local proton consumption by again doubling the enzyme loading (104 pmol - IO-TiO₂|FDH₅₂) and applying a more reductive potential (−0.8 V vs. SHE), the local pH increases, raising the current density to −3.0 mA cm⁻². However, the local pH then increases beyond the optimum due to the significantly higher current densities, therefore the bulk pH must again be lowered to 4.59 to further increase the enzyme activity. As the increase in local pH is slower in the less-confined IO structure, the local pH change can be observed in the chronoamperometry as an initial increase in current density, peaking at 1.2 h. The final electrolyte solution demonstrates current densities of −3.6 mA cm⁻², producing 0.55 ± 0.043 mmol cm⁻² of formate (55 ± 4.3 μmol cm⁻² h⁻¹) at a FE of (96 ± 8.7)% with a FDH-based TON
for formate of $1.0 \times 10^6$ and TOF of 28 s$^{-1}$. Thus demonstrating the significant 18-fold improvement that can be achieved compared to previously reported systems (IO-TiO$_2$|FDH$_{38}$, 38 pmol, 0.2 mA cm$^{-2}$) by simple adjustment of the experimental parameters.$^{39}$

![Figure 6](image1.png)

Figure 6. (a) CA of mesoITO|FDH$_{52}$ with an applied bias of −0.6 V vs. SHE in pH 6.39 (brown). CA of IO-TiO$_2$|FDH$_{54}$ with an applied bias of −0.6 V vs. SHE in pH 6.39 (orange). CA of IO-TiO$_2$|FDH$_{104}$ with an applied bias of −0.8 V vs. SHE in pH 6.39 (light green) and in the optimum electrolyte at pH 4.59 (dark green). (b) Experimental formate production and FE from CA after 10 h. Conditions: pH 6.39 [MOPS (86 mM), NaHCO$_3$ (50 mM), KCl (50 mM) and pH 6.39] and pH 4.59 [MOPS (100 mM), NaHCO$_3$ (1 mM), KCl (50 mM) and pH 4.59]. $DvH$ FDH$_{52}$ (52 pmol) and $DvH$ FDH$_{104}$ (104 pmol), CO$_2$ atmosphere, stirred, 25 °C.

**Conclusion**

The critical importance of understanding the local chemical environment of highly active confined enzymes in porous electrodes has been experimentally demonstrated. An independent FEM was also applied to predict the experimental currents, thus verifying the model and allowing the simulation and understanding of local concentration changes, an experimentally challenging feat in these complex multicomponent enzyme systems on porous electrodes. For the HER and the CO$_2$RR, the local pH was shown to increase around 2 pH units due to enzyme catalysis in a confined environment. Dramatic differences in activity were achieved without inhibitively large buffer concentrations, but instead through careful buffer
selection and manipulation of the electrolyte pH. In mesoporous electrodes, a 9-fold increase in the HER current density from −0.5 mA cm⁻² to −4.8 mA cm⁻² produced 50 ± 3 μmol cm⁻² h⁻¹ of H₂ and a 2-fold current density increase for CO₂ reduction, produced 21 ± 0.1 μmol cm⁻² h⁻¹ of formate. Finally, in an alternative architecture of inverse opal electrodes, even greater current densities were achieved through the combination of increased mass transport due to macroporous structuring, higher loading and more reductive potentials with an optimised electrolyte. For the HER current densities of −8.0 mA cm⁻² produced 80 μmol cm⁻² h⁻¹ of H₂ with a FE of 92% and for the CO₂RR current densities of −3.6 mA cm⁻² produced 55 ± 4.3 μmol cm⁻² h⁻¹ of formate, over 10 hours giving a total formate production of 0.55 ± 0.043 mmol cm⁻² with a FE of (96 ± 8.7)%. These figures demonstrate a 3-fold increase for the HER and an 18-fold increase for the CO₂RR on analogous reported electrochemical systems in which the local environment was not considered.¹²,⁵³ Through this work we have shown the ability to use experimental data and computational methods in tandem to analyze and optimise biological-artificial hybrid electrodes.

Electrolyte adjustment to mitigate the local pH changes of enzymes on a porous electrode has been shown as a simple method to maximise the product formation, important for fuel-forming applications. Whilst the precise conditions for each system will depend on the enzyme, current density and electrode architecture, we have demonstrated with the IO electrodes that the following guidelines can be applied to optimise any porous bioelectrochemical system. To achieve this the bulk pH should not be chosen for the ‘ideal’ enzyme activity pH (determined by solution assay), but so that the local pH changes lead the solution into the optimum range. For reduction reactions the local pH increases and therefore a lower pH should be selected. Whereas for oxidation reactions the local pH decreases, and a higher pH is appropriate. Then a buffer should be chosen to have good buffering at the local pH optimum, which in a perfect system will be the ‘idea’ enzyme activity pH. Further significant developments can be achieved with the utilisation of a less densely structured porous electrode to facilitate the mass transport of species. Moreover, as high current density bioelectrochemical systems reach the mass
transport limits of current electrode architectures, a shift towards more dynamic flow technology, such as gas diffusion electrodes, \cite{54-56} may be required to make advances in the future.

In enzymatic fuel cells the optimisation of the local environment could similarly improve the current performance of these bioelectrochemical devices. \cite{1,57,58} Low power output remains one of the key issues which hinders the wider application of enzymatic fuel cells. The densely porous electrodes employed in these systems, such as multi-walled carbon nanotubes, alongside the redox polymers often used to mediate electron transfer will lead to the development of large concentration gradients between the electrodes and the bulk. \cite{1,15,57-59} Whilst consideration of the substrate concentration across these electrodes has been made, the local pH and electrolyte composition has not been accounted for and therefore has the potential to significantly increase power output as shown here for bioelectrochemical fuel synthesis. \cite{46}

The negative effect of the local pH increase in enzymatic CO\textsubscript{2} reduction is enhanced compared to systems utilising synthetic catalysts as there is no concomitant beneficial change in selectivity against HER. This work demonstrates the increasing importance of studying enzymes as exemplary selective and specific molecular catalysts. As artificial molecular catalysts become more sophisticated, and hence more selective, the techniques and conclusions of this enzymatic research will become ever more applicable.

This study has demonstrated that the local chemical environment is distinctly different when highly active enzymes are confined at high loading under electrocatalytic conditions. This highlights the importance of distinguishing electrochemical techniques into two groups. Firstly, those which easily allow for mechanistic and fundamental enzyme studies, such as the use of unconfined, flat rotating disk electrodes which minimise mass transport effects and prevent the establishment of local environments. \cite{3} And secondly, stationary, porous electrodes which allow for significantly higher loading and current densities for product application purposes, but also establish local chemical environments distinct from the bulk solution. Whilst high
loading porous electrodes can be used in enzymology studies, the local environment changes must be accounted for to prevent misinterpretation of activity changes. A FEM has been shown to be a powerful technique for understanding fundamental experimental observations in highly complex and convoluted applications. This research prompts a variety of other implications not specifically addressed here, but of equal importance to the field, including the local concentration of salt species and electron mediators, the confinement effect of redox polymers, the local pH decrease in oxidation reactions, and local pH changes in live cell biofilms.

Supporting Information.

Theory and Simulation derivations, experimental procedures, SEM of the mesoITO and IO-TiO$_2$ electrodes, 3D representation of the H$_2$ase and FDH enzyme, supporting electrochemical experiments, simulation of the pH and buffer capacity as a function of the distance from the surface, the Good’s buffer concentration-activity solution and electrochemical assays.

Statement of Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

E.E.M., S.C. and E.R. designed the project. E.E.M. conducted the electrochemical experiments, S.C performed the computational modelling. A.R.O., A.M.C. and I.A.C.P. provided the enzymes and performed solution studies. E.E.M. and E.R. wrote the manuscript with input from all authors. E.R. supervised the project.
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Notes

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