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Current Opinion in Electrochemistry

Review Article

From protein film to single-entity protein electrochemistry

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Abstract

This mini-review discusses recent advancements in the single-entity electrochemistry technique for the analysis of catalytic activities of single redox protein molecules, highlighting papers of interest from the past three years. The diverse detection and experimental strategies, as well as the theoretical frameworks enabling the analysis of experimental data, are presented. Additionally, insights that can be obtained from comparing single-entity protein electrochemistry with protein film electrochemistry data are discussed.

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Current Opinion in Electrochemistry 2024, **45**:101532

This review comes from a themed issue on **Physical & Nano- Electrochemistry (2024)**

Edited by **Yige Zhou** and **Hang Ren**

For complete overview about the section, refer Physical & Nano-Electrochemistry (2024)

Available online 1 May 2024

https://doi.org/10.1016/j.coelec.2024.101532

Keywords

Single-entity electrochemistry, Single enzyme electrochemistry, Enzyme dynamics, Protein film voltammetry, Nanoconfinement.

Introduction

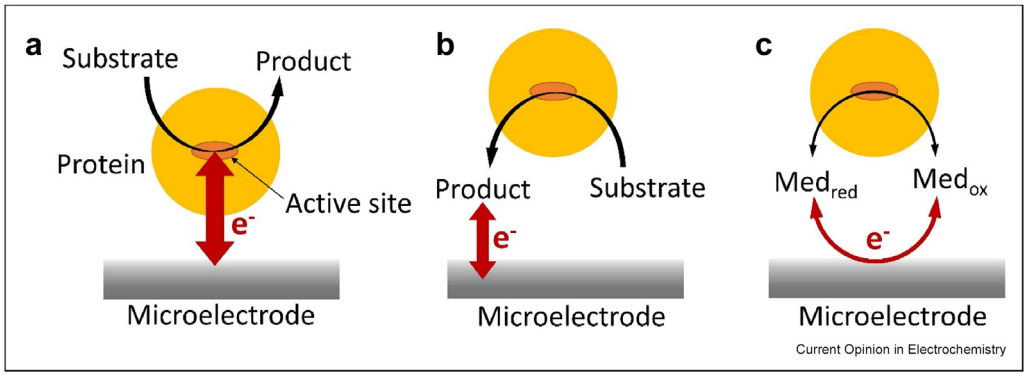
Redox proteins play a crucial role in numerous essential biological processes, including bioenergetics, elemental cycles, and metabolic processes. In these processes redox proteins function either as passive "wires" or transporters, transferring electrons from one site to another, or couple electron transfer with chemical reactions, serving as redox enzymes catalyzing oxidation–reduction reactions. Important biological functions, high selectivity, and efficiency in catalyzing various reactions have made redox enzymes an attractive target for mechanistic studies.

Electrochemical methods, which allow direct probing of electron transfer processes, today seem to be a natural choice for the functional investigation of many redox proteins [1,2]. However, until 1970 it was believed that it would not be possible to observe direct electron transfer (DET) between large protein molecules and an electrode [3]. In 1977, two independent reports by Eddowes and Yeh showed for the first time quasi-reversible voltammetry of cytochrome *c*, mitochondrial electron transfer protein, on bipyridyl-modified gold and tin-doped indium oxide electrodes, respectively [4,5]. A year later Berezin and Yaropolov demonstrated the detection of catalytic currents from redox enzymes, laccase and peroxidase, immobilized on carbon electrodes [6,7]. Following these first reports, electrochemical methods became more and more commonly used for studies of redox proteins, paving the way for the technique now widely known as protein film electrochemistry (PFE) [8,9].

In 2008, Lemay and colleagues pushed the limits of traditional PFE by monitoring the catalytic activity of just a few molecules of [NiFe]-hydrogenase immobilized on a gold nanoelectrode [10]. This breakthrough sparked the pursuit of measuring the activity of single redox protein molecules. We here refer to this method as single-entity protein electrochemistry (SEPE) to underscore its similarity to PFE, allowing for the measurement of activity in redox proteins with single-entity resolution. In 2016, our group demonstrated possibility of SEPE by detecting catalytic currents from single laccase molecules due to DET between the enzyme and the electrode amplified by the catalytic reaction of four-electron oxygen reduction [11]. The estimated turnover frequencies (k_{cat}) showed large fluctuations and on average were significantly higher than the values reported for solution assays. Compton's group reported measurements of catalase molecules by detection of the product of the enzymatic reaction between catalase and peroxide, O_2, at the electrode surface [12,13]. Again, the measured k_{cat} was much higher than the reported ensemble values.

Similar to the skepticism preceding the development of PFE methods, there is now a debate about the feasibility of detecting electron transfer between *single* enzyme molecules and electrodes, and about the utility of these measurements in understanding enzyme functioning [14–16]. To be fair, the skepticism is not without grounds. Presently, even with the best available

Figure 1



Detection methods in single-entity protein electrochemistry. (a) Direct electron transfer between the active site of the enzyme and the electrode amplified by the catalytic reaction is detected. (b) The product of the catalytic reaction occurring in solution is detected electrochemically on the electrode. (c) Electron transfer between the enzyme and the electrode mediated by a redox couple (Med_{ox}/Med_{red}) is detected on the electrode.

devices, the reliable measurement of only c. a. 2000 electrons is feasible [17]. This means that to detect currents from single enzyme molecules, the enzyme should exchange >2000 electrons per second with the electrode, which translates to a turnover frequency of greater than $2 \times 10^3/n$ (where n is the number of electrons participating in the catalytic reaction) molecules per second. Enzymes with such levels of catalytic activity are rare in nature, given that k_{cat} for biologically relevant redox enzymes commonly investigated by PFE methods range between 1 and 10,000 s^{-1}, with the majority falling in the middle of this range [18]. Moreover, in all reported SEPE experiments, determined turnover frequencies are much higher than the ensemble values, raising the question of whether these measurements uncover properties of enzymes previously unknown or are not reliable.

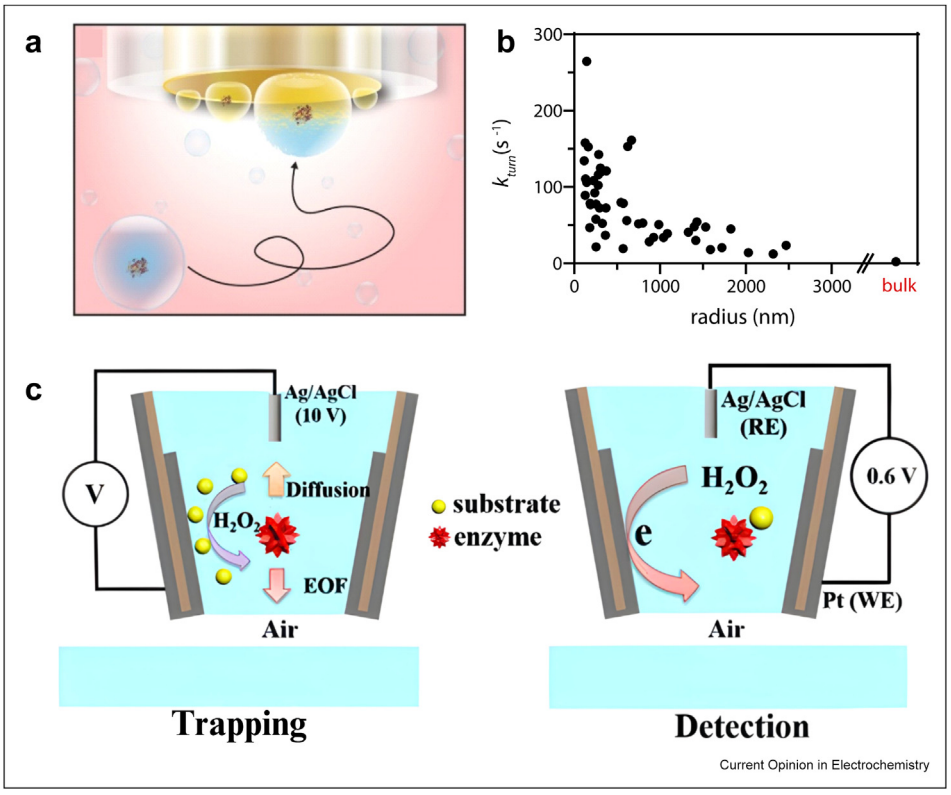
Two excellent reviews that discuss early SEPE reports in detail are available [15,16]. Additionally, a recent minireview by Chopin et al. discusses an alternative approach for detecting the electrocatalytic activity of single enzymatic entities using nano-scanning electrochemical microspore (SECM) imaging of immobilized entities [19]. Here, we focus on the developments in the field over the last 2–3 years following the first two reviews. We then discuss crucial information that can be extracted from SEPE (information not accessible by PFE), the details that can and should be correlated with PFE to validate the method, and the lessons we can learn from the progress in PFE that can be useful for SEPE development.

Recent developments in single-entity protein electrochemistry

Early SEPE reports demonstrated two approaches for detecting catalytic enzymatic activity: either by measuring DET under catalytic conditions when the single enzyme collides with the electrode (Figure 1a) [12,13], or by observing the electrochemical reaction of redox species generated by the enzyme's catalytic reaction in the solution (Figure 1b) [11,20]. Recently, a different detection method based on the principle of mediated electron transfer (MET) was introduced for detecting the catalytic activity of single enzyme molecules (Figure 1c) [21,22]. In MET detection, additional redox compounds, known as mediators, are employed to transfer electrons between the active site of the enzyme and the electrode. Mediators undergo electrochemical transformation on the electrode surface generating a current signal. Thus, the activity of a single G-quadruplex/hemin in peroxide reduction mediated by the benzoquinone/hydroquinone couple [21], and the glucose oxidation activity of adenine dinucleotide-dependent glucose dehydrogenase (FADGDH) mediated by ferrocyanide/ferricyanide [22], were measured.

Electrochemical studies of single enzymes under nanoconfinement have been reported in recent years (Figure 2) [23–25]. Nanoconfinement systems can be classified (but not limited to) into nanogap, nanochannel, nanopipette, and nanopore geometries. Conducting measurements under nanoconfinement provides a highly effective means to simulate a so-called ″crowded state″, in which proteins operate within biological systems, confined to the cytoplasm or internal spaces of organelles [26]. Moreover, nanoconfinement has been shown to significantly affect electrochemical reactions, altering mass transport and causing overlapped electrical double layers (EDL) and steric hindrance effects [23]. For example, the dimensions of confined spaces can lead to the exclusion of certain species based solely on their size, whereas overlapping diffuse EDL inside highly confined spaces can cause species to be either drawn into or expelled from the confined space [27]. This phenomenon can lead to

Figure 2



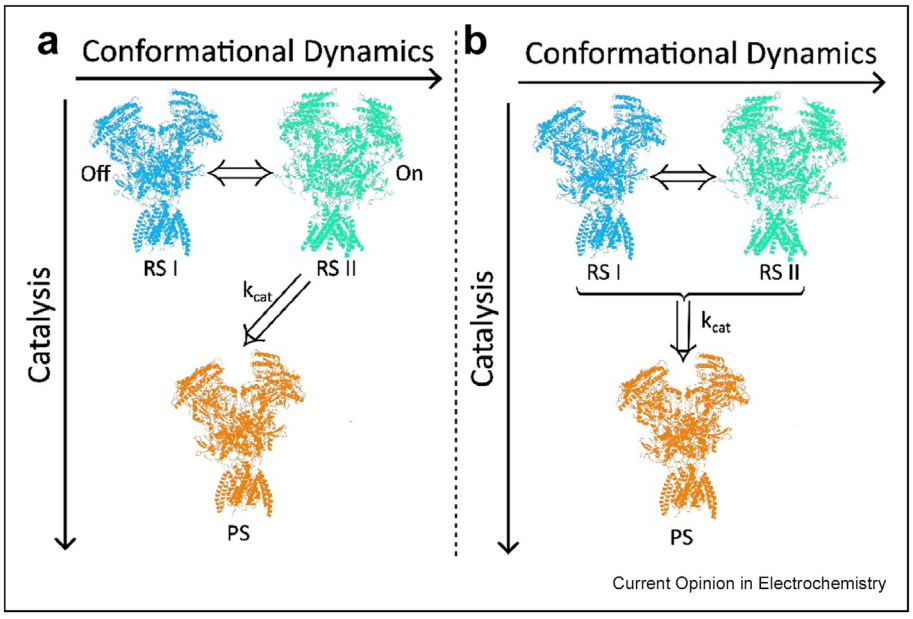
Single-entity protein electrochemistry under confinement. (a) Illustration of the sensing principle using confinement in nanodroplets on the example of FADGDH detection by the MET approach. Blue represents ferrocyanide, yellow ferricyanide, and pink 1,2-dichloroethane buffered solution. b. Dependence of the turnover frequency (k_{turn}) on the radius of the nanodroplet used for confinement. Reproduced with permission from Ref. [21]. c Schematic illustration of the sensing setup with conductive nanopipettes, using the example of glucose oxidase trapped by electro-osmotic flow. Reproduced with permission from Ref. [28]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

markedly different concentrations of analytes within the nanoconfinement space. In the case of enzymatic catalysis, this can result in turnover frequencies drastically different from those observed in solution. In addition, when the Debye length at the solid–liquid interface matches the size of the nanoconfinement space the EDL overlap and electromigration becomes a significant contributor to mass transport via electroosmotic flow [28]. For instance, in the absence of a supporting electrolyte, an amplified current as large as 2000-fold is observed due to electromigration and ion accumulation effects, resulting in highly sensitive enzyme measurements [28].

We would like to specifically mention several important reports on SEPE under nanoconfinement. Thus, FADGDH activity measured by the MET approach was achieved by trapping single enzyme molecules in 1 μm radius aqueous nanodroplets (attoliter, 10^{-18} L) suspended in 1,2-dichloroethane buffered solution (Figure 2a) [22]. Wang et al. reported detection of enzymatic activity of single glucose oxidase molecules (GOx) entrapped in 105 fL space of a conductive nanopipette by electrochemical detection of product of enzymatic oxidation of glucose in the presence of O_2, hydrogen peroxide [29]. The aqueous solution containing GOx was retained in the nanopipette by the oil sealing method [30]. Similarly, the activity of GOx down to 15 molecules confined in a conductive nanopipette was reported [31]. The single enzyme molecules were retained in the nanopipette by an electroosmotic flow induced by the applied voltage (Figure 2c). A common observation in all the reports is that the k_{cat} values increase with decreasing nanoconfinement volume and are significantly higher than ensemble-measured values (Figure 2b). This is not surprising as it is now recognized that many assumptions used in conventional enzyme kinetics which is used to measure ensemble k_{cat} values break down in the ″crowded state″ [32].

The role of conformational dynamic effects on enzymatic activity is one of the subjects currently being investigated in SEPE. There is an increasing recognition that ensemble-averaging methods, such as PFE, fall short of fully understanding the functioning of dynamic

Figure 3



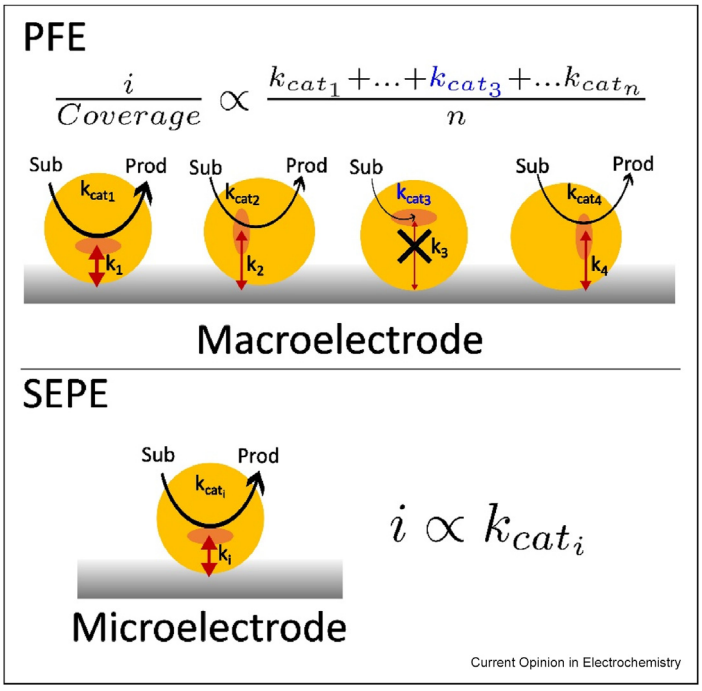
Schematic illustration of protein dynamics effects. RSI and RSII are reactant conformational states I and I, respectively. PS is a product state of the catalytic reaction. a. Enzyme conformational dynamics is coupled to the chemical reaction and therefore dynamic effects contribute to catalysis. b. There is no coupling between conformational dynamics and catalysis.

and complex protein molecules. While a debate persists on the extent to which conformational dynamics contribute to enzymatic catalysis (Figure 3)—with some esteemed scientists in the field emphasizing the lack of a solid proof for the role of dynamic effects in enzymatic catalysis—it is evident that single-molecule experimental methods such as SEPE are necessary to probe this phenomenon [33–35]. Compton's group proposed a theoretical model that enables the rationalization of experimentally observed k_{cat} in SEPE by dynamic fluctuations in enzymatic activity [36]. The authors concluded that the catalytic ability of single enzymes can be temporarily much higher than expected by the time-averaged Michaelis–Menten model, and these differences can be observed with the SEPE method. More recently, Ding et al. explored the conformational dynamics and kinetics of single catalase molecules during the enzymatic process under a magnetic field [37]. Using a theoretical model similar to those reported by Compton's group, the authors attributed the experimentally observed increased catalytic activity under magnetic fields to changes in the enzyme conformation. The circular dichroism and UV–vis spectra results verified that the magnetic field induces the unfolded and the loosened secondary structure of catalases. We recently developed an alternative model to rationalize the high catalytic activities of single catalytic molecules that does not require the incorporation of dynamic activity fluctuations [38]. Since catalase is a well-known diffusion-controlled enzyme and its active site is never saturated with the substrate, peroxide, it does not follow Michaelis–Menten kinetics. Instead, we modeled the enzymatic reaction as a diffusion-controlled reaction and introduced a partially active sphere geometry, considering the fact that not the entire enzyme is catalytically active but has a diffusion channel leading to an active site where the reaction occurs. The model allowed us to reproduce experimentally observed data with catalase and another diffusion-limited enzyme, superoxide dismutase, without the need to include conformational dynamics.

Insights from protein film electrochemistry

In a typical PFE experiment protein molecules are absorbed on a surface of an electrode forming a submonolayer protein film [39]. In principle, currents measured in PFE at a given applied potential and normalized to the number of active enzyme molecules (electrode surface coverage) can be compared with current signals in SEPE to validate SEPE results (Figure 4). However, there are two main problems with this approach. First, for the majority of enzymes, it is not possible to detect currents under non-catalytic conditions and therefore the exact surface coverage in PFE is usually not known [40]. Second, the protein molecules in PFE are randomly oriented on the electrode surface which is particularly problematic for DET current measurements. Some redox sites can be too far from the electrode to contribute to the measured catalytic current (Figure 4, top) [41]. These create problems when

Figure 4



Schematic illustrations of current signal generation in PFE (above) and SEPE (below). Red arrows denote the direct electron transfer step with different thicknesses illustrating different values of the electron transfer rate constants, k_i. Black arrows denote catalytic reactions at the active site of the enzyme with the rate constant k_{cati}. In the case of PFE, at large overpotentials, the current normalized to the surface coverage is proportional to the observed average rate of the catalytic reaction. This observed rate is an average over all protein molecules, even those that do not contribute to the current (k_{cat3}), due to unfavorable orientation and therefore slow interfacial direct electron transfer (k_3). In SEPE, each current signal at a large overpotential corresponds to the individual catalytic rate constant (k_{cati}) for the particular enzyme molecule interacting with the microelectrode. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

trying to correlate turnover frequencies measured in SEPE to data from PFE or, for that matter, to any ensemble measuring method. In any ensemble-averaging method, the number of active molecules generating a signal is not known. Attempting to correlate SEPE k_{cat} with the values measured by ensemble solution methods is even more challenging, as the rate-limiting step of the reaction, reflected in k_{cat}, can change when transitioning from homogeneous to heterogeneous kinetics. This was evident in our measurements with multicopper oxidases, where both the rate-limiting step and the electron transfer pathway to the active site changed when transitioning from the solution to the electrode-immobilized enzyme conditions [42,43]. This resulted in a significant increase in the k_{cat} value, which now is in agreement with our earlier single-molecule experiments [11]. Thus, the lack of activity correlation between ensemble and single-molecule measurements of redox proteins should not discourage researchers and, in our opinion, does not undermine the validity of SEPE measurements.

While the absolute enzymatic activity between PFE and SEPE cannot be compared, researchers presenting single-molecule data should carefully assess available PFE studies to ensure that there is no unjustified conflict between the provided results and ensemble electrochemical measurements. For example, Shen et al. reported measurements of the catalytic activity of single horseradish peroxidase (HRP) molecules in carbon-covered nanopipettes [44]. The authors observed oxidative and reductive current spikes in the presence of H_2O_2 that were attributed to DET between the enzyme and the electrode. This assignment is problematic because according to PFE measurements, HRP only exhibits reductive DET current due to the reduction of Compound I generated in the enzymatic reaction between HRP and peroxide by the electrode [45,46]. Foord et al. detected reduction current spikes upon collisions of catalase molecules with boron-doped diamond electrodes that were attributed to DET between the enzyme and the electrode [47]. These spikes were also detectable in the absence of the catalase substrate, H_2O_2, in solution but in the presence of dissolved O_2. From ensemble studies it is known, however, that it is not possible to detect DET if the distance between the active site and the electrode exceeds 15 Å [48,49]. For catalase used in this study the distance is ~30 Å [50]. Moreover, current signals due to the catalytic amplification could not be detected in the absence of substrate,

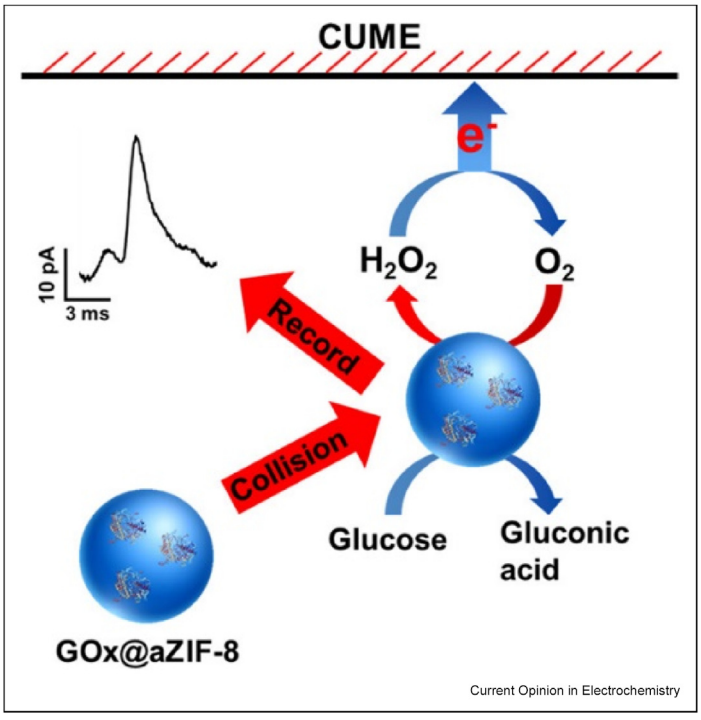
which for catalase is peroxide. Sánchez-Álvarez et al. observed both oxidative and reductive current spikes upon collisions of superoxide dismutase molecules, attributing them to DET between the enzyme and the electrode [51]. Superoxide dismutase does not require additional electron donors or acceptors for the catalytic reaction, as it catalyzes the dismutation of superoxide to O_2 and H_2O_2. Therefore, it is not clear how the electrode would participate in the reaction via DET, and this mechanism was not previously reported in PFE. These examples emphasize the importance of PFE data for the validation of SEPE results. The potential dependence of current signals observed in SEPE should correlate with PFE measurements. It is an important control experiment that must be performed in all SEPE measurements of single enzymes, especially based on DET detection. Moreover, inhibitors should have similar effects in SEPE as in PFE measurements, and their addition can be used to validate SEPE data.

Conclusions

In this mini-view, we have summarized recent trends in the field of single-entity protein electrochemistry, discussing proposed detection approaches, measurements under nanoconfinement, and the role of dynamic effects on enzymatic catalysis. We then discussed which data obtained by SEPE can and should be correlated with PFE data and which should not. Specifically, we debated why absolute values of turnover frequencies obtained under single-molecule conditions cannot be compared with turnover frequencies obtained by ensemble measuring techniques and should not be the reason to label SEPE data as erroneous. At the same time, it is unreasonable to assume that for enzymes for which it is not possible to detect DET under ensemble conditions, it can be detected in SEPE or catalytic activity towards different substrates not detectable by PFE can be observed under single-molecule conditions. SEPE measurements may record ″rare″ events that are generally not detectable by PFE methods. However, to claim this, rigorous probability and statistical treatment of data should be performed [14].

Traditional PFE involves studying the behavior of redox proteins through DET. However, the majority of reported SEPE measurements are based on either the detection of the product of enzymatic reaction or MET (Figure 1b and c vs Figure 1a). In these detection methods, the possibility of investigating enzymatic activity as a function of driving force, a unique feature of electrochemical methods that is not accessible by other experimental techniques, is not realized. This limitation is partially due to the low current resolution of SEPE, which is not sufficient for the investigation of the activity of single redox proteins commonly studied by PFE. Moving forward, we believe that utilizing bio-

Figure 5



Electrochemistry in bio-hybrid systems. Scheme illustrating the electrochemical detection of catalytic currents from a single zeolitic imidazolate framework nanoparticle incorporating 576 glucose oxidase molecules (GOx@aZIF-8) on a carbon ultramicroelectrode (CUME). Reproduced with permission from Ref. [50].

hybrid systems (Figure 5) [52–54], where a small *known* number of proteins are immobilized on well-defined nanoparticles such as conductive metal–organic frameworks [55,56] that are investigated upon their collisions with nanoelectrodes, can be an attractive halfway approach until sufficient resolution in SEPE measurements is achieved.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT 3.5 and Grammarly in order to check the grammar of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

A.S. and A.L.S. thank Göran Gustafssons Stiftelse (nr. 2215) for supporting this work. A.S. acknowledges the support from the Swedish Research Council (grant number N 2020-03262).

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