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Cyclic voltammetry for the study of microbial electron transfer at electrodes

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9.1 INTRODUCTION

The fact that certain bacteria can direct electrons to their outer surface, or transfer electrons from their outer surface to their inner compartments, makes it possible to measure the rate of electron flux from a living organism. When enzymes are interfaced with electrodes, either via their own direct mechanisms or mediators, the relationship between applied potential and current flowing at the working electrode can theoretically reveal rate-limiting processes, interfacial kinetics, and reaction stoichiometries (Armstrong 1999; 2005; Heering et al. 1998; Heering et al. 1997; Jeuken et al. 2002). The use of voltammetry to extract such unique information from bacteria is an emerging field that draws heavily from pioneering work developed first for cytochromes adsorbed to

electrodes (Eddowes and Hill 1977; Yeh and Kuwana 1977), and later to study enzymatic catalysis at electrodes (Hirst et al. 1996; Sucheta et al. 1993). Readers seeking deeper background of the electrochemical theory and models are directed to the discussions of Armstrong, Heering and Hirst, (e.g. (Armstrong 2005; Armstrong et al. 1997; Butt and Armstrong 2008; Léger et al. 2003)).

Microbial electron transfer to electrodes combines a series of irreversible enzymatic reactions (such as oxidation of acetate via the TCA cycle to yield NADH and reduced menaquinone), with a pathway of reversible electrochemical reactions that allow electrons to pass to the cell surface (Busalmen et al. 2008a; Schröder 2007). Electrons must then relay along a series of cytochromes, proteins, bound redox mediators, and/or soluble mediators, until they reach a reversible interfacial reaction at an electrode surface (within approximately 10 Å). A simplified scheme showing an idealized film of bacteria is shown in Figure 1.

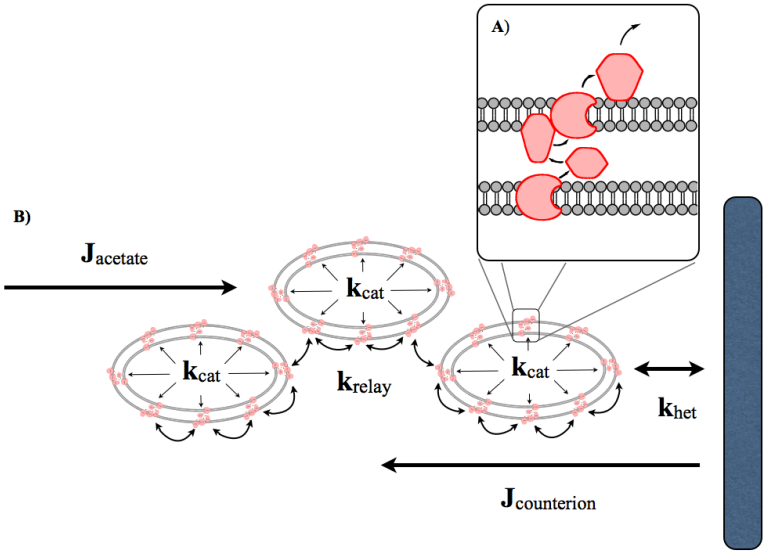

Figure 1 **A)** Cartoon showing possible paths of electron transfer across the inner and outer membranes of an electroactive bacterium. **B)** Schematic representation of mass transfer and kinetic limitations in an electrode-reducing biofilm in the absence of any soluble redox-active compounds. The total current is a function of: acetate flux ($\mathbf{J_{acetate}}$), irreversible metabolic reactions supplying electrons to the membrane ($\mathbf{k_{cat}}$), electron transfer around and between cells, relaying electrons to the interfacial contact point ($\mathbf{k_{relay}}$), interfacial transfer by proteins actually in contact with the electrode ($\mathbf{k_{het}}$), and counterion flux from the film ($\mathbf{J_{counterion}}$).

Voltammetry of living bacteria

In the simplest cases of irreversible chemical reactions occurring at an electrode, observed reaction rates are a function of the diffusion coefficient of the donor (how fast compounds can travel to the electrode), the standard rate constant of the reaction (how fast donor reacts when in contact with the electrode), and the electrode potential (which accelerates the rate, if electrons are available to hop from this terminal site). At flat planar electrodes, the rate constant and applied potential of most chemical reactions can be irrelevant, because diffusion quickly becomes rate limiting as the electrode is brought to a favourable potential. Such a combination of factors creates the familiar 'peak' in observed current as electrode potential is swept past the voltage where oxidation of the donor becomes favorable, because the donor is so rapidly depleted near the electrode that the surge in current cannot be sustained.

However, this is not the case with most enzyme-coated and bacteria-coated electrodes (Kano and Ikeda 2000; Marsili et al. 2008b). To appreciate this, one can calculate what current density a planar electrode should produce if a donor such as acetate could be rapidly and completely oxidized by a catalyst at the electrode surface. For example, the current of a theoretical rotating disk electrode oxidizing acetate can be calculated according to the Levich equation (Bard and Faulkner 2001), where current only depends upon the concentration of reduced substrate (e.g., 20 mM acetate), rotation rate, the kinematic viscosity of water, the number of electrons per acetate (8), and the diffusion coefficient of acetate (1×10^{-5} cm^2/sec).

One can see from this relationship that current produced is, in fact, not related to any aspect of the catalyst. It is only a function of how fast the electrode is rotated, which influences how fast substrate reaches the electrode. Thus, under conditions analogous to a slowly stirred solution, any electrode coated in a catalyst able to oxidize acetate should easily produce a current of about 50 mA/cm^2. This value is the 'mass transport limited' current, as it is limited only by how fast acetate can arrive at the surface. If bacteria on a surface are not a rate-limiting factor, one would expect similar current densities.

However, if we look at the best rates reported when wild-type strains of dissimilatory Fe(III)-reducing *Geobacter* species have been used to oxidize acetate at well-stirred, well-defined electrodes, values on the order of only ~0.5-1 mA/cm^2 are observed (Bond and Lovley 2003; Ishii et al. 2008; Marsili et al. 2008b; Min et al. 2005; Nevin et al. 2008; Reguera et al. 2006; Srikanth et al. 2008; Torres et al. 2007; 2008a; Torres et al. 2008b). From this, it is clear that acetate can diffuse to electrodes very quickly in comparison to how fast living cells appear to oxidize fuels and transfer those electrons to electrodes.

This calculation begs the question, if acetate is arriving at the electrode, why aren't bacteria consuming it? By varying the applied potential, and rate of potential change (scan rate), one can dissect this question further. For example, if the final reversible step ($\mathbf{k_{het}}$, in Figure 1) in which electrons hop to the electrode is sluggish (also described as having a small exchange current), it should respond predictably to a wide window of applied potentials, because potential can increase interfacial electron transfer rates (e.g., help the electrons hop the last gap to the electrode). If the reversible '$\mathbf{k_{relay}}$' pathway that feeds electrons to this final transfer point is slow, then faster scan rates under appropriate conditions could indicate the time required for electrons to arrive at the surface from more distant locations. Finally, if the irreversible reactions feeding electrons into the relay-and-interfacing pathway ($\mathbf{k_{cat}}$) is insufficient, we would expect a sharp response to applied potential, followed by a flat plateau in the current-potential relationship, as applied potential cannot accelerate an enzyme's rate beyond its V_{max}.

9.2 TURNOVER VS. NON-TURNOVER VOLTAMMETRY EXPERIMENTS

The remainder of this section will compare cyclic voltammetry experiments conducted under either *turnover* versus *non-turnover* conditions. Under *turnover* conditions, electron donor is provided to cells, causing an electron flow that can be varied as a function of electrode potential. The potential is typically changed very slowly (scan rates 1-10 mV/s), so that at each applied potential, all proteins involved in the pathway are oxidized and reduced ('turn over') multiple times. In *non-turnover* conditions, cells are first deprived of all electron donors, then electrons are pushed in and pulled out of cells by changing the electrode potential – inducing, at most, a single oxidation or reduction event by each redox center accessible to the electrode.

9.2.1 General considerations

Because diffusion can significantly affect voltammetry, any setup involving electrochemistry and bacteria should strive to create as consistent an environment as possible. For example, in most of the examples described in this chapter, *Geobacter sulfurreducens* cells are allowed to colonize a small electrode on the order of 1 cm^2, which has been polished to expose a uniform surface with features smaller than the bacteria themselves. In very early stages of growth, this produces an array of individual cells on a planar electrode. Analysis during this sub-monolayer phase is closest to the classical model of protein film voltammetry, where each cell behaves as an independent microelectrode surrounded by its own radial diffusion boundaries (Armstrong et

al. 1993; Picioreanu et al. 2007). Once cells begin to crowd, or touch each other, the system enters a regime more akin to a planar electrode, where diffusion may play a larger role. Key decisions to be made in setting up experiments are related to the techniques to be applied. Slow scan rate linear sweep voltammetry is relatively forgiving, as the slow potential steps minimize charging currents (Kim et al. 2007; Liu et al. 2005). To probe faster scan rates, apply pulse methods, or conduct impedance spectroscopy, one should employ a three electrode arrangement in which all connections are robust, electrodes are in close proximity to each other, and the working electrode surface is uniform (Marsili et al. 2008b; Srikanth et al. 2008).

As a final comment, one should note the reference used for reporting potentials in the literature. Biochemistry and microbiology are typically discussed in terms of E vs. the Standard Hydrogen Electrode (SHE), for comparison with standard values in biological textbooks and geochemical data. In contrast, it is common in electrochemistry to provide experimental data as collected, with E vs. Ag/AgCl or Hg/HgCl reference. As this review is intended for a biological audience, all values (including those taken from literature) are adjusted to E vs. SHE for easy comparison.

9.2.2 Voltammetry in the presence of substrates

As mentioned, the first cells attached to the electrode create the only situation similar to an array of individual microelectrodes. Allowing cells to grow as thicker cell layers introduces new factors such as film heterogeneity, long-range electron relay, cell-cell interactions, and diffusion through biofilms. Calculations (based on known rates of electron transfer), as well as biofilm images suggest that the transition from the sub-monolayer to multilayer stage (on polished electrodes) occurs by a current density of approximately 50 $\mu A/cm^2$ for *G. sulfurreducens*, (which can easily achieve a current density of 500-1000 $\mu A/cm^2$) (Marsili et al. 2008b). The fact that growth of *G. sulfurreducens* continues in an exponential manner near its maximum growth rate well beyond this monolayer phase strongly suggests that these first additional layers of cells are not causing a significant limitation in the cell-cell relay of electrons to the electrode. To date, no similar information is available for other electrode-reducing bacteria.

A typical catalytic voltammetry experiment can use such a thin film of bacteria. In the simplest case, the level of electron donor is provided in excess, which is easy to verify by increasing the concentration of donor available to cells. In the case of thin films of *Geobacter*, when acetate concentrations are > 5

mM, additional acetate has no effect on current density, nor do increased rates of stirring (Marsili et al. 2008b; Richter et al. 2008). These observations support the idea that the microbial film is not limited by supply of donor to the surface, and factors such as internal oxidation rates or cell-electrode electron transfer rates are dominant controlling influences.

The working electrode is switched to a starting potential (typically held for a brief resting period), then slowly stepped through a series of applied potentials until a maximum is reached, then swept back through that same range. As the goal is to not injure the cells, the potential window should be small enough to limit strong reducing or oxidizing potentials, but wide enough to bracket the oxidation processes of interest. The lowest potential applied is relatively easy to choose, as a current density of essentially zero should be achieved at the point where substrate oxidation is unfavourable (e.g., well below the midpoint potential of the donor). The highest potential applied depends on the response of the bacteria; again the goal is to sweep at least 100 mV above any potential where a change is observed (Bard and Faulkner 2001). Observations with most *Geobacter* strains typically reveal a flat plateau above potentials of +100 mV vs. SHE, allowing voltammograms to sweep from as narrow a window as -0.5 V to +0.2 V (Fricke et al. 2008; Liu et al. 2008). With *Shewanella sp.*, a gradual rise in current in the higher potential range, coupled with use of a lower potential donor, requires routine voltammetry to sweep from nearly -0.7 V to +0.4 V (Manohar et al. 2008; Marsili et al. 2008a). Each investigator should perform preliminary observations to determine an optimal window, demonstrate that repeated sweeps produce similar results, and verify that the process does not harm the cells.

Along with careful choice of the potential window, the scan rate also should be justified. An assumption during slow scan rate voltammetry is that each applied potential achieves a steady-state rate of turnover at that driving force; that it is reflecting physiological information about what the bacteria are capable of, under each imposed condition. Thus, a key definition of catalytic voltammetry is that moderate increases in scan rate do not alter the limiting current achieved at high potentials. Sweeping at 1 mV/s, followed by 2 mV/s, 5 mV/s, etc., and observing a similar response, primarily in the limiting current observed at higher potentials (corrected for the increase in capacitive current) demonstrates that the entire microbial film is being transitioned through a series of potentials slowly enough that diffusion is not affecting steady-state results. It can be desirable to sweep much more rapidly, but in such cases the goal is to ‘outrun’ catalysis or diffusion, to elucidate turnover or diffusion limitations within the film.

Voltammetry of living bacteria

Figure 2 shows representative data from a growing *G. sulfurreducens* biofilm, where the electrode potential was swept at 1 mV/s. In preliminary experiments, it was determined that sweep rates up to 5 mV/s did not alter the shape or features of the waveform, nor did additional acetate affect results (Marsili et al. 2008b). Analysis of these catalytic waves is simplified by plotting the derivative of each sweep as a function of potential, producing a nearly symmetrical peak (or series of peaks) centered at what is often termed the E_{hw} (half-wave potential) or E_{cat} (catalytic center potential) (see (Heering et al. 1998; Heering et al. 1997) for an example of the Butler-Volmer and Marcus theory underlying these and other analyses of catalytic waves).

What do these sigmoidal catalytic waves suggest about the physiology of electron transfer to electrodes by *Geobacter?* Starting from the zero current observed at low potentials (below the midpoint potential of acetate), the core reaction does appear irreversible. As potential rises above a threshold (around -0.2 V), the positive anodic current reflects continuous oxidation of acetate, and transfer of electrons to the electrode. This response rises nearly symmetrically around a midpoint of about -0.15 V. In this narrow region (between -0.2 and 0 V) the electrode potential (and likely, the potential of proteins on the exterior of cells) prevents interior reactions from operating at their maximal rate. In other words, between -0.2 and 0 V, the capacity of cells to generate electrons is in excess, but cells are limited by the availability of electron acceptor to take them and pass them to the electrode—the system has an excess of enzyme and is limited by the kinetics at the interface.

However, as the working electrode potential is raised as little as 0.05 V above this midpoint potential, the sharp response in anodic current ceases, and a plateau is reached. This flat region is important, because while interfacial reactions can be accelerated by increases in driving force (as described by Butler-Volmer or Marcus theory), enzymatic reactions have a characteristic V_{max}. The Nernstian response of the anodic current (rising symmetrically around a midpoint with a characteristic width of the derivative peak at half-height) is a hallmark of a system limited by the rate of enzymatic activity, not by interfacial kinetics of electrons hopping from terminal proteins to electrodes (Léger et al. 2003). This behavior would be expected from a system where proteins on the outer surface of cells rapidly equilibrate to a potential equal that of the electrode. If it has been previously demonstrated that diffusion to the electrode is not limiting (by varying levels of donor or stirring and finding no effect), then this relationship strongly suggests that enzymatic activity by the film of bacteria is limiting (a conclusion also reached in the discussion of the Levich equation).

As the potential is swept back across the same potential range, a similar voltammogram is observed, as one is essentially repeating the experiment. The only differences may be minor peaks that reflect redox centers accessible to the electrode, but whose redox status is not rate-limiting.

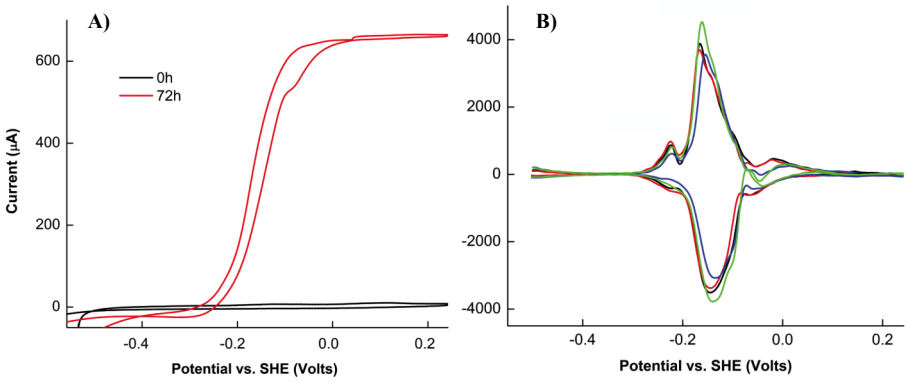

Figure 2. A) Cyclic Voltammetry (1 mV/s) of a *Geobacter sulfurreducens* biofilm after 72 h growth at +0.2 V vs. SHE on a 2.5 cm^2 planar graphite electrode. **B)** First derivative analysis of four replicate electrodes, revealing midpoint potentials within the catalytic wave.

Many voltammograms of bacteria, even of *G. sulfurreducens*, show more complex waveforms than the simple sigmoidal curve. Such results may show more pronounced peaks at the top of the waves, or a large 'surge' in anodic current during the forward scan that is not evident on the reverse scan. While it may be tempting to interpret such peak-like shapes as mediators or additional complexity, these features can be easily created by a mismatch between scan rates and diffusion rates, so that forward voltammograms begin to resemble diffusion-limited peaks at planar electrodes (Busalmen et al. 2008b; Yokoyama et al. 2002). This kind of behavior would also be expected when thick or porous electrodes are used, as donor concentrations could become depleted during the scan. An example of this kind of behavior was produced in voltammograms of a thin biofilm of *G. sulfurreducens*, simply by exposing it to saturating levels of acetate (producing a sigmoidal curve), vs. diffusion-limiting levels (producing peak-like features) (Marsili et al. 2008b).

This again points to the importance of scan rate; if the potential is raised rapidly to make the reaction favourable, and a large reservoir of donor is

temporarily available at the electrode, a surge in current will occur. But donor is consumed near the electrode in a Cottrellian fashion (current is proportional to $1/t^{1/2}$), and current decays back to a diffusion-limited rate, creating a 'peak'. Upon the reverse sweep, a more sigmoidal wave is obtained, producing a forward scan with a rapid onset and peak, and a reverse scan without this feature. Importantly, the midpoint potentials of the forward and reverse scans are not identical to those obtained with thin films, because they represent reactions occurring at a variety of local and transient concentrations.

A second 'non-standard' waveshape is that of *Shewanella*, which does not produce a plateau limiting current at higher potential, but continues to rise slowly between 0 and +0.4 V (Marsili et al. 2008a). In this case, one hypothesis is that free flavins secreted by cells (which have a midpoint potential of -0.21 V) are responsible for the early Nernstian response, which reaches a limiting current based on the diffusion rate of the soluble mediator. At higher potentials, cytochromes interfaced with the electrode (which have a higher midpoint potential) (Firer-Sherwood et al. 2008; Hartshorne et al. 2007) begin to contribute to electron transfer. The gradual rise in anodic current suggests that *Shewanella* direct electron transfer is a process with a slow interfacial rate of electron transfer, which requires significant overpotential to overcome.

9.2.3 Voltammetry in the absence of substrates

While catalytic voltammetry is obviously related to situations such as continuous electricity production in fuel cells, and produces easily measurable large signals, it represents the interplay between diffusion of donor, irreversible oxidations, and electron transfer. Voltammetry in the absence of electron donor can reveal the potentials of exposed redox proteins, and can often be used to estimate interfacial rate constants or long-range diffusion parameters (Armstrong 1999; 2005; Heering et al. 1998; Heering et al. 1997; Jeuken et al. 2002).

To achieve non-turnover conditions, cells must first be attached to electrodes (typically by growing to the desired density), then starved free of donors by incubating at positive potentials (Fricke et al. 2008; Xing et al. 2008). This can be problematic, as starving cells may downregulate electron transfer proteins, or detach cells from electrodes. For example, preliminary experiments with *G. sulfurreducens* have shown that starvation for 36 hours (which is needed to completely deplete intracellular stores of thick films) followed by addition of acetate often recovers ~50% of the original limiting current (Marsili et al.

2008b). However, once starved, many bacteria respond consistently to repeated scans at variable rates, to provide information about coverage, diffusion of electrons through the film, or interfacial kinetics. In contrast, other strains show a greater propensity to detach or die off, making even simple analysis unreliable.

If a stable, non-catalytic film can be obtained, an initial experiment is to perform a series of slow linear sweeps across the same potential range used for catalytic voltammetry. This should reveal a series of reversible peaks that, after baseline subtraction, are centered at characteristic midpoint potentials for each redox center. While a textbook characteristic of adsorbed redox agents is reversible anodic and cathodic peaks located at identical potentials, it is not uncommon for peaks from adsorbed proteins to be offset by 30-60 mV, possibly due to conformational changes, film heterogeneity, or overpotentials required to reach buried redox centers (Armstrong et al. 2000; Hirst and Armstrong 1998; Hirst et al. 1998).

To further analyze peak areas or peak heights, it is crucial (and often difficult) to subtract the baseline from non-turnover voltammograms. For an illustration of techniques used to extract the relatively small signals from background capacitance in protein film voltammetry, the reader can look to examples from the laboratories of Armstrong, Elliot, or Léger (Léger et al. 2003; Firer-Sherwood et al. 2008; Fourmond et al. 2009). In most of these approaches, polynomial or cubic spline baselines are fit between user-defined regions, in contrast to linear interpolation methods (Fourmond et al. 2009). In addition, collection of voltammograms across a range of scan rates using sterile (blank) electrodes can aid in estimation of correct baselines, especially with carbon electrodes.

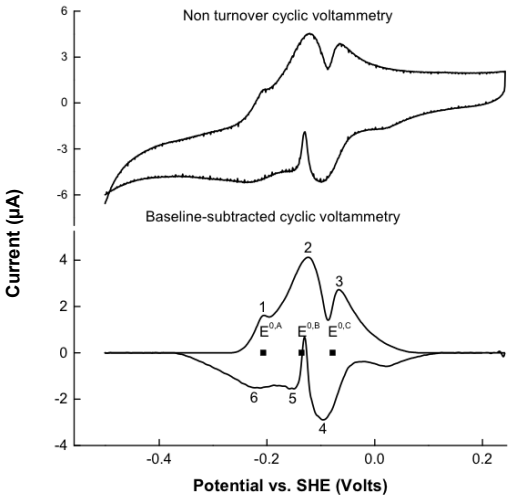

Figure 3. Raw data from *G. sulfurreducens* grown for 72h as a biofilm on a 2.5 cm^2 graphite electrode, starved for 36h in the absence of acetate, and subjected to cyclic voltammetry (1 mV/s) (top). Lower figure shows the baseline- subtracted voltammogram (using a cubic spline method in the free DOS program UTILS), and approximate midpoint potentials of putative detected redox centers.

The area under each peak indicates surface coverage of redox proteins, (Γ, in mol/cm^2), and can be calculated from the peak area (Q, in Coulombs) via $\Gamma = Q / (nFA)$ (Rusling and Forster 2003). To convert this to moles of protein/cm^2, one would need to make assumptions about the average number of redox centers/protein (as many cytochromes postulated to be involved in electron transfer are decaheme or greater). Early voltammetry of both *Geobacter* and *Shewanella* films have produced peaks consistent with a coverage of as much as 10^{-8}-10^{-9} mol electrons/cm^2, which, for an assumption of 10 e^- per protein, suggests 10^{-9}-10^{-10} mol protein are accessible to each cm^2. As a monolayer of 100,000 MW proteins can pack on a surface with a density of about 10^{-12} mol/cm^2 (Armstrong et al. 1997), these observations strongly suggest that redox centers beyond the electrode are rapidly able to accept electrons from the surface-associated members. If confirmed, these unexpectedly high coverage values may reflect relay of electrons to proteins into the periplasm, or even the quinone pool.

At very slow scan rates, all electroactive proteins interfaced with the electrode are assumed to oxidize and reduce, as the potential is slowly increased and decreased. In multicellular films, this requires again that a mechanism exists for electrons to be relayed to more distant proteins from the most closely surface-attached proteins. However, if the potential is changed quickly, more distant redox proteins may not have time to be oxidized. The more formal description of this phenomenon is a transition from thin-film behavior (where the film discharges as a single entity) to semi-infinite diffusion (where only a portion of the film, close to the electrode, discharges). The hallmark of this is a transition from the peak height being proportional to scan rate, to being a linear function of the square root of scan rate (Bard and Faulkner 2001; Fricke et al. 2008; Mano et al. 2006).

Looking to the future, scan rate analysis may be able to reveal information about interfacial electron transfer, or cell-cell relay properties. As scan rates are increased, the location of anodic and cathodic peaks shift in opposite directions. If the behavior has been confirmed to be thin-film (peak height proportional to scan rate), software or a simple fitting method (described by Laviron (1979)) can be used to estimate an average interfacial rate constant of electron transfer for an immobilized redox center (as k_0, with units in s^{-1}). For diffusional redox centers (such as shuttles), or for relay mechanisms showing semi-infinite diffusion, one can also use scan rate analysis based on the method of Nicholson and Shain, but the apparent rate constant (with units in cm/s) cannot yet be derived without further information such as the diffusion coefficient or

concentration of redox centers in the film (Nicholson and Shain 1964). Careful analysis of scan rate data, measurements of film discharge rates (chronocoulometry), impedance spectroscopy, and comparison with cells in the non-biofilm (sub-monolayer) stage will likely shed new light on the nature and limitations of cell-cell relay mechanisms, and separate them from cell-electrode electron transfer.

9.2.4 Concluding remarks

Considering the complexity of a self-assembling catalyst made of living cells, which performs a series of oxidations and electron transfer reactions that are linked to a series of proteins that naturally interface with electrodes, it is remarkable that techniques such as those described in this and other chapters can be as informative as they are. It should be stressed that, in contrast to the case of most protein film voltammetry, 3-D structures of proteins being studied are not yet known, the number of participating redox centers is an order of magnitude larger, enzymes and cells interact in a network of electron relay mechanisms, while parameters (such as temperature, pH, medium composition) can not be as widely varied to test hypotheses. In all cases, researchers should be cautious in applying theory, techniques, and models to living cells, and strive to create equally defined and controlled experimental conditions to better understand the electron transfer reactions catalyzed by these remarkable organisms.

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