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Perspective

Pushing the methodological envelope in understanding the photo/electrosynthetic materials-microorganism interface

Kiran Kuruvinashetti[1] and Nikolay Kornienko[1,*]

SUMMARY

Biohybrid photo/electrosynthetic systems synergize microbial metabolic pathways and inorganic materials to generate the fuels and chemicals to power our society. They aim to combine the strengths of product selectivity from biological cells and efficient charge generation and light absorption of inorganic materials. However crucial mechanistic questions still remain. In this review we address significant knowledge gaps that must be closed and recent efforts to do so to push biohybrid systems closer to applicability. In particular, we focus on noteworthy advances that have recently been made in applying state-of-the-art analytical spectroscopic, electrochemical, and microelectronic techniques to help pinpoint key complexities of the microbe-materials interface. We discuss the basic function of these techniques, how they have been translated over to study biohybrid systems, and which key insights and implications have been extracted. Finally, we delve into the key advances necessary for the design of next generation biohybrid energy conversion systems.

INTRODUCTION

The accelerating consumption of fossil fuels since the industrial revolution has resulted in the deterioration of earth's environment. As such, mitigating climate change, decreasing the levels of environmental pollution, and developing sustainable pathways to power tomorrow's society are amongst the most pressing challenges facing future generations (Chu and Majumdar, 2012; Lewis and Nocera, 2006). In the context of renewable energy, there has been much progress in technology development in generating electricity efficiently from solar, wind, and hydro sources (NREL, 2021). A central difficulty is converting the resultant intermittent electricity into energy dense fuels and chemical building blocks as replacements for fossil fuels because the catalytic chemistry has not yet been developed. Nature, on the other hand, has evolved elegant catalytic routes in using light, simple reducing/oxidizing equivalents and simple raw ingredients (e.g. CO_2, H_2O) to generate complex chemical products. Despite these strengths, natural systems powered by photosynthesis suffer from overall light to product efficiencies. Often natural systems are limited by incomplete light absorption and low throughput with overall solar to chemical/biomass efficiencies often typically situated below 1% as they are geared primarily for survival and proliferation (Blankenship et al., 2011).

Against this backdrop, there has been a rapid growth of research focusing on combining strengths of synthetic materials and natural catalysts into integrated biohybrid energy conversion systems (Kornienko et al., 2018). Specifically, this line of work aims to synergize the efficient light absorption and charge delivery of semiconductor photocatalysts and inorganic (photo) electrodes with electroactive microbes (Cestellos-Blanco et al., 2020). The chosen microbes are selected to take up the resultant charge from the inorganic material via various extracellular electron transfers (EET) pathways and perform the requisite complex catalytic chemistry through their evolved metabolic pathways, ideally generating products of high value at industrially relevant throughputs. A secondary advantage linked to microbial systems is that they contain built-in self-repair and reproductive mechanisms, alleviating the stability issues often limiting the use of isolated enzymes. With such biohybrid systems, the sustained, efficient and selective reduction of CO_2 to multi-carbon products is a longstanding goal. However, its achievement is still hampered by difficulties in seamlessly integrating the synthetic and biological components. We argue that overcoming these challenges will largely be made possible by the development of a fundamental understanding of the materials/biology interface aided through the innovation of analytical methods and thus, this is the focus of the present review.

[1]Department of Chemistry, Université de Montréal, 1375 Avenue Thérèse-Lavoie-Roux, Montréal, QC H2V 0B3 Canada

*Correspondence: nikolay.kornienko@umontreal.ca

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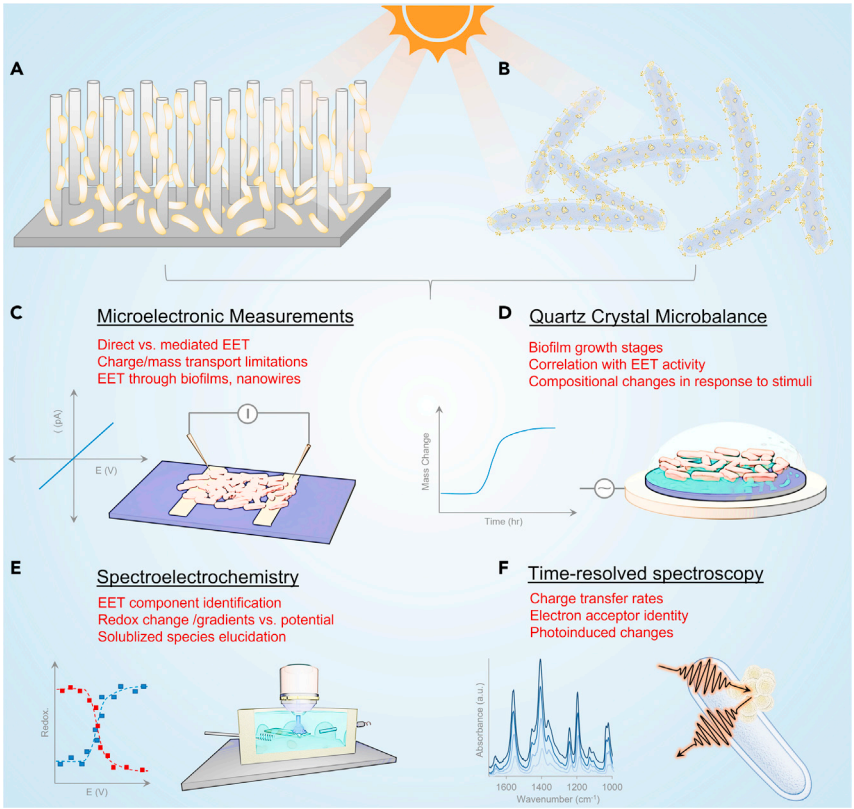

Figure 1. Overview of emerging techniques

(A and B) Biohybrid systems composed of electroactive microbes interfaced with (photo)electrodes (A) and synthetic light-absorbing particles synergized with functional microbes (B) will be the two principal types of systems focused on in this work.

(C–F) The emerging methods translated to probe biohybrid systems primarily include microelectronic measurements (C), QCM-based techniques (D), steady-state spectroelectrochemistry (E) and time-resolved spectroscopy (F), each of which can be applied to certain subsets of systems and offers unique mechanistic insights.

The systems that will be covered in this review are those consisting of either (photo)electrodes that donate charge to microbes who carry out the chemical catalysis (Figure 1A) or light-absorbing semiconductor nanoparticles directly interfaced with microbes who accept their photogenerated charges (Figure 1B). Although analogous systems featuring isolated enzymes in place of whole cells are of significant fundamental value (Cracknell et al., 2008; Ruff et al., 2020), this scope of this particular work is limited to microbial systems. Thus, the target of this review is to identify knowledge gaps in the function of biohybrid systems and subsequently detail the emerging techniques being adopted in this field to address these gaps. The key insights include those that help the following:

1. Understanding charge transfer mechanisms between inorganic and biological components
2. Identifying, then approaching, theoretical limits of macroscopic electrode performance
3. Pinpointing limiting factors in macroscopic biohybrid system performance
4. Rationally increasing synergy between inorganic and biological components

Against this backdrop, this work first will focus on the use of microelectronic devices and conductivity measurements which are increasingly used to decipher means of inter and intracellular charge transport and EET mechanisms (Figure 1C). Next, quartz-crystal microbalance (QCM) based techniques will be covered which provide insights into how the mass and rigidity changes of growing biofilms correspond to observed electrochemical activity (Figure 1D). Methods employing the use of steady-state vibrational and UV-Vis absorbance spectroscopy will then be discussed, which offer information regarding the proliferation of

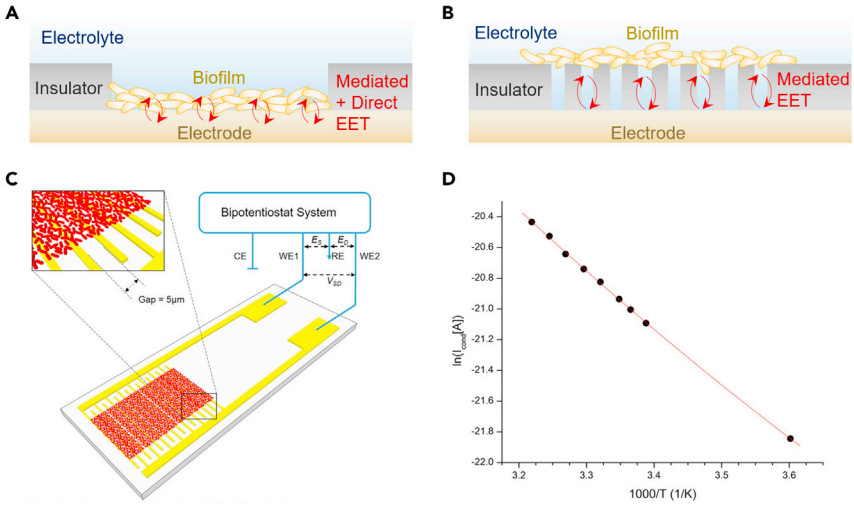

Figure 2. Microelectronic devices and conductivity

(A and B) The use of open electrodes (A) or those with exposed holes (B) enables the distinction between direct and mediated EET as the dominant mode (Jiang et al., 2010).

(C and D) Measuring conductivity between interdigitated electrodes (C) as a function of temperature can elucidate charge hopping as the dominant conductivity mode (D) (Xu et al., 2018).

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various cellular components implicated in EET pathways and redox changes of electroactive proteins as a function of location or applied potential (Figure 1E). Finally, the recent translation of time-resolved techniques to elucidate photoexcited charge transfer pathways and rates of various photoinduced processes will be the final topic (Figure 1F). Not every study focuses on functional biohybrid systems but rather aims to uncover mechanistic knowledge that can later be applied to such systems. Further, as some techniques are better geared toward measuring electrode or photocatalyst based biohybrids, the different sections will thus focus more on one particular type of system. To conclude, we provide future perspectives for steps necessary to take en route to realizing biohybrid systems in practical applications.

Microelectronic devices and conductivity measurements

We begin the discussion with the translation of microelectronic and conductivity techniques to probe charge transfer behavior of functional biofilms and isolated microbial cells. This knowledge is especially important because as EET mechanisms are still being elucidated (Kumar et al., 2017), charge transfer is often a factor limiting the throughput of biohybrid systems and rational means to maximize charge transfer rates thus underpins the development of next generation biohybrids. Ideally, the solar photon flux or charge flux from the inorganic component is limiting, rather than sluggish charge transfer between inorganic and biological components. Briefly, EET mechanisms largely consist of either 1) direct modes in which microbes and/or microbial nanowires directly transfer charge to/from electrodes. In contrast, 2) mediated EET modes often use soluble redox mediators such as flavins through other species such as cell-derived free enzymes have also been implicated (Deutzmann Jörg et al. 2015).

In this direction, microfabricated devices featuring (1) an open window with a conductive electrode accessible (Figure 2A) or (2) the conductive electrode covered by an insulator with only small open holes which were below the size of individual microbes (Figure 2B) were used to investigate EET pathways in *Shewanella oneidensis* MR-1 (Jiang et al., 2010). The results showed similar current outputs for both devices and that the currents rapidly decreased upon the exchange of media. Further, the current was not heavily dependent on the quantity of microbes directly adsorbed overtop of the microfabricated electrodes. Thus, this heavily implied that mediated EET was the dominant mode for this system. Alternatively, a field-effect transistor (FET) was used to probe EET pathways of *Clostridium ljungdahlii* microbes (Li et al., 2021), which do not possess genes coding for pili and cytochrome expression or quinone and hydrogenase secretion which are often used in the EET pathways. In an electrochemical cell under turnover conditions, the voltage across the source and drain electrodes, with the biofilm in between, dictated the availability and potential of

Table 1. Summary of information conferred from microelectronic device measurements and limitations of the experiments

Information	Challenges, limitations
Mediated vs. direct EET	Device fabrication, experimental throughput
Hopping vs. Metallic conductance	Translating from macroscopic to nanoscale
Single cell measurements, theoretical limits	Chemical/biological basis behind data

electrons. The availability of electrons was determined to be the dominant factor that resulted in the amount of catalytic current output and provided evidence for direct charge transfer as the principal mode of EET.

Source-drain type measurements have also been conducted as a function of temperature, to discern the mode of conductivity of conductive structures employed by *Geobacter sulfurreducens* (Yates et al., 2015). A pronounced temperature dependence in the conductivity, which could be subsequently fit to an Arrhenius model, provided evidence for a system in which charges hop via proximal cytochromes. In contrast, metallic conductivity would not have exhibited such temperature-dependent behavior. A similar line of studies was conducted with *S. oneidensis* MR-1 cells, also suggesting the presence of a charge hopping mechanism via a heme-based pathway (Figures 2C and 2D) (Xu et al., 2018). In a complementary work, individual microbial nanowires of *S. oneidensis* MR-1 were probed with a combination of nanofabricated electrodes and conductive atomic force microscopy (AFM) (El-Naggar et al., 2010). Having a microbial nanowire between two electrodes enabled the direct probing of its resistivity, found to be on the order of 1 $\Omega \cdot$cm while conductive AFM measurements were used to confirm the data. The deletion of cytochrome-coding genes resulted in mutants with non-conductive nanowires, demonstrating the involvement of cytochromes in the conductance mechanism.

Utilizing microelectrode methods can also enable single cell measurements, which are important to both understand diversity in performance between individual cells and to quantify upper efficiency limits from the extrapolation of single microbe measurements. To this end, microelectrodes are an important tool to interface with one cell at a time. The combination of real-time current measurements and optical tracking of *G. sulfurreducens* DL-1 on nanofabricated electrodes revealed steps in the current density that were correlated in time with the passing of a microbe over the electrode (Jiang et al., 2013). The consistency of the magnitude of these stepwise current jumps was evidence that they stem from single microbes. Finally, dividing the current by the cell volume and extrapolating to a macroscale electrode gave rise to a theoretical current density of $\sim 10^6$ A m^{-3}, a value higher by 100–1000× than previously attained at the time. This signifies that there is much room to improve the performance output of microbe-grafter electrodes via improving interfacial charge transfer. The EET efficiency of single microbes was further augmented by encapsulating *S. oneidensis* MR-1 cells with conductive polymer shells which ideally contact the EET machinery on the entirety of the cell exterior rather than just the part directly in contact with the electrode (Yu et al., 2020). Indeed, through correlating microelectrode currents with optical tracking, the polymer coated microbes exhibited currents of 292 ± 55 fA (or $\sim 2.5 \times 10^6$ electrons s^{-1}) per cell, which was more than 4 times greater than unfunctionalized microbes. As such, the results pave a tangible path forward to maximize macroscopic biohybrid performance via maximizing connectivity to individual microbes.

There are several important implications to the works cited above (Table 1). Although discerning between various modes of mediated and direct EET can be accomplished through porous electrodes or temperature-dependent conductivity and provide a good start for determining how to maximize the rates of these processes, there is still a significant gap between macroscopic electrode's current output and that measured for single cells. Although strategies like polymer encapsulation have been shown to help, knowledge of how to simultaneously optimize microbe 'wiring', local chemical environment, mass transport of reactants is not fully established. Further, the open question remains regarding the effects of discrepancies in the environment between those in microscopic single cell measurements (more open environment) and macroscopic electrodes (packed, extracellular matrix present). This is briefly summarized in Table 1.

Quartz-crystal microbalance

QCM measurements run an oscillating voltage through a piezoelectric quartz substrate and the resultant substrate's oscillation frequency is directly proportional to the mass adsorbed overtop of it (Sauerbrey, 1959).

Table 2. Summary of unique information and challenges of QCM measurements applied to biohybrid systems

Information	Challenges, limitations
Biofilm growth vs. time	Qualitative rather than quantitative
Hydration, ion/solvent movement in response to stimuli	Chemical/biological basis behind data
Window into electrochemically silent events	Discrepancies in environment, mass transport between QCM cell, standard reactor

Further, the application of dissipation measurements (essentially measuring the time it takes for the quartz to stop oscillating once the voltage application stops) enables the determination of the film rigidity, which in turn provides information of biofilm composition. As such, this line of measurements is especially important in correlating the growth and physical characteristics of biofilms to the performance (e.g. current density, product formation) of the resultant biohybrid system. Further, QCM is a useful platform in measuring how surface chemistry, charge, and morphology influence initial bacterial attachment and film growth en route to functional biohybrid electrodes. It must be mentioned that the highly viscoelastic (not rigid) nature of biofilms renders the data from QCM measurements qualitative rather than quantitative. For electrochemical QCM measurements, the piezoelectric quartz chip is covered with a thin film of a conductive material to serve as the working electrode, and the solution containing microbes/electrolyte/reactant is flowed overtop.

QCM measurements were initially used to correlate the growth of *Pseudomonas cepacian* cells with the biofilm mass, with non-linearities attributed to the formation of extracellular polymers (Nivens et al., 1993). In the context of electroactive microbes, QCM measurements were used to evaluate *G. sulfurreducens* biofilms with a customized cell that enabled spectroscopic measurements to be performed on the same electrode (Figure 3A) (Heidary et al., 2020). The goal of this work was to understand EET mechanisms in play during biofilm growth and operation in anodic and cathodic modes. A rapid increase of mass was noted as soon as the cells were injected into the media, whereas the current did not increase until 2 days later (Figure 3B). This was interpreted to signify that the delay in current was because of the necessity for the microbes to express cytochrome-based units used in their EET pathway. Further, switching from anodic mode (oxidation of acetate to CO_2) to cathodic mode (reduction of fumarate to succinate) did not significantly change the biofilm mass (Figure 3C), indicating that the decrease in reaction rates mainly stem from decreases in EET and/or metabolic rates of the microbes. Complementary QCM measurements of *G. sulfurreducens* biofilms also noted changes in film frequency during cyclic voltammetry cycles, suggesting a significant amount of solvent coming into/out of the biofilm during this process (Babauta et al., 2014). This signifies that the morphology and resultant abundance of solvent diffusion pathways may be a critical parameter in influencing biohybrid electrode performance.

Overall, these studies established that biofilm growth, EET machinery expression, and the onset of macroscopically measured currents are not always occurring simultaneously (Table 2). Although the lag and exponential phase periods of currents typically are used as proxies for understanding what is happening in a growing biofilm, these studies call to attention the importance of understanding what is occurring prior to any significant current outputs and raise important questions of how processes during this electrochemically silent period will affect performance measured afterward. A powerful line of experiments to this end could be the simultaneous utilization of QCM, electrochemical analysis, and perhaps spectroscopic probing during these early stages to get a better handle of the evolving materials-microorganism interface.

STEADY-STATE SPECTROSCOPIC PROBES

Vibrational spectroscopies are particularly useful in measuring the abundance, oxidation states, and chemical environments of EET-active components, en route to understanding their role in dictating biohybrid electrode performance. These types of measurements are readily adapted to standard electrochemical cells, with Raman and infrared measurements being conducted in reflection mode. Resonance Raman spectroscopy, in particular, is useful as the excitation wavelength can be tuned to match the absorbance of key species (e.g. heme units with well-defined absorption peaks) to enhance the resultant signals and consequently selectively probe these species. Further, even though the knowledge concerning EET pathways for more conventional systems is being rapidly solidified, a particular strength of these measurements is to translate the established methodology from model systems to new reactions and modes of operation

Table 3. Steady-state spectroscopy and its strengths and limits when applied to the interrogation of biohybrid systems

Information	Challenges, limitations
Identity of species involved in EET	Spectral interpretation
Chemical environment tracking	Not all species give rise to strong spectral features
Cellular response to electrochemical stimuli	Selective probing of species besides cytochromes

(e.g. *G. sulfurreducens* operating in cathodic mode). Further, inspiration is taken from nature and in particular, from studies of electrochemical interactions of these microbes with natural minerals or solubilized Fe species (Choi and Sang, 2016; Kato et al., 2013; Nakamura et al., 2013; Rosenbaum et al., 2011; Yao and Huang, 2016) to understand novel reaction pathways and EET modes in biohybrid systems.

The cytochromes and EET pathways of *G. sulfurreducens* adsorbed onto nanostructured silver electrodes were probed with surface enhanced resonance Raman spectroscopy (Millo et al., 2011). The change in the spectra attributed to cytochromes as a function of potential implicated these units in the microbes' EET process and the degree of spectral changes indicate that 90% of these units were electrochemically wired to the electrode. Raman spectroelectrochemistry was used to probe mixed culture *Marinobacter-Chromatiaceae-Labrenzia* (MCL) cathodes used to convert CO_2 to biomass (Yates et al., 2016). C-type cytochromes were implied to serve as the principal electron conduits in these biohybrid electrodes on the basis of the measured signatures of heme units in the Raman spectra. However, Fe–S clusters were also potentially detected, and their role in EET is still not fully determined (Yates et al., 2016). Raman spectroscopy, in combination with AFM measurements, was also used to probe *G. sulfurreducens* biofilms and in particular, the relation of cytochrome abundance with growth phases (Lebedev et al., 2014). AFM was employed to monitor biofilm formation while the Raman quantified cytochrome content. The experiments showed that cytochrome units are three times more abundant (on a per cell basis) in thicker biofilms, in which the cells also tend to form nanowire-like structures. This implied that these cells form cytochrome-containing nanowires responsible for their EET as a necessity to maintain electrical contact with the electrode in growing biofilms but that these structures are not as important for cells in the first layer which are in direct contact to the electrode.

In investigating less understood reaction pathways, resonance Raman spectroscopy probed the role of cytochromes of *G. sulfurreducens* biofilms operating in both anodic (oxidation of acetate to CO_2) and cathodic (reduction of fumarate to succinate) modes (Heidary et al., 2020). Electrochemically accessible cytochrome signatures were readily visible in the spectra and could be assigned to reduced and oxidized species as a function of potential (Figure 4A). Although cytochromes were abundant in anodic mode (Figures 4B and 4C), they were less present in cathodic mode, though still electrochemically accessible and their redox potential matched the midpoint of the CV curves under turnover conditions (Figures 4D and 4E). The decrease in cytochrome content was verified with UV-Vis absorbance, which showed significantly weaker absorbance bands of these species. Coupled to this decrease in cytochrome quantity in cathodic mode, Fe-containing nanoparticles were found to form on the cell surfaces (Figure 4F), thought to be a result of the precipitation of excess iron leached out form the cytochromes no longer in use. The data suggested that Fe species, solid or solubilized, may be in play in the cathodic mode EET pathways (Figure 4G).

Infrared spectroscopy is not necessarily tuned to any chromophore and can probe a range of redox active units, solubilized species, and protein structural changes rendering it a versatile tool in the context of biohybrid investigations. *Geobacter soli* biofilms, which performed both anodic (acetate oxidation) and cathodic (nitrate reduction) were investigated with infrared spectroelectrochemistry to gauge their EET pathways in each mode (Yang et al., 2017). Spectral signatures associated with cytochromes suggested that these units were active, whereas large changes in amide modes from proteins indicated that the electrochemical bias leads to significant conformational of membrane-bound proteins. Further, in comparing the microbe loaded anodes and cathodes, the different ratios of amide band changes (1600–1700 cm^{-1}) to the rest of the spectral features hinted at distinct, still to be determined, modes of EET between the two electrodes.

Emission based techniques with judiciously selected chemical probes are instrumental in probing for the existence or lack of specific species in the chemical environment of electroactive microbes. A recent

Table 4. Unique insights conferred from time-resolved measurements and the associated challenges that come with such experiments

Information	Challenges, limitations
Kinetics of photoinduced processes	Spectral interpretation
Identity of electron/hole acceptor	Translucent sample required, more difficult for photoelectrodes
Efficiency of natural/synthetic charge conduits	Charge flux from laser is much higher in typical experiment than photo(electro)chemical experiments

example entails the investigation of N_2-fixing *X. autotrophicus* and *Bradyrhizobium japonicum* microbe loaded nanowire electrodes (Lu et al., 2020). Although these species are O_2 sensitive, the use of the nanowire geometry led to O_2 gradients that permitted their growth in the O_2 deficient base of the electrodes. A Ru-based phosphorescent probe that selectively reacted with O_2 was employed and spatial mapping of its phosphorescence was the key in illustrating that under the experimental N_2-fixing conditions, O_2 was indeed excluded throughout the electrode. This work showed how simple geometric electrode design can dictate the immediate chemical environment of electroactive microbes. Spatially and temporally resolved measurements could be combined to extract the rates of various processes at a single cell level. To this end, *S. oneidensis* MR-1 cells were probed using a fluorescent membrane potential indicator via single cell fluorescence microscopy (Pirbadian et al., 2020). The fluorescence observed illustrated that the membranes of the cells in contact or close to the electrode experience hyperpolarization upon the application of a positive voltage. Further, tracking the response over time revealed that the cells reach their maximum levels of membrane hyperpolarization within 10–20 min. Overall, the sum of the results was consistent with a model involving solubilized flavins playing a key role in mediated EET.

In sum, spectroscopic techniques can probe a variety of important aspects, ranging from cytochromes participating in EET to local chemical environments (e.g. O_2 levels) (Table 3). A challenge here is in interpretation of new spectral features and unambiguously linking them to a species participating in EET (e.g. Fe–S clusters) or other relevant processes in the energy converting cycle. As such, new observations press for complementary experiments like the use of knockout mutants to strengthen hypotheses. Further, the use of new fluorescent probes that indicate the occurrence of various biological processes like membrane-bound enzyme activity would help immensely to understand what is occurring through electroactive biofilms as a function of distance from electrode, applied voltage, time, and more with a key end goal being to identify and eliminate key bottlenecks limiting the current and product throughput of biohybrid electrodes.

Time-resolved techniques

Techniques measuring changes over time are instrumental in understanding the entirety of the solar/electricity to fuels cycle within biohybrid systems. Among these, pump-probe spectroscopy, conventionally used to study solubilized chromophores and quantum dots, is uniquely situated to probe biohybrid systems exhibiting membrane-bound photosensitizers. In this line of measurements, a short visible pulse excites the light absorber and a secondary probe (visible or IR) measures the changes in the absorbance directly after the excitation, typically in the ps-ms range. The rates of change in the photocatalyst features provide information on the kinetics of charge transfer or recombination. In parallel, measuring the infrared region can provide information on the identity of the charge acceptor. The requirement for this type of measurement is simply having a semi-transparent solution which can be probed, readily met with most photochemical biohybrid studies, though is difficult to perform in electrode-based systems. The inspiration for much of this work stemmed from studies of enzyme-quantum dot hybrids, which represented a model system from which to learn and expand upon (Greene et al., 2012; Wilker et al., 2014).

The methodology from these earlier studies was thus translated to a system featuring *Moorela thermoacetica* with precipitated CdS particles on its surface that converted CO_2 to acetate when illuminated (Figure 5A) (Kornienko et al., 2016; Sakimoto et al., 2016). The principal question was how do photoexcited electrons on the CdS transfer over to the microbe? Biohybrids incubated with H_2 showed higher sustained photogenerated acetate yields and this was coupled with an increased expression of membrane-bound hydrogenase enzymes. Pump-probe measurements illustrated that photogenerated

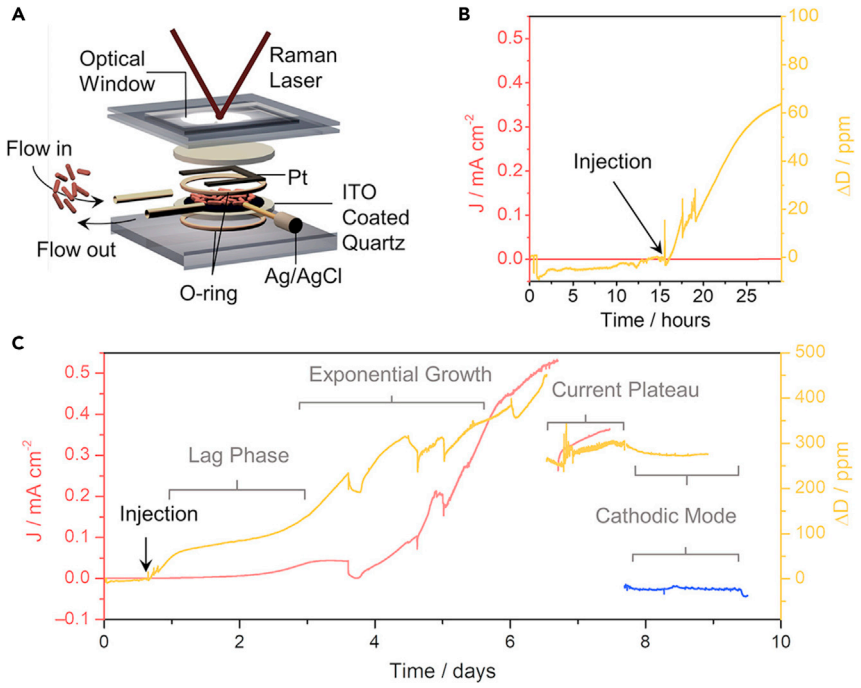

Figure 3. Quartz-crystal microbalance probes of biofilms

(A) Customized QCM cells enable correlating mass and viscosity changes with spectroscopic measurements (A) (Heidary et al., 2020).

(B and C) *G. sulfurreducens* biofilms increased in mass far before current increased (B) while switching to cathodic mode did not significantly alter the biofilm mass (C).

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electrons transfer out of the CdS faster when integrated with H_2-incubted cells, relative to glucose incubated samples, to an acceptor believed to be associated with this increased hydrogenase expression. Similarly, transient infrared spectra illustrated unique spectral photoinduced features in the H_2-incubated biohybrids. The sum of the data cumulated in a model involving hydrogenase enzymes as a key conduit that accept (directly or indirectly) photogenerated charges from CdS and generate H_2 as an entry point into their acetate producing metabolic pathway. In a similar line of research, *S. oneidensis* MR-1 microbes with periplasmic hydrogenase enzymes were integrated with $CuInS_2$/ZnS quantum dots through a simple co-incubation approach (Luo et al., 2021). This biohybrid system readily produced hydrogen when illuminated with visible light. Transient fluorescence and transient absorption lifetimes were both shorter in the hybrid system than for the quantum dots alone, indicating that the enzymes served as efficient acceptors of photogenerated charge. This was further verified through studies of mutants lacking the genes to express these enzymes, which featured substantially lowered hydrogen production.

Both steady-state and time-resolved photoluminescence were key in demonstrating the efficacy of reduced graphene oxide (RGO) as serving as an electron conduit between *S. oneidensis* MR-1 SW and Cu_2O nanosheets (Shen et al., 2020). Upon illumination, RGO carried photogenerated electrons from Cu_2O to the microbe, which then carried out hydrogen evolution via its hydrogenase enzymes. The composite system featured a lower intensity of steady-state photoluminescence than isolated components, indicating less radiative recombination and therefore that photogenerated electrons efficiently transferred from the Cu_2O to the cells. Complementary time-resolved photoluminescence revealed a longer luminescence lifetime which in this work was interpreted to indicate more efficient charge separation induced by the RGO.

In all, time-resolved spectroscopic techniques are beginning to shed light regarding the kinetics and charge pathways on relatively unknown systems (Table 4). While probing the semiconductor component is more straightforward and can be accomplished through photoluminescence and transient absorption techniques, more difficult questions remain in determining the identities of various charge accepting units.

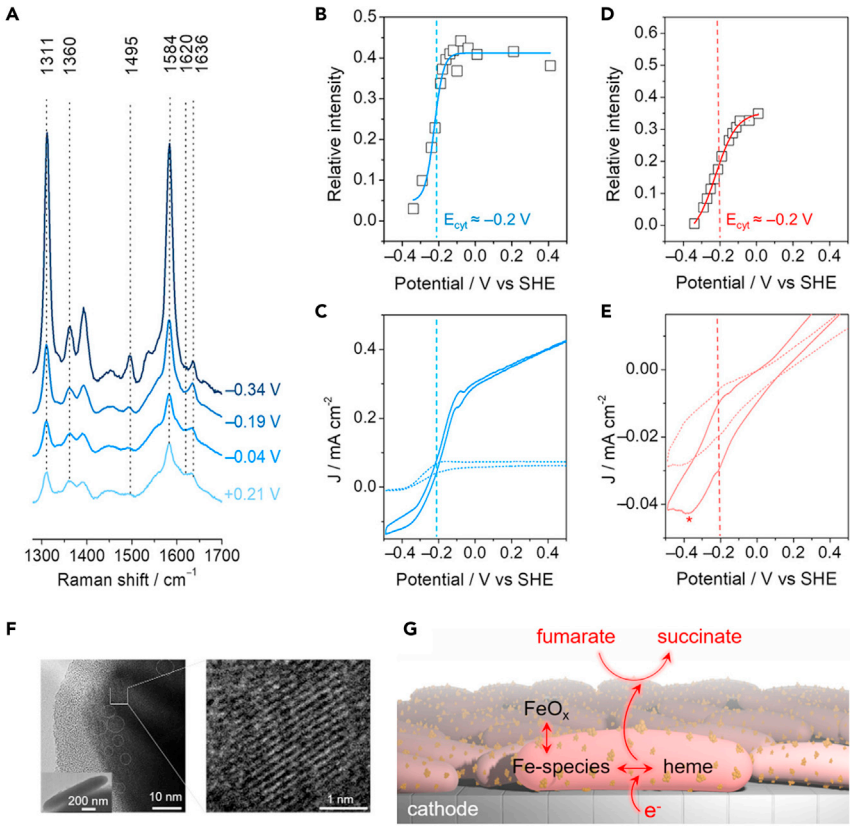

Figure 4. Steady state Raman investigations of biofilms

(A) Representative Raman spectra of *G. sulfurreducens* anode biofilms as a function of potential show signatures of reduced and oxidized heme units (A) (Heidary et al., 2020).

(B–E) The redox transition of the cytochromes matched the midpoints of biofilm CVs for both anode (B and C) and cathode (D and E) electrodes.

(F and G) The discovery of FeOx nanoparticles on the cell surfaces (F) after cathodic operation suggested their possible involvement in cathode mode EET (G).

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While vibrational spectroscopy has helped in this regard, unambiguous identification can be attained through a combination of isotope labeling, systematic screening (e.g. of semiconductor band gaps, surface chemistries …) and carefully performed studies of knockout mutants. Further, understanding of how the judicious placement of semiconductor nanoparticles on/in various parts of the selected microbes (Wei et al., 2018) or their photophysical character (Ding et al., 2019; Xu et al., 2021) affects charge transfer kinetics and pathways will open up avenues in rational design of next generation systems.

OUTLOOK

Throughout the discussion of these emergent techniques, a common theme emerges: each measurement often provides only a piece of the puzzle and the strongest claims made are usually those that are followed up by complementary measurements. That being said, we stress the importance of multimodal analysis of biohybrid systems. For example, if a particular protein was spectroscopically identified as potentially active in the energy conversion pathway, what happens if it is deleted from the cell's genome or selectively upregulated? Can these results be verified at a single cell level with microelectrode measurements? If integrated with semiconductor nanoparticles, how are resultant photoluminescence or transient absorption lifetimes affected?

Regarding spectroscopic measurements, identifying established proteins (e.g.) and their strong spectroscopic signatures is straightforward by now. A key challenge is the translation of these measurements to probe more ambiguous components and interpretation of the resultant spectra, all of which can be

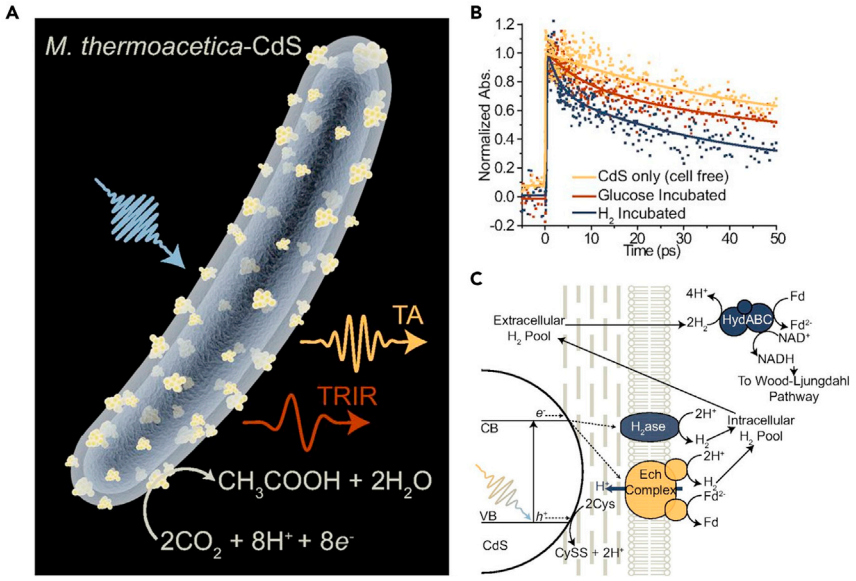

Figure 5. Pump-probe measurements of biohybrid systems

(A) *M. thermoacetica* – CdS biohybrids were probed with transient infrared and UV-Vis absorption spectroscopies (A) (Kornienko et al., 2016).

(B) Charge transfer was faster in H_2-incubated microbes featuring an increased quantity of membrane-bound hydrogenase enzymes.

(C) The results led to a model that features hydrogenase as a key unit in the sustained photochemical charge transfer pathway from CdS to *M. Thermoacetica*.

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aided through a combination of isotope measurements, knockout mutants, and theoretical modeling of various components contributing to the spectra in the complex ensemble. Further, while EET pathways have been identified for common microbes in established reactions such as acetate oxidation to CO_2, the same pathways may not necessarily be involved to the same extent when the reactions are changed. For example, cytochromes were deemed to be less abundant in *G. sulfurreducens* cathodic mode operation while Fe species may have been active (Heidary et al., 2020). It would be worthwhile to extend such studies to new frontiers such as microbe-induced polymerization reactions (Fan et al., 2020). In addition, combining spectroscopy with QCM during the early stages of biofilm formation will elucidate key points not captured with conventional electrochemical techniques. For QCM itself, developing models of frequency and dissipation signals would be helpful in converting the resultant data from qualitative to quantitative and integrating with spectroscopy will help bring a biological and chemical basis to the data (Table 1).

In a complementary direction, understanding the effects of the inorganic component on the function of the microbe is key toward approaching the rational design of materials-microbe interfaces. To this end, biohybrids consisting of *Clostridium autoethanogenum* with CdS particles on their surfaces that converted CO_2 to acetate under illumination were investigated (Jin et al., 2021). Transcriptional analysis illustrated activation of genes in Wood–Ljundahl pathway, as well as metal ion and flavin-binding proteins, indicating that likely involvement of flavins in a mediated EET route between CdS and the microbe. This hypothesis was further strengthened as adding external flavin mediators further augmented the light-driven CO_2 conversion rates. Similar work was performed on *M. thermoacetica*/CdS (Zhang et al., 2020). It would be interesting to extend such studies to systems where, for example, microbes are encapsulated in conductive polymers efficiently 'wiring' them to electrodes (McCuskey et al., 2020; Yu et al., 2020) or when the reaction solution is rationally optimized to maximize microbe packing and consequently current output (Su et al., 2020) and to cross-reference these results with the changes in EET machinery identified through the aforementioned spectroscopic techniques. Again, microelectrode measurements would assist greatly in disentangling between intrinsic improvements at a single cell level from engineering-level augmentation of factors like mass transport.

Finally, while attention is being increasingly put on studying biohybrid long-term viability and in turn minimizing effects of reactive oxygen species, quantitatively tracking these species with spatiotemporally resolved spectroscopic techniques and the better understanding the effects of protective elements (e.g. MOF shells or Au nanoclusters) (Ji et al., 2018; Zhang et al., 2018) would help translating this technology over to economic viability. In all, the rapid expansion of fundamental knowledge in electro/photoactive biohybrid systems, coupled to the growing maturity of their performance is a promising sign of the future growth of this field. While key challenges remain, a framework is laid out toward extracting the mechanistic basis from which to build upon in realizing the ambitious goals of the field.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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