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Review

Redox Electrochemistry to Interrogate and Control Biomolecular Communication

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SUMMARY

Cells often communicate by the secretion, transport, and perception of molecules. Information conveyed by molecules is encoded, transmitted, and decoded by cells within the context of the prevailing microenvironments. Conversely, in electronics, transmission reliability and message validation are predictable, robust, and less context dependent. In turn, many transformative advances have resulted by the formal consideration of information transfer. One way to explore this potential for biological systems is to create bio-device interfaces that facilitate bidirectional information transfer between biology and electronics. Redox reactions enable this linkage because reduction and oxidation mediate communication within biology and can be coupled with electronics. By manipulating redox reactions, one is able to combine the programmable features of electronics with the ability to interrogate and modulate biological function. In this review, we examine methods to electrochemically interrogate the various components of molecular communication using redox chemistry and to electronically control cell communication using redox electrogenetics.

INTRODUCTION

Communication has been defined as "reproducing at one point either exactly or approximately a message selected at another point" (Shannon, 1948). This definition was formalized during the telecommunications revolution and has since provided a paradigm to organize all communication processes, including parsing into semantic and physical components. This definition is easily applied to electronic communication: messages are semantically encoded and decoded according to simple decision trees, while communication components, such as transmitters, channel elements, and receivers handle the physical electronic relay (Figure 1A). As a result, the transmission of the encoded message as electrical current or electromagnetic radiation is easily received and decoded, so long as the recipient has access to the original decision tree. These processes can also be framed within the context of cell-cell communication wherein one cell transmits messages or information relevant to other members of the network through cues that both the sender and receiver contextually share.

We and others (Akyildiz et al., 2015; Kuscu et al., 2019; Rhee et al., 2012) suggest that communication theory can be used as an organizing principle to discuss the general roles for the molecular components that comprise a biological communication network. That is, in biomolecular communication, which is a subset of cell-cell communication, information is contained within a molecule's structure and conveyed by its transport. In this sense, a molecule's chemical and structural properties, as well as its mode of transport, represent means for encoding biological information. In Figure 1B, a message, such as population density in bacterial quorum sensing (QS), is encoded into the chemical and structural properties of autoinducer molecules, which are transported through an aqueous medium to other molecular receivers that are parts of nearby cells. Ultimately, the message's information is decoded by the processes of molecular recognition, such as binding to a transcriptional regulator. These formalisms enable a discussion of biomolecular communication at an abstract level.

One of the challenges in studying biological communication is that the encoding and decoding processes are the consequences of molecular and cellular computation that, in turn, is based on the simultaneous integration of multiple cues as well as a contextual history. Each molecular component can therefore participate in a multitude of encoding, transmission, reception, and decoding roles within a single system. This issue becomes even more difficult when either the components of the communication channel, or their

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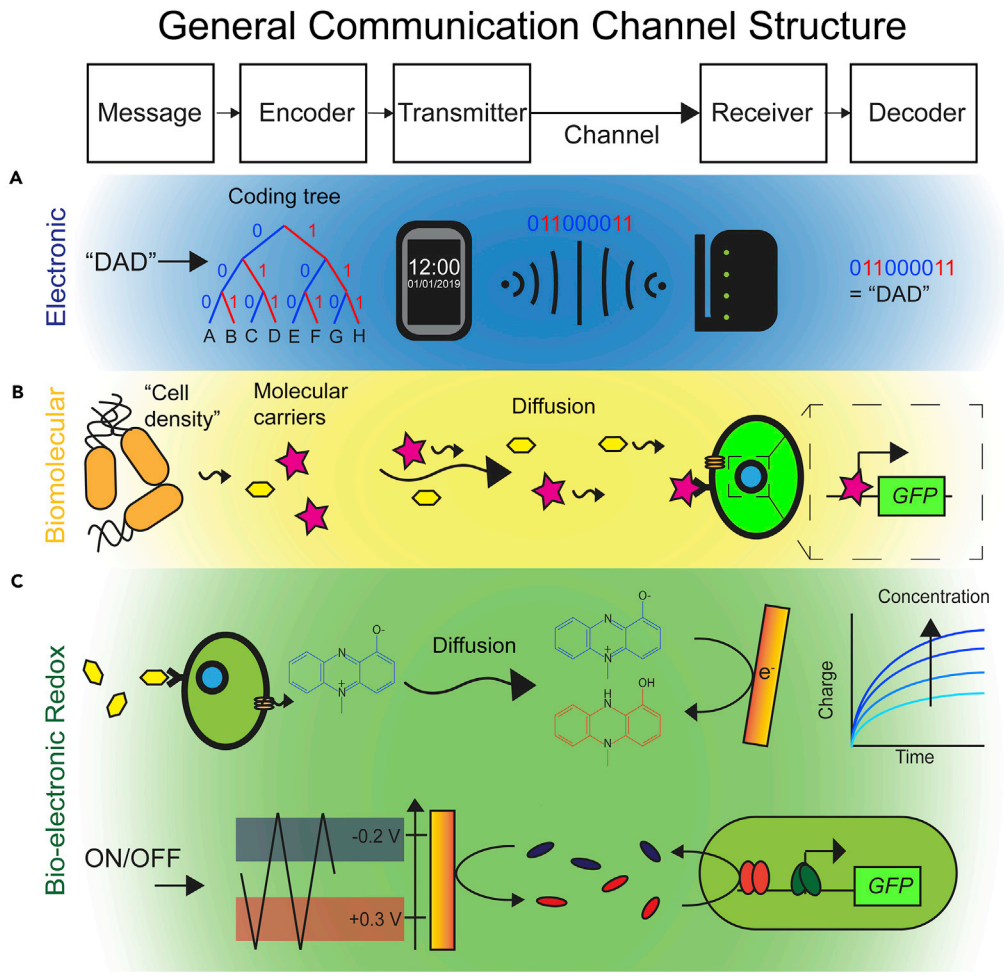

Figure 1. Communication Modalities

(A) Electronic messages are encoded into electromagnetic patterns using a formalized coding scheme and then transmitted through a communication channel (e.g., wires, air), where electron movements are detected by the conducting antenna and the messages are decoded using the same encoding scheme.

(B) Within biomolecular networks, cells encode messages into molecules, which are transported (e.g., diffusion, convection) and are received by communicating partners according to the characteristics of the message carrier. The message is contextually decoded, perhaps resulting in biological action such as the actuation of gene expression (GFP, depicted).

(C) Redox communication, already a prevalent mode of communication in biology, uses reduction and oxidation to transfer information between electronics and cells through molecular interactions. Here redox combines the key elements of both electronic and biomolecular communication systems. Within redox networks, molecular information can be received using electrodes, where it can be electronically decoded to recapitulate the molecular message. Similarly, electronic information can be encoded into molecular communication by reducing or oxidizing redox molecules that can be transmitted to cells to encode, transmit, and decode according to a pre-set biological paradigm.

interactions, are not properly represented. For example, bacterial QS (Delisa et al., 2001a, 2001b; Fuqua et al., 1994), although seemingly a simple consequence of a transmitted and perceived signal molecule, is complex, even requiring co-evolution of signal/receptor pairs for intraspecies communication (e.g., autoinducer-1 processes) (Whiteley et al., 2017) or multifaceted contextualization for interspecies communication (e.g., autoinducer-2 processes) (Pereira et al., 2013; Quan and Bentley, 2012). That is, whereas QS may seemingly communicate population density in one particular niche so that cells might reprogram their activity accordingly, it may communicate the local fluidic environment (Defoirdt, 2011; Kim et al., 2016; Luo et al., 2015, 2012; Mukherjee and Bassler, 2019; Servinsky et al., 2016) or metabolic conditions (Dandekar et al., 2012; Delisa et al., 2001a, 2001b) in other contexts. Cell fates might be completely different based on the same inputs, but set in different circumstances. Contextualization is similarly complex in the vast

interconnected communication networks of the mammalian immune, cardiovascular, and nervous systems (Webster et al., 2002; Weinberg et al., 2015; Xu et al., 2019). Thus, in natural settings, discerning the true message that is encoded by one or multiple signaling molecules is nearly impossible when viewed from an external window. For this reason, it is important to develop methodologies that insert the user into the context of the molecular communication network, so that the underlying encoding and decoding paradigms, as well as the communication channel, can be discerned.

We suggest that electronics can actually offer a unique vantage point for assessing and even controlling molecular communication between multiple cells because its devices are modular, it can be networked into large systems, and it uses established encoding and decoding processes. These attributes allow for simultaneous monitoring and probing of multiple components within a system to elucidate their functional roles. Perhaps even more importantly, electronics-like communication already exists between molecular components in biology through oxidation and reduction reactions, and this can be used to interrogate and control biological communication. That is, although freely moving electrons do not exist in biology, redox processes do transport electrons between molecular mediators (as well as with electronic interfaces). Redox reactions combine the unique characteristics of both networks (Kim et al., 2019) thus enabling electron-based communication that is transduced between the biomolecular and electronic domains through "redox channels" (see Figure 1C). Within biomolecular communication, redox molecules have unique structures and reactive moieties that determine their interactions with specific cellular receivers. These molecules can also be coupled to many non-redox communication forms through enzymes or other protein components. Similarly, changes in their oxidative states facilitate the movement of electrons within the molecular structures. Therefore, the structural changes of these molecules can be electronically interpreted or actuated, directly or indirectly, through standard electrochemical techniques. The combination of these elements allows for redox signals to directly or indirectly interact with all components of a molecular communication system, allowing the user to determine how a component creates or distributes a message.

In this review, we use communication theory as the organizing principle to discuss the general roles that molecular components play within a communication system, and further, we examine efforts to create redox channels to discern and control the molecular components involved in communication among cells. Our discussion focuses specifically on methodologies that allow for the electronic interactions, without diving into theoretical aspects of quantifying information transfer, as has been reviewed elsewhere (Mian and Rose, 2011; Rhee et al., 2012). Rather, we focus on practicable methodologies that enable electronics to bio and bio to electronics connectivity. We frequently use bacterial QS as a model communications network as it is a cogent exemplar of cell-cell communication. It is also experimentally tractable, societally important, and can be manipulated via tools of synthetic biology. We have separated this review into three sections—(1) efforts to access molecular components using electrochemistry, (2) efforts to control cell-cell communication using redox-based electrogenetics, and (3) in a final section, perspectives for closed-loop electronic feedback control of biological systems and areas for future research.

Accessing Cellular Communication

Electrode-Mediator Interactions

To electrochemically access molecular communication, several broad schemes have been developed. The goal is to determine the concentration, activity, or simply the presence of a signal molecule, thereby indicating what cues and components are used to encode and carry messages, and when they are contextually deployed. Redox signaling molecules, like hydrogen peroxide (Sies, 2017) or nitric oxide (García-Ortiz and Serrador, 2018), can be directly measured by an electrode using an appropriate combination of electrode materials and an applied potential. For example, hydrogen peroxide can be measured by applying an oxidizing or reducing potential and measuring the change in current when it is reduced to water or oxidized to oxygen. Because hydrogen peroxide and reactive oxygen species are elicited upon infection or wound development, an H_2O_2 measurement can indicate an elicited immune response (Liu and Zweier, 2001). That said many such molecules tend to be reactive in biological environments. As a result, these molecules can be short lived. To enable their electrochemical measurement, the electrodes should be placed locally (Gulaboski et al., 2019). Moreover, reactive redox molecules can potentially alter the message conveyed by reacting with and altering nearby macromolecules, for example, by breaking disulfide bonds. Conversely, stable redox molecules, like NADH (Ying, 2006) and ascorbate (Pignocchi and Foyer, 2003); reversible, redox cycling molecules like some phenolics (Rahman et al., 2006), phenazines (Okegbe et al., 2017), catecholamines (Ribeiro et al., 2016), and some of their precursors (Rivas and Solis, 1991); or redox-centered

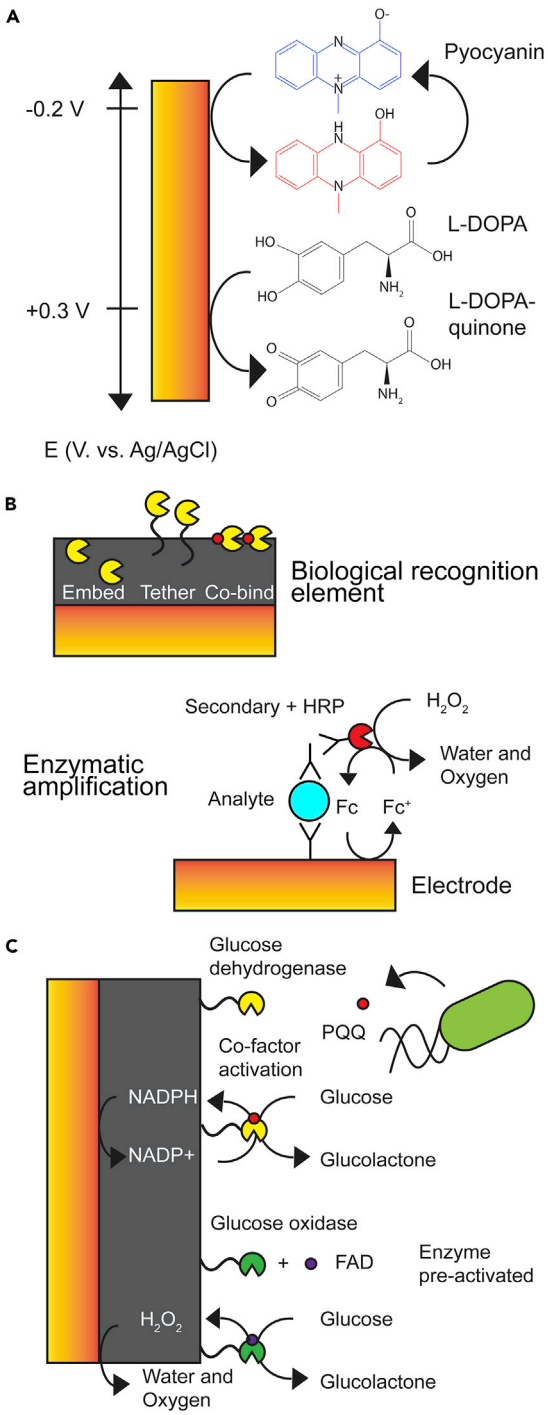

Figure 2. Electrochemical Methods to Detect Biomolecular Message Carriers

(A) Redox molecules can directly donate or accept electrons at defined Nernst potentials, producing a corresponding redox current. Some reactions are reversible, as is shown with pyocyanin, a secreted redox toxin of *Pseudomonas aeruginosa*. By comparison, L-3,4-dihydroxylphenylalanine (L-DOPA) typically undergoes a single oxidation reaction but can be oxidized further to eumelanin.

(B) Enzymes can be used to enhance recognition or to amplify a redox reaction. When used as a recognition element, the enzyme is often immobilized by being embedded in the electrode, tethered through a polymer, or co-bound with a tightly bound co-factor. Enzymes retained near the active surface provide for quick and efficient detection. Enzymes used for amplification are commonly linked to recognition units, like secondary antibodies, and consume a generic substrate to cycle a redox shuttle like ferrocene (Fc) with the electrode surface. These reactions are robust, allowing for multiple factors, like time, substrate concentration, and mediator concentration to be altered to enhance detection.

(C) Enzymes are used for direct or indirect recognition. For example, glucose dehydrogenase directly measures pyrroloquinoline quinone (PQQ), a cofactor secreted by bacteria that influences human metabolism, during conversion of glucose to glucolactone (indirectly indicated). Conversely, flavin adenine dinucleotide (FAD), a common cofactor involved in a wide array of metabolic pathways, can be detected indirectly by pre-treating glucose oxidase electrodes with biological samples and monitoring the burst of hydrogen peroxide generated in the presence of a set concentration of glucose.

proteins like azurin (Jeuken and Armstrong, 2001) can all be measured directly from samples without significant sample preparation (see Figure 2A). Many of these molecules are produced in response to particular cellular or environmental stimuli, allowing cells to coordinate survival responses (Laman Trip and Youk, 2020). The current produced from stable redox molecules is concentration dependent and can be enhanced by optimizing the electrode surface interactions. For instance, graphene-modified electrodes allow for favorable π-π stacking to enhance measurement of phenazines (Kim et al., 2013a). Mixtures of redox communication molecules can also be deciphered by dynamically altering the input potential, as

is commonly done in many voltammetry techniques (Dryhurst, 1982; Sandford et al., 2019), or through creative electrode design (Zhang and Lieber, 2016). Although these provide a modest level of selectivity for intercepting secreted molecular transmissions, the addition of enzymes and other recognition elements can enhance biosensor specificity toward single analytes.

To do this, enzymes both catalytically recognize substrates and exchange electrons with redox mediators, two functions that are often coupled to create a robust signal reception event. Such recognition elements can be thought of as communication filters, because they select specific elements within a communication channel (i.e., substrates) that will be able to influence further changes within the transmission/reception chain. It is also noteworthy that enzymes can refine or distort the message by catalyzing the conversion of substrates in a communication chain to a new message carrier(s) (i.e., the catalytic products), thus representing a basic form of information processing. Many redox enzymes catalyze reactions with the help of electron donors or acceptors like NAD+/NADH or oxygen (Monti et al., 2011). All the products involved in catalysis act as a relay between the enzymatic filter and future recipients of the original molecular message. An excellent example is the commercial glucose biosensor, which indirectly monitors insulin dysbiosis by measuring blood sugar levels (an indirect message carrier) with the enzymes glucose oxidase or glucose dehydrogenase (Ferri et al., 2011). The redox signals that are produced by these enzymes are hydrogen peroxide or superoxide, in the case of glucose oxidase, and NADPH in the case of glucose dehydrogenase. As hydrogen peroxide and superoxide are reactive and short lived their role as message relay vectors to an electrode receiver must fit within a spatiotemporally tight window. For this reason, electrode functionalization by covalent attachment, polymer entrapment, or cofactor binding of the enzyme to the electrode surface (see Figure 2B) can localize the redox reaction and improve signal detection (Gregg et al., 1991; Heller, 1992). Enzymes used in this way are biological recognition elements because they only act on specific cues. That said many assays use enzymes as both recognition elements and signal amplifiers (Figure 2B). Here, the original molecular signal is amplified by activating or localizing the catalytic activity of an enzyme, which then generates a multitude of new message carriers by converting a non-signaling substrate into a relevant product. Enzyme amplifiers typically undergo direct oxidation/reduction within their catalytic sites and need additional redox mediators to restore their catalytic activity—the recycling of catalytic activity is subsequently the source of amplified detection. Such assays benefit from enzymatic molecular specificity as well as highly efficient relay. Here, stable relay molecules, such as ferrocene (Fc), shuttle the molecular information to the electrode. For example (Figure 2B), horseradish peroxidase is commonly linked to antibodies for use in electronic enzyme-linked immunosorbent assays (ELISA), which is a group of highly sensitive and modular assays that present unique advantages when compared with their optical analogs (Ricci et al., 2012). Last, some enzyme co-factors, like flavin adenine dinucleotide (FAD), are redox active and enable direct or mediated electron transfer to electrode surfaces. These co-factors are comparable to ferrocene within electrochemical ELISAs, except they are biologically active and often modulate the activity of recipient enzymes. Among these co-factors, pyrroloquinoline quinone (PQQ) is used to recycle the redox state of quinoprotein enzymes like glucose dehydrogenase (Laurinavicius et al., 2002). Interestingly, flavins and PQQ are secreted molecules that can serve to shuttle electrons (Marsili et al., 2008) or activate enzymes in other species (Kasahara and Kato, 2003). Each activity can be thought of as relaying a message to further intra- and extracellular recipients. These co-factors can be used for the detection of cellular secretions of both substrates or co-factors by either analyzing the overall redox current generated by enzymatic catalysis after a period of time or measuring the instantaneous rate changes in current generated at fixed substrate concentrations (see Figure 2C).

Cell-cell communication can be more complex, as noted earlier, in that it can be difficult to assign the consequences of molecular communication to a single component's concentration or activity. Redox signaling provides both an example and a solution for this problem. For instance, when hydrogen peroxide is generated at a wound site, its signaling function recruits immune cells, but it also oxidizes nearby biomolecules, such as cysteine or quinone residues on proteins, and these can result in systemic changes throughout the immune system (Antunes and Brito, 2017). In these cases, assigning information content to a single redox signal can be difficult, when perhaps the relevant message is instead contained within the collective oxidation-state of a biological system. One way to get more information out of a system is to probe in parallel with a variety of indicators, in this case with different redox mediators, which are small molecules that reversibly exchange electrons between biological molecules and the electrode surface. Here, the mediators have distinct properties that select for particular molecular interactions that can span multiple analytes, thus conveying a global "redox context" of a biological network. These mediators can either be covalently

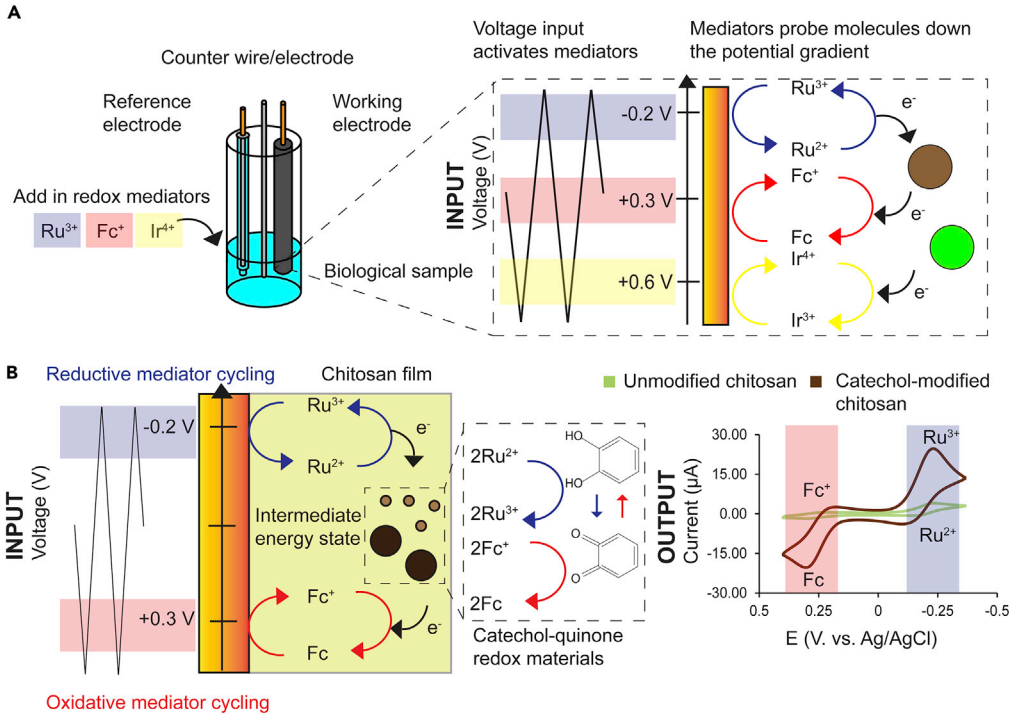

Figure 3. The Use of Redox Mediators to Probe Complex Biological Systems

(A) Redox probing involves the addition of a select number of stable, reversible redox mediators to a biological sample to act as an intermediary between the sample and electrode. During the probing process, an input wave activates the mediators at their respective Nernst potentials through redox reactions. The mediators in turn transfer electrons with the sample down a potential gradient. As a result, an output wave is generated that depicts the general movement of electrons through the sample.

(B) Redox probing is useful for characterizing a material's properties, like synthetic catechol-chitosan (shown) or natural melanins (Kim et al., 2020), ingested antioxidants (Lee et al., 2014), and humics in soils (Kim et al., 2014b, 2011). Catechol-quinone molecules within non-conducting chitosan can store and discharge electrons based on interactions with mediators. As is depicted, by picking mediators that straddle the energy state of the material, a redox cycling reaction is created that simultaneously amplifies the reduction current of the low oxidative potential mediator and the oxidation current of the high oxidative potential mediator by directing the electron flow. These techniques can be used to identify the energetic landscape of redox couples on materials, as well as their ability to act as redox buffers in biological communication systems.

attached to a surface (Du et al., 2014) to continuously turnover the biological-electrode interface or they can be purposely added to samples to probe biological signatures in a manner analogous to sonar (Kang et al., 2018a; Li et al., 2017) (see Figure 3A). In the latter, the mediators are oxidized/reduced at an electrode surface and diffuse into a sample where they interact with various redox-active moieties by donating or receiving electrons. The mediators then diffuse back to the electrode becoming re-oxidized or reduced when the electrode cycles through the characteristic voltage associated with each state, thus producing a measurable change in current. As a result of these interactions, redox peak currents associated with each mediator's redox state are amplified, attenuated, or unperturbed based upon the type of redox interaction with the sample. This can be viewed as the "redox context." For example, we found that when the oxidative stress, and thus the oxidative state, was enhanced within a serum sample, electrons accumulated within the high oxidative potential mediator iridium, thus increasing the measured oxidative peak current produced from electrochemical probing of the mediator (Kim et al., 2017). This experiment demonstrates the general principle that electrons flow from low oxidative potential molecules, to higher oxidative potential redox molecules. Coupled interactions, such as the unbalanced enhancement of only the reduction peak of a mediator, without enhancement of the oxidative peak, or vice versa, indicates electron accumulation or depletion within a biological sample. Similarly, when using multiple mediators, unbalanced enhancements down a potential gradient, such as the enhancement in a reductive peak of a low oxidative potential mediator coupled with another unbalanced oxidative enhancement of a high oxidative potential

mediator, can indicate redox cycling with an intermediate redox couple energetically positioned between the two mediators (Kim et al., 2013b). Signal processing analysis of these interactions can illustrate a generalized encoding structure of communication, and when used alongside complex voltage inputs (Adamson et al., 2017), additional molecular components like enzymes (Léger and Bertrand, 2008), and other analytical methodologies can help untangle how localized messages reverberate throughout a biological channel. When combined within spatially organized arrays, these techniques can also be used to study the spatial dynamics of larger systems of cells, such as bacterial biofilms (Bellin et al., 2014), mammalian spheroids (Abe et al., 2020), or other systems.

Furthermore, we and others have shown how redox-mediated electrochemical probing is used to analyze and couple biological communication within materials, largely by interacting with redox-active components (reviewed in Kim et al., 2014a). Such materials represent reservoirs or depots of redox interactions, thus allowing for integrated interpretations of changes to a redox network over a confined period of time. Interestingly, redox-inactive materials can be conjugated with redox mediators such as catechols and quinones, and these are easily fabricated at discrete locations (Liu et al., 2010). We have suggested that these materials can serve as a form of bioelectronic memory to locally address where specific communication events happen within a spatial domain, such as within the spatial resolution of an electrode array or gel (Wu et al., 2020). That is, in these storage devices, a non-conducting material, such as catechol-modified chitosan, serves as a redox sink to sequester electrons away from the electrode. Then, soluble redox mediators can charge and discharge the catecholic redox sink through reversible interactions with both the catechol and quinone moieties and an electrode in response to voltage inputs or perhaps other biological redox interactions. Here, two mediators, such as ruthenium, a low oxidative potential mediator, and ferrocene, a higher oxidative potential mediator, straddle the oxidative potential of a quinone in the gel. As a result, the electrons from ruthenium are donated to quinone groups, forming catechols, and the electrons are removed by ferrocene by re-conversion of catechols to quinone groups. As exogenously provided mediators will only cycle at specific voltage inputs, and therefore will only remove electrons or donate electrons to the gel based on the electrochemical settings, the material integrates biological communication into the reduced/oxidized distribution of its catechol/quinone moieties over a course of time. As a result, this system allows interpretation of how a communication channel is altered as the result of a succession of events. For example, we have previously used this system to determine how enzymes like tyrosinase alter catecholic accumulation within chitosan gels depending upon the presence or absence of additional secreted phenolic molecules (Liu et al., 2014).

We note that many naturally produced materials are redox-active as a result of multiple signaling and metabolic events. The purposeful addition of redox mediators to electrode/materials systems is especially useful when analyzing these natural materials, which are often found as large molecular aggregates dispersed throughout biological networks (see Figure 3B). In these analyses, a series of redox mediators are added to biological samples under controlled conditions to "reverse engineer" the type of redox couple within the material, the overall amount of the couple, and its Nernst potential. Examples of the large molecular aggregates that have been analyzed using this method include humic substances from environmental samples (Aeschbacher et al., 2010) and melanins (Kang et al., 2018b), which belong to a diverse grouping of biological polyphenols that play roles in protecting cells from oxidative stress (Kim et al., 2020) and shuttling electrons during respiration (Tan et al., 2019; Turick et al., 2002). Each natural material, therefore, represents an example of how biological systems alter communication channels, including as a result of prior events. In this way, the creation or degradation of redox materials like humics within soil represents one way cells perceive and relay information, such as by the secretion of polyphenols to restructure the local microenvironment, subsequently altering the ensuing communication events (Lipczynska-Kochany, 2018).

Cell-Based Interactions

Cells have exquisite ability to detect and interpret molecules. By exploiting the tools of synthetic biology many cells, typically prokaryotes, have been engineered to precisely detect and report on the presence of molecules ranging from heavy metal contaminants to clinical biomarkers (Wan et al., 2019). Moreover, beyond translating molecular cues into redox-based outputs, redox-based responses have been used to kill pathogens and recruit immune functions to wound sites, for example. By engineering cells to provide redox-based outputs, engineered cell networks can be linked to electronics.

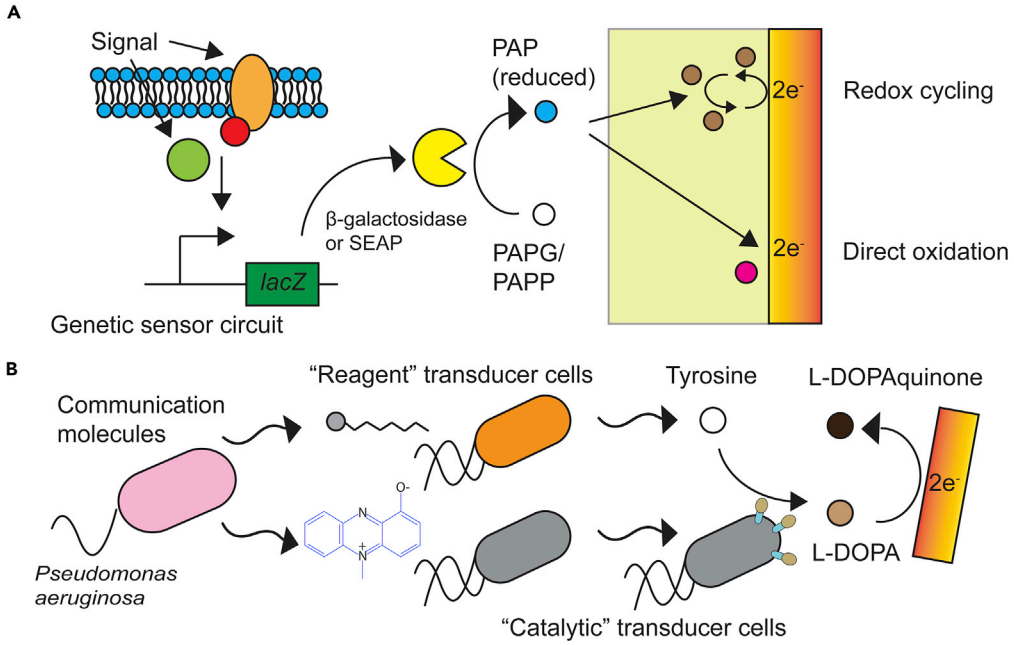

Figure 4. Cellular Bio-electronic Communication Systems That Use Redox Reactions

(A) Synthetic gene circuits can be used as detection systems by linking the transcriptional regulation of an enzyme to the presence of an analyte molecule. β-Galactosidase and secreted alkaline phosphatase (SEAP) both enzymatically catalyze the generation of 4-aminophenol (PAP) from their respective redox-"silent" substrates. PAP, in turn, can be directly oxidized, or reversibly cycled with materials, to link cellular transcription to an electrochemical signature.

(B) Redox reactions can be split between multiple cell populations to detect multiple communication molecules simultaneously. In this scheme, "reagent" transducer cells produce the enzymatic substrate, the amino acid tyrosine, whereas "catalytic" transducer cells produce the corresponding enzyme, tyrosinase. Using this method, two communication molecules can be detected simultaneously by electrochemically detecting the enzymatic product L-DOPA.

The principle advantage of using cells as engineered elements within a redox communication channel is their ability to naturally decode the holistic message contained in molecular mixtures based upon natural or engineered biomolecular circuits. This enables the creation of "sentinel" cells to gather molecular information that is being transmitted between neighboring or even distant cells (based on signal transport). To provide an example, because cells natively process environmental stresses and can be engineered for molecule-specific activation, cell-based biosensors can quickly report on exposure to harmful substances (e.g., herbicides or insecticides within water samples, Funabashi et al., 2005; Riangrungroj et al., 2019; VanArsdale et al., 2019) and other such stressors. Methods that rely on global stresses (e.g., oxidative stress) are well suited for rapid threat detection on mobile electronic devices before turning to more classical, laboratory-centric chemical methodologies to determine the exact chemical species. In this section we will review examples of electrochemical cell-based sensors, which, through redox, produce electronic signatures in response to communication molecules.

Many cell-based electrochemical sensors rely on enzymes to generate a redox molecule, which is subsequently detected at an electrode surface (see Figure 4A). In this manner, these systems are comparable to enzyme detection schemes, except that the recognition unit is replaced by a genetic element contained within, or introduced into, a living cell (Biran et al., 1999; Matsui et al., 2006; Wang et al., 2017). Therefore, these systems can be used to monitor the role various transcriptional elements play within information transduction. β-Galactosidase is a convenient reporter enzyme encoded by the *lacZ* gene that has been widely used in genetic and biomolecular assays. Numerous systems exploit the cleavage of the redox-"silent" substrate 4-aminophenyl-β-galactopyranoside (PAPG), releasing the redox mediator 4-aminophenol (PAP). Thus, by placing transcriptional regulation of the *lacZ* gene in response to a molecular cue, one can generate a rapid and simple electrochemical output. As an example, we created sensor cells with a *lacZ* gene circuit to electrochemically monitor bacterial QS mediated by the QS autoinducer-2 (AI-2), an intra-

and interspecies signaling molecule. This process was mathematically modeled to efficiently compare the electrochemical method to the well-established Miller assay (Tschirhart et al., 2016). One advantage of cell-based molecular detection is that cells can reproduce, allowing for the creation of "living" sensors. Cells containing these circuits can be electrodeposited with alginate (Cheng et al., 2011) and chitosan (Kim et al., 2014c) to create hydrogel-based living materials. We have also used these methods to embed cells on an electrode in an alginate-chitosan multilayer to monitor AI-2 QS (non-redox active) through a *lacZ* gene circuit, which in turn produced a PAP signal that was enhanced by redox cycling with the catechol-chitosan layer (Liu et al., 2017). Circuits like these can be used within devices to monitor the reception of molecular communication by various genetic elements.

A similar enzyme-based system uses secreted alkaline phosphatase (SEAP), which catalyzes the hydrolysis and transphosphorylation of phosphoric acid monoesters. In a reaction similar to β-galactosidase and PAPG, SEAP can cleave the redox-silent substrate p-aminophenyl phosphate to release the redox-active PAP. While this system is commonly fused to antibodies and used to detect cell surface receptors in immunosorbent assays (Takahashi et al., 2009), transcriptional regulation of SEAP can be linked to a variety of transcription factors in eukaryotic cells enabling cell-based sensing (Shiku et al., 2009). Unlike β-galactosidase, SEAP can be secreted into a localized area, which, when coupled with techniques like scanning electrochemical microscopy (SECM), enables spatially dependent electrochemical measurements. For instance, SECM can be used to monitor single-cell responses in transfected HeLa cells to monitor a wide group of communication signals within patterned cultures (Murata et al., 2009). SECM techniques can also be coupled with additional electrodes, quantum dots, and redox mediators to create an enhanced, multiplexed output based on biological redox cycling within a sensor array to enhance spatial sensitivity (Lin et al., 2009; Pumera et al., 2007). For these reasons, SEAP transcriptional circuits, in connection with SECM, are useful for discerning how communication spreads throughout a population or a series of locations.

Another mode by which cells translate molecular communication into redox signals is through the direct synthesis, secretion, and/or catalytic conversion of redox molecules or moieties. One such example is the oxidation or nitrosylation of tyrosine residues, which is prevalent in immune responses (Frederickson Matika and Loake, 2014; González-Santoyo and Córdoba-Aguilar, 2012) and melanin formation (Slominski et al., 2004). These reactions can be electrochemically monitored by coupling tyrosine with tyrosinase (Rivas and Solis, 1991) or laccase (Rodríguez Couto and Toca Herrera, 2006). When compared with the previously discussed cell-based detection, these systems relay information through the time-dependent generation of the substrate, enzyme, and redox product. Therefore, both the amount of substrate and the catalytic reaction contain information about the nature of a system. In our recent work (VanArsdale et al., 2020), we split the tyrosine-tyrosinase redox reaction between two cell populations wherein each population needed a specific input and the coupled responses led to a single output (resulting in an AND logic gate, Brophy and Voigt, 2014). We used this to eavesdrop on *Pseudomonas aeruginosa* virulence signaling indicated by the secretion of both a QS autoinducer and a phenazine toxin (see Figure 4B). We linked cell-surface display of tyrosinase (Gustavsson et al., 2016; Hörnström et al., 2019) caused by the presence of redox toxin pyocyanin, with metabolically overproduced tyrosine in response to autoinducer-1 (AI-1) QS signal N-(3-oxo-dodecanoyl)-l-homoserine lactone. When both signals were present, the surface-displayed tyrosinase on the first population catalytically converted the secreted tyrosine from the second population into L-3,4-dihydroxyphenylalanine (L-DOPA), which was measured through oxidative voltammetry. This technique demonstrated a simple method to couple multiple cell populations within a network to monitor cell-cell communication signals in real time. Furthermore, because two independent input signals were required, a robust output was produced that minimized leaky signal and false-positives.

Many forms of cell-cell communication, however, take place in poorly accessible areas unsuitable either for continual extraction or for re-fueling, like the bottom of the ocean. In these instances, an ideal cell-based communication channel would be able to generate its own power or be able to act as a sensor devoid of any substrate fuel. Native species that are suitable for these types of channels are known as electrigens/exoelectrogens (Logan et al., 2019; Teravest and Ajo-Franklin, 2016), because they naturally couple cellular respiration to the oxidation and reduction of metals that can be used as electrodes. As a result, these species are commonly deployed in biofuel cells to generate electricity through interactions with organic matter (see Figure 5A). Exoelectrogens use two broad methods to move electrons: (1) redox shuttles and (2) redox conductive structures. Within redox shuttle producing exoelectrogens, the molecular message is

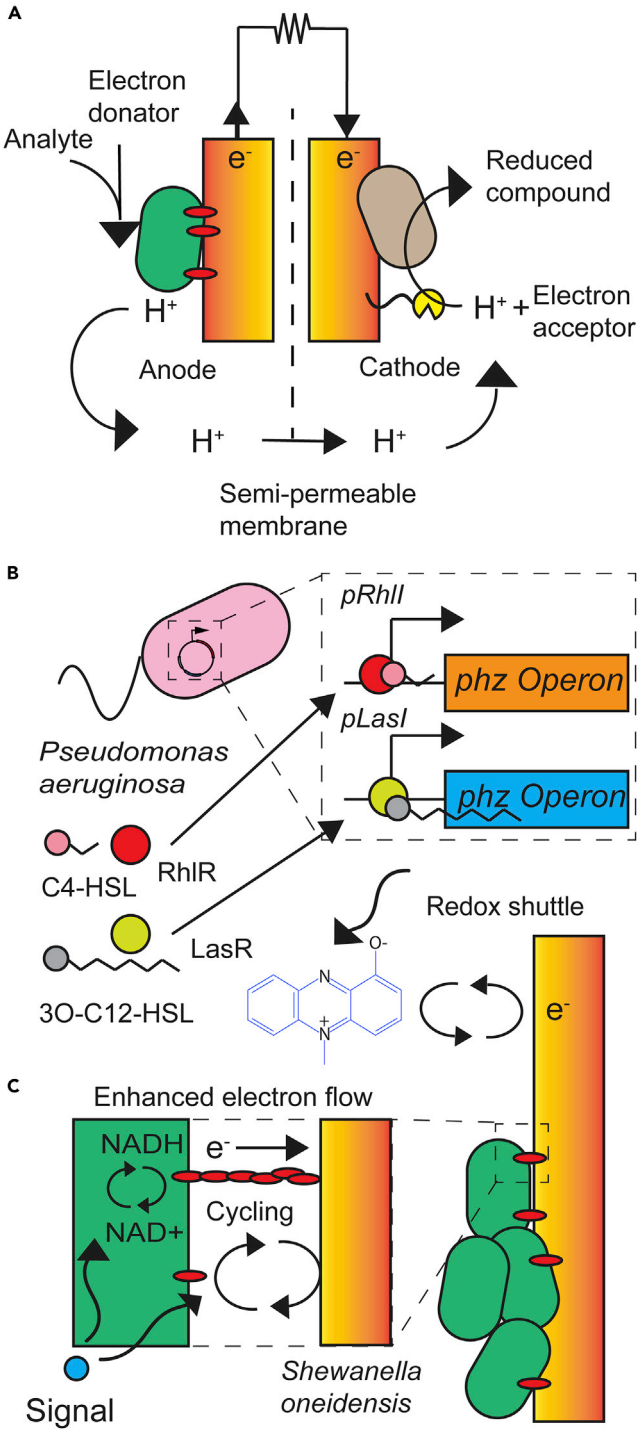

Figure 5. Detecting Communication Molecules Using Exoelectrogens

(A) In biofuel cells, cellular respiration with an electron donor in an anodic chamber is coupled to a corresponding synthetic reaction catalyzed by either an enzyme or another microbe at a biocathode. Each chamber is separated by a semi-permeable membrane to prevent chamber mixing to ensure unidirectional electron flow. A resistor is placed between both chambers to alter the voltage drop and thus power a device. These devices can be used for detection by linking either the respiratory or synthetic reactions to the presence of an analyte, alongside additional electronic circuit design.

Figure 5. *Continued*

(B) Exoelectrogens like *Pseudomonas aeruginosa* produce soluble redox shuttles to aid in their respiration processes. Using synthetic biology, the metabolic synthesis of these molecules can be linked to the presence of communication molecules, like the acylhomoserinelactones C4-HSL and 3O-C12-HSL.

(C) Dissimilatory metal-reducing species, like *Shewanella oneidensis*, use cytochromes to directly shuttle electrons to metal surfaces through a redox wire. These electron shuttling systems can be used to detect biomolecules either through transcriptional regulation of the protein components or by enhancing electron flow, either through interaction with the cytochrome turnover of metabolic reductants or by increasing electron turnover by diffusive redox cycling with the electrode.

interpreted by the cell, and consequent change occurs in the concentration or redox cycling frequency of a secreted, extracellular shuttle (Glasser et al., 2017). *Pseudomonas aeruginosa* is a common shuttle-producing exoelectrogen often used in wastewater fuel cells because it produces soluble redox shuttles as part of its QS communication cascade (Dietrich et al., 2006). These shuttles enable *P. aeruginosa* biofilms to respire by diffusively connecting the biofilm to the electrode through reversible redox reactions (Venkataraman et al., 2010; Wang et al., 2013). The secretion of the shuttle pyocyanin was previously adapted in engineered *P. aeruginosa* to function as an AND logic gate for detection of both the *lasR*- and *rhlR*-recognized AI-1 cell signals (Li et al., 2011) (see Figure 5B). Redox shuttles are also convenient for lowering overpotentials (Yong et al., 2011) and expression in heterologous species (Schmitz et al., 2015), and recent work suggests that they can be synthetically designed (Mutter et al., 2019). Exoelectrogens that produce conductive structures within biofilms use multi-heme nanowires and redox gradients to shuttle electrons (Strycharz-Glaven et al., 2011; Yates et al., 2016). Many of these organisms are anaerobic bacteria that reside in organic matter of soil and aquatic environments (Bond, 2002) and have been utilized in a wide variety of biofuel cells (Tender et al., 2002; Ueki et al., 2016). In terms of detecting communication molecules, the components used to transfer electrons to extracellular acceptors, like the cytochrome metal respiratory system (Mtr) of *Shewanella oneidensis,* can be used to create novel biosensors either through transcriptional regulation or through enhanced respiration (see Figure 5C). Similar to pyocyanin, previous work has demonstrated that these components can be transcriptionally regulated to create an AND logic gate for detecting QS signals (Hu et al., 2015). Others have taken advantage of the ability of cytochromes to drastically enhance molecular redox cycling to detect sub-nanomolar concentrations of pyocyanin (Yang et al., 2017) and riboflavin (Si et al., 2016). Last, others have combined *Shewanella oneidensis* with other fuel cell species to detect metabolic secretions like lactate (Zeng et al., 2019). Each of these examples works when a molecule, or a group of molecules, is present, and the current generated from the biofilm increases. Although these techniques have yet to be commercially deployed, exoelectrogens offer the unique possibility to create self-powered biosensors (Yates et al., 2017) and living materials (Bird et al., 2019) that can autonomously operate in challenging locations like the seafloor (Tender et al., 2008; Zhou, 2015). The components from these microbes can also be expressed in heterologous species (Jensen et al., 2010), or can be rationally engineered (Atkinson et al., 2019) to create powerful production hosts (Glaven, 2019).

Controlling Cell-Cell Communication

Compared with molecular detection, far fewer efforts have been developed that enable electronic devices to *control* cell-cell molecular communication via genetic manipulation. Newly referred to as "electrogenetics," these techniques draw from similar methodologies that seek to control native metabolic activity in electroactive cells through electronic stimulation (Chu et al., 2020; Hirose et al., 2018; Hongo and Iwahara, 1979). Within redox electrogenetic systems, a message is encoded into a user-controlled signal that a cell receives and decodes either using its endogenous communication apparatus or an engineered function. Devices that can stimulate and control cell-cell communication have historically been linked to cells through ionic currents or through the proteins and cells that regulate ionic currents, such as variants of the patch-clamp technique (Hamill et al., 1981) or the many natural and engineered light-sensitive ion channels and proteins within optogenetics (Deisseroth, 2011; Kolar and Weber, 2017). In contrast, direct electronic integration, in the form of directed electron flow, has remained elusive despite the centrality of redox signaling throughout biology (Jones and Sies, 2015) and the scalability of electrochemistry for biomanufacturing. In this section, we will review efforts for controlling cellular signaling by the electrochemical transmission of information through redox reactions.

One of the earliest efforts was developed by Fussenegger and coworkers who engineered mammalian cells to respond to the oxidative conversion of ethanol to acetaldehyde (Weber et al., 2008) (see Figure 6A). They achieved this by heterologous expression of the transcriptional activator AlcR from *Aspergillus nidulans* to

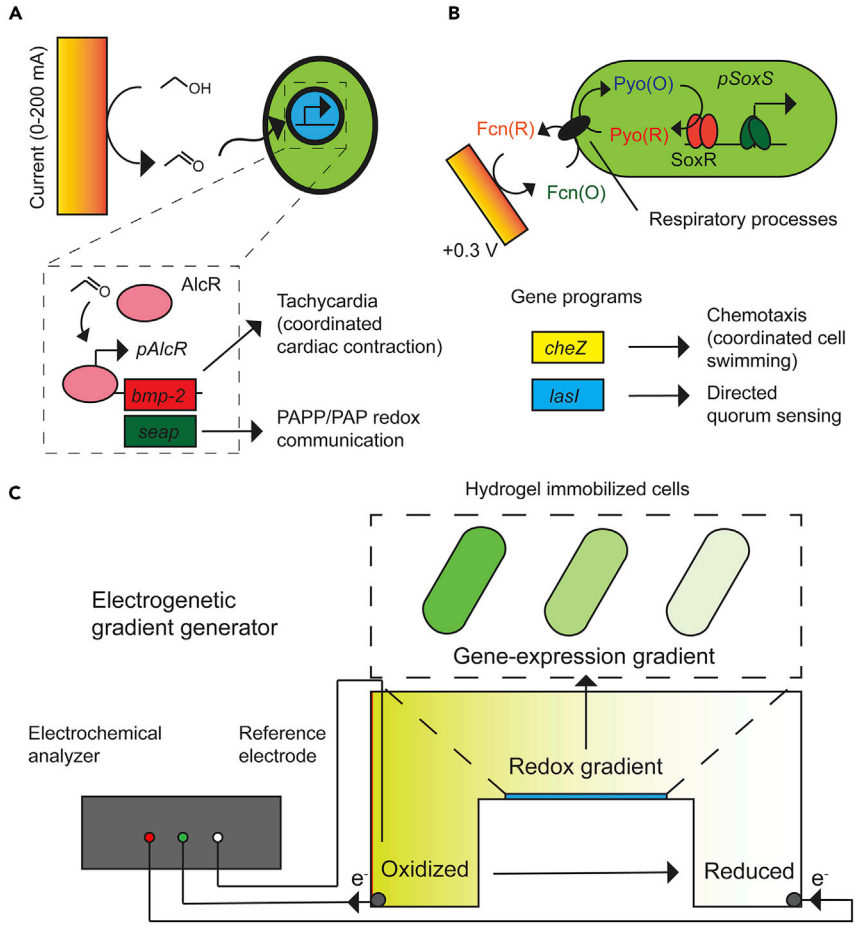

Figure 6. Electrogenetic Control of Cellular Communication

(A) An acetaldehyde-sensing system from *Aspergillus nidulans* was heterologously used to link the electrochemical conversion of ethanol to acetaldehyde to the transcriptional regulation of a synthetic gene circuit in mammalian cells (Weber et al., 2008). This system was used to control the contraction rate of cardiomyocytes, demonstrating synthetic control of tissue-level behaviors.

(B) The superoxide sensitive (SoxS) gene circuit from *Escherichia coli* was used to create an electrogenetic system using the redox mediators pyocyanin and potassium ferrocyanide (i.e., Fcn(R) (Tschirhart et al., 2017)). Pyocyanin initiates expression of genes downstream of the *soxS* promoter by oxidizing the SoxR transcription factor. Pyocyanin is then oxidatively cycled through cellular respiration processes, which are linked to the electrochemical system through potassium ferricyanide (i.e., Fcn(O)), the oxidative couple to Fcn(R). As a result, the amount of Fcn(O) generated at the electrode determined the extent of SoxR transcriptional activation. Electrogenetic control was coupled to the expression to the autoinducer-1 synthase LasI and the motility protein CheZ to control cell communication and movement, respectively.

(C) We coupled the SoxRS electrogenetic system to a deactivated-Cas9 transcriptional activator to create an electrochemically controlled CRISPR system (eCRISPR). Using an electrogenetic gradient generator in which immobilized cells are placed between an anode and cathode, differential genetic responses were encoded based on the position of individual cells. At the anode, Fcn(R) is oxidized to its Fcn(O) couple. Conversely, at the cathode Fcn(O) is reduced back to Fcn(R). The resulting gradient enabled a spatially controlled pattern of electrogenetic activation between the two chambers.

regulate gene expression of the aldehyde-inducible promoter pAIR. By combining with a device, they coupled the electronically controlled current (and thus the generation of acetaldehyde from an ethanol substrate depot) to the transcriptional response of engineered cells. In their demonstration, the acetaldehyde-actuated cells altered the contractile frequency of engineered rat cardiomyocytes in a cell network, thus demonstrating the ability to electrochemically alter the function of molecular signaling components. This system first coined the term "electrogenetic," which has since been subsequently adopted by us

(Tschirhart et al., 2017) and others (Hirose et al., 2019) to describe electron-mediated control over engineered gene circuits.

Our group expanded the redox electrogenetic toolbox by creating a direct electron linkage instead of relying upon voltage-dependent degradation of ethanol: we incorporated electrically actuated redox mediators to directly actuate genetic regulation of the *soxRS* stress regulon of *Escherichia coli* (Tschirhart et al., 2017). The key difference between this and the acetaldehyde system is the direct exchange of electrons between the electronic and biological components. As a result, a message can be directly programmed as the number of electrons passed between the electrode and the cellular environment. In our system the SoxR transcription factor was activated by pyocyanin, the previously mentioned redox toxin produced by *P. aeruginosa* (see Figure 6B). Under aerobic conditions, pyocyanin is generally in its oxidized state where it can cycle between the cell and dissolved oxygen to continually actuate *soxRS* gene transcription. In an anaerobic environment, gene expression is initiated by oxidized pyocyanin, which, without oxygen, can only cycle with respiration associated molecules connected to the reduction of potassium ferricyanide (i.e., Fcn(O)), a commonly used biological redox mediator (Pasco et al., 2000). As a result, the transcriptional upregulation of the *soxS* promoter became directly linked to the concentration of Fcn(O), which was reversibly generated by electrochemically oxidizing potassium ferrocyanide (i.e., Fcn(R)). In terms of communication, this system was shown to control a variety of bacterial behaviors, including QS, cellular motility, and protein production all by altering the amount of charge (i.e., the number of electrons) input into the system.

To expand the capabilities of redox-enabled electrogenetics and to foster a bioelectronics molecular communication paradigm, we developed an electronic-CRISPR (eCRISPR) system that brings into play an array of CRISPR functions, enabling for more extensive intracellular control (Bhokisham et al., 2020). Moreover, as CRISPR works in a variety of cell types, eCRISPR was developed so that by simply switching guide RNA sequences electronic control was shown to work in different bacteria, including *E. coli* and *Salmonella enterica serovar typhimurium*. Specifically, we adapted a deactivated-Cas9 transcriptional activator (Bikard et al., 2013) to act as a transcriptional switch. We used this to activate and silence specific gene targets including pleiotropic sources of genomic "noise." These advances, when considered in sum, enabled the creation of an electronically controlled redox communication device to direct multiple populations simultaneously. As a demonstration, we showed how messages could be electronically encoded into voltage inputs and transmitted to eCRISPR-modified cells by electrochemically programming Fcn(O) concentration gradients within a redox communication channel. We were able to guide bacterial gene expression with spatiotemporal control (see Figure 6C). We suggest that eCRISPR offers unique opportunities, especially in low-oxygen environments, where the availability of electron acceptors can be electrochemically altered to control and pattern gene expression, such as in microaerobic or anaerobic bioreactor environments (Schmitz et al., 2015) or even the mammalian digestive tract (Jones and Neish, 2017).

Closing the Loop and Future Directions

Cell-Based Biohybrid Devices

Ultimately, one goal for embedding electronics within cell communication networks is to electronically direct physiological function for a variety of medical and engineering applications. For example, multiple groups have proposed the idea of sentinel therapy (Kojima et al., 2016), in which engineered "sentinel" cells respond to and mediate disease or user-mediated signals (Mansouri et al., 2019). The acquisition of a disease signal, such as pathogen QS molecules, or immune signals like nitric oxide or hydrogen peroxide, can be used to activate a genetic program that seeks out and destroys pathogens or heals lesions (McKay et al., 2018; Virgile et al., 2018). Naturally, there are significant concerns relative to the long-term containment of such sentinel populations, or the ability of these cells to later integrate into homeostatic environments. A noteworthy example is the current state of chimeric antigen receptor T cell therapy, which has a high-prevalence of post-treatment dysbiosis in the form of disease relapse and cytokine release syndrome, which suggests both over-active and under-active integration into the native immune system by the engineered population (Prasad, 2018). Techniques that can rationally control cell activation and population size could revolutionize cell-based therapies.

An alternative strategy to unregulated sentinel therapy is to create a system of biohybrid devices that can monitor and control sentinel cell activation through a central electronic network (see Figure 7A). In the case

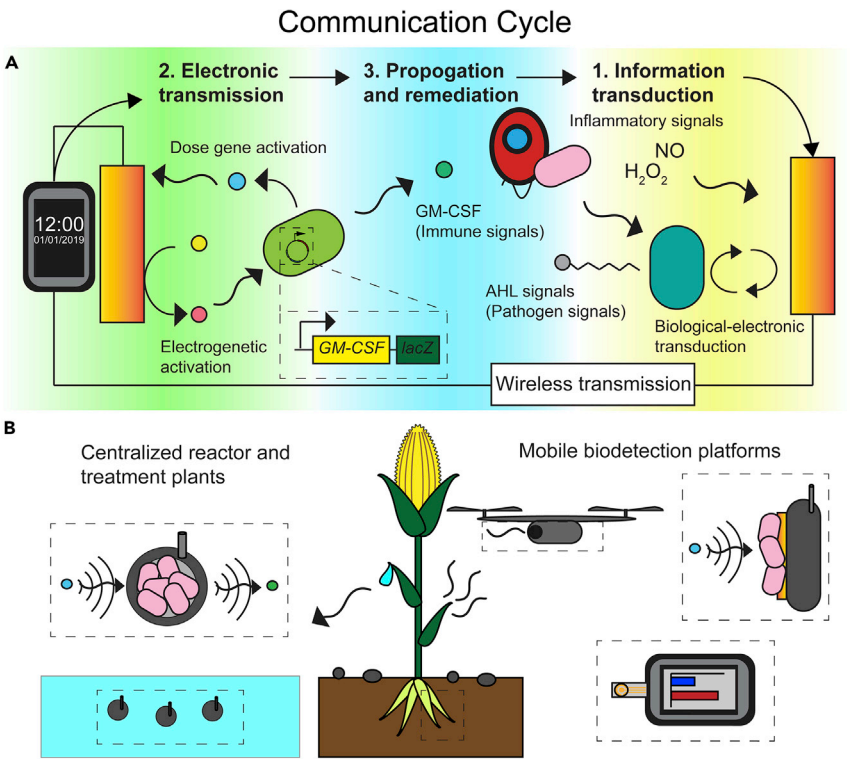

Figure 7. Future Opportunities to Create Closed-Loop Biohybrid Communication Devices

(A) Electronic systems can be used to monitor and control sentinel cell mediation of therapeutics within a communication cycle. These processes can be broken into three basic elements colored yellow, green, and blue: 1. (Yellow) There is an initial information transduction of pathogen and immune signals by an electrochemical measurement that is wirelessly transmitted to a central processor (e.g., hydrogen peroxide and nitric oxide are inflammatory signals, AHLs are pathogen biomarkers). 2. (Green) The processor initiates electrogenetic activation of the sentinel population according to the need of the patient (e.g., electrogenetic dosing and activation of a pro-inflammatory probiotic is indicated by generation of a redox reporter detected near the activating electrode). 3. (Blue) The sentinel populations' action begins to act against the pathogen invasion (e.g., pro-inflammatory secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) from the electronically activated probiotic enhances immune clearance of the pathogen), thus altering the signals that will be acquired in the first step (yellow), closing the communication control loop.

(B) Bioelectronic molecular communication can be used to monitor and mediate agricultural problems through centralized treatment centers and in mobile detection schemes. To the left of the corn plant, centralized platforms can use biological-pods specialized to sense various compounds in water treatment reservoirs that collect agricultural run-off and initiate synthesis of remedial compounds to prevent further water pollution. Mobile detection schemes, shown to the right of the corn plant, can be used by drones or agricultural workers to detect soil conditions, crop needs, plant molecular signals, and the containment of chemical herbicides and insecticides from air, water, and soil samples to create dynamic and personalized field management plan.

of gastrointestinal (GI) diseases, ingestible electronic devices could be deployed to sense GI tract biomarkers, as is depicted in the yellow region. Many of these devices already exist, some of which have used cells as sensor elements (see review Beardslee et al., 2020). These devices could then electronically activate sentinels to treat disease (depicted in the green and blue regions), all while continuing to relay back information about the treatments' successes. This idea was well encapsulated in a recent work that used a new ionic form of electrogenetic control to regulate insulin production within type 1 diabetic mice from an implanted device (Krawczyk et al., 2020). In effect, the wireless control of these implanted devices inserts the human user as an intermediate controller in the response of engineered sentinels to disease states, thus minimizing risks and allowing for patient-specific treatments. This strategy also enables novel, patient-guided therapeutics in which it is the user who decides what is a suitable outcome, based upon both their personal preferences and linked professionals who monitor outcomes through the internet.

In the more immediate future, electronic integration with cell-cell communication has immediate applications in agriculture, environmental remediation, and biomanufacturing (see Figure 7B). Here, molecular communication between crops and their environment contain information regarding soil nutrient content, the containment of chemical treatments, and expected product yields per acre (Bhattacharyya and Jha, 2012; Calvo et al., 2014; Richardson et al., 2009). Mobile devices equipped with cellular sensors might be used by humans or even robotic drones to sample the environment to discern plant needs by location, limiting the use of chemical herbicides and their unintentional drift by liquid or gas into nearby fields (Henry et al., 2004). Centralized treatment plants can then monitor and mediate chemical run-off using networked biological-pods, ("bPods"), equipped with electrochemical sensors, engineered cells, and wireless transmitters (Stine et al., 2020). Similar devices, or in-line microfluidic devices (Shang et al., 2019), could also be used to electronically monitor molecular communication in biomanufacturing with the potential to monitor multiple media components simultaneously, and to direct metabolic use of engineered strains (Stephens et al., 2019; Tsao et al., 2010). Each of these systems demonstrates that either in single devices, or networked devices, there are immediate applications for electronically actuated biomolecular communication.

Although the future holds great promise, there remain several fundamental barriers for engineered electrogenetic device integration with native cellular systems. Some of these issues are not unique to the cell-cell communication paradigm, such as the need for high-throughput screening, integrated databases, rational workflows, etc. (Benner and Sismour, 2005). A key aspect of electronic integration with cell-cell communication is its systemic and contextual nature as noted earlier. Specifically, a biological response to any input, electronic or not, is entirely context dependent (Terrell et al., 2015). "Smart" electronic interfaces allow us to interact with cell-cell communication within a shared context, but subsequent analysis is needed to know the limitations of this form of integration. We have used information theory primarily as means of structurally categorizing molecular components with larger systems, whereas the mathematical branch of information theory has previously revealed how cell-cell communication networks link population responses (Suderman et al., 2017), intracellular cross talk (Rowland et al., 2012), or a combination of both elements (Cheong et al., 2011), to limit the potential range of outcomes and alleviate errant noise. Similar analyses could elucidate the limits of electrochemical communication channels. Such an analysis would also be key in determining the major participants in the information flow through a single cell (Pierobon et al., 2016), and throughout a synthetic communication network (Harper et al., 2018). This issue is fundamental to redox communication, which uses semi-specific molecular interactions. This problem, however, is not isolated to redox signaling but applies to biological communication in general. As was previously mentioned, QS responses are complex integrations of a variety of inputs, including non-specific host interactions and the general physical aspects of the environment (Darch et al., 2018). Similarly, glycomics and glycoproteomics, two massive signaling networks, use redundant, semi-specific signals to create integrated cellular responses (Mian and Rose, 2011). Network perspectives both of natural cell-cell communication and engineered bio-electronic communication will be key to determining how to integrate redox channels into complex synthetic and therapeutic systems.

Conclusion

There is immense promise in the use of redox to both interrogate and electronically control cellular communication in that it will open a wide variety of engineering applications. We have highlighted attempts to intercept information from cellular communication via molecular exchange, as well as efforts to electronically control cell-cell communication. These systems allow for molecular components within communication systems to be systematically studied. They should also enable the creation of bio-electronic communication systems. Future work that embeds these biological mimics of network technology into greater systems will enable the electrosynthesis of complex products (Glaven, 2019), integrated user-controlled biological devices (Kim et al., 2019), and perhaps user-defined cellular consortia (McCarty and Ledesma-Amaro, 2019). We believe electronic integration with cellular communication will be crucial in the development of integrated cellular technologies (Akyildiz et al., 2015). In the end, this technology ultimately will help define future movements in biotechnology and beyond, expanding the current "Internet of things" to include the "Internet of biological things."

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AUTHOR CONTRIBUTIONS

J.P. researched literature and edited manuscript and figures; E.V., G.F.P. and W.E.B. wrote and edited the manuscript.

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