Microscale and Nanoscale Electrophotonic Diagnostic Devices

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Abstract

Detecting and identifying infectious agents and potential pathogens in complex environments and characterizing their mode of action is a critical need. Traditional diagnostics have targeted a single characteristic, *e.g.* spectral response, surface receptor, mass, intrinsic conductivity, *etc.* However, advances in detection technologies have identified emerging approaches in which multiple modes of action are combined to obtain enhanced performance characteristics. Particularly appealing in this regard, electrophotonic devices capable of coupling light to electron translocation have experienced rapid recent growth and offer significant advantages for diagnostics. In this chapter, we explore three specific promising approaches that combine electronics and photonics: (*a*) assays based on closed bipolar electrochemistry coupling electron transfer to color or fluorescence (*b*) sensors based on localized surface plasmon resonances, and (*c*) emerging nanophotonics approaches, such as those based on zero-mode waveguides and metamaterials.

Introduction

The first line of defense against the scourge of infectious agents centers on detection of pathogens at the point of care. Consequently, detecting and identifying infectious agents have attracted an enormous amount of attention, and molecular diagnostics, particularly those which exploit recent advances in nucleic acid detection technology, *e.g.* PCR (Elnifro et al. 2000), high-(throughput sequencing (Shendure and Ji 2008), *etc.* have come to the fore. At the same time there is a general realization that nucleic acid amplification brings with it the unwanted baggage of increased diagnostic complexity, prolonged analysis times, and even problems with clinical sensitivity and selectivity (Jung et al. 2010). Furthermore, genetic analyses are insufficient to address general pathogen challenges, since pathogen phenotype is not uniquely determined by the genotype, alone. Therefore, a fully competent analysis should address the complete spectrum of pathogen characteristics, including the abilities to: (1) locate and adhere to host tissues; (2) harm the host, for example by exotoxin secretion; and (3) evade the immune system of the host. These are especially important in light of new emerging pathogens. Thus, developing methods to rapidly and accurately identify potential pathogens in complex environments and to characterize their mode of action is critical, and the technological figures-of-merit that must be achieved are daunting.

Against this backdrop, a number of new technologies have been thrown into the breach – including both whole cell detection and molecular recognition. For example, surface capture of bacteria is a mainstay in a number of assay formats in which different capture agents (lectins, antibodies, recognition peptides, aptamers, *etc.*) have been used to pull-down bacteria to a surface. Once captured the bacterium can be detected, for example, by fluorescence (Delehanty and Ligler 2002), surface plasmon resonance (Fratamico et al. 1998), optical waveguide resonance (Rowe-Taitt et al. 2000), amperometry (Gau et al. 2001), surface enhanced Raman scattering (Grow et al. 2003), optical resonators (Watts et al. 1994), mass spectrometry (Bundy and Fenselau 1999), and electrochemical impedance (Varshney et al. 2007). While these approaches are useful, they typically exploit a single mode of action. At the same time a new set of detection technologies is emerging in which multiple modes of action are combined to yield detection schemes with enhanced performance characteristics. In particular, microscale and nanoscale electrophotonic devices – structures capable of coupling light to electron transfer and/or translocation - have experienced rapid growth over the last decade and offer significant advantages for diagnostic platforms. In this chapter we will highlight three new approaches to combining electronics and photonics which have substantial diagnostic promise: (*a*) closed bipolar electrochemistry (Liu et al. 2016b) coupling electron transfer to color, fluorescence, *etc.*; (*b*) localized surface plasmon resonance (LSPR) sensors (Shinohara et al. 2015); and (*c*) emerging nanophotonics approaches, such as those based on non-classical optics, such as zero-mode waveguides (ZMW) (Eid et al. 2009) and metamaterials (Hendry et al. 2010).

II. Background

II.A. Bipolar Electrochemistry.

General principles. Bipolar electrochemistry, which couples redox reactions at the opposite poles of a non-powered (*i.e.* unconnected) metal in contact with an electrified fluid, has opened new avenues of electrochemical sensing, **Figure 1(a)** (Fosdick et al. 2013). Two driving electrodes at opposite ends of the fluid medium produce a potential gradient in electrolyte solution, which maps onto the isopotential of the bipolar electrode (BPE) creating interfacial potential differences between the opposite poles of the BPE

and the electrolyte solution in contact with them (Mavre et al. 2010). This potential difference enables the coupling of anodic and cathodic reactions at the two ends (poles) of the BPE. Since BPEs can be fabricated in a variety of sizes, shapes, and topological forms, numerous applications have been described, including material preparation and fabrication (Ulrich et al. 2008), molecular sensing (Fosdick and Crooks 2012), micro swimmers (Wang et al. 2006) and bipolar electrode focusing (Perdue et al. 2009).

Open, **Figure 1(a)**, and closed, **Figure 1(b)**, BPEs have both been explored (Fosdick et al. 2013). Open BPEs typically employ a single channel, and the anodic and cathodic poles are in the same solution environment, with coupled redox reactions occurring at the opposite poles (Mavre et al. 2010). In this geometry, ionic current constitutes a large fraction of the total current between driving electrodes. Because there is no external connection, BPE arrays, consisting of a large number of individual BPE electrodes, can be controlled with one set of driving electrodes (Chow et al. 2009). Therefore, open BPEs are good candidates for wireless electrochemical sensing in fluidic environments. In contrast, closed BPEs are analogous to series-coupled electrochemical cells.(Guerrette et al. 2012) The anodic and cathode poles in closed BPE systems are in contact with physically separate (and possibly chemically distinct) solutions. Because the two compartments are physically separated, the BPE provides the only path for electron transfer between the two coupled half reactions.(Guerrette et al. 2012) Therefore, closed BPEs may employ chemically distinct analytes and reporters, thus physically segregating the detection from readout steps, greatly minimizing background interference. Also, the reporter system can be regenerated and reused many times. Thus, closed BPEs can implement remote detection (Xu et al. 2016b), reducing cross talk between analyte and reporter solutions and eliminating the need for a fluidic path containing the BPE (Xu et al. 2016c).

Optical readout strategies. While early bipolar electrochemistry research largely focused on coupling redox reactions and measuring the resulting Faradaic current (Loget et al. 2013), recent applications have demonstrated that converting electric to optical signals can be a powerful approach to improve sensitivity, simplify instrumentation design, and suppress signal crosstalk (Zhang et al. 2017). Here, we will highlight

recent developments using optical readout strategies, including those based on electrochromic, electrofluorigenic, and electrochemical plasmon effects, all of which allow electron transfer at one pole of a closed BPE to be read out by an optical reporter system at the other pole.

Electrochromic readout is achieved when an electron transfer reaction in the reporter cell results in a colorimetric change in the closed BPE (Liu and Crooks 2012). For example, we developed an electrochromic sensing platform based on coupling redox reactions in an analytical cell with a redox-sensitive colorimetric indicator, methyl viologen (MV), in the reporter cell (Xu et al. 2016a). The whole detection system was designed to work with battery power and cell-phone color readout as a proof-of-concept of device simplicity and portability. Electrofluorigenic readout can be realized by exploiting redox-sensitive fluorescence. *e.g.* in resorufin, to achieve ultrasensitive detection. In this approach, redox reactions in the analytical cell were coupled to the electrochemical transformation of weakly emissive resazurin to strongly emissive resorufin in the reporter cell. The effect is versatile, as was demonstrated in two different geometries: nanopore recessed disk-multiscale BPEs (Ma et al. 2015) and parallel interdigitated electrode BPEs (Xu et al. 2015). Besides the high sensitivity, both detection systems are capable of fluorescence imaging, which can be used to explore heterogeneous electrochemical phenomena. Looking to the future, we are developing a closed BPE approach that uses localized surface plasmon resonance (LSPR) readout to combine the simplicity of electrochromic and the sensitivity of electrofluorigenic assays. In this strategy, simple galvanic electrodeposition is used to shift the LSPR wavelength in order to monitor the reaction in the analytical cell.

II.B. Localized Surface Plasmon Resonances.

General principles. Localized surface plasmon resonances constitute an optical effect observed particularly in noble metal (*e.g.,* Au, Ag, Cu) nanoparticles, which possess a dielectric constant with a negative real and a small positive imaginary component.(Willets and Van Duyne 2007) When the frequency of incident radiation matches the resonant oscillation frequency of free electrons in the nanoparticles, electrons oscillate coherently, **Figure 2(a)**, resulting in a surface plasmon resonance. Because the nanoparticles size is typically ≤ $\lambda/10$, the induced resonance is highly localized around the

metal nanoparticles, *i.e.* a localized surface plasmon resonance.

Construction and characterization. Metallic nanoparticles can be chemically synthesized in solution as well as patterned onto substrates using lithography.(Willets and Van Duyne 2007) Combining the advantages of these two methods, nanosphere lithography (NSL) (Hulteen and Van Duyne 1995), allows large scale fabrication of periodic arrays of metallic nanoparticles. Modified NSL methods have been used to create different patterns on substrates such as modulated nanopore arrays (Fu et al. 2016) and nanopillar arrays (Hall et al. 2013). NSL-fabrication makes ideal substrates for LSPR spectroscopy (Haes and Van Duyne 2004). The extinction of metal nanoparticle arrays is monitored using a uv-visible spectrometer in either transmission or reflection. The LSPR can be tuned based on the material, size, and shape of the nanoparticles, as well as the surrounding environment, including both solvent and adsorbates. This sensitivity to the environment has been exploited for biomolecular sensing applications (Hall et al. 2011a), with the key being the manipulation of the local dielectric environment and the resulting shift in the LSPR wavelength, $\Delta\lambda$, **Figure 2(b)**.

Selectivity. LSPR sensing is inherently nonspecific - anything adsorbed onto the sensor surface can shift the LSPR wavelength. Thus, to create a selective LSPR sensing platform, recognition agents with high affinities towards their specific targets are incorporated in the sensor design. A broad range of recognition agents include: biotin and (strept/neutr)avidin (Haes and Van Duyne 2002), antibody-antigen pairs (Hall et al. 2011b), lectin and carbohydrates (Cai et al. 2017), enzyme-substrate pairs (Sekretaryova et al. 2014), siderophores (Doorneweerd et al. 2010), bacteriophages (Tripathi et al. 2012), as well as oligonucleotide-based recognition systems, such as aptamers (Urmann et al. 2016), antimicrobial peptides (Mannoor et al. 2010), and other DNA sequences. These recognition agents are used to target small molecules (Muguruma et al. 2011), proteins (Bertok et al. 2013), bacterial lysates (Taylor et al. 2006), bacterial spores (Pestov et al. 2008), intact bacterial cells (Hu et al. 2018), viral proteins (Nidzworski et al. 2017), viral DNAs (Dong et al. 2015), and intact viruses (Chang et al. 2018).

II.C. Nanophotonics.

At the extreme sensitivity limit, the direct electrochemical observation of single electron transfer events in biological systems is a severe challenge. The problem arises, because the generated currents are at, or below, the noise floor of the measurement. Thus, the principal approaches to measuring single molecules electrochemically have relied on amplifying the signal, for example by redox cycling (Zevenbergen et al. 2007). Alternatively, converting redox processes to photon emission events, *i.e.* luminescence, can circumvent the noise floor problem, a fact that was exploited by Xie, who studied single redox enzymes, using the redox-sensitive florescence of flavin adenine dinucleotide (FAD) to monitor enzyme turnover in single molecules of cholesterol oxidase (Lu and Xie 1999).

Although the electrochemical behavior of single molecules has been characterized, observing single electron transfer events is a much more challenging objective (Byers et al. 2015). Lemay *et al.* observed single enzyme electrochemistry in nanolithographic structures producing fA-scale currents commensurate with the electron transfer activity of ~10 molecules (Hoeben et al. 2008). They pioneered the use of a nanometer-spaced anode and cathode on opposite faces of a nanofluidic channel, allowing them to observe correlations between successive single molecule electron transfer events (Zevenbergen et al. 2009).

Another kind of nanophotonic platform – the electrochemical zero mode waveguide (E-ZMW) – consists of a small (d < 100 nm, typ.) cylindrical perforation in a thin metal film, capable of trapping optical radiation, *viz*. **Figure 3** (Levene et al. 2003). The trapped radiation can interact with molecules contained in the zeptoliter-scale (1 zL = 10^{-21} L) volume bounded by the radiation field within the nanopore. The small volumes enclosed by the active region of the E-ZMW, their close proximity to the working electrode, and the small distances over which diffusive mass transport occurs combine to give the E-ZMW some powerful properties, including the ability to observe single electron transfer events using simultaneous optical and electrochemical measurements. Although zero mode waveguides may be constructed as single nanopores, more commonly they are fabricated as a parallel array of nanopores, which is advantageous, because (1) the average occupancy of individual pores may be controlled by concentration, (2) the electrodes in each individual pore are connected to those in all the other pores, so

they can all be controlled at the same potential, E_{appl}, and (3) the electrochemical behavior is integrated over the entire array. The central characteristic of these architectures is that single molecule spectroscopic and electrochemical data can be acquired simultaneously. Thus, fluorigenic molecules, such as the flavoenzymes whose FAD cofactors are strongly fluorescent in the oxidized state and nearly dark in the reduced state ($FADH_2$) (Kao et al. 2008), can be used to follow single electron transfer reaction events by measuring correlated electrochemical and spectroscopic (fluorescence) events at the single enzyme molecule level.(Zaino et al. 2015)

III. **Point-of-Care Devices**

Point of care (PoC) devices are an increasingly important subject for researchers, as the need for diagnostic tools to address under-served populations grows. In response to the need for a control standard for PoC devices, particularly for use in low-resource and developing areas, the World Health Organization (WHO) has established criteria for the manufacture, operation, and efficacy of PoC devices via the Sexually Transmitted Diseases Diagnostics Initiative (SDI). These are known as the ASSURED criteria (Peeling et al. 2006):

- Affordable
- Sensitive
- Specific
- User-friendly
- Rapid and robust
- Equipment-free
- Deliverable to end-users

Specific values for ASSURED criteria vary based on control standards for the specific immunoassay (Yager et al. 2008). Generally, to be considered a viable PoC tool, these devices should have high sensitivity and specificity, be able to withstand storage temperatures in excess of 30ºC, and be simple enough to use that there is little need for extensive training or equipment. In addition, manufacture of PoC devices should be cost-effective while maintaining the test's diagnostic repeatability and reproducibility between production lots. Standards for external quality assurance, which would monitor quality and

viability of the PoC tests, vary by country or region and through national or international bodies such as the European Commission or the U. S. Food and Drug Administration (Kosack 2017).

Lateral flow assays (LFA) are one of the few PoC diagnostic tools that have approached ASSURED criteria, according to the WHO. LFAs commonly utilize either a double antibody sandwich assay or inhibitive immunoassay for analysis. The paper-based dipstick design is most common, wherein a conjugate pad contains dried reagent and a reaction membrane contains control and test lines for analyte determination. Sample mobilizes the reagent—containing conjugate antibody (sandwich assay) or antigen (competitive assay) and signal molecule—and is wicked to the test and control lines which contain the appropriate conjugate antibodies to capture the analyte for the assay. LFAs are straightforward to use, but they are challenged with multiplex assays, typically being constrained to run multiple single assays in parallel. The most well established LFAs are used for the detection of human chorionic gonadotropin (hCG, the human pregnancy hormone), infectious diseases (HIV, hepatitis B), cardiac markers (troponin C, creatinine kinase-MB, myoglobin), and malignancies (Cheng 2016).

Emerging technologies, such as imaging and image analysis, microfluidics, and nanotechnologies in PoC device design have opened new possibilities and posed new challenges (Yager et al. 2006). Advanced cameras and image analysis algorithms render LFA-based diagnostics less labor-intensive, although they require trained operators. Microfluidics allows for small sample volumes and the automation of sample preparation without a highly trained operator and nanotechnologies allow for novel labeling techniques for easier imaging and detection. However, despite their effectiveness, designs using these advances often struggle to adhere to the low-cost manufacturing needs of end-users and commonly require external equipment operations. Paper-based microfluidic devices go some way in solving the issues of high cost and complicated operation (Yetisen et al. 2013). Multilayer paper-based microfluidic assemblies can integrate programmable valves and switches through the use of mechanical force (Martinez et al. 2010). Fluidic timing and metering can be achieved by the introduction of hydrophobic films in microchannels with ±6% of total wicking time precision (Noh and Phillips 2010), while fluidic batteries have shown the ability to power a red LED(λ_{em} = 630 nm) up to 8 min by varying the amount of electrolyte and battery

configuration with maximum short circuit current and open circuit voltage values ranging from 660 µA and 1.3 V for one galvanic cell to 722 µA and 5.0 V for a 16-cell battery (Thom et al. 2012). Thus, these methods have the potential to automate sample treatment and function of the device, though they have yet to be widely adopted by the medical devices industry.

IV. **Micro- and Nano-enabled Electrophotonic Assays**

IV.A. Whole cell detection.

An alternative to PCR-enabled DNA detection and identification of pathogens and infectious agents relies on whole-cell detection (Hu et al. 2018). A label-free and semi-quantitative optical sensing platform was recently developed by our group using whole-cell mediated LSPR wavelength shifts. This versatile sensing platform has the potential to detect a broad range of potential pathogens simply by switching the analyte-specific recognition agents. Here we focus on applications to whole cell bacterial detection, using either aptasensors and siderophore-based devices. In both biosensors, we have achieved a balance in the detection speed (~3 h), sensitivity (1 *P. aeruginosa* cell, and 80 *A. baumannii* cells), and dynamic range (10-10^7 cfu mL^{-1}), which are essentials for an ideal biosensor (Hu and Bohn 2017).

Aptasensor. Aptasensors use aptamers - single strand oligonucleotides with unique secondary and tertiary structures to bind target analytes with high affinities (K_d from nM to pM) as recognition agents (Iliuk et al. 2011). Specific aptamers are selected against their analytes (*e.g.,* whole bacterial cells as represented by bacterial membrane proteins) through a process called systematic evolution of ligands by exponential enrichment (SELEX) (Wilson and Szostak 1999). Importantly, the affinity of aptamers towards their specific targets is comparable to that of antibody-antigen pairs. Moreover, aptamers are stable in a wide range of pH, temperature, and buffer conditions.

In the whole cell LSPR based aptasensor,(Hu et al. 2018) a *Pseudomonas aeruginosa* specific aptamer (Wang et al. 2011), was utilized to detect intact bacterial cells. The overall strategy involves recognition of the surface-bound aptamer by a bacterial membrane transporter protein (Sandy and Butler 2009b). Because the recognition agent (aptamer) is bound to the surface, the bacterium becomes immobilized in

close proximity to the sensing volume of the LSPR elements (nanotriangles). *P. aeruginosa* was chosen as the model microorganism, because there is an urgent need to develop a rapid and accurate diagnostic tool to combat increasing antimicrobial resistance (*Antimicrobial Resistance* 2018). General sensor chip design is shown in **Figure 4(a)**. The aptasensor is based on LSPR response of an hexagonal array of gold nanotriangles, **Figure 4(b)**, which was fabricated using NSL. Sensor chips were subsequently modified with a mixture of biotinylated polyethylene glycol (Bt-PEG) thiol and PEG thiol (1:3, v:v), neutravidin, and biotinylated aptamer (Bt-aptamer) in a sandwich format. PEG thiol was used to prevent non-specific adsorptions onto the sensor surface; Bt-PEG thiol was chosen to bind to neutravidin, which was further linked to Bt-aptamer. This carefully designed surface modification was used to efficiently capture whole cell *P. aeruginosa*. After careful processing, with special attention paid to removing unwanted agents, *e.g.* salts, non-specifically bound macromolecules, *etc.*, and to preventing bacterial cell lysis, the bacteria containing sensor chips, **Figure 4(c)**, were gently washed, dried, and characterized in a uv-visible spectrometer with an integrating sphere. The whole-cell LSPR aptasensor exhibits an astonishing sensitivity, down to a single bacterial cell, with a linear range from 1 - 1000 colony-forming-units (cfu) mL^{-1}, as well as a clinically relevant detection range of 10 – 10^5 cfu mL^{-1}. Additionally, this aptasensor is selective for *P. aeruginosa* strain PAO1 over strain PA14, *Escherichia coli*, and *Staphylococcus aureus*. Currently, the detection time is limited to ~3 hours by the need for careful inactivation of live bacteria. However, the detection time can be reduced to ~ 1 hour or less in clinical settings. In addition, the LSPR aptasensor chips were shown to be stable in ambient conditions for over 2 months, raising the possibility of their use in hostile climates, such as those found in many resource limited areas.

Siderophore-based devices. To demonstrate the versatility of the whole cell bacterial sensing platform, the same sensor chip and basic surface modifications are being utilized in a siderophore-based sensor. Siderophores are a class of small (500 - 1500 Da) molecules synthesized by bacteria, fungi, and plants to acquire ferric iron (Fe^{3+}) with high affinity ($K_f > 10^{30}$) from the environment (Hider and Kong 2010). Due to the low availability of free ferric iron in the environment (10^{-9} to 10^{-18} M) as well as in serum (10^{-24} M), and the fact that ferric iron is essential for key biological processes in almost all microorganisms, plants, and

animals, microbes and plants produce siderophores to chelate ferric iron when the intracellular iron concentration is below ~10^{-6} M (Miethke and Marahiel 2007). More than 500 siderophores have been discovered, of which 270 structures have been identified, falling into three main classes of siderophores: catecholates, hydroxamates, and (α-hydroxy)carboxylates based on the iron binding moieties (Hider and Kong 2010). "Mixed-type" siderophores have also been identified. Different siderophores exhibit various affinities towards ferric iron, ranging over ~30 orders of magnitude. Fe(III) scavenged from the environment (in the form of siderophore-Fe^{3+} complex) is then recognized and actively transported through the microbial cell membrane into the cytoplasm with the assistance of various receptor, binding, and transport proteins.(Sandy and Butler 2009a)

In the siderophore-based LSPR biosensor being developed by our group, we utilize a laboratory synthesized siderophore, specifically a biscatecholate-monohydroxamate mixed ligand siderophore with three polyethylene glycol repeating units linked to a biotin, as a recognition agent in order to target *Acinetobacter baumannii*, a top priority pathogen by the World Health Organization (WHO) in 2017 *(WHO Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed* 2017). Specifically, NSL-fabricated Au nanotriangle arrays were modified with Bt-PEG thiol/PEG thiol (1:3, v:v), neutravidin, Bt-siderophore, and iron (III) acetylacetonate. *A. baumannii* was added and incubated, allowing the bacteria to recognize and bind to the Bt-siderophore-Fe^{3+} complexes on the sensor surface. Bacteria attempt to actively transport the complex through their cell membranes, but given that the Bt-siderophore-Fe^{3+} complexes are anchored onto the sensor surface, the result is that the *A. baumannii* is pulled from solution and immobilized. Initial work on the LSPR siderophore-based sensor has yielded promising results. LSPR response has shown that the siderophore-based biosensor can recognize and detect *A. baumannii* down to 400 cfu mL^{-1} (equivalent to 80 cells), with a dynamic range from 0.4 x 10^3 to 0.4 x 10^7 cfu mL^{-1}. The decreased sensitivity compared to the aptasensor can be explained by: (1) impurities in the synthesized Bt-siderophore, and (2) the inherent affinity of this siderophore recognition system. Additionally, the selectivity and longevity of this siderophore-based biosensor are currently under investigation.

IV.B. Molecular biomarker detection.

Closed bipolar electrochemical assays. Taking advantage of closed BPE (CBE) structures opens up new approaches for electrochemical assays. Redox reactions at the poles (driving electrodes) of the BPE enable electron transfer throughout the entire system, so species with redox activity can be detected straightforwardly within a CBE configuration. CBE systems were initially used for voltammetric studies, in which electrochemical reactions on bipolar microelectrodes and nanoelectrodes were coupled in a CBE architecture and were shown to exhibit different voltammetric responses (Cox et al. 2012; Guerrette et al. 2012). Taking advantage of fluorigenic redox reactions as reporters, they also introduced fluorescence-enabled electrochemical microscopy (FEEM) for imaging transient and heterogeneous electrochemical processes by coupling fluorescence microscopy to CBE structures (Oja and Zhang 2014). This technique enables the use of fluorescence microscopy to observe conventional electrochemical processes on large electrochemical arrays with proper choice of fluorescence probes. Wang and colleagues integrated microfluidics with CBE system to develop a dual-channel mode electrochemiluminescence (ECL) sensing microchip (Luo et al. 2013). In their serial CBE configuration, voltage drop across the ECL reporting cell changed as a function of the conductivity in the analyte cell, thus establishing a quantitative relation between ECL response and the sample conductivity (Zhang et al. 2016c). The use of a serial CBE structure also enables all the oxidants, reductants, or related chemical probes to be determined in different reservoirs in a single device (Zhang et al. 2014). CBEs constructed from interdigitated electrode arrays (IDEAs), which support the coupling of independent redox reactions in a CBE structure were developed by our group. When coupled to fluorescence imaging, this approach allows a diverse set of spatially varying electrochemical phenomena to be explored (Xu et al. 2016c). Extending this idea, rapid, portable, and cost-effective measurement of indicator absorption was used to implement a CBE-enabled method for electrochemical sensing of redox-active biomarkers based on the electrochromic response of MV reporter and rendered in a field-deployable format (Xu et al. 2016b). This method produced a linear relationship among the analyte concentration, the quantity of MV generated, and the colorimetric response, yielding a limit of detection of 1.0 μM. Electrochemical detection has also been performed in a split BPE design, by coupling the electrochemical cell with a LED as a signal readout to provide a low-

cost analytical tool with high throughput for portable analysis (Zhang et al. 2015). In addition to sensing applications, BPE structures have also been used for electrocatalyst screening (Zhang et al. 2016b).

Indirect CBE assays. In the context of diagnostic assays, we define "indirect" as those redox reactions that happen in the analytical cell with the assistance of a redox mediator and/or redox enzyme. As mentioned above, model redox-active analytes like ferricyanide and ferrocene have been used in model CBE sensing assays (Xu et al. 2016a). In order to extend the CBE sensing assays to general biosensing applications, and recognizing that many biologically relevant analytes, *e.g.* metabolites, such as glucose, lactate and uric acid, are not redox active, it is imperative to develop indirect redox processes that can be used in place of direct CBE assays. For biomarkers that are not redox active, electrochemical mediators can be employed to couple enzymatic recognition reactions to the electrode as specific mediator-electrocatalyst pairs (Xu et al. 2017).

The detection of glucose has been used to illustrate the working principle of indirect CBE assays. Glucose, which is not directly redox-active, was mixed with glucose oxidase (enzyme) and ferricyanide (mediator), and then detection was accomplished by the enzyme-catalyzed oxidation of glucose to produce gluconic acid, converting the glucose oxidase back to its reduced form. Reduced glucose oxidase then reacts with ferricyanide to regenerate oxidized glucose oxidase and ferrocyanide. In order to complete the redox cycle, a sufficiently oxidizing potential is applied to working electrode to convert ferrocyanide back to ferricyanide, the resulting current either being measured directly in an amperometric assay or coupled to an MV-containing reporter cell to develop an electrochromic response. Owing to the regeneration of glucose oxidase (oxidized form) and ferricyanide, the reaction continues as long as glucose is present in the mixture. For glucose, a linear response was observed up to 1 mM with a limit of detection (LOD) of ~ 180 μM. More importantly, the scheme outlined here is general for non-redox active analytes that can react with the aid of an oxidoreductase enzyme and electrochemical mediators. As an example, LODs of 110 and 180 μM were obtained for uric acid and lactate, respectively, both of which are sufficient for routine clinical monitoring.

To extend the capability of device for multiplex operation, the indirect CBE assay has been modified by integrating multiple (three) sets of detection chemistries into a single device, to accomplish the simultaneous determination of glucose, lactate, and uric acid, as shown in **Figure 5**. This indirect CBE assay was used to simultaneously detect 2.5 mM each of glucose, lactate, and uric acid. An obvious purple color was observed in the presence of analytes, confirming that multiple analytes can be determined simultaneously and independently by color change in a single multiplex device. Further enhancing the utility and PoC readiness of the device, the analytical cell was fabricated as a disposable, paper-based carbon electrode without any pretreatment. Importantly, both the analytical cell and the reporter cell can be optimized separately. For example, the sensitivity of biomarker detection can be tuned by using reporter cells with different volumes. Since electrochemical reactions are quantitative coulometrically, the number of absorbers ($MV^{+\bullet}$) generated is proportional to analyte concentration, but the absorbance (color change) in the reporter cell is proportional to $MV^{+\bullet}$ concentration. Thus, decreasing the reporter cell volume enhances the sensitivity of the measurement, demonstrating the potential of CBE to test biomarkers with different detection ranges.

Other (future) biomarkers. Besides the above metabolites, other biomarkers can be detected with the CBE strategy. Typical protocols rely on efficient modification of the working electrode with a biomolecular recognition agent, which recognizes and captures the target analyte, producing an electrochemical signal which is read out (Kimmel et al. 2012; Turner 2013; Labib et al. 2016). Numerous biomarkers, including proteins (Wu et al. 2013), DNA/RNA molecules (Wu et al. 2012), and pathogens (Liu et al. 2016a), have been successfully detected by CBE assays. Zhang and co-workers demonstrated fluorescence-enabled CBE assays, which has proven to be capable of recording the transient extracellular vesicle release of neurotransmitter molecules, *e.g.* dopamine (Cox et al. 2012; Oja Stephen and Zhang 2015). Xu and co-workers used ECL-based CBEs to detect several important biomarkers, including HL-60 tumor cells (Zhang et al. 2016a), and prostate-specific antigen (Wu et al. 2015).

Recently, our laboratory has begun to develop integrated CBE-based biosensing platforms aimed at the early and accurate diagnosis of sepsis, a potentially life-threatening syndrome caused by infection.

Several biomarkers are used in clinical tests to diagnose and manage the severity of patient's illness, *e.g.* lactate, C-reactive protein, the liver enzymes ALT and AST, creatinine, procalcitonin (PCT), and bilirubin (Reinhart et al. 2012). There is hope that a multiplex assay ported to a PoC device, by reducing the time to accurate diagnosis, can have a substantive impact on patient outcomes. It is a challenging task detect multiple analytes simultaneously in a portable device. For example, there are enormous concentration differences exhibited by the diagnostic biomarkers in the blood of patients, *e.g.* lactate and PCT are millimolar and nanomolar, respectively, thus making multiple analyte detection (much less quantitation) within one device is extremely complicated. However, the combination of relevant enzyme chemistries and indirect CBE assays, gives impetus to our efforts to solve these problems, and preliminary results indicate that CBE assays have the potential to screen phenotypes that require accurate multiple-biomarker assays in a point-of-care format.

V. **Conclusions**

The inclusion of microscale and nanoscale construction principles into electrophotonic devices has generated a new set of diagnostic devices with improved performance characteristics relative to existing PoC approaches. These diagnostics exploit the capacity of small-scale metal-dielectric assemblies to manipulate electromagnetic radiation and electron transfer reactions simultaneously, making it possible to couple electron transfer events to optical emission, absorption, and scattering readouts, thereby creating new ways to assay for pathogens from the molecular scale up to whole cells. Thus, we continue to look to advances in the previously separate fields of high sensitivity analytical electrochemistry and nanophotonics for exciting new opportunities to be exploited.

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