ACCOUNTS of chemical research

Article

Cite This: Acc. Chem. Res. XXXX, XXX, XXX−XXX

pubs.acs.org/accounts

Achievements and Challenges for Real-Time Sensing of Analytes in Sweat within Wearable Platforms

Published as part of the Accounts of Chemical Research special issue "Wearable Bioelectronics: Chemistry, Materials, Devices, and Systems".

Michael C. Brothers,[†,‡] Madeleine DeBrosse,[†,§,∥] Claude C. Grigsby,[†] Rajesh R. Naik,[†] Saber M. Hussain,[†] Jason Heikenfeld,[§] and Steve S. Kim*[,†]

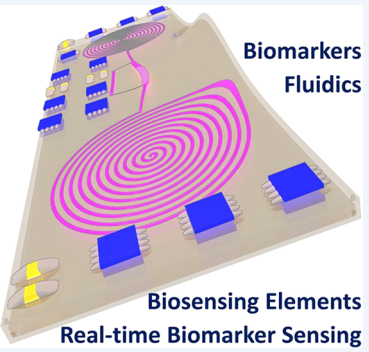
[†]711th Human Performance Wing, Air Force Research Laboratory, Wright-Patterson AFB, Ohio 45433, United States

[‡]UES Inc., Dayton, Ohio 45432, United States

[§]Department of Electrical Engineering and Computer Science, University of Cincinnati, Cincinnati, Ohio 45221, United States

[∥]Oak Ridge Institute for Science and Education (ORISE), Oak Ridge, Tennessee 37830, United States

CONSPECTUS: Physiological sensors in a wearable form have rapidly emerged on the market due to technological breakthroughs and have become nearly ubiquitous with the Apple Watch, FitBit, and other wearable devices. While these wearables mostly monitor simple biometric signatures, new devices that can report on the human readiness level through sensing molecular biomarkers are critical to optimizing the human factor in both commercial sectors and the Department of Defense. The military is particularly interested in real-time, wearable, minimally invasive monitoring of fatigue and human performance to improve the readiness and performance of the war fighter. However, very few devices have ventured into the realm of reporting directly on biomarkers of interest. Primarily this is because of the difficulties of sampling biological fluids in real-time and providing accurate readouts using highly selective and sensitive sensors. When additional restrictions to only use sweat, an excretory fluid, are enforced to minimize invasiveness, the demands on sensors becomes even greater due to the dilution of the biomarkers of interest, as well as variability in salinity, pH, and other physicochemical variables which directly impact the read-out of real-time biosensors.



This Account will provide a synopsis not only on exemplary demonstrations and technological achievements toward implementation of real-time, wearable sweat sensors but also on defining problems that still remain toward implementation in wearable devices that can detect molecular biomarkers for real world applications. First, the authors describe the composition of minimally invasive biofluids and then identify what biomarkers are of interest as biophysical indicators. This Account then reviews demonstrated techniques for extracting biofluids from the site of generation and transport to the sensor developed by the authors. Included in this discussion is a detailed description on biosensing recognition elements and transducers developed by the authors to enable generation of selective electrochemical sensing platforms. The authors also discuss ongoing efforts to identify biorecognition elements and the chemistries necessary to enable high affinity, selective biorecognition elements. Finally, this Account presents the requirements for wearable, real-time sensors to be (1) highly stable, (2) portable, (3) reagentless, (4) continuous, and (5) responsive in real-time, before delving into specific methodologies to sense classes of biomarkers that have been explored by academia, government laboratories, and industry. Each platform has its areas of greatest utility, but also come with corresponding weaknesses: (1) ion selective electrodes are robust and have been demonstrated in wearables but are limited to detection of ions, (2) enzymatic sensors enable indirect detection of metabolites and have been demonstrated in wearables, but the compounds that can be detected are limited to a subset of small molecules and the sensors are sensitive to flow, (3) impedance-based sensors can detect a wide range of compounds but require further research and development for deployment in wearables. In conclusion, while substantial progress has been made toward wearable molecular biosensors, substantial barriers remain and need to be solved to enable deployment of minimally invasive, wearable biomarker monitoring devices that can accurately report on psychophysiological status.

BIOMARKERS IN MINIMALLY INVASIVE BIOFLUIDS FOR WEARABLE SENSORS

Performing minimally invasive biomarker sampling is crucial for building wearables that monitor physiological events that impact human performance (i.e., dehydration, fatigue, and disease) in real time. The authors are interested in detecting biomarkers that are predictors of performance, including identification of fatigued personnel. Biomarkers for human performance have been identified in blood. For example, elevations in creatine kinase, insulin-like growth factor 1, and urea have been associated with fatigue in competitive athletes.[1] Most studies to-date have monitored biomarkers in blood; it is hypothesized that changes in biomarker levels, with some time delay, will

Received: November 3, 2018

Downloaded via UNIV OF CINCINNATI on January 28, 2019 at 20:25:30 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

© XXXX American Chemical Society

DOI: 10.1021/acs.accounts.8b00555
Acc. Chem. Res. XXXX, XXX, XXX−XXX

eventually be observed in minimally invasive biofluids, such as eccrine sweat.[2] Eccrine sweat is preferred among minimally invasive biofluids since it contains few interfering agents and can be collected without contact with mucosal layers, minimizing discomfort.

Simultaneously, eccrine sweat (sweat) has multiple challenges compared to sampling more invasive biofluids. Sweat, being an excretory fluid, varies in salinity (10−100 mM) and pH (4.5−7.0).[3] Previous work has demonstrated the impact of both salinity and pH on sensor outputs and how they impact sensor measurements.[4] Therefore, the wearable device must contain a suite of sensors that measure and account for variations in the physiological biofluid and the subsequent impact on sensor output. Additionally, sweat typically contains lower concentrations of biomarkers of interest than blood or interstitial fluid (ISF) due to filtration. Cortisol, a hormone known to be implicated in acute and chronic stress, has been reported to have an order of magnitude difference in total concentration between blood and sweat[5] but a similar concentration of the free analyte (unbound to transcortin). Glucose concentrations are ∼100× lower in sweat (Table 1); other biomarkers of interest that are typically found at picomolar concentrations in blood have not been quantitated in sweat but are likely to be diluted by 100× or more in sweat due to filtration by the tight junctions in the extracellular matrix (ECM) (Table 1).[6,7] Only very small polar molecules, such as ammonia or water, or small lipophilic molecules, such as steroids, will readily pass through the cellular membrane lining the sweat gland, while small charged ions will be isotonically secreted through the ECM before being reabsorbed in the reabsorptive duct. Small metabolites, such as lactate, in some instances, will be produced locally by the cells during metabolism and therefore will not correspond to blood levels.[2] However, most other charged or larger (>1000 Da) biomarkers will have to move extracellularly through the ECM into sweat. The tight junctions in the ECM effectively act as an analyte filter, explaining the reduced concentrations of biomarkers found in sweat.[6] Since the filtering effect should be constant at constant sweat rates, sweat levels of analytes should also be indicative of serum levels. The reduced concentration of biomarkers in sweat causes real-time sensors to work near their limit of detection or requires analyte extraction or concentration within the device to increase analyte levels to be more compatible with biosensors.

FLUIDIC INTERFACE BETWEEN BIOFLUIDS AND BIOSENSORS

The rate of sweat production varies, even within an individual, dependent upon hydration level, heat stress, and exertion, thus confounding analyte measurement. Human skin contains 10s to 100s of sweat glands per square centimeter. Assuming a rate of 1 nL/gland/min for prolonged sweating, sweat is produced at 10s to 100s of nanoliters of sweat per minute per square centimeter.[3] The authors have discovered that under normal conditions, sweat rates are too unpredictable for continuous sampling. Currently, sweat is chemically stimulated using pilocarpine,[8] which activates the muscarinic receptors within the sweat through topical application and delivery using iontophoresis. However, pilocarpine is rapidly metabolized and produces a localized sweating response for ∼90 min. The Heikenfeld lab has demonstrated that carbachol,[9] an acetylcholine mimic, activates the sweat response for a prolonged period (half-life of 4−8 h). The prolonged sweat response can be ascribed to the enhanced stability of the carbamate bond in carbachol versus the ester in

Table 1. Properties and Concentrations of Selected Biomarkers in Sweat versus Other Biofluids

	Na	K	lactate	glucose	cortisol	drugs	cytokines	antibodies
MW (Da)	23	19	90	180	362	most 100s	>5000−10000s	∼100000s
lipophilicity	very low (ion)	very low (ion)	very low (ion)	low (polar)	high	many are lipophilic	very low	very low
blood plasma	135−145 mM	3.5−5 mM	0.5−10 mM	4.1−6.9 mM	100s of nM total; 10s of nM unbound fraction	most equiv. to unbound in plasma	pM to nM	varies significantly, total ∼0.4−16 mg/mL
interstitial fluid	similar to plasma	similar to plasma	similar to plasma	similar to plasma	unbound is similar to plasma	many equiv. to unbound in plasma	80% of plasma	15−25% of plasma
saliva	10s mM	10s mM	0.1s to 1s mM	∼1% of plasma	unbound is similar to plasma	many equiv. to unbound in plasma	local production dominates	local or very highly diluted
sweat	∼10s of mM	∼5−15 mM	∼5−10s mM	∼1% of plasma	unbound is similar to plasma	Many equiv. to unbound in plasma.	<0.1% of plasma	local or very highly diluted

DOI: 10.1021/acs.accounts.8b00555
Acc. Chem. Res. XXXX, XXX, XXX−XXX

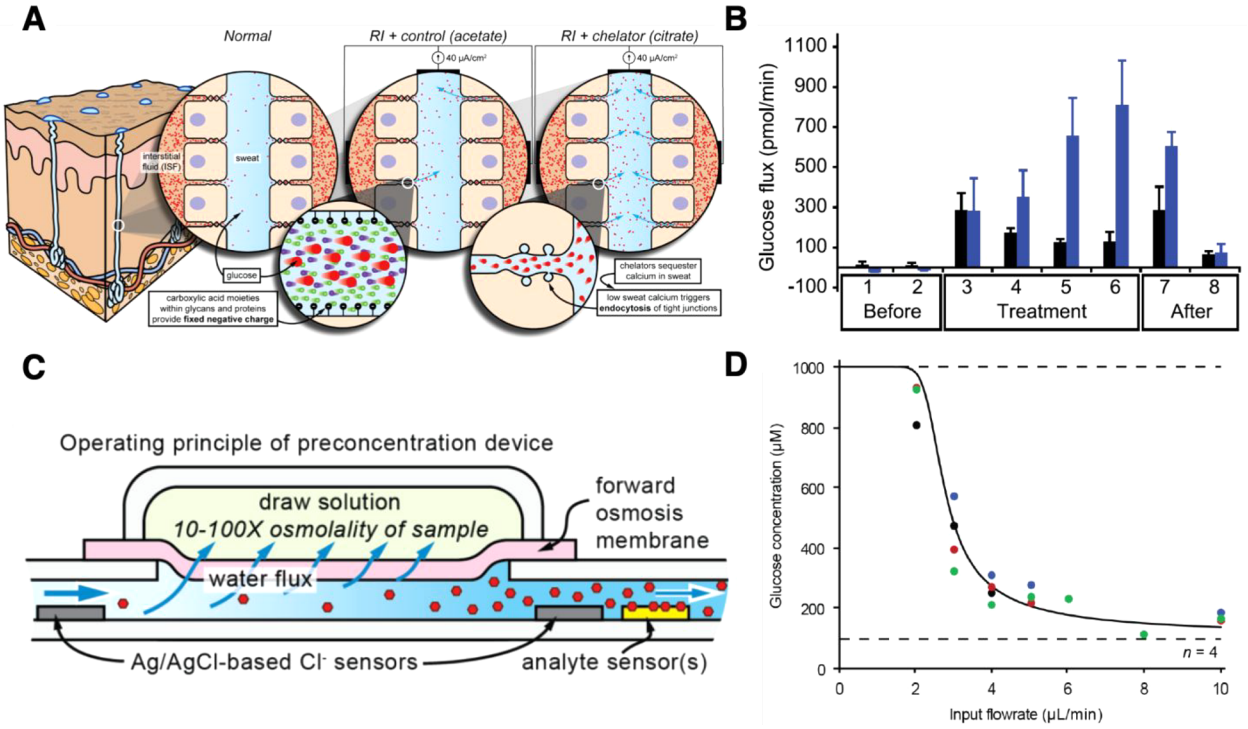


Figure 1. Methods for increasing analyte concentrations in sweat: (A) Reverse iontophoresis in the sweat gland triggers electroosmosis due to negatively charged glycans. Addition of chelating agents causes reversible endocytosis of the ECM, enabling enhanced analyte flux. (B) Approximately 30 min of reverse iontophoresis in the presence of chelating agents causes 5−10× increase in glucose flux compared to iontophoresis alone. (C) Osmotic preconcentration devices use a water permeable membrane and a draw solution to cause controllable concentration until the sample is isosmotic to the draw solution. (D) Biofluid flow rate influences the degree of concentration. Reproduced with permission from refs 7 and 11. Copyright 2018 and 2019 Public Library of Science.

acetylcholine, preventing hydrolysis by acetylcholine esterase. Questions remain about the chemical composition of chemically induced sweat versus heat-induced sweat, and no publications to date have characterized analyte variations between the two methodologies. It is our working hypothesis that most analytes enter through a sweat-rate independent pathway, and thus the mechanism of sweating should not matter[7] and only analyte dilution is caused by increased sweating. Therefore, the authors believe that it is critical to either calculate the dilution of analytes between the ISF and the sweat or determine the ratio of biomarkers to report on the physiological state; the authors prefer the later approach.

The low concentration of analytes in sweat provides challenges for real-time sensors. Reverse iontophoresis[7] has been used to enhance analyte extraction from the analyte rich ISF to sweat by promoting electroosmotic flow, similar to an approach demonstrated by the Glucowatch.[10] In short, reverse iontophoresis occurs upon application of a DC current between two electrodes on the skin, causing the flow of anions toward the electrochemical cathode and the flow of cations toward the electrochemical anode. Since the ECM contains negatively charged glycosamingoglycans within a narrow channel, creating a high ratio of surface charge to volume, a capacitive double layer exists between the glycans and the sodium ions, enabling electroosmosis. The net effect of electroosmosis is analyte extraction, as uncharged analytes migrate toward the electrochemical cathode (Figure 1A).

However, the amount of energy (0.5 mA/cm^2) required to extract even low concentrations of small biomolecules such as glucose is incompatible with low power, wearable devices. In order to enhance analyte flux while minimizing energy consumption, the Heikenfeld lab has found that iontophoretic delivery of a chelating agent can reversibly break apart the tight junctions through sequestration of calcium, causing endocytosis of calmodulin proteins into early endosomes (Figure 1A). The same electric field is used to produce reverse iontophoresis at the anode, causing electroosmosis. The net result is a substantial increase in the flux of analytes due to reduced filtration by the ECM at a power usage 1/10th of the Glucowatch (0.04 mA/cm^2)[7] (Figure 1B). It is speculated that other larger analytes, such as proteins, can be similarly enhanced in concentration, thereby enriching analyte concentrations in sweat without piercing the dermal barrier.

The Heikenfeld lab is simultaneously exploring complementary methodologies to concentrate samples using membrane based concentration techniques that rely on water flux due to an osmotic gradient.[11] In short, an ion impermeable forward osmosis membrane from Fluid Technology Solutions (FTS Rainstick) is placed between the sample and the osmotic draw, causing water flux to the draw solution until the sample and draw solution are isotonic (Figure 1C). This mechanism enables the extent of concentration to be controlled, but at the expense of concentrating interfering salt ions simultaneously, practically limiting analyte concentration to ∼10× at appropriate flow rates (Figure 1D). However, if only the ratio of biomarkers is important, then a salt permeable membrane can be used, enabling continual concentration, and thus the potential for 100× or more concentration of analytes. Through proper design of the membrane, channel length, and osmotic draw solution composition, the system can be optimized for the desired application. The system can also be tuned to balance the inherent trade-off between time-resolution and analyte concen-

DOI: 10.1021/acs.accounts.8b00555
Acc. Chem. Res. XXXX, XXX, XXX−XXX

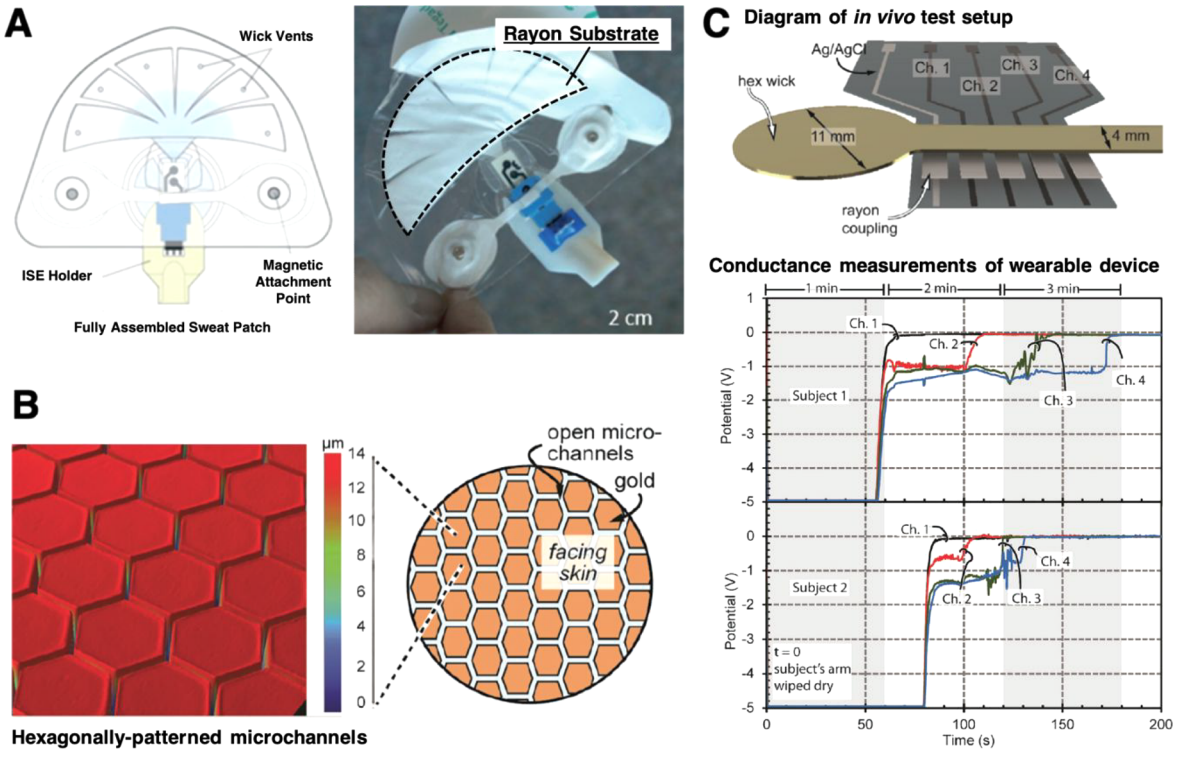


Figure 2. Mechanisms for fluidic transport: (A) Rayon transports liquids from skin along fiber walls to sensors. (B) Hex wick enables nanoliter transport using hydrophilic substrates in patterned microchannels. (C) Attachment of the device to a sweating arm enabled measurement of the speed of wicking as a function of time by relating time of observed potential change to linear distance in an array of electrodes. Reproduced with permission from refs 12 and 13. Copyright 2018 Royal Society of Chemistry.

tration due to the increased fluid requirement for increasing levels of preconcentration.

Sensing platforms are often placed either face-up on the skin surface or behind a protective layer in order to reduce electrochemical and mechanical noise, as well as to prevent abrasion of the sensing elements. A fluidic interface is necessary to control fluid flow and enable temporal resolution at a single-point electrochemical sensor. Most commonly, paper fluidics have been deployed in wearables because of their low cost, ease of implementation, inherent flexibility, and wicking properties. In one exemplary case of a wearable device, regenerated cellulose (rayon) is used as a highly absorbent, thin pad for ion sensing (Figure 2A).[12] The sensor suite is placed off the skin but fluidically connected through the rayon to enable highly selective and robust detection of ions on the skin. The Heikenfeld lab has shown three main obstacles with this approach: (1) the configuration contains ~10 μL of fluidic dead volume between the skin and the sensor, causing time delays between sweat production and sensing, (2) regenerated cellulose can act as a reservoir of analytes, causing errors in measurements, and (3) over the time course of the measurement, sample evaporation is likely to occur.[13]

To reduce the dead volume between the skin and the sensor, the Heikenfeld group developed an open-faced, hexagonal wick that operates at less than 100 nL/cm^2, using a highly hydrophilic, flexible platform. In short, the open microfluidic platform uses capillary flow through open microfluidic channels to transport liquids. The key to this platform is to use either high aspect ratio channels, which are difficult to manufacture, or highly hydrophilic channels. The Heikenfeld group used easy to manufacture 10 μm wide, 15 μm deep channels embedded into a hexagonal pattern, which creates redundancy in the fluidic path and thus removes the impact of defects in the pattern (Figure 2B). For channels generated with imperfect corners, a contact angle below 35° must be maintained to enable capillary flow through the channels and junctions.[13] The key to obtaining this contact angle was (1) deposition of gold on the channel followed by (2) functionalization with a peptide containing multiple thiols to form a highly stable coating on the gold substrate due to avidity principles. The peptide also contained >0.5 carboxylic acid moieties per residue to provide a high charge density at the substrate−liquid interface. Unmodified peptides are generally recognized as safe and thus can be in dermal contact. Additionally, they are relatively inexpensive to synthesize compared to alternative polythiol molecules. Both optical and conductance measurements demonstrated nanoliter fluidic transport (Figure 2C). Conductance measurements were acquired through placement of a series of electrodes on the hex wick and measuring the time needed to observe a change in voltage potential, demonstrating primitive sensor integration as well as fluid transport. Thus, by functionalizing an inorganic material with a biological element, the properties could be manipulated to enable small volume transfer necessary to enable real-time sensing.

BIOSENSING RECOGNITION ELEMENTS AND TRANSDUCERS

Real-time sensing requires specific recognition of the analyte of interest. Ions are most readily detected using an ionophore recognition element (RE). Ionophores are molecules that are primarily hydrophobic but have a polar binding pocket whose shape and charge density enables selectivity to the ion of interest (Figure 3A). Commercially available ionophores have been demonstrated to typically have at least 2 to 3 orders of

DOI: 10.1021/acs.accounts.8b00555
Acc. Chem. Res. XXXX, XXX, XXX−XXX

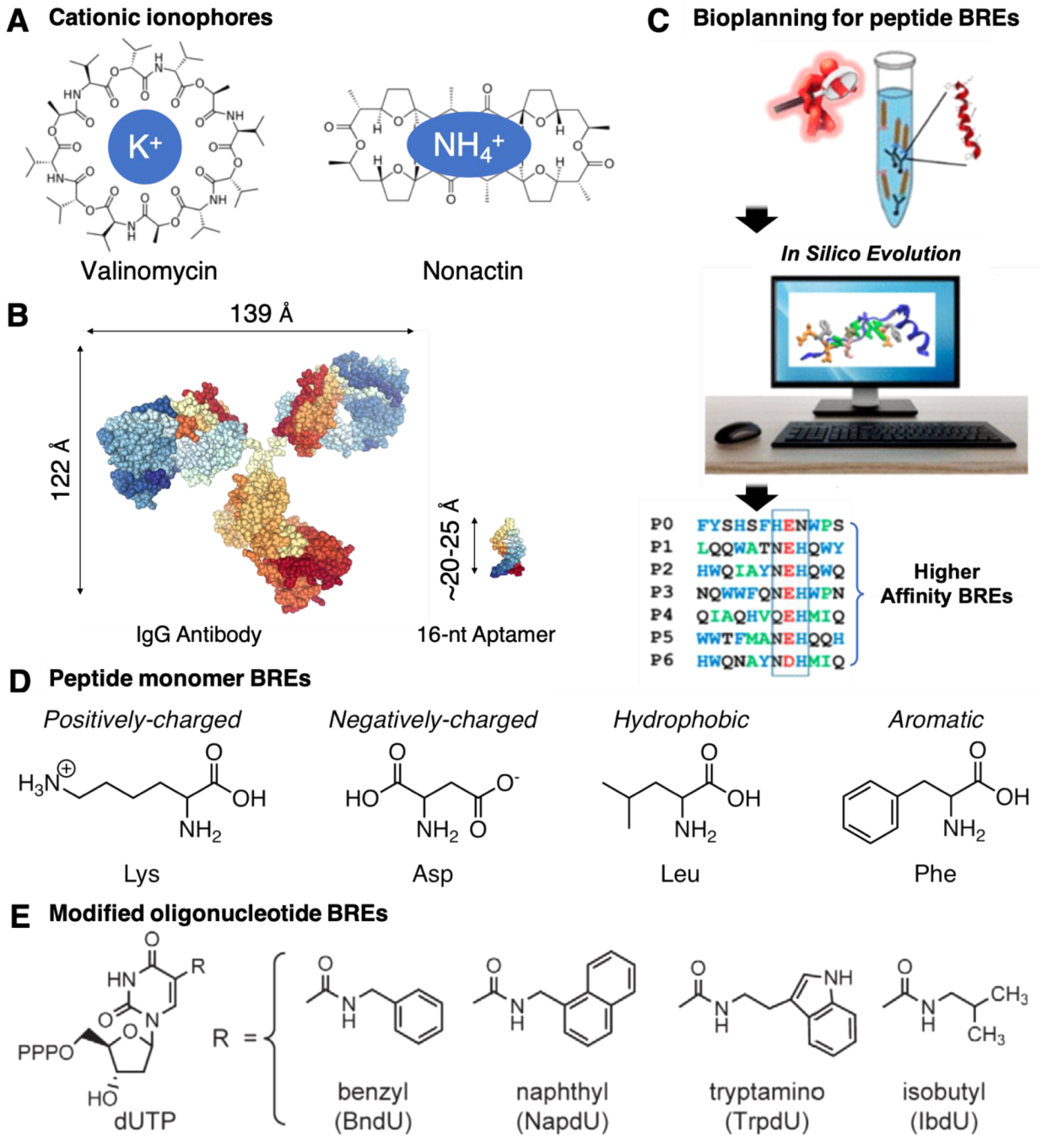


Figure 3. Rationale behind REs: (A) Ionophores for cations contain ether and carboxyl groups in ion binding pocket. (B) Cartoon of standard BREs to scale demonstrating differences in size. (C) Combined computation and experimental process for optimization of BREs to targets of interest. (D) BREs containing diverse functional groups enable selectivity and sensitivity innately for peptides, or (E) using modified oligonucleotides for aptamers. Reproduced with permission from ref 16, Copyright 2018 American Chemical Society, and ref 17, Copyright 2010 Public Library of Science.

magnitude selectivity toward the ion of interest versus other ions[14] upon proper formulation of a cocktail containing poly(vinyl chloride) (PVC), detergents, ionophores, and additional agents to minimize noise. Small molecules that are part of the metabolic process are most readily detected using protein biorecognition elements (PBREs). Enzymes are a subclass of PBREs that in some instances directly or indirectly produce molecules that can be detected through electrochemical means. Enzymes typically work through a lock and key mechanism where binding of the substrate causes a reversible or irreversible catalytic conversion, followed by release of the substrate. The inherent selectivity and production of a measurable output make enzymes ideal biorecognition elements (BREs) when available that can be implemented in current wearable platforms.

Detection of large molecules, steroid hormones, and non-steroid hormones are typically performed using either PBREs or synthetic BREs (SBREs). PBREs, including the 150 kDa antibody (Figure 3B), show strong affinity and selectivity to the target analyte. However, their size and stability issues have caused exploration of smaller fragments. Nanobodies, or single domain antibodies, are subfragments of antibodies from the

DOI: 10.1021/acs.accounts.8b00555
Acc. Chem. Res. XXXX, XXX, XXX−XXX

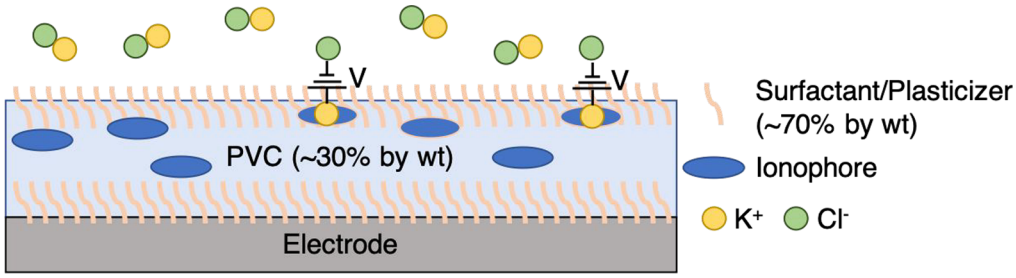


Figure 4. Solid-state ISEs consist of plasticized PVC doped with ionophores deposited on an electrode. Binding of the ionophore to the ion creates a charge separation, resulting in a measurable potential corresponding to analyte concentration.

variable chain region[15] that retain binding specificity with improved stability. SBREs have been used in biosensors to mimic the binding activity of naturally occurring biomolecules. Peptides and oligonucleotide aptamers are appealing as they can be selected for from large combinatorial libraries (>10^9 random sequences), designed *in silico* (Figure 3C) and synthesized with non-native nucleic acids or functional groups (Figure 3B). Recently, Naik and co-workers used *in silico* approaches to increase the binding affinity of a troponin-binding peptide (K_d = 0.23 nM) and then demonstrated its efficacy using localized surface plasmon resonance (LSPR) and electrochemical impedance spectroscopy (EIS) sensing platforms.[16] Additionally, screening of combinatorial libraries has revealed unique target specific BREs against viruses, bacteria, and human performance biomarkers.[16] The synthetic short peptides have advantages over other BREs with regards to prolonged stability, while retaining binding specificity due to the diversity of functional groups (particularly charged and hydrophobic) on the side chains (Figure 3D). Complimentarily, aptamers with modified functional groups that mimic PBREs (Figure 3E)[17] can also provide high selectivity and sensitivity toward analytes.

Novel carbon and 2D materials are promising sensing elements due to their conductive properties and band gaps that can be exploited for molecular sensing. Carbon nanomaterials, including carbon nanotubes and graphene, are useful as transduction elements in wearable electronic sensors. These nanomaterials have robust electronic properties, as well as a high surface-to-volume ratio, without any compromise on the mechanical properties that wearable sensors require.[18] However, coupling BREs to carbon and 2D sensing transducers imposes significant challenges, primarily due to their atomically thin and nanometer sized dimensions. The authors have led the discovery of BREs that bind directly to the sensing materials, including carbon nanotubes,[19,20] graphene,[21] and MoS_2[22] using phage display, and further extended their research scope to develop the tools for probing the biotic−abiotic interface. For example, the authors revealed that the interactions between monolayer graphene and peptides are (1) not influenced by their underlying substrate, (2) affected by the transducing graphene quality, and (3) not dependent on the number of graphene layers.[23] Overall, thorough understanding of the biotic−abiotic interface is expected to open the path toward building molecular-level controlled bio−nano hybrid biosensors, and enable specific analyte sensing through functionalization with BREs.

REAL-TIME SENSING TECHNOLOGIES FOR BIOMARKER DETECTION IN WEARABLE DEVICES

Wearable sensors will enable real-time monitoring of biomarkers; each sensing platform listed below works only for specific classes of analytes, each with their unique challenges toward implementation in real-time biosensing. For this Account, we will refer to real-time monitoring as any methodology that operates for at least 8 h and provides time-resolved measurements at least every 15 min. Sensor platforms to determine binding of two macromolecules for a real-time wearable sensor are difficult, as they must be (1) highly stable (i.e., operating in an unadultered biofluid), (2) portable, (3) reagentless, (4) continuous, and (5) responsive in real time. We have down-selected to those methodologies that we have pursued and in many cases demonstrated through this collaboration for detection of ions, small molecules, and proteins and discuss exemplary results and remaining challenges.

Detection of ions has been pursued and demonstrated using ion selective electrodes (ISEs). ISEs are formed by placing an ion-selective membrane on top of a conducting electrode and measuring the change in potential upon binding of the ion of interest; the electronic potential varies as the ion of interest is partitioned into and out of the hydrophobic phase through binding to its ionophore, generating charge separation (Figure 4). This results in a potential that can be measured and correlated to concentration using the Nernst equation.[14,24] ISEs have been used in wearable devices for measurement of sodium,[12,25] potassium,[12] calcium,[26] and pH.[26] Minimizing measurement drift is critical to accurate sensing through (1) selection of mechanically and chemically robust materials, (2) preconditioning of the ISE in a reference fluid to hydrate the surface, and (3) accounting for residual drift during analysis. Typically the Ag/AgCl reference electrode is used because of its simplicity and in conjunction with the ISE used to report on salinity; however, since it is by definition a chloride sensor, it is a poor choice in sweat, as anions including chloride, lactate, and carbonate[27] are known to have order of magnitude changes and not be covariant. Therefore, solid-state reference electrodes, including those using ionic liquids are preferred; only one exemplary device[12] (Figure 2A) has deployed this critical component.

Small molecule detection to date has been demonstrated most commonly using enzymatic sensing. Enzyme based sensing is ubiquitous in the form-factor of the glucose test strip and relies upon the enzyme to convert the analyte of interest directly or indirectly into a molecule that can be sensed. In general, the substrate embeds itself into the active site, causes a catalytic conversion mediated by a redox cofactor, and then releases from the binding pocket.[28] In the most common enzymatic sensors, $NADH/FADH_2$ are produced as a byproduct of certain enzymatic reactions (Figure 5A). These redox cofactors then react with and reduce redox mediators (i.e., Prussian blue), which can then be directly measured at the electrode surface by application of an oxidation potential. Currently, wearable

DOI: 10.1021/acs.accounts.8b00555
Acc. Chem. Res. XXXX, XXX, XXX−XXX

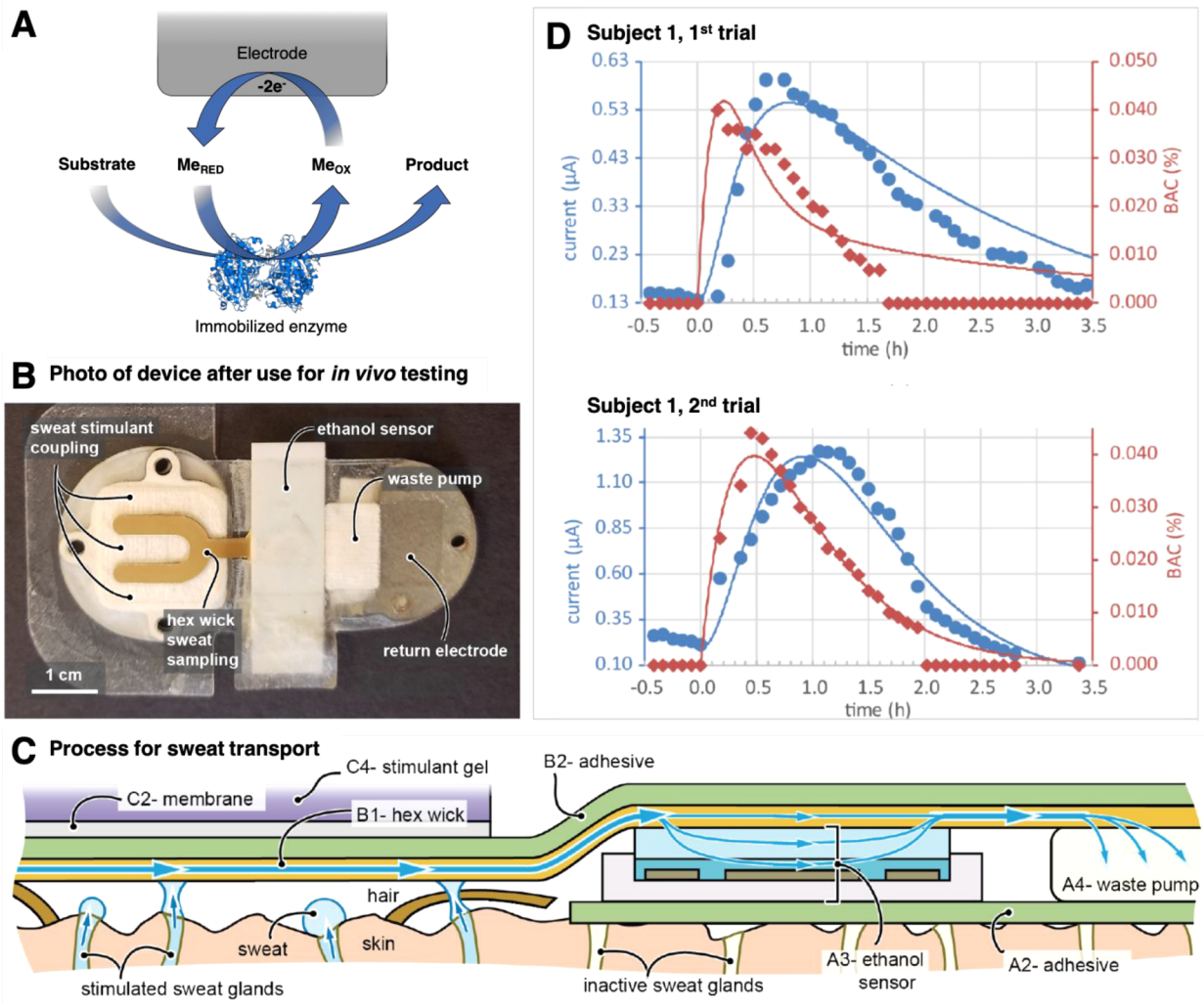


Figure 5. Demonstration of an integrated real-time, enzymatic sensor for ethanol: (A) Enzymatic sensors measure NADH/$FADH_2$ generated upon enzymatic catalysis, converting cofactors NAD^+/FAD to the reduced state. Electrochemical reduction of NADH/$FADH_2$ to NAD^+/FAD is then measured using chronoamperometry. (B) Picture and (C) schematic of the integrated device: (1) agarose gel with carbachol (C4) for sweat stimulation, (2) hex wick for fluid transport (B1), (3) alcohol oxidase immobilized in PDMS coupled via fumed silica to the hex wick (A3), and (4) waste pump containing desiccant (A4). (D) Measurement of alcohol breath values in two subjects correlates with sweat values with an offset of 10s of minutes, identifying the time for an ingested analyte to be observed in eccrine sweat. Reproduced with permission from ref 31. Copyright 2018 Royal Society of Chemistry.

enzymatic sensors have been demonstrated using $FADH_2$ cofactors for lactate,[29] glucose,[29,30] and ethanol[31] (Figure 5A). In a more recent study, pH, temperature, and humidity corrections were applied, resulting in more accurate correlations between sensor output and biochemical concentration.[30] In order to provide the first demonstration of a nontrivial, real-time sensor directly correlated to the physiologic state in a wearable device, the Heikenfeld lab integrated many of the components listed in previous sections, primarily sweat stimulation using carbachol, fluidic transport using a hexagonal wick, and an alcohol oxidase BRE, into an integrated sensing device (Figure 5B,C). Chemically stimulated sweat was generated via iontophoresis in order to maintain a near-constant flow rate across the sensor for the duration of the experiment. Fumed silica or silica in gelatin was used to couple the hex wick[13] to the enzymatic sensor; fumed silica was chosen due to its porous nature that allows permeation of small molecules, such as ethanol and water, through the matrix to the enzymatic sensor. Through careful selection and integration of device components, the first data was demonstrated that correlates sweat ethanol to breath ethanol, with a measured lag time of 10s of minutes according to sweat sensor measurements versus breathalyzer measurements (Figure 5D). This for the first time provided real-time data demonstrating the correlation of sweat to other biofluids for an ingested compound, as well as the time for an ingested analyte to enter sweat. More complex sensors need to be developed to integrate into this platform, as well as methodologies to enable detection of nontrivial biomarkers. Regardless, this work served as the apex of over 5 years of collaborative efforts between the authors.

While these initial proof of concept tests are promising, to date, no enzymatic sensor that compensates for all confounding factors has been demonstrated in a wearable device. Correcting for all physiological variability is imperative in excretory fluids versus fluids in homeostasis and is discussed in detail in the patent literature.[32] In short, enzyme structure and function is sensitive to changes in pH, resulting in changes in protonation states of amino acids, modifying conformation or reactivity, thus impacting enzyme activity. Enzymes by definition consume target analytes and thus are sensitive to analyte flux. Therefore

DOI: 10.1021/acs.accounts.8b00555
Acc. Chem. Res. XXXX, XXX, XXX−XXX

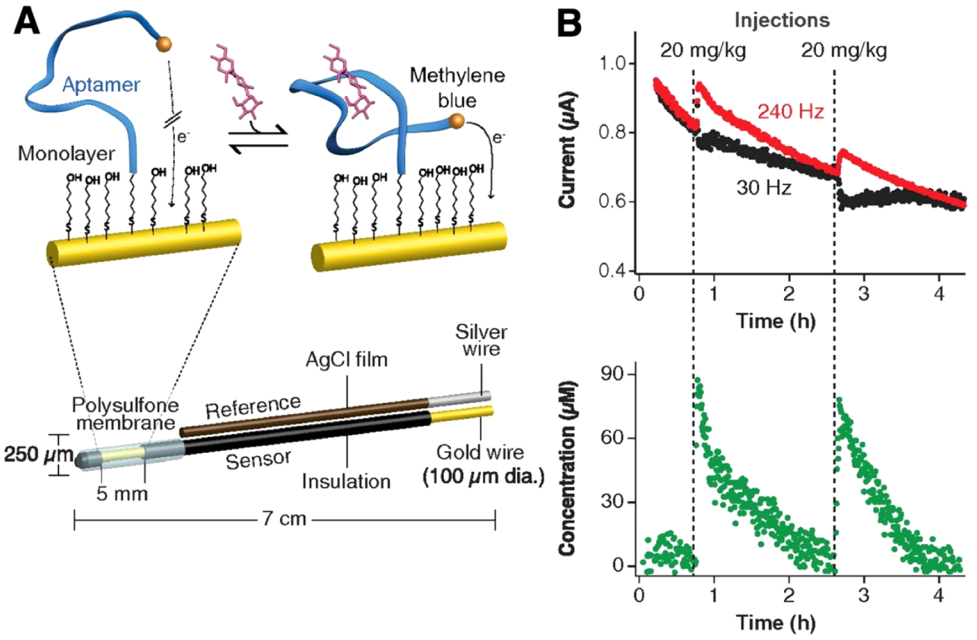


Figure 6. E-AB/Impedance based sensors for real-time measurement of analytes: (A) A conformation change upon binding causes the tethered redox tag to move closer to or further away from the surface, impacting the rate of charge transfer. Application of a polysulfone membrane over sensor enabled measurement of kanamycin in rats by reducing biofouling. (B) Measuring at multiple frequencies enables drift correction through reporting the ratio of current. Reproduced with permission from ref 36. Copyright 2017 National Academy of Sciences.

changes in flow rate will accompany a change in sensor output; an increase in flow rate results in higher enzyme/sensor output, while a decrease in flow rate results in lower enzyme/sensor output. Finally, changes in salinity will impact intermolecular forces, thus modifying binding and release enzyme kinetics, modifying the output. All of these factors must be accounted for, using either a table or algorithms, to report accurate analyte concentrations.

Most biomarkers of interest, including proteins and steroid hormones, cannot be detected to date through enzymatic sensors or ISEs. Therefore, the authors are pursuing sensors using electrochemical techniques,[16] including EIS and square wave voltammetry (SWV), because of their promise as reagentless detection techniques to enable real-time detection. Two approaches have succeeded in making this technique reagentless: (1) electrochemical aptamer-based sensors (E-AB) (or derivatives thereof) formed by directly tethering a redox tag to the BRE[33,34] and (2) coupling of a redox tag to a SAM containing BREs.[35] The former approach relies on the BREs undergoing a change in conformation or dynamics as a function of analyte binding. When the redox tag is placed in an opportune spot, the distance between the redox tag and the surface changes upon binding, thus modifying the resistance to charge transfer (R_{CT}) (Figure 6A). To date, E-AB sensors have not been demonstrated in a wearable device. However, the Plaxco group measured kanamycin concentrations in an ambulatory rat for hours using multifrequency SWV; sampling different time points in the kinetic oxidation of methylene blue and reporting the ratio enables correction for sensor drift and thus real-time measurements of kanamycin ingestion and clearance (Figure 6B).[36] The alternative approach has been demonstrated to-date only on *E. coli*,[35] where binding of *E. coli* to maginin caused a decrease in impedance as the tethered redox tag is constrained to the sensor surface.

Physiological variability in sweat creates additional challenges for EIS sensing modalities. Changes in pH impact the redox potential of commonly used redox reporters that transfer protons. In response, the authors have identified redox tags that are pH-independent that operate at low voltages.[37] Additionally, sensor measurements and changes in R_{CT} due to target detection are often masked by nonspecific binding. To mitigate this, the following strategies have been deployed: (1) filtration using a polysulfone membrane (Figure 6A), (2) immobilization of the E-AB in a collagen matrix,[38] and (3) deposition of antifouling, zwitterionic phosphocholine head groups on the sensor surface.[39] Both collagen and polysulfone membranes prevent fouling by preventing proteins from physically approaching the sensing element surface; however, this limits their use for detection of small molecules. Zwitterionic antifouling coatings, in contrast, use highly charged, inherently charge-balanced surfaces to attract water to form a hydration shell that prevents hydrophobic interactions. Limitations still exist to sensor stability due to spontaneous oxidation of the gold−thiol bond; the use of polythiols has been demonstrated to improve longevity and resiliency to sample acquisition in sensors[33] and on gold surfaces.[13] Finally, EIS-based sensors have the greatest dynamic range when the BRE on the sensing element has a dissociation constant (K_D) close to the concentration of analyte in the biofluid. However, BREs designed for low K_D (sub-picomolar) typically decrease off-rate kinetics and thus require hours for complete dissociation, causing a time lag for real-time monitoring. Local depletion of biomarkers at the sensor also becomes problematic. For example, 1 μL of sample containing 1 nM analyte contains only 6.02×10^8 analytes; in contrast even a 1 mm^2 probe contains $\sim 10^{12}$ BREs on the surface. Therefore, larger samples of biofluids must be sampled, the flow rate must be accounted for in sensor response, or the number of BREs must be minimized.

CONCLUSIONS

Real-time biosensing in sweat is in many ways the "Holy Grail" of health monitoring but presents multidisciplinary challenges

DOI: 10.1021/acs.accounts.8b00555
Acc. Chem. Res. XXXX, XXX, XXX−XXX

that require diverse solutions. Substantial improvements in sweat handling and transport have enabled the first demonstration of a real-time sensor in conjunction with a predictable biological event, demonstrating the ability for sweat biomonitoring to be predictive of the physiologic state. However, substantial research remains through collaborative efforts to improve sensing technologies and capabilities to enable the end goal of minimally invasive, real-time health monitoring that will result in real-time monitoring of human performance using biomarkers. More specifically, additional work is needed to identify, validate, and quantitate biomarkers in sweat, to optimize sweat generation, transport, and processing, to identify BREs toward analytes of interest, and to develop highly robust sensing platforms that can operate in the field. Only then can human performance monitoring be realized, enabling mitigation strategies to be implemented proactively, thus improving outcomes for human health and performance.

AUTHOR INFORMATION

Corresponding Author

*E-mail: Steve.Kim.13@us.af.mil.

ORCID

Rajesh R. Naik: 0000-0002-7677-928X
Jason Heikenfeld: 0000-0001-5778-8343
Steve S. Kim: 0000-0002-9519-077X

Notes

The authors declare the following competing financial interest(s): Jason Heikenfeld is the CSO and founder of Eccrine Systems, a company developing wearable devices for sweat sensing.

Biographies

Michael Brothers is a Research Scientist and Technical Program Manager at the 711th Human Performance Wing, AFRL, and UES Inc. researching chemical and biological sensors. Dr. Brothers has expertise in biowarfare agents, novel devices, and sensors.

Madeleine DeBrosse is an engineering Ph.D. student at the University of Cincinnati. Ms. DeBrosse is working on novel device design in collaboration with AFRL. Madeleine has experience in nanotoxicology and novel devices.

Claude Grigsby, Technical Advisor for the Analytics and Biosciences Division of the 711th Human Performance Wing, AFRL, is an analytical chemist, with 27 years of experience in environmental sample analysis and mass spectrometry-based biomarker discovery.

Rajesh R. Naik obtained his Ph.D. in the field of molecular biology from Carnegie-Mellon University. Currently, he is the Chief Scientist of the 711th Human Performance Wing of the Air Force Research Laboratory.

Saber Hussain is the Core Research Area lead for molecular sensing and physiology at the 711th Human Performance Wing, AFRL, and led efforts for elucidating the biomolecular interactions of nanomaterials. Dr. Hussain has expertise in toxicology and sensor development.

Jason Heikenfeld is the Assistant Vice-President of Commercialization and Professor of Engineering at the University of Cincinnati and is a cofounder of Eccrine Systems Inc.

Steve Kim is a Research Physical Scientist at the 711th Human Performance Wing, Wright-Patterson AFB, OH. Dr. Kim obtained his Ph.D. from the University of Connecticut (2007). Dr. Kim has expertise in biosensor development for the USAF.

ACKNOWLEDGMENTS

All authors acknowledge Drs. Benji Maruyama, Ahmad Islam, Joseph Slocik, and Ariana Nicolini at the Materials and Manufacturing Directorate, U.S. Air Force Research Laboratory for their helpful discussion. All authors acknowledge funding support from the U.S. Air Force Research Laboratory. J.H. acknowledges funding from the National Science Foundation and the industrial members of the Center for Advanced Design and Manufacturing of Integrated Microfluidics (NSF I/UCRC award number IIP-1738617).

REFERENCES

(1) Hecksteden, A.; Skorski, S.; Schwindling, S.; Hammes, D.; Pfeiffer, M.; Kellmann, M.; Ferrauti, A.; Meyer, T. Blood-Borne Markers of Fatigue in Competitive Athletes - Results from Simulated Training Camps. *PLoS One* **2016**, *11*, e0148810.

(2) Moyer, J.; Wilson, D.; Finkelshtein, I.; Wong, B.; Potts, R. Correlation Between Sweat Glucose and Blood Glucose in Subjects with Diabetes. *Diabetes Technol. Ther.* **2012**, *14*, 398−402.

(3) Sonner, Z.; Wilder, E.; Heikenfeld, J.; Kasting, G.; Beyette, F.; Swaile, D.; Sherman, F.; Joyce, J.; Hagen, J.; Kelley-Loughnane, N.; Naik, R. The Microfluidics of the Eccrine Sweat Gland, Including Biomarker Partitioning, Transport, and Biosensing Implications. *Biomicrofluidics* **2015**, *9*, 031301.

(4) Hianik, T.; Ostatná, V.; Sonlajtnerova, M.; Grman, I. Influence of Ionic Strength, pH and Aptamer Configuration for Binding Affinity to Thrombin. *Bioelectrochemistry* **2007**, *70*, 127−133.

(5) Jia, M.; Chew, W.; Feinstein, Y.; Skeath, P.; Sternberg, E. Quantification of Cortisol in Human Eccrine Sweat by Liquid Chromatography - Tandem Mass Spectrometry. *Analyst* **2016**, *141*, 2053−2060.

(6) Heikenfeld, J.; Jajack, A.; Feldmen, B.; Granger, S.; Gaitonde, S.; Begtrup, G.; Katchman, B. Peripheral Biochemical Monitoring: A Critical Review of Analyte Access for Interstitial Fluid, Saliva, and Sweat. *Nat. Biotechnol.* **2019**, submitted for publication.

(7) Jajack, A.; Brothers, M.; Kasting, G.; Heikenfeld, J. Enhancing Glucose Flux into Sweat by Increasing Paracellular Permeability of the Sweat Gland. *PLoS One* **2018**, *13*, e0200009.

(8) Yang, Y.; Gao, W. Wearable and Flexible Electronics for Continuous Molecular Monitoring. *Chem. Soc. Rev.* **2019**, DOI: 10.1039/C7CS00730B.

(9) Simmers, P.; Li, S. K.; Kasting, G.; Heikenfeld, J. Prolonged and Localized Sweat Stimulation by Iontophoretic Delivery of the Slowly-Metabolized Cholinergic Agent Carbachol. *J. Dermatol. Sci.* **2018**, *89*, 40−51.

(10) Tierney, M.; et al. Clinical Evaluation of the GlucoWatch Biographer: A Continual, Non-Invasive Glucose Monitor for Patients with Diabetes. *Biosens. Bioelectron.* **2001**, *16*, 621−629.

(11) Jajack, A.; Stamper, I.; Gomez, E.; Brothers, M.; Begtrup, G.; Heikenfeld, J. Continuous, Quantifiable, and Simple Osmotic Preconcentration and Sensing within Microfluidic Devices. *PLoS One* **2019**, *14*, e0210286.

(12) Alizadeh, A.; Burns, A.; Lenigk, R.; Gettings, R.; Ashe, J.; Porter, A.; McCaul, M.; Barrett, R.; Diamond, D.; White, P.; Skeath, P.; Tomczak, M. A Wearable Patch for Continuous Monitoring of Sweat Electrolytes during Exertion. *Lab Chip* **2018**, *18*, 2632−2641.

(13) Twine, N.; Norton, R.; Brothers, M.; Hauke, A.; Gomez, E.; Heikenfeld, J. Open Nanofluidic Films with Rapid Transport and No Analyte Exchange for Ultra-Low Sample Volumes. *Lab Chip* **2018**, *18*, 2816−2825.

(14) Bühlmann, P.; Chen, L. Ion-Selective Electrodes with Ionophore-Doped Sensing Membranes. In *Supramolecular Chemistry: From Molecules to Nanomaterials*; Gale, P., Steed, J., Eds.; Wiley-Liss: Hoboken NJ, 2012; pp 2539−2579.

(15) Dmitriev, O.; Lutsenko, S.; Muyldermans, S. Nanobodies as Probes for Protein Dynamics in Vitro and in Cells. *J. Biol. Chem.* **2016**, *291*, 3767−3775.

DOI: 10.1021/acs.accounts.8b00555
Acc. Chem. Res. XXXX, XXX, XXX−XXX

(16) Xiao, X.; Kuang, Z.; Slocik, J.; Tadepalli, S.; Brothers, M.; Kim, S.; Mirau, P.; Butkus, C.; Farmer, B.; Singamaneni, S.; Hall, C.; Naik, R. Advancing Peptide-Based Biorecognition Elements for Biosensors Using *in-Silico* Evolution. *ACS Sensors* **2018**, *3*, 1024−1031.
(17) Gold, L.; Ayers, D.; Bertino, J.; Bock, C.; Bock, A.; Brody, E.; Carter, J.; Dalby, A.; Eaton, B.; Fitzwater, T. Aptamer-Based Multiplexed Proteomic Technology for Biomarker Discovery. *PLoS One* **2010**, *5*, e15004.
(18) Bandodkar, A.; Wang, J. Non-Invasive Wearable Electrochemical Sensors: A Review. *Trends Biotechnol.* **2014**, *32*, 363−371.
(19) Pender, M.; Sowards, L.; Hartgerink, J.; Stone, M.; Naik, R. Peptide-Mediated Formation of Single-Wall Carbon Nanotube Composites. *Nano Lett.* **2006**, *6*, 40−44.
(20) Kuang, Z.; Kim, S.; Crookes-Goodson, W.; Farmer, B.; Naik, R. Biomimetic Chemosensor: Designing Peptide Recognition Elements for Surface Functionalization of Carbon Nanotube Field Effect Transistors. *ACS Nano* **2010**, *4*, 452−458.
(21) Kim, S.; Kuang, Z.; Slocik, J.; Jones, S.; Cui, Y.; Farmer, B.; McAlpine, M.; Naik, R. Preferential Binding of Peptides to Graphene Edges and Planes. *J. Am. Chem. Soc.* **2011**, *133*, 14480−14483.
(22) Muratore, C.; Juhl, A.; Stroud, A.; Wenbi Lai, D.; Jawaid, A.; Burzynski, K.; Dagher, J.; Leuty, G.; Harsch, C.; Kim, S.; Ngo, Y.; Glavin, N.; Berry, R.; Durstock, M.; Derosa, P.; Roy, A.; Heckman, E.; Naik, R. Printed Biomolecular Templates for 2D Material Patterning. *Appl. Phys. Lett.* **2018**, *112*, 233704.
(23) Kim, S.; Kuang, Z.; Ngo, Y.; Farmer, B.; Naik, R. Biotic-Abiotic Interactions: Factors That Influence Peptide-Graphene Interactions. *ACS Appl. Mater. Interfaces* **2015**, *7*, 20447−20453.
(24) Hu, J.; Stein, A.; Bühlmann, P. Rational Design of All-Solid-State Ion-Selective Electrodes and Reference Electrodes. *TrAC, Trends Anal. Chem.* **2016**, *76*, 102−114.
(25) Emaminejad, S.; Gao, W.; Wu, E.; Davies, Z.; Yin Yin Nyein, H.; Challa, S.; Ryan, S.; Fahad, H.; Chen, K.; Shahpar, Z.; Talebi, S.; Milla, C.; Javey, A.; Davis, R. Autonomous Sweat Extraction and Analysis Applied to Cystic Fibrosis and Glucose Monitoring Using a Fully Integrated Wearable Platform. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 4625−4630.
(26) Nyein, H.; Gao, W.; Shahpar, Z.; Emaminejad, S.; Challa, S.; Chen, K.; Fahad, H.; Tai, L.; Ota, H.; Davis, R.; Javey, Al A Wearable Electrochemical Platform for Noninvasive Simultaneous Monitoring of Ca^{2+} and pH. *ACS Nano* **2016**, *10*, 7216−7224.
(27) Bijman, J.; Quinton, P. Lactate and Bicarbonate Uptake in the Sweat Duct of Cystic Fibrosis and Normal Subjects. *Pediatr. Res.* **1987**, *21*, 79−82.
(28) Zhu, Z.; Garcia-Gancedo, L.; Flewitt, A.; Xie, H.; Moussy, F.; Milne, W. A Critical Review of Glucose Biosensors Based on Carbon Nanomaterials: Carbon Nanotubes and Graphene. *Sensors* **2012**, *12*, 5996−6022.
(29) Gao, W.; Emaminejad, S.; Nyein, H.; Challa, S.; Chen, K.; Peck, A.; Fahad, H.; Ota, H.; Shiraki, H.; Kiriya, D.; Lien, D.-H.; Brooks, G.; Davis, R.; Javey, A. Fully Integrated Wearable Sensor Arrays for Multiplexed in Situ Perspiration Analysis. *Nature* **2016**, *529*, 509−514.
(30) Lee, H.; Song, C.; Hong, Y.; Kim, M.; Cho, H.; Kang, T.; Shin, K.; Choi, S.; Hyeon, T.; Kim, D. Wearable/Disposable Sweat-Based Glucose Monitoring Device with Multistage Transdermal Drug Delivery Module. *Sci. Adv.* **2017**, *3*, e1601314.
(31) Hauke, A.; Simmers, P.; Ojha, Y.; Cameron, B.; Zhang, T.; Twine, N.; Brothers, M.; Gomez, E.; Heikenfeld, J.; Ballweg, R. Complete Validation of a Continuous and Blood-Correlated Sweat Biosensing Device with Integrated Sweat Stimulation. *Lab Chip* **2018**, *18*, 3750−3759.
(32) Heikenfeld, J. C.; Bertrand, J. A.; Brothers, M. C.; Gomez, E. Accurate Enzymatic Sensing of Sweat Analytes. International Patent WO2018057695 A1, 2018.
(33) Phares, N.; White, R.; Plaxco, K. Improving the Stability and Sensing of Electrochemical Biosensors by Employing Trithiol-Anchoring Groups in a Six-Carbon Self-Assembled Monolayer. *Anal. Chem.* **2009**, *81*, 1095−1100.
(34) White, R.; Phares, N.; Lubin, A.; Xiao, Y.; Plaxco, K. Optimization of Electrochemical Aptamer-Based Sensors via Optimization of Probe Packing Density and Surface Chemistry. *Langmuir* **2008**, *24*, 10513−10518.
(35) Li, Y.; Afrasiabi, R.; Fathi, F.; Wang, N.; Xiang, C.; Love, R.; She, Z.; Kraatz, H. Impedance Based Detection of Pathogenic E. Coli O157:H7 Using a Ferrocene-Antimicrobial Peptide Modified Biosensor. *Biosens. Bioelectron.* **2014**, *58*, 193−199.
(36) Arroyo-Currás, N.; Somerson, J.; Vieira, P.; Ploense, K.; Kippin, T.; Plaxco, K. Real-Time Measurement of Small Molecules Directly in Awake, Afmbulatory Animals. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 645−650.
(37) Bertrand, J.; Hanley, B.; Brothers, M. Biosensors with Ph-Independent Redox Moieties. International Patent WO2018067521 A1, 2018.
(38) Santos-Cancel, M.; White, R. Collagen Membranes with Ribonuclease Inhibitors for Long-Term Stability of Electrochemical Aptamer-Based Sensors Employing RNA. *Anal. Chem.* **2017**, *89*, 5598−5604.
(39) Liu, X.; Xiao, T.; Wu, F.; Shen, M.; Zhang, M.; Yu, H.; Mao, L. Ultrathin Cell-Membrane-Mimic Phosphorylcholine Polymer Film Coating Enables Large Improvements for In Vivo Electrochemical Detection. *Angew. Chem., Int. Ed.* **2017**, *56*, 11802−11806.

DOI: 10.1021/acs.accounts.8b00555
Acc. Chem. Res. XXXX, XXX, XXX−XXX