Science & Society

TRANSPARENT PROCESS OPEN ACCESS

EMBO reports

Electricity, chemistry and biomarkers: an elegant and simple package

The potential of electrochemical biosensors for developing novel point-of-care diagnostics

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COVID-19 has been of the deadliest pandemic in recent history that has caused millions of deaths worldwide and a massive strain on healthcare resources. Patients in intensive care units were at risk of secondary bacterial infections due to invasive mechanical ventilation, and many of them were preventatively treated with broad-spectrum antibiotics. This will most likely further increase the development of antibiotic resistance (ABR), a natural, evolutionary response in microbes to antibiotic exposure. With the drastic increase in antibiotic usage during the COVID-19 pandemic to prevent secondary bacterial infections, we can anticipate the emergence of new multi-drug-resistant strains over the next few years (Mahoney *et al*, 2021).

Even before the pandemic, the burden of ABR due to excessive overuse of antibiotics, especially in human health care and agriculture, had created a massive public health problem. The annual deaths caused by drug-resistant bacteria are expected to reach 10 million by 2050. It also has a massive economic impact as patients infected with multi-drug-resistant pathogens need more expensive antibiotics and necessitate longer hospital stays. The annual cost of treating ABR infections is estimated to be US$20 billion globally, and the expected annual GDP loss will exceed US$100 trillion by 2050.

Preventing the spread of ABR depends heavily on antibiotic stewardship through controlled prescription. The key to achieving this is effective diagnostics to answer three clinical questions: Does the patient have a bacterial infection? Does the infection warrant treatment with antibiotics? Which antibiotic should we use? However, conventional diagnostics to answer these questions —either genotyping or ELISA—are cumbersome, slow, and costly and require trained personnel. Beyond the requisite facilities and personnel to perform these assays, genotypic methods cannot determine antimicrobial susceptibility and cannot detect emerging resistance patterns for which a gene has not yet been defined.

> *"A rapid, cheap, and reliable point-of-care diagnostic system would, therefore, enormously help to curb the spread of antibiotic resistance."*

These limitations are exemplified by one of the most prevalent sources of bacterial infections: urinary tract infections (UTIs). There are an estimated 150 million UTIs annually globally, which cause US$6 billion in healthcare expenditures. More than 90% of UTIs are ABR, and nearly 30% of women diagnosed with a UTI report a recurrent infection within six months. Current diagnostics are essentially useless to clinicians, as they require centralized facilities and take several days to yield results. As a result, many UTIs are routinely treated with broad-spectrum antibiotics. A rapid, cheap, and reliable point-of-care diagnostic system would, therefore, enormously help to curb the spread of antibiotic resistance. In short, we need something similar to the widely used glucose test for diagnosing bacterial infections.

Point-of-care diagnostics

Diagnostic testing in general has significantly improved the quality of health care, as it provides the physician with important specific health metrics about the patient that cannot be obtained from visual observation or physical examinations. Still, most diagnostic tests are limited to clinical settings and require specialized laboratories and ponderous, expensive equipment. Furthermore, the need for transporting samples to centralized laboratories and extensive sample preparations result in long wait times that can delay critical decisions. A prominent example is sepsis, a life-threatening immune response to an infection. Multiple blood biomarkers can be used to diagnose sepsis and inform treatment strategies, but their levels fluctuate quickly, which makes rapid detection critically important.

An important solution to reduce cost and turn-around time is point-of-care diagnostics: tests that can be performed on site without the need for trained personnel or specialized equipment. Point-of-care testing immediately detects a biomarker with only minimal sample processing. The two most notable commercialized point-of-care tests are the at-home pregnancy test and the blood glucose test. The latter has significantly improved diabetes management and thereby life expectancy and quality of life for millions of patients by allowing them to monitor their blood glucose levels in real time. The treatment of many other conditions could be similarly improved by point-of-care diagnostics. However, despite the importance of rapid testing in sepsis,

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DOI 10.15252/embr.202255096 | EMBO Reports (2022) 23: e55096 | Published online 21 April 2021

bacterial infections, and other critical illnesses, point-of-care diagnostic tests beyond the pregnancy and glucose tests remain few and far between.

Here, we describe the science behind point-of-care diagnostic tests and further developments to expand such tests to infectious diseases. Specifically, we discuss advances in electrochemical sensing, with the glucose meter as a case study, because electrochemical biosensor technology has many benefits such as low-cost assembly, rapid detection, quantitative readout, and extremely low limits of detection. We believe that this technology could become the gold standard for a wide range of point-of-care diagnostics to address the problems discussed above—differentiate between viral and bacterial infections, identify bacterial pathogens, determine their susceptibility to multiple antibiotics, and measure the patient's immune response.

Biosensors for point-of-care diagnostics

The pregnancy test and the glucometer are both biosensors, meaning they rely on a biorecognition element—a protein, a peptide, a nucleic acid sequence, or even a whole cell—to detect the analyte, that is, the molecule of interest. The key components involved are analyte recognition, signal transduction, and readout. The two most common configurations for point-of-care biosensors are lateral flow assays, such as the pregnancy or the COVID-19 antigen test, and electrochemical tests, such as the glucose sensor.

Lateral flow tests are paper-based assays for the visual detection of an analyte (Zamani *et al*, 2021a). The most common ones operate based on antigen-antibody interactions, though nucleic acids can also be used for detection. After the sample is applied to the sample pad, it flows to the conjugate pad, where the analyte of interest first binds to gold nanoparticles labeled with specific antibodies. The sample continues to flow down the test strip, and the analyte again binds to antibodies on what is called the test line which generates a colored visual readout. In the case of the pregnancy test, the analyte of interest is the hormone human gonadotropin (hCG); it is detected directly in urine in less than 30 min. COVID-19 antigen tests detect SARS-CoV-2 antigens from nasopharyngeal samples.

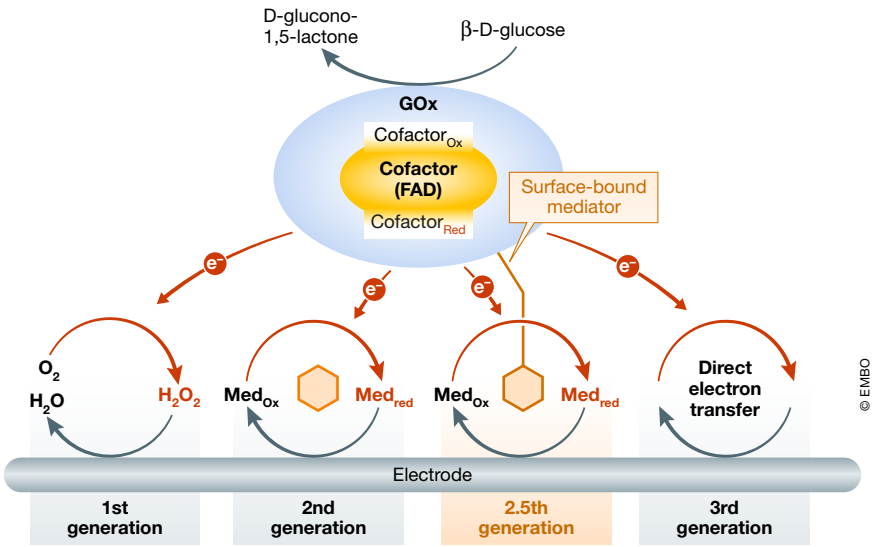

Figure 1. Evolution of the glucose sensor.

Glucose sensors are generally based on the glucose oxidase enzyme (GOx) interacting with a mediator to convert glucose to glucono-lactone. Original glucose sensing schemes were based on the conversion of molecular oxygen to hydrogen peroxide, which was then converted to water at the electrode. The second generation used a small-molecule redox-active mediator that could be reduced by GOx and then oxidized at the electrode. The mediator was originally in solution but eventually immobilization on the electrode to prevent mass transport limitations in the sensor. Third-generation sensors utilize direct electron transfer from the enzyme to the electrode. Adapted from Suzuki *et al* (2020).

The major benefits of lateral flow assays are that they are affordable and portable and do not require any specialized equipment. Disadvantages include that they are inherently qualitative, so they cannot determine, for instance, the viral load of a given disease, which is critical for instance for treating HIV. They also suffer from a lack of sensitivity. The at-home pregnancy test, for example, is an order of magnitude less sensitive than laboratory-based assays to detect hCG from blood (Cole, 2014). Similarly, COVID-19 antigen tests are less sensitive than the alternative PCR assay and, therefore, can give false-negative results if used early in the course of the disease, when viral loads are too low for lateral flow-based detection. Finally, lateral flow tests are not suitable for detecting blood biomarkers because it would require a prior purification step to separate the biomarker from the blood.

Enzymatic electrochemical tests: the glucometer

Electrochemical tests, which detect an analyte of interest by measuring the current of relevant redox reactions, are often superior to lateral flow tests owing to their higher sensitivity and quantitative nature combined with simplicity and affordability. There are various types of electrochemical tests, and the most common ones for blood-based tests are enzymatic electrochemical biosensors and affinity-based electrochemical biosensors.

Most commercialized glucometers are enzymatic biosensors, which directly and indirectly detect electrons generated by the metabolism of glucose by the enzyme glucose oxidase (Fig 1; Clarke & Foster, 2012; Zozulia *et al*, 2018; Suzuki *et al*, 2020). The first generation of enzymatic glucose biosensors, commercialized in 1975 as the 23A YSI analyzer, used oxygen as the electron acceptor that is converted to hydrogen peroxide. However, there were significant technical challenges that prevented this technology from being translated into an at-home test. For one, it was sensitive to the oxygen concentration at the electrode surface. Second, due to the requisite applied potential to reoxidize hydrogen peroxide to molecular oxygen, the test needed to be operated at high voltage, which decreased its specificity. Finally, the test was expensive as it used platinum electrodes.

The second-generation glucose sensors replaced oxygen with another electron acceptor to serve as a conduit between the

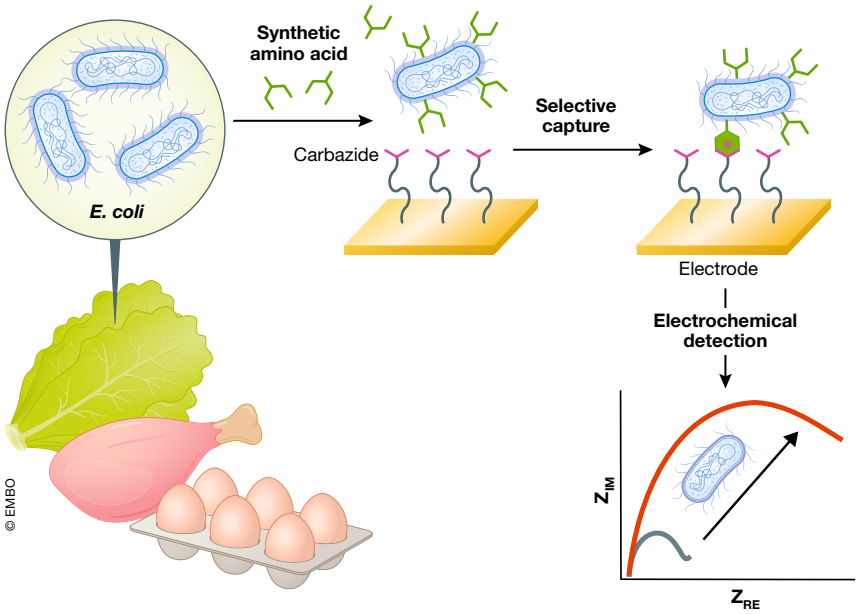

Figure 2. Detection of bacteria.

Pathogenic *Escherichia coli* pose a significant threat to public health, both as a major cause of urinary tract infections and foodborne illnesses. We can capture and detect *E. coli* on a disposable gold electrode through the initial addition of a synthetic amino acid to a sample. Viable *E. coli* incorporate the amino acid through peptidoglycan remodeling, and we can use this amino acid as a chemical handle to covalently capture cells at an electrode.

enzyme's active center and the electrode surface. This development combined with advances in screen-printing technology paved the way for the first commercial at-home glucose test, called ExacTech, in 1987. It used an enzyme electrode that contained glucose oxidase and ferrocene as an electron transfer mediator. Then enzyme metabolizes glucose in the sample and reduces ferrocene, which is reoxidized by the electrode. This generates a current proportional to the concentration of the glucose present in the original sample; a simple amperemeter is sufficient to quantify this. This sensor required lower reduction potentials, which decreased interference by other molecules and increased the specificity compared with the first-generation glucose sensor (Fig 1).

Second-generation glucometers also employed glucose dehydrogenase instead of glucose oxidase, as oxidase-based sensors are sensitive to oxygen concentrations at the electrode surface. For example, Roche developed the AccuChek Advantage that uses glutamate dehydrogenase (GDH) and pyrroloquinoline quinone (PQQ; Newman & Turner, 2005). It is more sensitive than glucose oxidase but was found to be susceptible to interference by maltose or galactose.

Despite their commercial success, second-generation biosensors still suffered from poor stability and toxicity of the mediator. This inspired the third-generation glucometers, which rely on direct electron transfer (DET) between the enzyme and the electrode. It also requires an even lower reduction potential than many common mediators, further increasing the specificity of the assay. However, DET between the electrode surface and glucose oxidase—as it has a higher sensitivity for glucose—is challenging, because the active site is buried deep within the enzyme. Various immobilization techniques have been developed to decrease the distance between the active site and the electrode surface, which, however, may decrease the enzyme's catalytic activity.

Despite these challenges, commercial electrochemical biosensors have greatly improved diabetes management, and they have consistently monopolized the US$5 billion per year market for more than two decades. The next step for this technology is further optimization so that these sensors can be used for continuous glucose monitoring. This has been shown to improve diabetes management and a lower risk of low blood glucose crises by sounding an alarm upon abnormal glucose levels. Such sensors generally monitor glucose levels every 10 min for up to 72 h. The Dexcom G5 Mobile is currently the only approved model for making treatment decisions (Lee *et al*, 2021). All other models must be verified manually with readings from conventional blood glucose meters to calibrate the system.

New applications for electrochemical biosensors

The evolution of the glucose sensor required decades and significant investment even for detecting a small molecule that is prevalent in high concentrations in human blood with an enzyme that has natively evolved to oxidize it. We anticipate that the development of equivalent technologies to detect or diagnose infectious disease will require similar time and investments. However, clever detection schemes combined with new platform technologies could help to decrease the time for detection and increase specificity. My group, for instance, works on developing electrochemical sensors to detect infectious disease agents by harnessing the inherent activity of microbes to capture them on an electrode surface (Fig 2; Klass *et al*, 2021); improving electrodes for detection by decreasing their cost and difficulty of manufacture (Zamani *et al*, 2021c); and incorporating biological amplification to increase selectivity and sensitivity (Zamani *et al*, 2021b). Our ultimate goal is to improve global health equity by decreasing the financial barriers to testing (Sofen & Furst, 2020; Castle *et al*, 2021; Zamani *et al*, 2021b).

Pathogenic strains of *Escherichia coli* associated with both UTIs and foodborne illnesses cause millions of sick cases and thousands of deaths each year. Diagnostic platforms to detect pathogenic *E. coli* must work in a variety of environments including farmland and fields, processing facilities, restaurants, clinics, and homes. As electrochemical detection offers a rapid and inexpensive alternative to traditional detection and quantification methods such as PCR and ELISA, significant effort has been devoted to developing such biosensors. Most of these rely on antibody- or aptamer-based recognition, but incorporating these molecules increases the cost and complexity, decreases stability, and relies on non-covalent interactions for detection, which can lead to unreliable results.

We developed an electrochemical sensor to detect *E. coli* from complex food samples

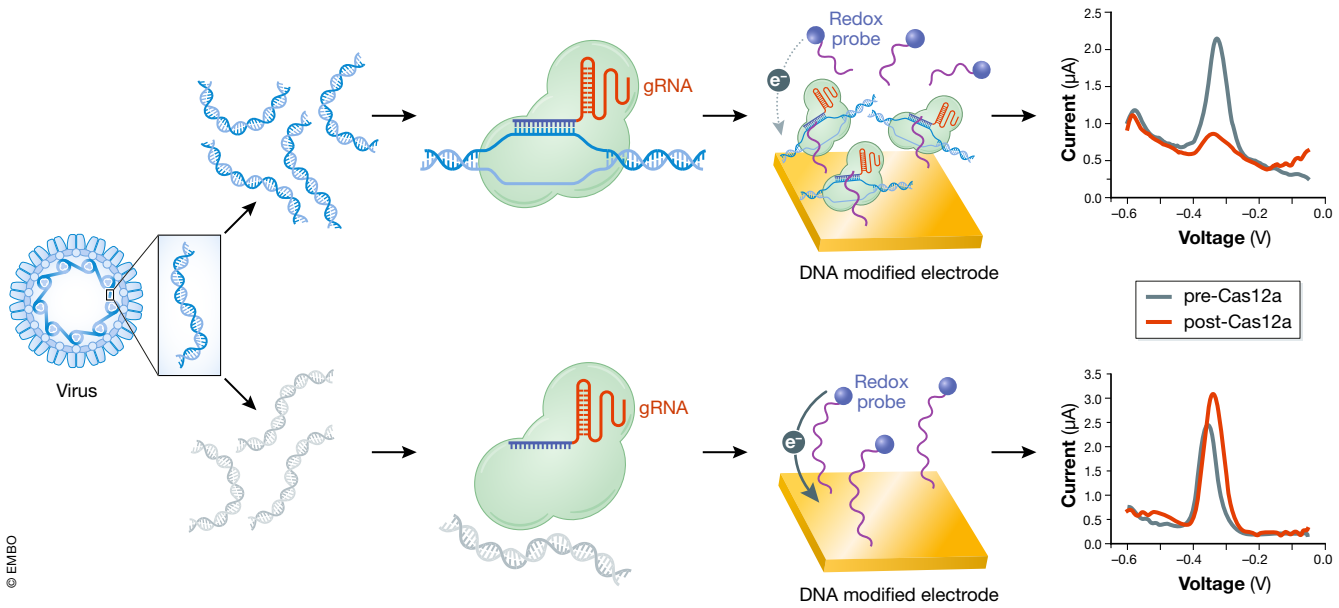

Figure 3. Workflow for human papillomavirus detection.

Viral genetic material is amplified isothermally. The amplicons can then be used to activate a CRISPR-Cas12a endonuclease enzyme. The activated enzyme will cut DNA on an electrode, turning the electrochemical signal off. If the Cas12a is not activated, the signal remains on.

and body fluids by covalent capture of the microbes at the electrode surface (Klass *et al*, 2021). The overall workflow involves the addition of a synthetic amino acid to a sample. *E. coli* rapidly incorporate this molecule if they are alive—thus, this platform further selects for live bacteria—and eventually displays proteins with the synthetic amino acid on the cell surface. The amino acid also contains a reactive head group—a boronic acid—that reacts with a carbazide to form a covalent bond through a bioorthogonal coupling to prevent nonspecific reactions with native components in the sample.

We then modified a commercially available disposable electrode with a thiolated carbazide, which reacts with the labeled *E. coli* to covalently capture them at the electrode surface. This overall platform has two main advantages over conventional biosensors: the cells must be alive to incorporate the synthetic amino acid, which enables specific capture even from very complex environments; and the cells are covalently captured at an electrode surface, which increases the durability of the platform. With this workflow, we are able to obtain a detection limit of 12 *E. coli*/ml in synthetic urine. Additionally, the platform can detect endogenous *E. coli* from contaminated food and feline urine with higher sensitivity and lower inherent error than the gold standard method of colony counting. Because the platform is generated using commercial disposable electrodes, it is anticipated to be easy to translate this technology into commercial applications.

> *"As electrochemical detection offers a rapid and inexpensive alternative to traditional detection and quantification methods [. . .], significant effort has been devoted to developing such biosensors."*

Improving electrodes

Commercial disposable gold electrodes, as those used in our *E. coli* sensor, are generally fabricated through screen printing of gold-doped ink through a mask. Screen-printed electrodes (SPEs) require that the inks are doped with other materials; these additives can alter the morphology of the gold and can interfere with biosensor function. Additionally, even for SPEs, most electrode fabrication protocols require complex equipment that must be housed in a clean room, which significantly increases the cost. To circumvent these issues, we developed and implemented a protocol to generate disposable electrodes using 24 karat gold leaf, the same material that is used as edible decorations. Importantly, it is very thin and therefore inexpensive, while it maintains the quality of gold without the addition of dopants.

These electrodes can be fabricated in nearly any setting as it involves making a sticker out of the gold leaf that can be applied to a transparent backing as the working and counter electrodes (Zamani *et al*, 2021b,c,). The sticker is cut from gold leaf on adhesive backing using a small cutter plotter. Silver paint can be added to generate a pseudoreference electrode. The whole process is very fast—it takes minutes excluding the time for adhesives to dry—and inexpensive: the total cost per electrode is approximately 50 cents. These electrodes have the key advantages of reusable, high-quality, crystalline gold surfaces but with significantly lower cost and easy production, which makes them ideal for low-resource settings.

We have also observed that these gold-leaf electrodes provided more reproducible data with lower limits of detection as compared to the commercial ones (Zamani *et al*, 2021c). Despite the fact that several iterations of gold-leaf electrodes have already

been reported in the literature, we found no reports of direct comparisons between gold-leaf electrodes and commercial screen-printed electrodes, especially for biosensing applications. We, therefore, performed a head-to-head comparison between these gold-leaf electrodes and two prevalent forms of gold SPEs: hot-annealed and cold-annealed that yield different morphologies. The electrodes were analyzed using atomic force microscopy and scanning electron microscopy to directly compare the micro- and nano-scale features on the surfaces, which we found to have significant differences. Based on these differences, we further compared the ability of these electrodes to detect DNAse I endonuclease activity. We observed significant differences in our ability to detect this enzyme on DNA-modified electrodes based on the underlying electrode morphology, with our gold-leaf electrodes outperforming both types of the SPEs (Zamani *et al*, 2021c).

"Continued efforts in our laboratory and others are developing point-of-care sensors to detect not only infectious diseases, but in the case of bacterial pathogens, to determine antibiotic resistances too."

Incorporating biological amplification to increase the selectivity and sensitivity

We used these gold-leaf electrodes to develop a sensor for human papillomavirus (HPV) that disproportionately impact women in low-resource settings; these areas also generally lack the resources, personnel, and facilities to perform conventional laboratory-based tests. HPV infects millions of people and causes nearly all cervical cancer cases with two strains in particular (HPV 16 and 18) responsible for more than 70% of all cases. This cancer is the fourth most common cancer globally in women and is easily cured if diagnosed early.

Importantly, the distribution and burden of infection is not equal. In resource-rich settings, five laboratory tests are currently FDA-approved to detect HPV DNA; these tests generally cost between US$30 and 75 per test, but the equipment required costs tens of thousands of dollars. Not surprisingly, more than 90% of cervical cancer-related deaths occur in resource-limited settings. Using the previously described gold-leaf electrodes, we developed a workflow that combines an isothermal nucleic acid amplification and CRISPR-based detection for HPV DNA from clinical samples (Fig 3). This workflow has 100% sensitivity and 89% specificity for HPV-18 from clinical samples (Zamani *et al*, 2021b). It also emphasizes the importance of combining biological activity from the CRISPR enzyme activation with optimized platforms to develop technologies that can be eventually translated to commercial systems.

Future developments of electrochemical biosensors for point-of-care testing

The success of the commercial glucometer illustrates the potential of electrochemical diagnostics. We have built on this success and combined improved electrochemical platforms with detection strategies that harness the inherent activity of biomolecules. The advantages of the combination of these strategies to move electrochemical diagnostics into the realm of infectious disease sensing are obvious. Continued efforts in our laboratory and others are developing point-of-care sensors to detect not only infectious diseases, but in the case of bacterial pathogens, to determine antibiotic resistances too. This knowledge is essential to enable the requisite antibiotic stewardship.

Acknowledgements

We would like to acknowledge the MIT Research Support Committee for supporting this work. M. Z. was supported through a training grant in environmental toxicology from the National Institute of Environmental Health Sciences (NIEHS) from the National Institutes of Health (NIH), Grant # T32-ES007020.

Disclosure statement and competing interests

The authors declare that they have no conflict of interest.

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