Revolutionizing Phenotypic Antimicrobial Susceptibility Testing: Lightning-Fast Techniques Based on Cutting-Edge Electrochemistry

Pragadeeshwara Rao R*, MRSC

Biosafety Lab, Amity Centre for Nanomedicine, Amity University Uttar Pradesh, Noida, UP, 201313

*Corresponding email: rprao@amity.edu / r.p.rao@outlook.com

Abstract.

Due to the ongoing concern for the development of rapid and accurate antimicrobial susceptibility testing (AST) methods in the field of clinical microbiology, several novel AST systems have been developed to replace the traditional gold standard AST techniques, which are time-consuming and can lead to delayed susceptibility guided therapy outcomes. In this review, the focus is on the phenotypic assays based on electrochemistry that are used to determine bacterial susceptibility towards antibiotics. Electrochemical techniques have gained significant interest in the field of AST due to their rapidity, sensitivity, and specificity, although it is important to note that some of these methods may have limitations regarding the specific classes of antibiotics and bacteria that they can be applied to, which researchers can address by fine-tuning the techniques or incorporating additional controls. By offering significant advantages over traditional AST methods in terms of rapidity and accuracy, electrochemical phenotypic assays could lead to improved patient outcomes.

Introduction

Antimicrobial resistance, as a phenomenon, is the culmination of a complex evolutionary process that occurs within the challenging milieu of microorganisms. It is the result of genetic changes or adaptations that arise as a response to the selective pressures exerted by antimicrobial agents. This lead to the restricted used of antibiotics as novel antibiotics development and use is not widespread[1]. As we are aware, the current gold standard methods for antimicrobial susceptibility testing (AST) are notoriously laborious, often requiring extended periods of time to generate results that are not readily available within a single working day. Consequently, healthcare practitioners may resort to broad-spectrum antimicrobial agents as an initial intervention while awaiting the laboratory's results from antibiogram testing to guide the selection of a more tailored antimicrobial therapy. This approach, while expedient, is not without its limitations, and can contribute to the rise of antimicrobial resistance as well as lead to a host of adverse outcomes for patients. As such, there is a pressing need for the development and implementation of more efficient and reliable AST methods that can deliver timely and accurate results to support optimal antimicrobial

prescribing practices[2]. In addition, it is imperative that stringent limitations be placed on the accessibility of antibiotics to populations in developing nations. This is yet another influential element in the proliferation of antimicrobial resistance; however, this issue lies outside the purview of this review. When conducting a gold standard antimicrobial susceptibility test (AST), phenotypic methods are frequently favoured as the initial approach in routine clinical laboratory evaluations. This preference is due to the fact that these methods offer a more comprehensive understanding of antibiotic resistance, and can assist in directing treatment options by providing minimum inhibitory concentration measurements that can be correlated with achievable serum levels.[3–5] A comprehensive examination of the assay for gold standard phenotypic and genotypic antimicrobial susceptibility testing is beyond the purview of this review. I would recommend consulting the works of Alex van Belkum et al[6] and Kira J. Fitzpatrick et al[7], as they offer comprehensive coverage of these techniques in the literature. Typically, the onset of infection arises from the successful adaptation of a pathogen within the host cells and normal microflora through the secretion of virulence factors. This results in the manifestation of symptoms that can aid in the dissemination of the disease. The identification of pathogens responsible for infections can be achieved through various microbiological tests, such as direct detection and culture. Microscopy-based direct detection is generally preferred for detecting pathogens that are difficult to culture, such as *N. gonorrhoeae* and *M. leprae*. Conversely, culture-based techniques are used for detecting and characterizing samples containing pathogens. While *in vivo* and *ex vivo* culture methods exist, *in vitro* culture utilizing artificial media is commonly employed as a definitive identification technique to determine antimicrobial susceptibility and for epidemiological studies. Antimicrobial susceptibility testing is designed to observe inhibited microbial growth in the presence of antimicrobial agents, which can either be bacteriostatic (causing reversible inhibition) or bactericidal (causing death). The clinical and laboratory standard institute or the European Committee on Antimicrobial Susceptibility Testing provides guidelines and thresholds for the determination of minimum inhibitory concentration (MIC). However, it is important to note that antimicrobial susceptibility testing is a probabilistic process as the relationship between MIC and clinical pharmacodynamics/pharmacokinetics for each case is complex and often does not

MIC (minimum inhibitory concentration)

It is the lowest concentration of an antimicrobial agent that causes inhibited growth.

The observer (via AST): observes fewer colonies or diminishing of turbidity in comparison to positive controls. More often the planktonic cells at MIC can grow if plated without antimicrobial agent or upon their removal.

MBC (minimum bactericidal concentration)

It is the lowest concentration of an antimicrobial agent that causes cell death.

The observer (via AST): observes no colonies or absence of turbidity. More often the planktonic cells at MBC cannot grow if plated without antimicrobial agent or upon their removal.

guarantee therapeutic success or significantly improve the therapeutic outcome[4]. The gold standard method is a universally accepted, definitive, and dependable technique for measuring a specific phenomenon or outcome. It is generally regarded as the most accurate and trustworthy method of measuring a particular phenomenon, and it is often used as a benchmark against which other methods or measurements are evaluated. The use of a gold standard allows researchers to assess the accuracy and efficacy of new methods by comparing them to a known, established standard. In the context of antimicrobial susceptibility testing (AST), there are several gold standard methods that are considered the most accurate and dependable ways to determine the susceptibility of bacterial strains to antibiotics. The initial methods developed for AST are phenotypic in nature and involve techniques that rely on the growth of the microorganism being tested. For those microbes that do not grow readily, special, or enriched media containing growth factors must be used. Among the commonly used phenotypic methods are broth dilution, agar dilution, disk diffusion, and gradient diffusion (Etest®). The first AST method was the ditch plate technique, which was introduced by Alexander Fleming in 1924[8]. This was modified by cutting wells[9] into agar, and the principle of this technique was developed further by the cylinder plate (also known as the Oxford cup or Heatley cup assay[10]). Following this, Fleming's second contribution to modern AST was the broth dilution technique using turbidity as an end-point[11], and later using pH as an indicator instead of turbidity[12]. In the 1940s, Schmith & Reymann described an agar dilution AST method for gonococci[13]. However, the MIC estimates from agar dilution were too time-consuming and cumbersome. Subsequently, many variants of diffusion and dilution techniques were reported by various researchers[9]. Thus, the AST gradually evolved from the ditch plate method, and it became evident that there was a need to standardize AST as many variables affected the test results. In 1968, the National Committee for Clinical Laboratory Standards (NCCLS), now known as the Clinical and Laboratory Standards Institute (CLSI), was established to create a consensus standard, guidelines, and best practices for AST in clinical laboratories. Although there are several gold standard techniques used for the determination of antimicrobial susceptibility testing, these methods are generally comprised of either manual or automated approaches. Phenotypic gold antimicrobial susceptibility testing involves techniques that rely on the growth of the microorganism, and those microbes that do not readily grow require the use of special or enriched media containing growth factors. In summary, the gold standard methods for AST are recognized as the most accurate and reliable ways to determine the susceptibility of bacterial strains to antibiotics, and they provide a benchmark against which other methods are compared to determine their validity and reliability[4].

Electrochemical Phenotypic Antimicrobial susceptibility testing

The emerging electrochemical antimicrobial susceptibility testing draws inspiration from the established gold standard AST techniques. The detection principles underlying these methods rely on the monitoring of analyte changes that occur at the interface between the analyte and substrate, facilitated by Electrochemical Impedance Spectroscopy, Cyclic Voltammetry, Square Wave Voltammetry, Pulsed Voltammetry, etc., A central classification scheme that distinguishes between *Faradaic and non-Faradaic currents* serves to differentiate among these sensors based on the principle that governs their operation and measurement. In general, phenomenon that occurs at the interface between metal and solution involves the transfer of charges or electrons, which can lead to oxidation or reduction. This type of reaction is subject to *Faraday's law*, which states that the amount of chemical reaction that results from the flow of current is directly proportional to the amount of electricity passed. Because of this fundamental relationship, such reactions are classified as faradaic processes. However, it is important to note that under certain conditions, there may be a range of potentials at the electrode-solution interface where no charge-transfer reactions occur. This phenomenon is known as the "overpotential" or "overvoltage" region, where the electrode potential is too high or too low for the charge transfer reaction to occur. In this region, the electrode does not contribute to the electrochemical reaction, and the reaction is controlled by other factors such as mass transfer or chemical reaction kinetics. This is because these reactions may be thermodynamically or kinetically unfavourable. Thus, the occurrence of faradaic processes is not an inevitability, but rather is contingent on the specific conditions and properties of the system under investigation. The *Non-faradaic* processes are chemical reactions or phenomena which occur at the interface between an electrode and a solution during an electrochemical reaction, yet they do not entail the transference of charge or electrons across the interface. Instead, such processes implicate changes in the structure of the interface, alongside the adsorption and desorption of species onto or from the electrode surface. Examples of non-faradaic processes encompass adsorption and desorption, diffusion, conformational changes, and capacitive charging. It is imperative to duly consider *non-faradaic* processes during electrochemical experiments, for they may wield a notable influence upon the behaviour of the cell. Although *non-faradaic* processes do not entail the transfer of charge, they may still affect the kinetics of the *faradaic* reaction and the overall behaviour of the cell. Thus, it is essential to take into account the effects of *non-faradaic* processes on the overall behaviour of the electrochemical cell, even in

In electrochemical analysis, several variables affect the rate of an electrode reaction. These variables can be broadly categorized into five categories: electrode variables (Material, Surface area, Geometry, Surface condition), mass transfer variables (diffusion, convection, Surface concentration, Adsorption), solution variables (Bulk concentration of electroactive species, Concentration of other species such as electrolyte, pH, ion, Solvent), external variables (Temperature, Pressure, Time), and electrical variables (Potential, Current, Quantity of electricity).

instances where only *non-faradaic* processes are extant. This may simplify the analysis of the cell's behaviour. Proceeding onwards, during an electrochemical experiment, a solution houses a working electrode and a reference electrode. An external power supply is employed to alter the potential difference between these electrodes. This variation in potential can facilitate the transference of electrons across the interfaces between the electrode and solution, thus producing a current flow in the external circuit. The number of electrons that are transferred is directly linked to the degree of chemical reaction transpiring at the electrode. The presence of bacteria can exert an influence upon charge transfer, mass transfer, diffusion and redox reactions transpiring at the solution and electrode interface[14]. These effects may consequently impact the rate of electron flow and ultimately manifest within the resultant current-potential curve. In biological systems, the flow of electrons oftentimes correlates with metabolic processes and thereby plays an integral role in the functioning of microorganisms. Through a thorough comprehension of the association between charge transfer reactions and the resulting current-potential curve, valuable insights may be collected regarding the interplay between bacteria and their surrounding environment. Such insights serve as pivotal factors towards comprehending the involvement of microorganisms in biogeochemical cycling and towards designing novel methodologies for bioremediation. In the present review, we direct our attention towards assessing antimicrobial susceptibility testing in relation to these underlying principles. The magnitude of electron flow, or current, is quantified in units of amperes (A), with one coulomb of charge per second being equivalent to one ampere. Through plotting current as a function of potential, a current-potential (i vs. E) curve can be generated. This curve conveys insightful details concerning the reactions taking place at the interface of the electrode and solution. Proper comprehension of these curves is fundamental to electrochemistry, much like the comprehension of ATP utilization is a vital concept in comprehending biological systems. Therefore, to comprehend the behaviour of microorganisms in the electrochemical cell, one must bear in mind that electrochemical behaviour is governed by several parameters, including electrode potential (E), current (I), concentration (C), electrode area, mass transfer, and electrode geometry. These parameters can be controlled, or one or two parameters can be kept constant to monitor the behaviour of the electrochemical cell due to the analyte, bio entities such as mammalian cells, or in our case microorganisms. Phenotypic approaches have a rich history in classical microbiology as a means to determine the antibiotic susceptibility of bacterial strains. Such approaches are grounded in the culture of bacteria on a suitable medium, which is subsequently observed at a later stage to ascertain the bacteria's response to specific antibiotics. Nonetheless, advances in microsystems engineering and lab-on-a-chip technologies have surpassed the limitations of traditional agar plate or liquid culture methods, allowing for more sensitive probing of bacterial growth. As a result of these technological advancements,

a broad range of sensor technologies with varying degrees of sample or microfluidic sample handling and integration have emerged. The development of such diverse sensor technologies has paved the way for a promising research field that investigates the use of electrochemical approaches to determine antibiotic susceptibility. These approaches typically entail measuring the changes in the electrical signals or properties of bacterial cells as they grow and metabolize in the presence of antibiotics, thereby enabling the rapid determination of antibiotic susceptibility, thus we will cover herein their advancement in techniques in this review. A.C. Ward et. al[15]., developed a technique with the use of electrochemical impedance spectroscopy (EIS) to determine antimicrobial susceptibility testing (AST). due to the high sensitivity of EIS, it is well suited to measuring AST and monitoring biofilm formation. The article discusses two distinct approaches to bacterial detection and analysis. Firstly, the use of a sensor to detect *S. aureus* in chronic wound infections is proposed. The sensor operates based on the electrochemical properties of wound exudate, which are similar to fetal calf serum, allowing for rapid detection of the bacteria in just 30 minutes. Additionally, efforts have been made to reduce the cost of the sensor by minimizing the amount of substrate and using conductive ink. Following this, Stuart Hannah et al[16,17] presents(Figure 1b) a novel approach for determining antibiotic susceptibility by modifying an electrode surface with agarose gels. The hydrogel interface between the electrode and the electrolytes was generated using the common polysaccharide agarose, which has a porous nature. They were able to use this method to detect differences in susceptibility within just 45 minutes, by observing bacterial growth and inhibition on agarose-containing growth media and monitoring changes in response to antibiotics for *MRSA*. The use of gel-antibiotic modified electrode sensors to monitor inhibited and uninhibited bacterial growth is a relevant example of this approach. This technique has been used to probe the antibiotic susceptibility of *MRSA* and *E. coli*[17]. The authors note that this method could be applied to other bacteria as well and could prove to be a valuable tool for rapid antibiotic susceptibility testing. Overall, the use of hydrogel interfaces in combination with electrochemical sensors provides a promising avenue for developing new and more effective methods for detecting and analysing bacterial growth and antibiotic susceptibility. Derrick Butler et. al[18], describes an interesting approaches for measuring bacterial growth and metabolism(Figure 1C). The technique involves the use of 3D interdigitated microelectrodes integrated with a gel-based bacterial culture. This technique relies on measuring the non-faradaic impedance of the microelectrodes and is able to differentiate between live and dead cells. The microelectrode design increases the sensing area almost three-fold and is capable of detecting 10 cells/mcL of *E. coli* in just one hour. However, the article notes that there are limitations to this method, and the clinical viability of this work has yet to be demonstrated. Additionally, the study only explores the differentiation between live and dead cells and does not provide data on

minimum inhibitory concentrations (MIC) against antibiotics. Daniel C. Spencer et. al[18]. introduced a novel approach to AST(Figure 1d), which they called "iFAST." This method is based on microfluidic impedance cytometry, a well-established label-free technique used for characterizing mammalian cells, but adapted to mimic fluorescence-activated cell sorting (FACS). iFAST measures changes in the biophysical properties of bacteria after exposure to antibiotics, allowing for rapid and sensitive AST. The technique works by measuring the electrical properties of single particles as they flow between microelectrodes in a microfluidic chip, allowing for measurement at the single-cell level. The authors suggest that the simplicity of this measurement technique makes it a promising candidate for a new generation of rapid tests in the clinical laboratory. In addition to its ability to detect antibiotic resistance, iFAST can also monitor sub-populations of bacteria that may be resistant or tolerant to antibiotics, providing real-time data on the emergence of resistance. However, the clinical feasibility of population analysis has not been demonstrated in this study. While the iFAST approach shows considerable promise, a fully-realized, miniature, and integrated rapid AST system is required to reliably predict AST for a range of pathogens and resistance mechanisms. Nonetheless, this study provides a valuable contribution to the development of improved therapies and a better understanding of complex bacterial phenotypes. The conductometric approach developed by Xuzhi Zhang et al[19] represents a promising method for antibiotic susceptibility testing (AST) due to its non-contact nature and suitability for handling resistant and biosafety level 3 pathogens(Figure 1a). The authors of this study validated the conductometric culture-suspension-sensor (CCS) AST method, utilizing *E. coli* and *V. parahaemolyticus* as microorganism models and enoxacin, florfenicol, ampicillin, kanamycin, and sulfadiazine as antibiotic probes. The results indicated that the CCS AST method was clinically valid, with MICs obtained through the CCS AST assays being higher than those obtained through the standard broth microdilution (BMD) AST assays in 72% of cases. The CCS AST method offers numerous advantages, including affordability, accuracy, sensitivity, and user-friendliness, and serves as proof-of-concept for a phenotypic AST method based on the multichannel CCS. The authors suggest that this approach is superior to the BMD AST method in terms of simplicity, sensitivity, and user-friendliness. Additionally, the CCS AST method provides an automated way to perform AST assays, making it a promising high-throughput tool. The accuracy of this new method for standard bacterial strains and isolates ranges from 68.8% to 92.3%. It is expected that this approach will provide clinical laboratories with a versatile platform for rapid MIC determination of diverse types of microorganisms, including adherent species. The conductometric system utilized in the CCS AST method can also be easily reproduced in the laboratory and can even be configured in a capillary format. Yichao Yang et al.[20] have developed a novel microfluidic device that shows promise for urinary tract infection (UTI) diagnosis(Figure 2C). The device monitors fluctuations in electrical

resistance of E. coli in the presence and absence of antibiotics within a microfluidic channel. In order to determine its clinical validity and suitability for use, further testing is required in the form of focused parametric studies. Domingo-Roca et al.[20] have described a portable 3D printed impedance-based biosensor that offers a promising approach for rapid antibiotic susceptibility testing. The customized electrode design allows for gel modification, enabling the detection of bacterial growth, death, and cell growth inhibition in just 90 minutes using a sample volume as low as 100uL. Electrochemical impedance spectroscopy was utilized to identify growth profiles and confirm antibiotic susceptibility of *Escherichia coli* and *Pseudomonas aeruginosa*. Antibiotic sensitivity determination was possible in just 90 minutes following overnight culture, significantly faster than the current gold standard that takes 24-48 hours. The gel-modified electrochemical sensor supports prokaryotic cell adhesion and can be used to induce cellular growth and death. This biosensor is a significant advancement in the field of antibiotic susceptibility testing, and it has the potential to improve clinical outcomes by enabling timely administration of appropriate antibiotic therapy. Giampaolo Pitruzzello et al.[21] conducted a innovative study to demonstrate the capability of impedance spectroscopy to measure signatures of single bacteria, with the time of action of antibiotics affecting the duration of the test(Figure 2b). The method was also assisted by microfluidics. However, it is important to note that bacteria must be in a metabolically active state at the time of the test, which is a requirement shared by most of the techniques discussed in this review article which can be troublesome in certain scenario. Thus, impedance spectroscopy has shown great promise in the field of antimicrobial susceptibility testing, and there have been many exciting developments in this area in recent years, combining impedance spectroscopy with other techniques, such as dielectrophoresis, can further enhance its capabilities. Dielectrophoresis can be used to manipulate the movement of bacteria and other particles using electric fields[22](Figure 2a), which can help to improve the sensitivity and specificity of impedance measurements, and with a results provided within 1hrs. here are also many other potential applications of impedance spectroscopy in microbiology and biomedicine, such as for monitoring cell viability, detecting bacterial infections, and studying the effects of drugs and other compounds on cells and tissues[23–26]. Also, Impedance spectroscopy enables the detection of significant parameters such as the bacteriostatic or bactericidal characteristics of novel and existing antimicrobial compounds[27] and whole cell detection of vancomycin susceptibility[28].

Let us now shift our attention to a class of predominantly *faradaic technique* that can be employed to measure antimicrobial susceptibility. This technique involves monitoring the respiration of microorganisms and can be achieved using redox

mediators. The basic principle of this approach is to measure the changes in electrical current that result from the respiration of microorganisms. In the presence of redox mediators, such as ferricyanide, the electrons released during respiration can be transferred to the mediator, resulting in a change in the mediator's redox state. This change in redox state can be detected using a variety of electrochemical techniques, such as amperometry or cyclic voltammetry[29]. Gretchen Tibbits and their colleagues introduced an innovative technique that utilized phenazine methosulfate as a mediator for a diverse array of pathogens and antibiotic classes. Their findings demonstrated that they were able to consistently classify strains as antibiotic-resistant or -susceptible in a remarkably short timeframe of less than 90 minutes for methodology development and under 150 minutes for blinded tests. It is worth noting that this technique offers an efficient and reliable approach to determine antimicrobial susceptibility and has potential applications in clinical settings[30].Resazurin, a frequently utilized redox indicator in cell viability assays, has emerged as a reliable technique for detecting viable cells by way of sequential reduction to resorufin and dihydroresorufin. The reduction reaction is dependent upon the cell's growth phase and concentration and can be measured via a reduction in the current peak. This methodology has been demonstrated to be effective for the identification of resistant and susceptible bacteria in a lab-on-a-chip format, with a reporting time of one hour[31]. Justin D. Besant et al. have reported on this approach, highlighting its potential for rapid and efficient antimicrobial susceptibility testing. Yang Gao and colleagues have devised an alternative technique which, in essence, quantifies the extracellular electron transfer (EET) from bacteria to an electrode in a microbial fuel cell, thereby furnishing results in five hours[32]. Rongshuai Duan et. al[33] presents a study showcasing the detection of resistant *Escherichia coli* bacteria by employing Methylene Blue as an electron acceptor to observe bacterial respiration. Additionally, they utilized graphene ink to augment the electrochemical response for enhanced detection. In another study Xuyang Shi et.al.[34] demonstrated AST via dual platform application: simultaneous detection-ID of bacteria and Rapid AST (RAST) (Figure 3a). The detection of *E. coli* in a whole blood

The term 'gating voltage' is used to refer to the potential difference that is applied to an electrochemical cell in order to control the rate at which ions move between the two electrodes. ***Gating voltage*** is an important parameter in electrochemical processes, as it controls the rate at which electrons flow from one electrode to the other, and thus affects the rate of reaction. When an electric potential is applied to a cell, ions move from one electrode to the other to make both electrodes have the same electric potential. If a potential difference between the two electrodes is large enough, ions will move rapidly between the two electrodes, leading to a rapid increase in the rate of reaction. By controlling the gating voltage, the rate of reaction can be controlled. Gating voltage can also be used to control the extent of a reaction. By increasing the gating voltage, the rate of reaction can be increased, which can lead to a higher yield of the desired product. On the other hand, decreasing the gating voltage can lead to a decrease in the rate of reaction, which can result in a lower yield of the desired product.

sample containing *E. coli* and *L. innocua* served as proof-of-concept for the simultaneous detection-ID process. By performing a 204-minute sample-to-result AST, this technique also provides a proof-of-concept example of Rapid AST being superior to state-of-the-art methods. In principle the method uses an immunoassay platform to identify bacteria in samples without the need for culture enrichment. Horseradish peroxidase (HRP), a redox enzyme, is immobilized to the working electrode (WE) through a sandwich immune complex in the detecting platform. The gating voltage, V_G, creates an electric field at the solution-enzyme-electrode interface, which lowers the electron tunnel barrier and improves the electrochemical signal between the electrode and HRP. As a function of V_G, the signal current is amplified. The results show that the platform can provide a quantitative assessment of the susceptibilities of two strains of *E. coli* to ampicillin and chloramphenicol after two hours of antibiotic exposure. In the future, they hope to develop a test kit based on the platform described here, consisting of a set of detection electrodes individually immobilized with the antibody of one of the most common pathogens. The availability of specific antibodies is a potential weakness of this platform. According to a thorough search of antibody manufacturers, antibodies for the most encountered pathogens are already commercially available. The future development of this platform will necessitate extensive quality assurance of each antibody pair for a specific pathogen or strain. For each type of bacteria, a different working electrode needs to be constructed. The challenge in clinical viability of this technique is that it requires labelling antibodies on the surface of electrode, the exploration on various antibiotic panel needs to be explored along with clinical samples. Yi Liu and colleagues have demonstrated that electrochemical detection of pyocyanin (PYO), a redox-active bacterial metabolite secreted by *Pseudomonas aeruginosa*, can rapidly ascertain the critical ciprofloxacin level required for bactericidal deactivation, in as little as 2 hours in antibiotic-treated growth media. To improve detection sensitivity for PYO, nanoporous gold, modified with a self-assembled monolayer, is employed to mitigate interference from oxygen reduction while simultaneously maintaining a low charge transfer resistance level and preventing electrode fouling within biological sample matrices[35]. Cristina Ocaña et. al,. have developed a simple, rapid, and cost-effective multichannel system for monitoring bacterial pH changes over time. The system employs miniaturized, all-solid-state pH sensors with a Ta_2O_5 sensitive layer to record pH changes resulting from bacterial growth in small volume Eppendorf test tubes. This approach is particularly valuable for evaluating microbial metabolic activity and antibiotic response. While blood pH can be assessed using electrodes or point-of-care instruments such as blood-gas analysers, these methods can be prohibitively expensive and complicated. The team successfully characterized the system using *E. coli* as a model bacterium and demonstrated its ability to detect increasing concentrations of viable *E. coli* in undiluted culture medium within just 5 hours of assay time, with a detection limit of

less than 10^3 CFU/mL[36]. Priyanka Mishra et. al.[37] also implemented resazurin as a redox mediator to facilitate antibiotic susceptibility testing. Using the differential pulse voltammetry technique, electrochemical changes in peak current values were monitored, with interpretation based on these changes. Their findings reveal a novel approach that enables detection of 10^4 cells/mL in less than 4 hours for *K. pneumoniae* and *E. coli*, with results compared to gold standard AST. This methodology demonstrates promise for rapid AST and the use of a Pt-coated bio-electrode as a biosensor shows potential for analysing metabolic activity and monitoring the response of different antibiotics on bacterial cell viability. Another study reports the monitoring of riboflavin a redox protein secreted by the viable *E. coli* cells[38]. In a study conducted by Benjamin Crane et. al.,[39] the utility of resazurin as redox mediators was explored for the detection of *Escherichia coli* with the goal of developing a diagnostic tool for urinary tract infection(Figure 3c). Notably, the study employed bulk modification of screen-printed electrodes during the carbon-graphitic ink screen printing process to incorporate resazurin into the electrodes. The detection sensitivity of resazurin was found to be as low as 15.6 µM via differential pulse voltammetry, in certain cases this strategy can alleviate the issue with mediators inferring with antibiotics. Conversely, the scholarly investigation conducted by Adam Bolotsky et. al.,[40] details the direct modification of screen-printed electrodes through the deposition of resazurin, thereby generating a crystalline formation(Figure 3b). This one-step approach enables the synthesis of a novel and exceptionally stable organic crystalline layer which exhibits redox activity. Furthermore, they have demonstrated the sensor as a reagent-free electrochemical sensor for in situ detection of bacterial metabolic activity with a response time of 60 min. Owing to its reagent-free nature, this methodology allows for direct measurement of bacterial activity, but it can be prone to certain inferring antibiotics. Furthermore, the work reported by Ritu Das et. al.,[41] presents an electrode modification technique is employed utilizing a facile alginate gel that has been impregnated with carbon nanodots(Figure 3d). The objective of this approach is to enable monitoring of bacterial growth kinetics as a function of pH., Many other techniques use similar strategies of electrode modification with multiwalled carbon nanotubes[42,43] and graphene[44]. In our group, R Pragadeeshwara Rao et al.[45] proposed a variant of electrochemical antimicrobial susceptibility testing assay developed by Kamonnaree chotinantakul[29] and peter Ertl[46] that tracks changes in redox mediators in the presence of bacteria. The bacterial response (gram-negative *E. coli* and gram-positive *B. subtilis*) to various antibiotics is monitored using electrochemical measurement via cyclic voltammetry in the electrochemical antimicrobial susceptibility test (EAST). A bacterium has intrinsic redox properties that serve as a signature of its viability; this redox signature is used as a parameter for monitoring bacterial susceptibility using an electrochemical assay known as EAST. This method yields results for drug efficacy, half-life period, and IC_{50},

among other things. Within 15 minutes of antibiotic incubation, whereas conventional methods require hours to days to see results. Also, this study suggests the changes in electrode kinetics with the use of nanomaterial could improve the performance of AST. In principle the method detects the redox behavior of *E. coli* and *B. subtilis* before and after undergoing antibiotic stress by monitoring changes in ferricyanide as redox mediators. To study the kinetics of bacterial response to various antibiotics, an electrochemical cell configuration with optimal concentrations of carbon nutrient and electron shuttle is developed (of various mechanisms of action). Increased sensitivity to EAST can be achieved by using a bacterial-friendly L-CeONP coated electrode, indicating that miniaturization will improve EAST efficacy. The author concluded that a bacteria-friendly L-CeONP-coated electrode could increase sensitivity toward EAST, implying that enhanced electron transportation rate (ETR) by the bacteria friendly semiconducting nano particles, would improve sensitivity toward EAST and hence would improve EAST efficacy. As for the clinical viability of the method, it can only interpret bactericidal activity, and clinical applicability requires the examination of a variety of clinical samples. The clinically viability solution is currently being developed as an extension of this work. Many studies are also devoted to the development of the sensor platform as the commercial instruments are expensive and they attempted to make a low cost potentiostat[47]. Carine R. Nemr et. al[48] have advanced an innovative approach to detecting methicillin-resistant *Staphylococcus aureus (MRSA)* in clinical samples such as nasal swabs. Their work involves utilizing an antibody-based capture probe, which can accurately report the endpoint of *MRSA* directly from these samples. This approach holds significant promise for clinical application, particularly as a primary antimicrobial susceptibility marker.

Conclusion and Summary.

Antimicrobial resistance (AMR) is a significant global threat to public health, leading to increased morbidity, mortality, and economic burden. It is clear that current rapid AST solutions and automated AST systems do not have sufficient evidence to determine if they have a significant impact on mortality, length of hospital stay, or clinical response rates when compared to the gold standard AST[48]. Thus, rapid antibiotic susceptibility testing (AST) technologies could have the potential to improve patient outcomes and reduce the threat of AMR by providing results in a matter of hours with reduced turnaround time, guiding susceptibility-guided therapy, and reducing overuse or misuse of antibiotics. Electrochemical AST systems have been increasingly reported in published studies, demonstrating reliable and fast AST determination in a phenotypic manner in the range of <60 min AST time as seen above with the potential to even detect single-cell level ASTs. Electrochemical approaches have great potential for widespread deployment due to their low cost, ease of manufacture, and high analytical sensitivity. However, for deployment in LMIC, low-cost portable electrochemical analysers will be needed. The lack of standardization,

limited range of pathogens and antibiotics being tested, cost, limited clinical evidence, and turnaround time are the reasons for the limited use of rapid AST solutions and automated AST systems in the current clinical setting. In certain cases, the novel electrochemical development itself will have a significant cost, and in most cases, follow-up clinical trials are not taken up by the same groups. The future of Rapid AST lies in personalized medicine, where individual patients' bacterial infections can be treated with tailored antibiotic therapies based on their unique susceptibility profiles. Biosensing device development is needed to address challenges such as distinguishing polymicrobial samples, detecting drug resistance levels, and using a redox reagent-free or biocompatible reagent-enabled strategy. 3D printing technology is expected to be seen further in future for AST and can be used to fabricate multiple biorecognition elements on a single electrode, and the technique should cover a broad dynamic range for the quantification of drugs. We have seen few studies reported using 3D printing technologies, but we are yet to see the use of 4D printing technologies in electrochemical AST systems.

Acknowledgements: I convey my utmost gratitude to Amity University Uttar Pradesh and our esteemed department HOI for their unwavering assistance and encouragement in facilitating the successful progression of this endeavour.

Funding: This work was supported by a TARE fellowship funded by the Science and Engineering Research Board (grant number TAR/2020/000329).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References:

1. Marston, H. D., Dixon, D. M., Knisely, J. M., Palmore, T. N. & Fauci, A. S. Antimicrobial resistance. *JAMA - J. Am. Med. Assoc.* (2016) doi:10.1001/jama.2016.11764.
2. O'Neill, J. Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations, 2014. **4**, (2014).
3. Khan, Z. A., Siddiqui, M. F. & Park, S. Current and emerging methods of antibiotic susceptibility testing. *Diagnostics* vol. 9 (2019).
4. Davidson, S. S. *Davidson ' s Princples and Practice of Medicine*. *Davidson's principles and practice of medicine* (2010).
5. Jorgensen, J. H. & Ferraro, M. J. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clin. Infect. Dis.* **49**, 1749–1755 (2009).

6. van Belkum, A. *et al.* Innovative and rapid antimicrobial susceptibility testing systems. *Nature Reviews Microbiology* vol. 18 (2020).

7. Fitzpatrick, K. J. *et al.* Progressing Antimicrobial Resistance Sensing Technologies across Human, Animal, and Environmental Health Domains. *ACS Sensors* vol. 6 (2021).

8. A comparison of the activities antiseptics on bacteria and on leucocytes. *Proc. R. Soc. London. Ser. B, Contain. Pap. a Biol. Character* **96**, (1924).

9. Reddish, G. F. PRESENT TRENDS IN METHODS OF TESTING ANTISEPTICS. *Ann. N. Y. Acad. Sci.* **53**, (1950).

10. Abraham, E. P. *et al.* FURTHER OBSERVATIONS ON PENICILLIN. *Lancet* **238**, (1941).

11. Fleming, A. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B.influenzae. *Br. J. Exp. Pathol.* (1929) doi:10.1038/146837a0.

12. Poupard, J. A., Rittenhouse, S. F. & Walsh, L. R. The evolution of antimicrobial susceptibility testing methods. *Advances in Experimental Medicine and Biology* vol. 349 (1994).

13. Petro, J. SULPHONAMIDE RESISTANCE IN GONORRHŒA. *Lancet* **241**, (1943).

14. Mitchell, P. The protonmotive Q cycle: A general formulation. *FEBS Letters* (1975) doi:10.1016/0014-5793(75)80359-0.

15. Ward, A. C. *et al.* Identification and characterisation of Staphylococcus aureus on low cost screen printed carbon electrodes using impedance spectroscopy. *Biosens. Bioelectron.* **110**, (2018).

16. Hannah, S. *et al.* Rapid antibiotic susceptibility testing using low-cost, commercially available screen-printed electrodes. *Biosens. Bioelectron.* **145**, (2019).

17. Hannah, S. *et al.* Development of a Rapid, Antimicrobial Susceptibility Test for E. coli Based on Low-Cost, Screen-Printed Electrodes. *Biosensors* **10**, (2020).

18. Spencer, D. C. *et al.* A fast impedance-based antimicrobial susceptibility test. *Nat. Commun.* **11**, (2020).

19. Zhang, X. *et al.* Affordable automated phenotypic antibiotic susceptibility testing method based on a contactless conductometric sensor. *Sci. Rep.* **10**, (2020).

20. Yang, Y., Gupta, K. & Ekinci, K. L. All-electrical monitoring of bacterial antibiotic susceptibility in a microfluidic device. *Proc. Natl. Acad. Sci. U. S. A.* **117**, (2020).

21. Pitruzzello, G., Johnson, S. & Krauss, T. F. Exploring the fundamental limit of

antimicrobial susceptibility by near-single-cell electrical impedance spectroscopy. *Biosens. Bioelectron.* **224**, (2023).

22. Swami, P., Sharma, A., Anand, S. & Gupta, S. DEPIS: A combined dielectrophoresis and impedance spectroscopy platform for rapid cell viability and antimicrobial susceptibility analysis. *Biosens. Bioelectron.* **182**, (2021).

23. Ramírez, N., Regueiro, A., Arias, O. & Contreras, R. Electrochemical impedance spectroscopy: An effective tool for a fast microbiological diagnosis. *Espectroscopía impedancia electroquímica, Herram. eficaz para el diagnóstico rápido microbiológico.* **26**, (2009).

24. Hosseini, S. N., Lazarjan, V. K., Akram, M. M. & Gosselin, B. CMOS Multi-Frequency Lock-in Sensor for Impedance Spectroscopy in Microbiology Applications. in *20th IEEE International Interregional NEWCAS Conference, NEWCAS 2022 - Proceedings* (2022). doi:10.1109/NEWCAS52662.2022.9842207.

25. Krukiewicz, K. Electrochemical impedance spectroscopy as a versatile tool for the characterization of neural tissue: A mini review. *Electrochemistry Communications* vol. 116 (2020).

26. Xu, Y. *et al.* A review of impedance measurements of whole cells. *Biosensors and Bioelectronics* vol. 77 (2016).

27. Bancalari, E., Martelli, F., Bernini, V., Neviani, E. & Gatti, M. Bacteriostatic or bactericidal? Impedometric measurements to test the antimicrobial activity of Arthrospira platensis extract. *Food Control* **118**, (2020).

28. Norouz Dizaji, A. *et al.* Electrochemical-based '"antibiotsensor"' for the whole-cell detection of the vancomycin-susceptible bacteria. *Talanta* **234**, (2021).

29. Chotinantakul, K., Suginta, W. & Schulte, A. Advanced Amperometric Respiration Assay for Antimicrobial Susceptibility Testing. *Anal. Chem.* **86**, 10315–10322 (2014).

30. Tibbits, G., Mohamed, A., Call, D. R. & Beyenal, H. Rapid differentiation of antibiotic-susceptible and -resistant bacteria through mediated extracellular electron transfer. *Biosens. Bioelectron.* **197**, (2022).

31. Besant, J. D., Sargent, E. H. & Kelley, S. O. Rapid electrochemical phenotypic profiling of antibiotic-resistant bacteria. *Lab Chip* **15**, (2015).

32. Gao, Y., Ryu, J., Liu, L. & Choi, S. A simple, inexpensive, and rapid method to assess antibiotic effectiveness against exoelectrogenic bacteria. *Biosens. Bioelectron.* **168**, (2020).

33. Duan, R., Fang, X. & Wang, D. A Methylene Blue Assisted Electrochemical Sensor for Determination of Drug Resistance of Escherichia coli. *Front. Chem.* **9**, (2021).

34. Shi, X., Kadiyala, U., Vanepps, J. S. & Yau, S. T. Culture-free bacterial detection and identification from blood with rapid, phenotypic, antibiotic susceptibility testing. *Sci. Rep.* **8**, (2018).

35. Liu, Y. *et al.* Minimum bactericidal concentration of ciprofloxacin to Pseudomonas aeruginosa determined rapidly based on pyocyanin secretion. *Sensors Actuators, B Chem.* **312**, (2020).

36. Ocaña, C., Brosel-Oliu, S., Abramova, N. & Bratov, A. Low-cost multichannel system with disposable pH sensors for monitoring bacteria metabolism and the response to antibiotics. *Instrum. Sci. Technol.* **49**, (2021).

37. Mishra, P. *et al.* Rapid antibiotic susceptibility testing by resazurin using thin film platinum as a bio-electrode. *J. Microbiol. Methods* **162**, (2019).

38. Singh, A. K., Jaiswal, J. & Dhayal, M. Platinum disc electrode for in-situ electrochemical inactivation of bacterial growth in culture media. *J. Electroanal. Chem.* **868**, (2020).

39. Crane, B. *et al.* Rapid antibiotic susceptibility testing using resazurin bulk modified screen-printed electrochemical sensing platforms. *Analyst* **146**, (2021).

40. Bolotsky, A. *et al.* Organic redox-active crystalline layers for reagent-free electrochemical antibiotic susceptibility testing (ORACLE-AST). *Biosens. Bioelectron.* **172**, (2021).

41. Das, R. & Singh, N. Exploring electrochemistry of carbon nanodots and its application in noninvasive bacterial growth monitoring. *Biosens. Bioelectron.* **144**, (2019).

42. McLeod, J., Stadler, E., Wilson, R., Holmes, A. & O'Hare, D. Electrochemical detection of cefiderocol for therapeutic drug monitoring. *Electrochem. commun.* **133**, (2021).

43. Ren, Y. *et al.* Rapid detection of antibiotic resistance in Salmonella with screen printed carbon electrodes. *J. Solid State Electrochem.* **24**, (2020).

44. Li, C. & Sun, F. Graphene-Assisted Sensor for Rapid Detection of Antibiotic Resistance in Escherichia coli. *Front. Chem.* **9**, (2021).

45. Rao R, P. *et al.* Rapid Electrochemical Monitoring of Bacterial Respiration for Gram-Positive and Gram-Negative Microbes: Potential Application in Antimicrobial Susceptibility Testing. *Anal. Chem.* acs.analchem.9b04810 (2020) doi:10.1021/acs.analchem.9b04810.

46. Ertl, P., Robello, E., Battaglini, F. & Mikkelsen, S. R. Rapid Antibiotic Susceptibility Testing via Electrochemical Measurement of Ferricyanide Reduction by Escherichia c oli and Clostridium s porogenes. *Anal. Chem.* **72**,

4957–4964 (2000).

47. Butterworth, A., Corrigan, D. K. & Ward, A. C. Electrochemical detection of oxacillin resistance with SimpleStat: a low cost integrated potentiostat and sensor platform. *Anal. Methods* **11**, (2019).

48. Anton-Vazquez, V., Hine, P., Krishna, S., Chaplin, M. & Planche, T. Rapid versus standard antimicrobial susceptibility testing to guide treatment of bloodstream infection. *Cochrane Database Syst. Rev.* **2021**, (2021).

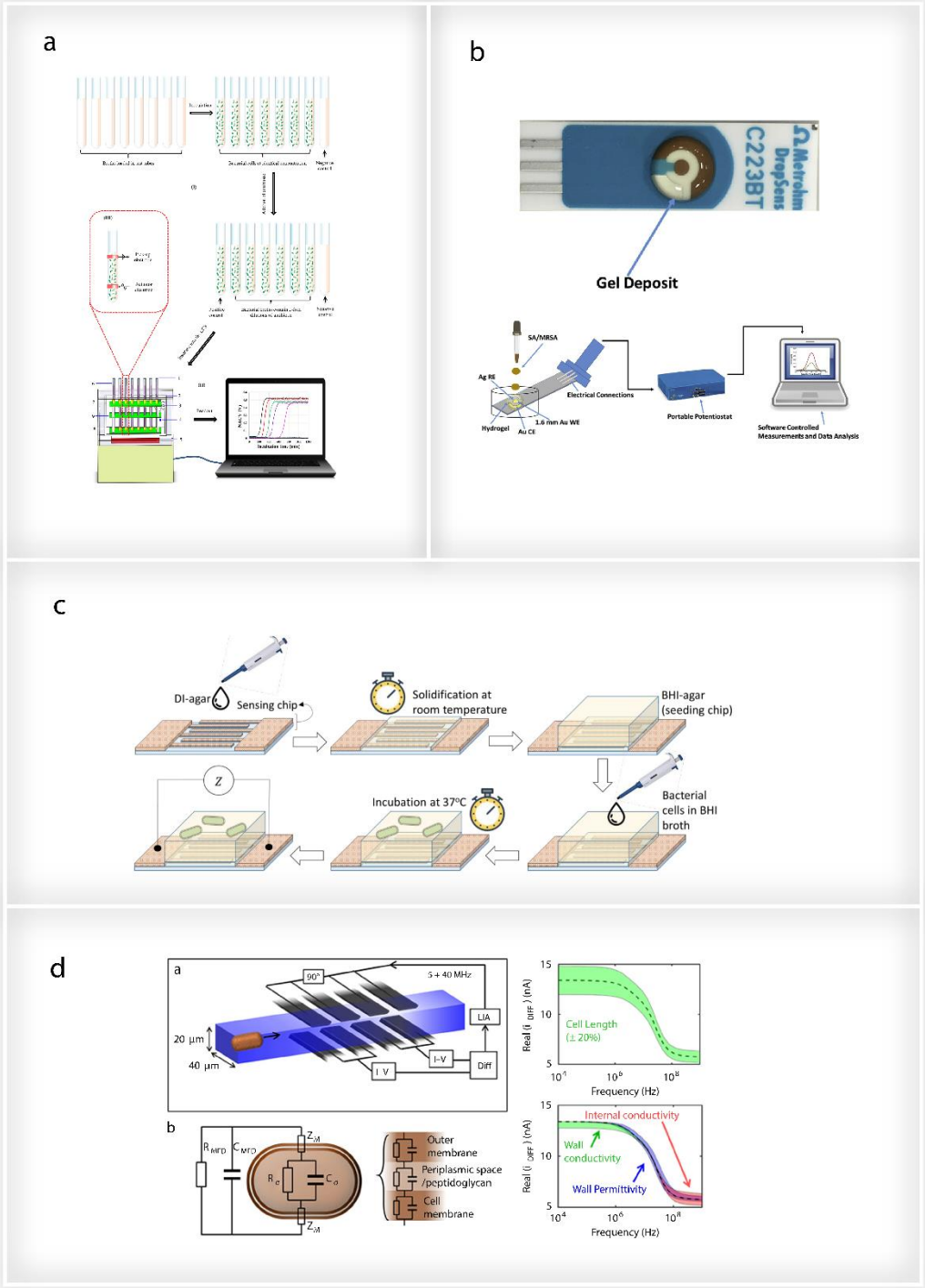

Figure 1. ***a)*** *The workflow of contactless conductometric assay for AST is depicted and is adapted with permission from Xuzhi Zhang et al. Copyright © 2020.* ***b)*** *the image shows hydrogel-coated three-electrode system utilized for the antibiotic susceptibility testing (AST) of S. aureus (SA) and MRSA against amoxicillin and oxacillin. This figure has been adapted with the permission of stuart Hannah et. al. and is copyrighted © by Biosensors and Bioelectronics in 2021.,* ***c)*** *Plots the experimental scheme of agar integrated 3D microelectrodes for AST adapted with permission from D. butler et. al. copyright © 2018 Biosensors and Bioelectronics.,* ***d)*** *Presents the multi-electrode microfluidic impedance chip with equivalent electrical circuit adapted with permission from Daniel C. Spencer et. al. Copyright © 2020.*

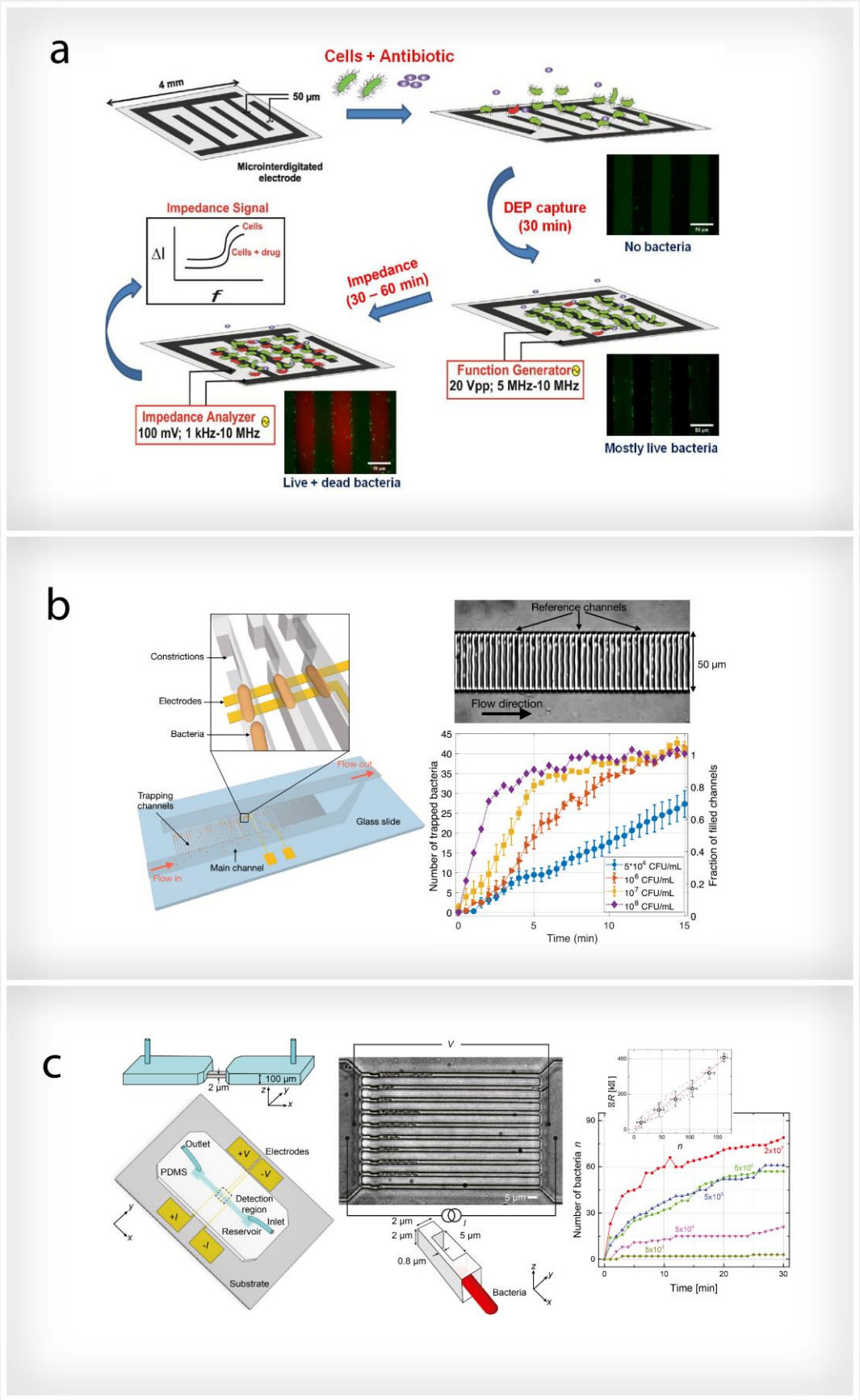

Figure 2. a) Representation of the principle of operation of DEPSIS biosensor adapted with permission from Pragya Swami et. al., copyright © 2021. b) Presents the microfluidic trapping channels used to achieve single-cell resolution, adapted with permission from Giampaolo pitruzello et. al., copyright © 2023 Biosensor and Bioelectronics. ***C)*** *microfluidics device setup for the detection of electrical resistance fluctuations as a result of bacteria, adapted with permission from Yichao Yang et. al., copyright © 2020.*

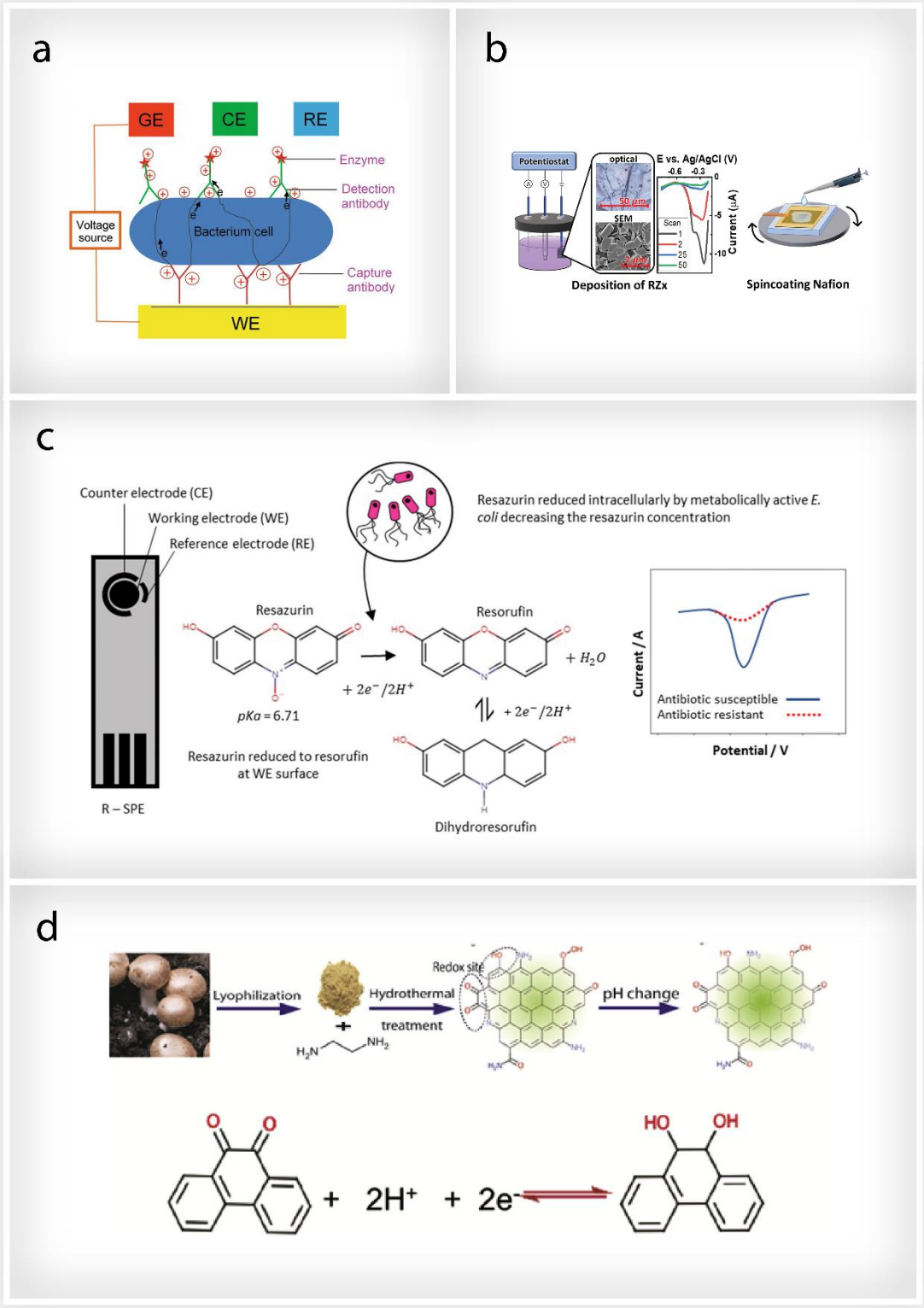

Figure 3. ***a)*** *The detection platform based on FEED is presented, which explains the operational principle and the application of the gating electrode adapted with permission from Xuyang Shi et. al. copyright © 2018,* ***b)*** *Presents the fabrication of Resazurin modified electrode via differential pulse voltammetry of ORACLE-AST, adapted with permission from Adam Bolotsky et. al. Copyright©2020,* ***c)*** *Presents the working principle of resazurin based phenotypic AST for bulk modified resazurin screen printed electrodes, adapted with permission from Benjamin Crane et. al. Copyright©2021, and* ***d)*** *Shows the process of preparing carbon dots and highlights their redox activity, which is attributed to the presence of redox active quinone sites within their structure, adapted with permission from Ritu Das et. al. copyright©2019.*