Electrochemical antibiotic susceptibility testing – an emerging approach for fast and accurate determination of antibiotic effect in complex samples

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Abstract

With the emergence of drug resistant infections it is becoming increasingly important to develop technologies which accelerate and enhance prescription of antibiotics. For routine hospital testing in advanced economies, gold standard analysis techniques usually identify the correct antibiotic for treatment of an infection in 12-72 hours. Current techniques are highly automated, can handle many samples but are inflexible, can be time consuming, and are not amenable to point of care use. Electrochemical approaches lend themselves very well to antibiotic susceptibility testing (AST) because of the possibility of developing low cost miniaturised systems which can be deployed at the point of need or as high throughput systems which can be used in centralised hospital laboratories. This article will detail recent advances in the field of electrochemical AST and offer commentary on the current state of the field and the prospects for translation of electrochemical AST systems into real world use.

Introduction

The problem of antimicrobial resistance (AMR) is global healthcare challenge. It manifests as increased resistance to and increasing minimum inhibitory concentrations (MICs) for antimicrobial drugs. Clinically, this manifests as increasing levels of morbidity and mortality due to increasing prevalence of drug resistant infections [1], [2]. To exemplify the problem, as noted in a review carried out for the UK Government into AMR, Lord Jim O'Neill noted that without action, by 2050, AMR will account for approximately 10 million deaths per year and will kill more people than cancer and diabetes in combination [3]. Reducing the impact of AMR means targeting specific areas, which include: new antibiotics, behaviour and policy change, better prescription of antibiotics, and new diagnostic technologies. New diagnostic technologies mean antibiotic stewardship [4] can be improved and electrochemical AST has a role to play in this effort. To reduce the emergence and spread of AMR it will be necessary to prolong the useful lifetime of existing antibiotics and crucially, delay the development of resistance for antibiotics of last resort and any new compounds which will be deployed to address the challenge of AMR.

Bacterial infections are very common and drive the widespread and routine prescription of antibiotics. The discovery of antibiotics in combination with their low cost, convenience and effectiveness was one of the major scientific achievements of the twentieth century, leading to a new age in medicine, making possible routine surgery, complex surgery, chemotherapy, and reducing infant and maternal death rates. Infections can vary in a great deal of ways. The pathogen, can be Gram negative or Gram

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positive (if bacterial), but it could also be a fungal species and the possible sites of infection are numerous including: the urinary tract, the respiratory tract, the gastrointestinal tract, the blood (bacteraemia), the cardiovascular system, the brain along with external wounds and injuries. This range of infectious organisms, multitude of infection sites, and range of possible antibiotics can make drug prescribing into a complex process. Guidelines and methodologies exist to assist 'empiric' antibiotic prescribing practices [5] but is widely acknowledged that the process can be enhanced through development of new technologies which better guide this process and which make more likely the targeted prescription of narrow spectrum antibiotics as opposed to broad spectrum antibiotics.

Determination of antibiotic susceptibility by electrochemical means is an exciting and flourishing area of research. As previously stated, the nature of electrochemical measurements (high sensitivity) coupled to the possibility for low cost and miniature systems makes the approach well suited to AST measurements. In the following sections, two particularly common approaches to AST determination are discussed (genotypic and phenotypic AST) along with some examples of assays that have been developed through use of novel electrode modifications.

Genotypic Approaches

Development of AMR in bacteria correlates with changes to the nucleic acid composition of a pathogen. Acquisition of new genes, from exchange of plasmids or mutations to the existing bacterial chromosome, facilitate the development of resistance mechanisms which allow bacteria to continue growing in the presence of antibiotics. Hence, to confirm the presence of resistance and therefore the likely effectiveness of certain antibiotics, studies have reported development of electrochemical biosensors which measure for the presence of particular resistance genes in samples. Examples include two studies which developed sensing solutions for detecting resistance to oxacillin, a common narrow-spectrum beta-lactam antibiotic. The first study detailed the optimisation process of label-free impedimetric biosensors. They identified two critical factors determining the sensitivity of the overall system: (i) the components in the self-assembled monolayer suitable for biorecognition, and (ii) the roughness of the electrode surface which, combined, were demonstrated to play a key role in sensor sensitivity.[6]. The second showcased how a simple isothermal amplification technology (recombinase polymerase amplification – RPA) can be used to detect oxacillin resistance in combination with a horse radish peroxidase (HRP) labelled thymine molecule. The polymerase enzyme used in the assay is able to incorporate the HRP labelled thymines into surface tethered probes, and successful detection of oxacillin resistance leads to an increase in the amperometric signal due to the close proximity of HRP-labelled amplicons at the surface [7]. In a similar approach to the work reported on impedimetric detection of oxacillin resistance, the detection of colistin resistance using, the *mcr-1* gene was demonstrated using a sensor placed in a temperature controlled flow cell and showed how control of temperature can influence assay signal and ultimately improve sensitivity [8]. An interesting trend in the literature is to assess the effect of antimicrobial compounds on samples of bacteria over a short incubation time and via the use of surface tethered oligo nucleotides, measure changes in the abundance of nucleic acids as a result of antibiotic exposure. Use of probe sequences which can identify organisms (e.g. 16S rRNA) are particularly popular and studies have been published which demonstrate this sensing principle for *Enterobacterales* and *Pseudomonas aeruginosa* [9], and *Mycobacterium smegmatis* [10]. Another interesting approach for improving the sensitivity of genotypic sensor systems is device miniaturisation. This is because smaller sensors can give improved signal-to-noise and enhanced mass transport resulting in increased sensitivity to nucleic acid hybridisation on the sensor surface. With this theme in mind, a nanoelectrokinetic sensor for Methicillin-resistant *Staphylococcus aureus* (MRSA) genomic DNA was showcased which can detect specific genes from MRSA with dielectrophoretic enrichment to accelerate hybridisation and

impedimetric read out of hybridisation [11], and a simple microfabricated microelectrode sensor was employed to show a 10-fold increase in a DNA hybridisation assay for a key gene in *Mycobacterium tuberculosis* (TB) [12]. An important consideration in AMR is the environment; it is known that as well as hospital environments, the natural environment is a source of selective pressure which can result in development of drug resistant organisms. One area where this is particularly the case is for the water course and so groups have looked at the detection of important antibiotic resistance genes (e.g. beta-lactam hydrolase) in water samples, again using DNA hybridisation which can be read out in a simple label-free manner using impedance [13]. Whilst genotypic AST has some advantages, there are a number of crucial disadvantages which include DNA amplification steps such as PCR can significantly lengthen time to result, detection of a resistance gene does not always infer phenotypic resistance meaning the organism may appear resitant but in fact be susceptible, bacteria in a given sample may be detected but could have been dead at sample collection, multiplexing can be difficult and next generation sequencing has emerged in recent years as the dominant technology for genotyping. These disadvantages explain the current popularity of phenotypic testing which will be covered in the next section.

Phenotypic Approaches

Phenotypic approaches for rapid determination of AST take their inspiration from classical microbiology techniques, where bacteria are cultured on suitable media and growth observed at a later stage. Due to advances in microsystems engineering and lab-on-a-chip technologies, it is possible to probe bacterial growth more sensitively than using a traditional agar plate or liquid culture. This has led to a great diversity of sensor technologies with differing degrees of sample/microfluidic sample handling and integration. A number of electrochemical approaches to determining AST have been reported and firstly electrochemical impedance spectroscopy (EIS) will be covered. The EIS technique allows for the AC perturbation of an electrochemical cell and measurement of the associated current response to allow measurement of several important parameters, including the solution resistance, double layer capacitance, charge transfer resistance, and Warburg impedance. Due to the richness and high sensitivity of the EIS approach it is possible to probe several aspects of cellular growth, meaning that the technique is well suited to AST measurements and monitoring of biofilm formation [14]. A relevant example is the modification of an electrode surface with agarose gels and the subsequent use of gel-antibiotic modified electrode sensors to monitor inhibited and uninhibited bacterial growth, therefore inferring antibiotic sensitivity (example work is shown in Figure 1). This approach so far has been used to probe the antibiotic susceptibility of MRSA [15] and *E. coli* [16] in a simple and cost-effective manner through the use of gel modified, screen printed electrodes and without sample handling, enrichment, or system integration. The agarose modification approach has also been demonstrated with microelectrode devices for the measurement of bacterial metabolism [17]. Impedimetric measurements are particularly well suited to measurement of bacterial growth because the technique is sensitive, label free and provides information about the resistive and capacitive properties of the system under test. This enables several aspects of bacterial growth, such as membrane permeability, cell lysis and accumulation of cells to be monitored with minimal measurement complexity such as use of labels or wash steps.

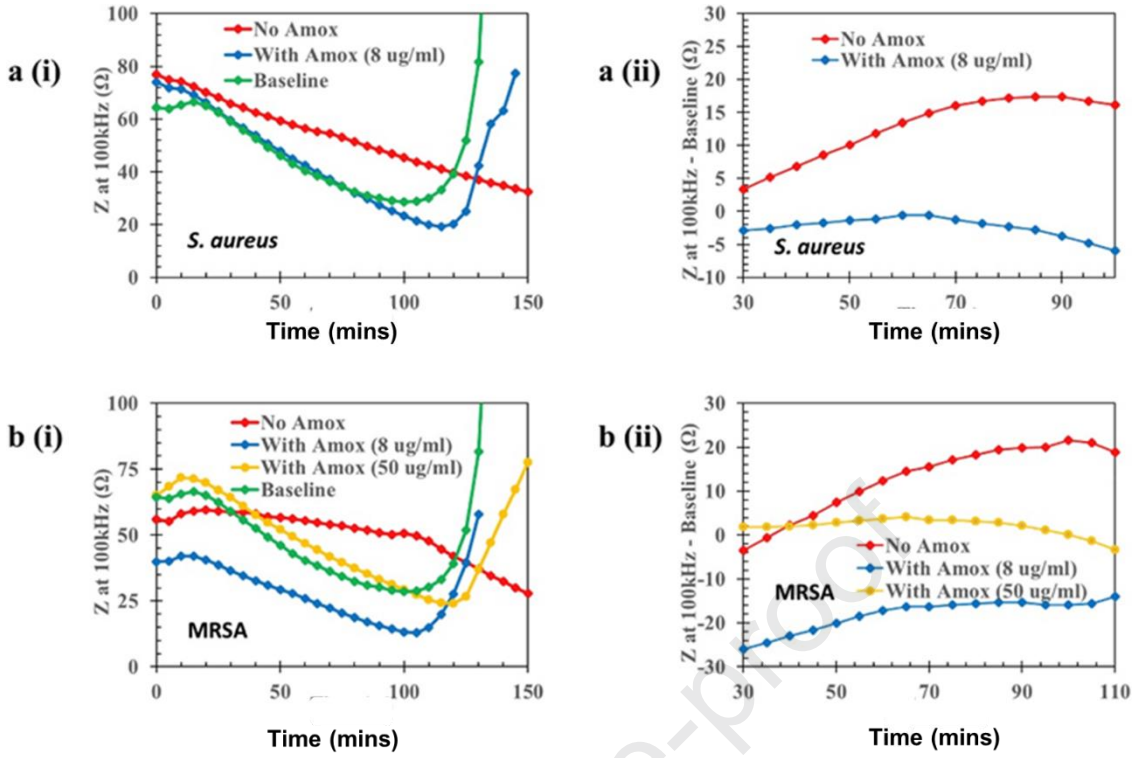

Figure 1. Example growth curves from an impedimetric AST system developed through modification of screen-printed sensors with antibiotic-gel combinations in order to reveal growth profiles for drug-susceptible staphylococcus aureus (*S. aureus*) and drug-resistant (MRSA) organisms in the presence and absence of amoxicillin [15]**. A(i) and B(i) show raw impedance growth curves. A(ii) and B(ii) show baseline subtracted impedimetric growth curves with upward gradients being indicative of growth and static or descending gradients being indicative of inhibition of growth. Detecting MRSA is crucial for good clinical management of drug resistant infections. The pathogen is routinely screened for in UK Hospitals and its presence determines isolation and discharge decisions. Developing fast and low cost testing solutions would dramatically enhance clinical workflows relating to this troublesome drug resistant pathogen.**

Other impedimetric systems offer more comprehensive integration and effective merging of detection, culturing, and separation technologies. A particularly elegant example of an integrated impedimetric system is 'iFAST', which stands for impedance-based Fast Antimicrobial Susceptibility Test [18]. The device (summarised in Figure 2) measures changes in the electrical and morphological properties of many thousands of single organisms at high throughput using microfluidic impedance cytometry. The system, as reported, utilises a sample developed by overnight incubation and within 2 to 3 minutes of loading onto the device can measure changes resulting from antibiotic exposure. The authors demonstrated that susceptibility profiles can be established for a wide range of antibiotics with a number of different modes of action. Other AST systems have been devised with similar operating principles to the iFAST system. Whilst impedance can provide useful information about several key electrochemical parameters, it is possible to conduct simpler measurements. Several studies have been published showing the use of conductometric measurements for *E. coli* susceptibility determination [19], and resistance measurements in channels on a microfluidic chip [20] to measure susceptibility.

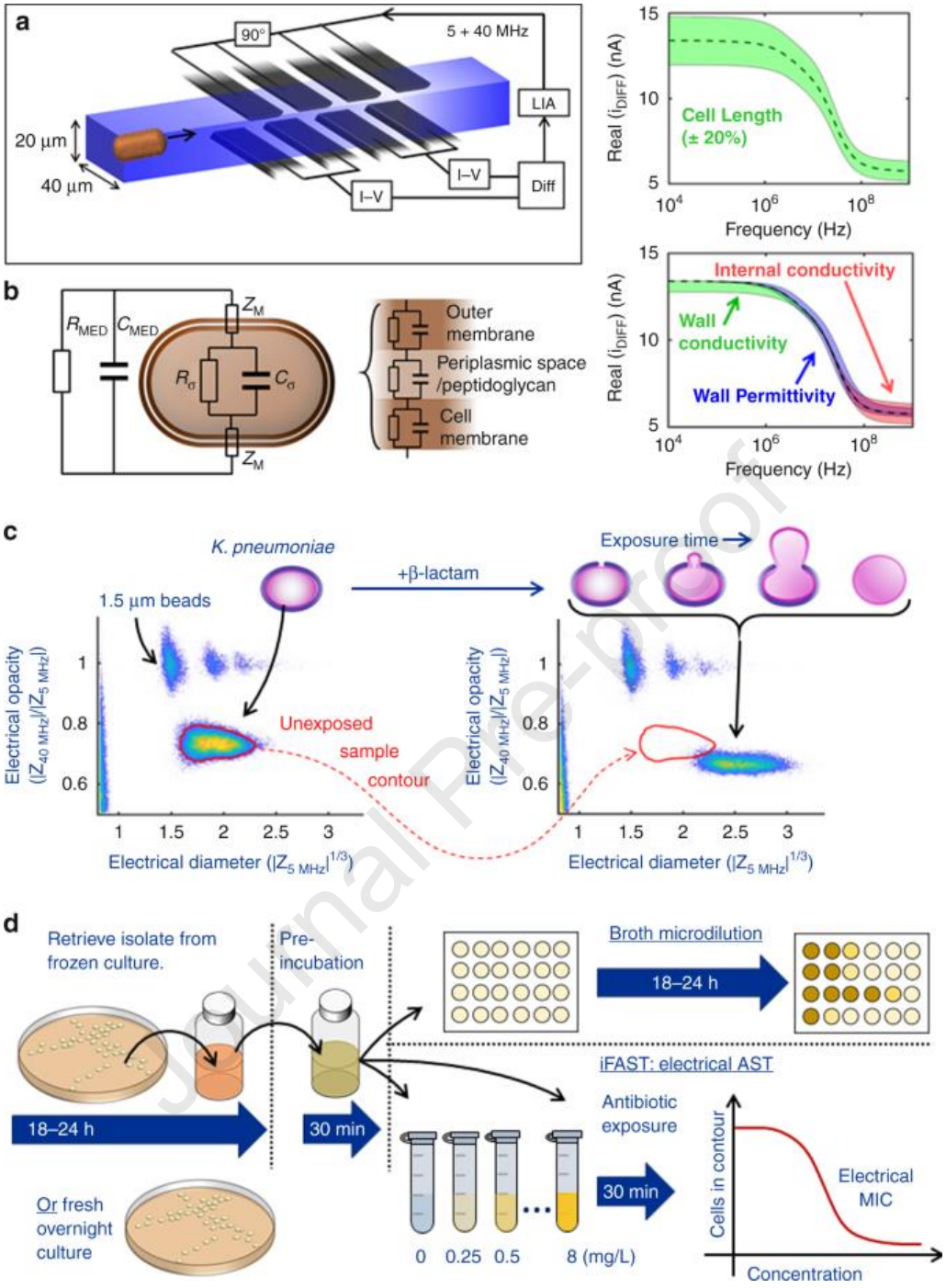

Figure 2. Overview of the 'iFAST' system used to measure changes in the electrical responses of bacterial populations [18]**. (A) Multi-electrode microfluidic impedance chip. Cells flow one-by-one between sets of electrodes and are measured simultaneously at two frequencies using a lock-in-amplifier (LIA). (B) Equivalent electrical equivalent circuit model for a Gram-negative bacteria, and a simulated spectrum of the Real part of the impedance vs. frequency highlighting frequency windows wherechanges in cell properties become apparent. (C) Impedance scatter plot of bacteria (K. pneumoniae, 10,000 events) together with 1.5-µm diameter polystyrene beads (with doublets and triplets) that are used as reference particles. The x-axis is the cube root of the impedance (proportional to diameter) measured at a frequency of 5 MHz. The y-axis is the electrical opacity, a measure of membrane/cell wall properties normalised to cell volume. This is measured at 40 MHz where the electrical properties of the cell wall and membrane are most apparent (see b). Two data**

sets are pre- and post-exposure to Meropenem at the clinical breakpoint for 30 min at 37 °C. In the scatter plot, the red contour defines the initial cell population. The diagram illustrates the change in cell properties following exposure to a β-lactam antibiotic as the cell wall breaks down (reduction in opacity) and the bacteria swell (increase in volume). (D) Experimental methodology for the impedance-based Fast Antimicrobial Susceptibility Test (iFAST). An actively dividing culture is prepared and incubated for 30 min with antibiotics. Polystyrene beads are added and the sample is measured for 3 min to determine the electrical MIC.

Moving on from impedimetric sensors, another popular area within electrochemical phenotypic measurements are respiration-based assays. In these systems, bacterial metabolism is measured, often through a redox mediator and correlated with antibiotic effect. A particularly sophisticated example is a study which measured oxidation of potassium ferrocyanide, where ferricyanide was used as an electron acceptor from living bacteria [21]. The technology was demonstrated with *Burkholderia pseudomallei,* which is a particularly antimicrobial-resistant pathogen that causes lethal melioidosis in tropical climates. Another nice example of this type of assay was the measurement of external electron transfer through a liquid medium using phenazine methosulfate as the electron carrier. The technology was demonstrated with *Acinetobacter baumannii, Staphylococcus aureus, Escherichia coli, and Klebsiella pneumoniae.*The times-to-result were 90 minutes in culture/development samples and 150 minutes for blinded samples [22]. Taking the respiration concept and integrating it into a lab-on-a-chip format is particularly effective for AST determination. For instance, it has been shown that a microfabricted chip with microchannels for capture and culture of bacteria enables measurement of resazurin reduction to resorufin (as a proxy for viability). The approach was demonstrated with urine samples and following culture to identify the pathogen. Results were possible within 1 hour of culture on the device [23]. Approaches akin to microbial fuel cell systems have also been reported where measurement of the total accumulated energy and its correlation with gold standard MIC assays was demonstrated using the common pathogen *Pseudomonas aeruginosa* [24]. Methylene blue has been used as a redox probe to measure microbial metabolism and in the process showing that sensitivity of four *E. coli* isolates to ciprofloxacin, gentamicin, and ampicillin could be readily established [25]. In addition to these methods highlighted, several other studies have reported viable routes to determining AST in a phenotypic manner [26]–[33].

Electrode Modifications

An important subclass of phenotypic electrochemical AST systems are those involving at least one electrode modification. Such electrode modifications can vary greatly in complexity, but broadly attempt to either improve electron transfer properties in order to enhance sensitivity, or directly incorporate a redox reporter molecule so that bacterial metabolic status can be directly probed on a pre-modified sensor. Here, two particularly strong examples stand out. Both make use of the previously mentioned molecule resazurin and the measurement of its reduction to resorufin by bacterial metabolism (as a proxy for cell viability). The first study demonstrates the production of bulk modified screen-printed carbon electrodes where graphitic ink is modified with resazurin to produce a screen printed sensor which through differential pulse voltammetry (DPV) measurement can reach a limit of detection for resazurin of 15.6 µM. The devices are then used to monitor growth of *E. coli* ATCC® 25922 in the presence and absence of gentamycin sulphate towards eventual application in urinary tract infection (UTI) detection [34].

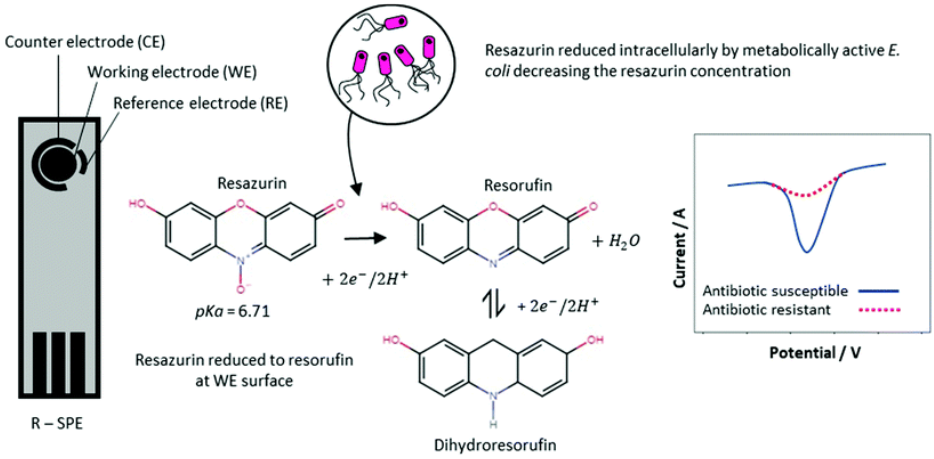

Figure 3. Pictorial scheme describing the production and use of bulk-modified screen-printed sensors for monitoring antibiotic susceptibility [34]**. The bulk modified electrodes permit the measurement of resazurin as a proxy for cell viability. For susceptible bacteria the reduction wave is present because viable bacteria are not present and so the resazurin within the bulk modified surface remains unconverted. For resistant bacteria which are actively growing the wave is absent because the available resazurin on the electrode surface has been converted by the metabolically active organisms able to grow in the presence of antibiotics.**

The second example again utilises a sensor based on direct modification with resazurin with the work reporting the development of organic redox-active crystalline layers for reagent-free electrochemical antibiotic susceptibility testing (ORACLE-AST). The system developed is used to demonstrate the measurement of *E. coli* K-12 treated with two antibiotics, ampicillin and kanamycin. The study reported a detection capability of 60 mins for prediction of antibiotic susceptibility profile [35]. A prominent modification of electrodes for the purpose of phenotypic AST determination is the use of carbon nanomaterials with examples including carbon nanodots [36], multi-walled carbon nanotubes [37 & 38], and graphene [39] all being deployed in AST systems. Other well-designed examples of AST systems produced with complex electrode modifications include direct modification of gold with vancomycin [40] and L-lysine-functionalized cerium oxide nanoparticle coated indium tin oxide to monitor bacterial viability in the presence of a range of relevant antibiotics [41].

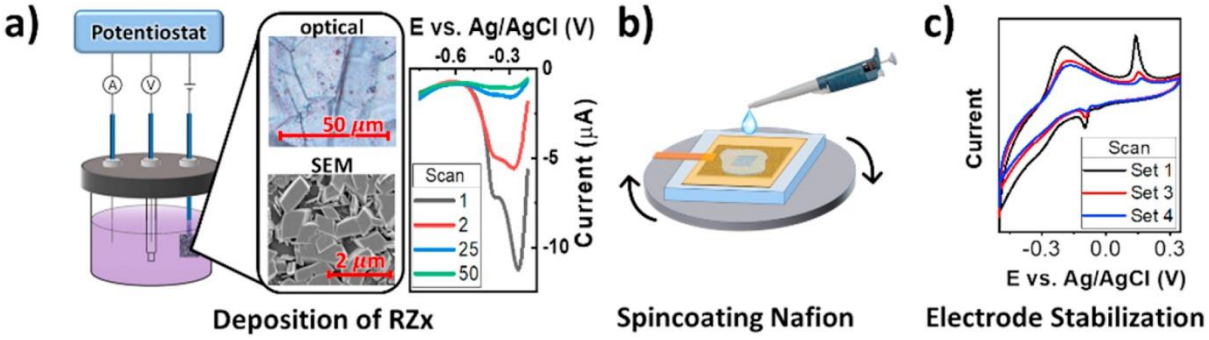

Figure 4. Scheme depicting the ORACLE-AST device which has been shown to effectively monitor drug susceptibility for *E. coli* K12 [35]**.**

There is great diversity into the approaches taken to determine AST electrochemically. Some studies, which do not easily fit our categorisation but are noteworthy, include: hardware developments to measure oxacillin resistance in a low cost manner [42], determination of bacteriostatic vs bactericidal

effects using impedimetric sensing [43], combining impedance with dielectrophoretic sample handling for AST determination [44], and nanoparticle-mediated capture and electrochemical detection of MRSA [45].

Summary and Conclusions

With the increasing occurrence of AMR and recent developments of lab-on-a-chip technologies, in the last five years there have been considerable increases in the number of published studies reporting electrochemical AST systems. This increasing interest means that there is a groundswell of evidence in the literature demonstrating, with high certainty, that reliable AST determination can be performed in a phenotypic manner in the range of 30 to 60 minutes. Electrochemical approaches have great potential for widespread deployment because of their low cost, ease of manufacture, and high analytical sensitivity and will almost certainly lead to development of commercially viable technologies which find clinical use because they greatly improve on existing techniques. One significant barrier that exists at the moment is the requirement to hit sub 10 minute times to result so that an AST result can be offered across the time frame of a doctor's consultation. This limit exists because of the sometimes low level of pathogen in a sample, the analytical sensitivity of the device, and the doubling time of the bacteria in question. Further into the future, meeting these challenges of high sensitivity and reducing the time to result from 30-60 minutes to 5-10 minutes is a key objective for those working in this field. A key driver for this is mandatory testing prior to antibiotic prescription. There is already a requirement in a handful of countries to have a confirmatory test before an antibiotic can be prescribed. This evolving and growing policy will eventually become the norm in most countries (around 2030) and it is therefore crucial that suitable technologies are developed to make this emerging requirement feasible.

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***of special interest**

****of outstanding interest**

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4.5 hour assay which uses functionalised nanoparticles to selectively capture bacteria of interest for determination by electrochemical means. Assay principle is demonstrated with a key pathogen that has critical implications for hospital admission, departure and isolation decisions.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: