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Review

Innovations in point-of-care electrochemical detection of pyocyanin

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ABSTRACT

Pseudomonas Aeruginosa (*P. Aeruginosa*) is a prevalent and opportunistic bacterium which frequently causes severe nosocomial infection. Current clinical detection methods are limited in terms of necessary time and equipment. Increasingly, *P. Aeruginosa*'s electrochemically active virulence factor pyocyanin has facilitated its rapid detection, primarily through voltammetric methods such as cyclic voltammetry (CV), differential pulse voltammetry (DPV) and square wave voltammetry (SWV). A comprehensive background on the synthesis and pathogenicity of *P. Aeruginosa* via pyocyanin (PyoC), is included herein. Innovative electrochemical sensor design and modification is reviewed, with a focus on screen-printed electrodes, paper-based devices, arrays, surface modification and biorecognition elements such as aptasensors.

Contents

1. Introduction

1.1 Nosocomial infection

Nosocomial, or healthcare-associated infections (HCAIs) are defined by the World Health Organisation (WHO) as: "An infection acquired in hospital by a patient who was admitted for a reason other than that infection" [1,2] or "An infection occurring in a patient in a hospital or other health care facility in whom the infection was not present or incubating at the time of admission, [including] infections acquired in the hospital but appearing after discharge [or] occupational infections among staff of the facility" [3]. With the WHO evaluating that around 15 % of hospitalised patients will suffer from a HCAI, they can be considered prevalent within the health care systems [4]. With an estimate 1.4 million people suffering from HCAI-related complications globally and 8.9 million HCAIs estimated to occur annually within European hospitals and long-term care facilities (LTCFs) HCAIs can be considered a global health crisis [5]. Extended hospitalisation as a result of HCAIs is the most significant contributor to the considerable economic burden these infections place upon the health care [6–8]. The use of additional hospital resources and equipment creates an imbalance within the healthcare system, which sees funds towards treating potentially preventable conditions [4]. Fig. 1 highlights the burden to the healthcare system as a result of some of the most prevalent HCAIs.

This review aims to compare a number of electrochemical sensors for the detection of *P. Aeruginosa*, via its redox active pyocyanin (PyoC) virulence factor. A comprehensive overview of the different sensor types available is provided, alongside their benefits and limitations in regard of the use of point-of-care devices for PyoC detection, and finally assessment of the potential for new research within this field is explored.

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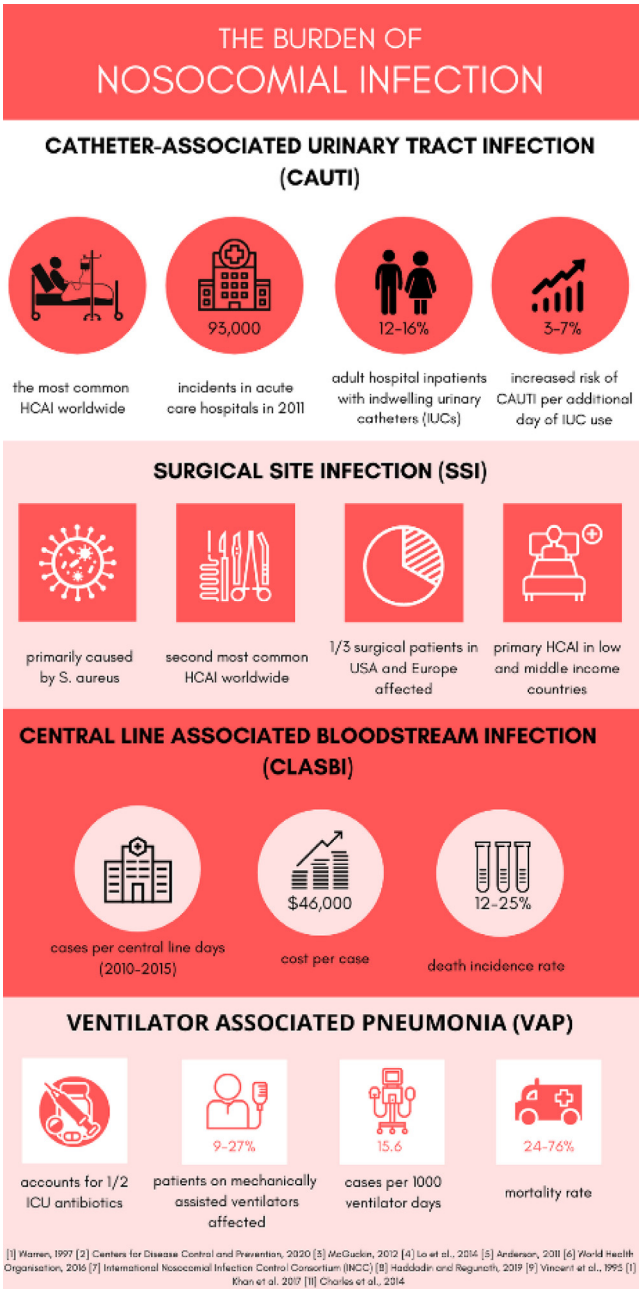

Fig. 1. Morbidity and mortality regarding the most prevalent nosocomial infectionsNosocomial pathogens comprise bacteria, viruses, protozoans and fungal parasites, though bacteria are most prevalent, constituting approximately 90 % of all infections [9,10]. Common agents contributing to HCAIs include enterococci, Streptococcus spp., S. aureus, Klebsiella pneumonia (K. pneumonia), Escherichia coli (E. coli) and Pseudomonas aeruginosa (P. aeruginosa) [11].

1.2 Pseudomonas Aeruginosa

P. Aeruginosa (shown within Fig. 2*)* is an opportunistic pathogen which frequently causes severe nosocomial infections, resulting in high mortality rates [12–20]. It is the fourth most common bacteria responsible for hospital acquired infections in Europe [21]. From 2016 to 2017, such infections cost the United Kingdom's National Health Service (NHS) in the region of £2.1 billion [22].

This gram-negative bacterium tends to thrive amongst patients whose immune systems are compromised [23] and is often considered the most perturbing bacteria associated with cystic fibrosis (CF) lung infections, as well as blood stream and chronic surgical and burn wound infections. Amongst CF patients, 80 % will suffer from a *P. Aeruginosa* infection at some point in their lives [24]. These infections tend to be highly inflammatory as they progress (refer to Fig. 3), leading to death in over 90 % of infected CF patients [25]. Immunocompromised patients are at higher risk of developing sepsis, ventilator associated pneumonia (VAP), urinary tract infections (UTI) and surgical site infection (SSI), to which *P. Aeruginosa* is a key contributor [26]. In these cases, mortality rates may reach as high as 76 %, depending on which pathogens are prevalent [24]. The presence of *P. Aeruginosa* is known to contribute to the delay of healing in chronic wounds. Wolcott *et al.* and Hogsberg *et al.* stated that for patients receiving split thickness skin grafts to treat chronic leg ulcers, *P. Aeruginosa* presence acted as a predictor of skin graft outcome, impacting the patient recovery both physically and emotionally [27,28].

Increasingly, *P. Aeruginosa* has displayed inherited and acquired resistances to many antibiotics [12,15–18]. Efforts have been made by the Centres for Disease Control and Prevention (CDC) and the Infectious Diseases Society of America (IDSA) to stem the spread of drug-resistant pathogens via diagnosis and monitoring, with an aim to minimise the spread of antibiotic resistant strains [29]. Early *P. Aeruginosa* detection is vital for successful treatment, whilst the bacteria can still be successfully treated with antibiotics, though this has proven difficult due to the lack of sensitive detection methods available at this early stage [30,31].

As previously stated, *P. Aeruginosa* is a gram-negative bacterium, whose outer membrane contains Protein F (OprF), which behaves as a porin, reducing permeability and endowing the microbe with a high antibiotic resistance [32]. This contrasts with gram-positive bacteria, which are multi-layered and more susceptible to antibiotics. To facilitate movement and display chemotaxis, *P. Aeruginosa* utilises a single flagellum, which also attaches to host tissues and promotes invasion during the early stages of infection [33]. Type IV pili [34], polar filaments comprised of homopolymers from pilin [35], endow *P. Aeruginosa* with the ability to bind to mucosal surfaces and epithelial cells. Fig. 2 (a) shows a scanning electron microscope (SEM) image of *P. Aeruginosa*, whilst Fig. 2 (b) depicts a computer-generated image of *P. Aeruginosa*, based on SEM imaging.

1.3 *P. Aeruginosa* biofilms

Biofilms have been defined as a mucilaginous accumulation of bacteria, suspended in an extracellular polymeric substance (EPS) matrix [37–39]. Often, biofilms will irreversibly attach to a surface, with particular success at a solid–liquid interface [37]. Their tenacity within a clinical environment derives from the use of implantable medical devices, which are associated with 60–70 % of HCAIs [40]. Antibiotic treatment of biofilm infections can reverse symptoms which are caused by the expulsion of planktonic cells but cannot kill the biofilm itself [41]. Therefore, symptoms are capable of recurring, ultimately rendering surgical intervention necessary [42].

Biofilms are readily produced by *P. Aeruginosa*, and greatly contribute to its pathogenicity [43]. Whilst other bacteria establish biofilms through cell division [39], *P. Aeruginosa* form microcolonies, using type IV pili to facilitate congregation [44–46]. Immune response of the host is subdued, furthering the progression of infection and limiting antibiotic efficacy [42]. PyoC is also known to be linked to biofilm formation [47,48].

1.4 PyoC

PyoC (Fig. 4) is the virulence factor produced by *P. Aeruginosa,* whose intrinsic redox activity presents the opportunity to monitor its presence and correspond this to the present of the *P. Aeruginosa* pathogen. Furthermore, it also acts as a quorum sensing (QS) molecule for

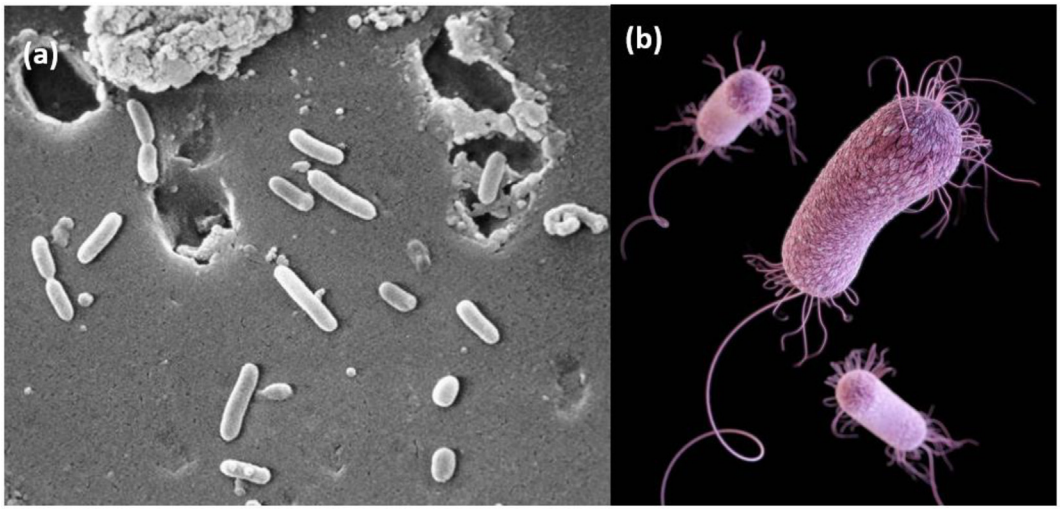

Fig. 2. (a) SEM image of P. Aeruginosa and (b) 3D computer-generated image of P. Aeruginosa bacteria J. Oosthuizen, Journal, 2013. P. Aeruginosa is characterised as a rod-shaped, monoflagellated bacterium of 1–5 μm in length and 0.5–1.0 μm in width [36].

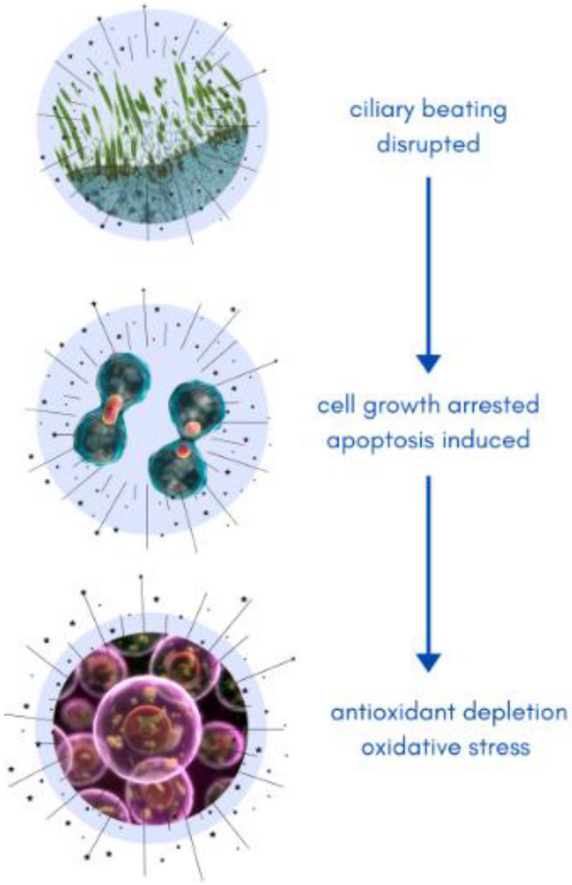

Fig. 3. Progression of P. Aeruginosa infection.

the pathogen. Several studies have confirmed its exclusive secretion by *P. Aeruginosa,* which uniquely carries the protein-encoding genes required for its synthesis [49–53]. QS, which occurs during wound colonisation, allows the bacteria to intuitively control gene expression, thus controlling virulence. As bacterial virulence is increased, host physiology is damaged, contributing to an increased risk of infection. It has been reported that QS results in PyoC being synthesised by 96–98 % of *P. Aeruginosa* strains [54,55].

PyoC has the molecular formula $C_{13}H_{10}N_2O$ and is defined as 'an iminium betaine 5-methylphenazin-5-ium, substituted at position 1 by an oxidanyl group.' It may exist in one of three states: oxidised, monovalently reduced or divalently reduced [56]. The redox activity of PyoC endows it with the ability to act as an electron shuttle, disrupting the redox homeostasis of host cells by relieving them of electrons which are then donated to oxygen (O_2), causing generation of reactive oxygen species (ROS) such as superoxide (O_2–) and hydrogen peroxide (H_2O_2). This results in cell damage and ultimately cell death [57]. Whilst PyoC has oxidative effects on other organisms such as *E.coli*, *P. Aeruginosa* itself appears immune to this effect, due to limited redox-cycling [58]. PyoC demonstrates promise as a diagnostic biomarker for the rapid identification of nosocomial infections involving P. Aeruginosa infection [51,52]. It has been reported that QS results in PyoC being synthesised by 96–98 % of *P. Aeruginosa* strains [54,55]. All *P. Aeruginosa* isolates studied by Sismaet *et al.* produced measurable concentrations of PyoC, with increasing severity of symptoms and cases of comorbidity as PyoC concentrations rose [18]. PyoC, has been observed in the μM and mM ranges dependent upon the analysed sample type, with sputum samples noted as containing up to 130 μM and the mM range observed within ear infection secretions [60].

PyoC redox activity arises from the reversible phenazine transformations at approximately −0.18 V and −0.28 V vs SCE and the non-reversible phenolic oxidation at ~0.85 V [54]. However the phenolic oxidation of PyoC has been observed to result in polymerisation which in turn leads to an increase in peak height and current with increasing scan rates (see Fig. 5). Electrochemical initiated polymerisation results from the two polymeric forms resultant from the phenolic oxidation combining with the monomeric PyoC within solution. Induction of this polymerisation is not desired as it limits the ability to effectively monitor PyoC within a given sample, and as such it is typical of electrochemical studies for the detection of PyoC to focus upon the phenazine transformations by limiting the scanned potential range to below 0.1 V [54]. Thus identification and monitoring of PyoC is made via the monitoring of the reduction peak at ~-0.2 V (Fig. 5).

1.4.1 Biosynthesis of PyoC

PyoC is known to be extremely pathogenic, causing inflammatory physiological effects to host tissue which leads to infection amplification and comorbidity [18,61]. Ciliary dysfunction (shown within Fig. 3) impairs mucociliary clearance, a vital line of defence for the

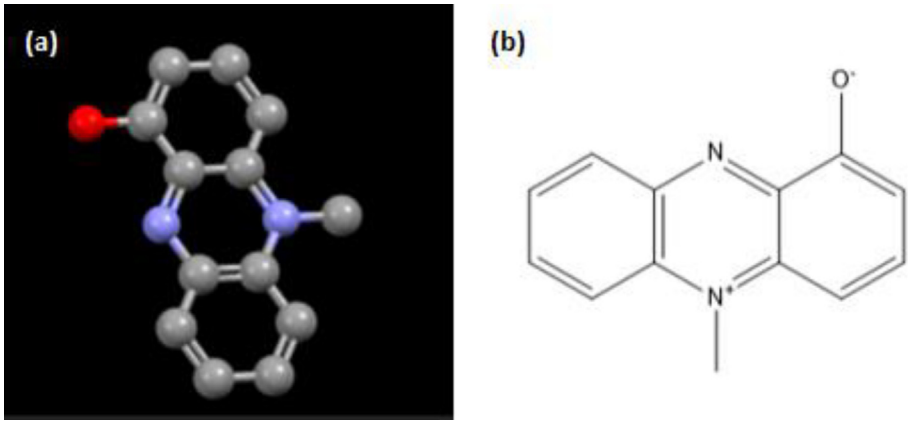

Fig. 4. (a) 3D and (b) 2D representations of pyocyanin structure.

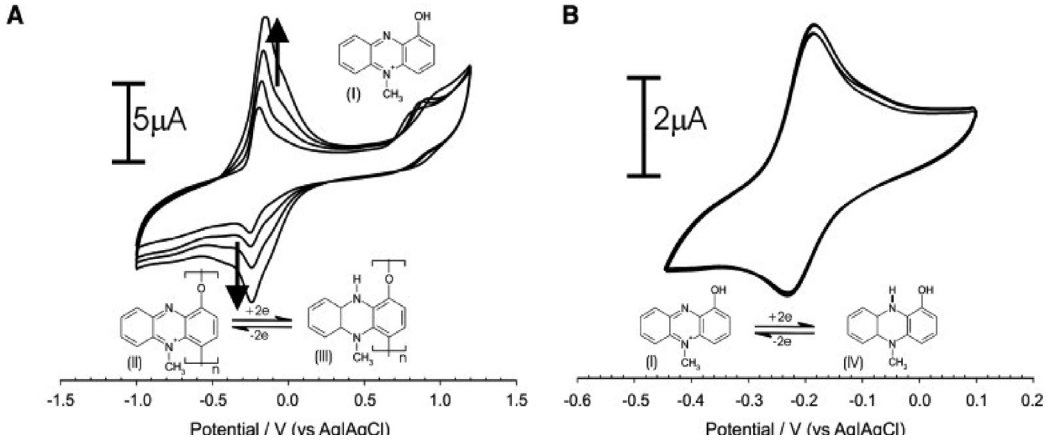

Fig. 5. Example CV showing the redox activity of PyoC where (A) show pyocyanin polymerisation due to high anodic potentials at 0, 10, 20 and 30 cycles. (B) shows pyocyanin detection by preventing polymerisation by limited scan range to − 0.45 V to + 0.1 V for 0, 10, 20 and 30 cycles. Reproduced from ref. 64 with permission from Elsevier copyright 2010.

lungs [62], and causes chronic nose, ear, sinus and chest problems [63–65]. Low concentrations of PyoC induce cellular senescence, with apoptosis occurring at higher concentrations. Inhibition of cell growth and replication impedes tissue repair and advances morbidity and mortality [66]. Antioxidant depletion results in the formation of free radicals, which, in turn, leads to oxidative stress [67]. Furthermore, the immune response of the host is attenuated and the switch from acute to chronic infection is promoted [68].

PyoC is biosynthesized from phenazine-1-carboxylate. 5-methylphenazine-1-carboxylate is an unstable intermediate, whose reactive nature facilitates the two-step conversion of phenazine-1-carboxylate to PyoC. This process is regulated by PhzM and PhzS, which are protein-encoding genes [69,70]. The toxicity of PyoC can be largely attributed to its redox activity, which causes depletion of cellular antioxidants such as NADH and glutathione [71]. It also modifies cytosolic concentration of calcium, causing disruption of ion transport regulation, ciliary beating, and mucus secretion by airway epithelial cells [72]. The mechanisms by which PyoC initiates pathogenesis are variable. It may inhibit nitric oxide synthase [73–75] or form a complex to interact with endothelium-derived relaxing factor or nitric oxide (NO). NO is vital for blood flow and blood pressure control, as well as immune function [76]. PyoC also inhibits epidermal cell growth [77] and lymphocyte proliferation [78], has antibiotic properties against other microorganisms [79], and influences the acquisition of iron by pseudomonads [80].

P. Aeruginosa-derived phenazines may also prompt alveolar macrophages to produce interleukin-8 (IL-8) and leukotriene B4. These are known as neutrophil chemotaxins; they populate the airways with neutrophils, inciting an inflammatory response and neutrophil-mediated tissue damage [81,82]. Phenazines are thought to boast such a broad range of biological activity due to their ability to undergo redox cycling in the presence of molecular oxygen and reducing agents. This causes a build-up of toxic O^{2-} and H_2O_2 and ultimately leads to oxidative cell injury or death [83–85]. PyoC can also act synergistically with pyochelin, a siderophore, and with transferrin cleaved by *P. Aeruginosa* or neutrophil-secreted proteases in infected lungs. This catalyses the formation of $OH^{\cdot}$, a highly cytotoxic agent which causes damage to pulmonary endothelial cells [83,85].

1.4.2 Oxidative stress of PyoC

Oxidative stress is defined as an imbalance between free radical production and antioxidant defences [67]. The term ‘free radical’ references any atom, molecule or ion which possess as at least one unpaired valence electron [86]. This imbalance infers increased chemical activity, which can lead to tissue injury [67]. The instability of free radicals leads to their high reactivity, and have a tendency to react with nonradicals, which are far more abundant within the human body. Possible targets include all biological macromolecules, proteins, lipids, nucleic acids, and carbohydrates [86].

When a radical reacts with a nonradical, a free radical chain reaction is initiated, new radicals are formed, which can then go on to react with other macromolecules creating a cascading reaction. Prominent examples of this are protein damage and lipid peroxidation. The hydroxyl radical ($OH^{\cdot}$) is understood to be the most potent oxidant within the body and displays an extremely short half-life. It can attack most biological molecules and initiate the propagation of these free radical chain reactions. Oxygen may accept an electron to form O_2-, which in itself is not particularly reactive with a weak oxidative potential. It is much more successful species for the reduction of iron complexes (e.g., cytochrome *C*). However, it can dismutase to form H_2O_2 [66], a means by which PyoC has induced oxidative stress in both epithelial [87] and endothelial cells [88].

Mohamed *et al.* studied the effects of oxidative stress on *P. Aeruginosa* in mice. The susceptibility of *P. Aeruginosa* to various antibiotics was altered upon exposure to H_2O_2, with minimum inhibitory concentrations (MICs) either increasing or decreasing. QS genes were found to be expressed at a significantly lower level in H_2O_2-stressed cells. Overall, oxidative stress exposure reduced *P. Aeruginosa* pathogenicity in the host [89]. Previous studies had reported an increase in *P. Aeruginosa* host virulence in response to oxidative stress [47,90]; however, this can likely be explained through experimental differences [89]. It is also important to consider that bacterial defences may be increased by low levels of oxidative stress, whilst increasing levels can markedly damage bacterial cells, due to excessive ROS production, per the hierarchical oxidative stress model [91].

PyoC has been shown to contribute to the unusual tenacity of *P. aeruginosa* infections [47]. PyoC-associated ROS have been linked to cellular phenomena which enhance *P. Aeruginosa's* capacity for survival [92]. *P. Aeruginosa* pathology is also increased by weakening of host defences, via depression of mucociliary transport [93,94] and the initiation of bronchoconstriction if nebulized into the airways [95].

Considerable antioxidant defence mechanisms are in place which act to protect the body from free radical attack. Gutteridge described an antioxidant as "any substance that, when present at low concentrations, compared with those of the oxidizable substrate, considerably delays or inhibits oxidation of the substrate." Catalase, dismutase and peroxidase enzymes are key cellular antioxidant defences [96]. Furthermore, the ability of mitochondrial cytochrome oxidase to act as a catalyst in the electron transport chain (ETC) without the release of ROS greatly decreases potential for intracellular production of free radicals [97].

2 Detection methods

Given the swift progression of *P. Aeruginosa* infections, rapid detection in situ is vital for efficacy of treatment. Current bacterial identification methods typically take 24 h or more to perform; whilst newer molecular and biochemical identification methods improve upon this delay, they require a sufficient number of cells for investigation, thus requiring an incubation period of several hours [98]. Some clinics utilise polymerase chain reaction (PCR) identification, which can give quantitative results and requires less timely analysis (~1 h+). However, this technique requires extensive sample preparation, as well as expensive reagents, which contribute to its limited clinical availability [99]. Processing results of this technique can also take several hours and a degree of expertise in the technique required, deeming it inadequate for employment as point-of-care clinical testing [43]. Absorbance and spectroscopic methods offer robust results; however, their operation can be complex for those not specialised in these analytical techniques. PyoC must first be extracted from samples through liquid extraction with chloroform and subsequently purified, which requires trained technicians and the use of hazardous solvents, meaning an appropriately equipped laboratory must be used. Surface-enhanced Raman spectroscopy (SERS) can also be utilised for PyoC detection. This technique enables the enhancement of Raman signals via plasmonic nanostructures on the surface of the substrate [100–102]. Its high sensitivity [103–105], rapidity, and reliability [101] as an analytical tool have led to multiple SERS sensors being studied for the detection of PyoC. SERS has also been used to study the formation of P. Aeruginosa biofilms by monitoring PyoC signals [106–107]. The need for this extensive sample preparation processes also contributes to longer detection times of these techniques [99]. Liquid chromatography-mass spectrometry (LC-MS), often considered the gold standard detection system in a number of fields, can produce quantitatively reliable results; however, expansive instrumentation is required, a fully equipped laboratory, specialised personnel to perform and supernatant extraction from the growth medium [30]. Microbiological colony identification is another commonly employed method, though it suffers from low sensitivity and still requires the use of costly equipment and reagents. Additionally, the process is laborious, increasing time and labour costs [108]. Automated instrumentation techniques, may initially appear a promising alternative but they call for plate culture preparation to obtain a bacterial colony, incurring a lead time of 18–24 h [109,110]. Considering the current limitations facing these detection methods the need for the development of a simple, sensitive PyoC detection method becomes clear. Results must be achieved rapidly and in a cost-effective manner, without any need for pre-treatment or sample incubation.

Electrochemical methods may present a solution to bridge this gap. Their high sensitivity and rapidity lend themselves well to efficient analyte detection. Techniques such as cyclic voltammetry (CV) and differential pulse voltammetry (DPV) are simple and cost-effective, especially compared with the current detection methods which have been previously described. CV is used extensively within the field of electrochemistry. Its applications are far reaching and include the study of simple redox processes and the characterisation of multi-electron transfer processes [111]. Since biosensor performance relies on redox activity, CV is well suited to their characterisation [112]. DPV is also an effective analytical tool, which is more sensitive than CV for lower analyte concentrations [113]. It enables in-depth study of chemical reactions, including their mechanisms, kinetics and thermodynamics, and can also be used for quantitative analysis [114]. Increasingly, the electroactive nature of PyoC has been exploited to enable electrochemical detection methods, a comprehensive summary of such methods is presented within Table S1 [100]. The increased interest surrounding the electroactive nature of PyoC and its exploitation in electrochemical detection strategies has resulted in a number of recent reviews on this topic [115–119]. Various studies have utilised PyoC as a *P. Aeruginosa* biomarker due to its inherent redox activity [18,43,54,59,70,99,120–125]. Its excellent electrochemical activity, due to its substituted phenazine structure, makes the toxin especially applicable for detection using cyclic voltammetry (CV) [126]. The electrochemical signals produced may also be amplified using redox activation or cycling, which would enable ultrasensitive detection of diagnostic biomarkers [127].

The study of electrochemical biosensors is increasing in popularity. These sensors are cheap to produce, simple to operate and can be extremely sensitive. Their portability, speed of operation and operational simplicity renders them applicable for incorporation into point-of-care devices. As such, they offer the potential for real-time diagnosis and monitoring without the need for pre-treatment or purification proceedures [23,115,123,128–130].

2.1 Screen-printed electrodes

Screen-printed electrodes (SPEs) are an attractive and increasingly prevalent option for low-cost, disposable biosensors and thus ideally suited toward point-of-care devices. Their manufacture involves the layered deposition of ink onto a solid substrate, through a screen shaped to the required geometry. Often, SPEs will feature a three-elec-

trode configuration (working (WE), reference (RE) and counter (CE) electrodes) which lends itself to electrochemical analysis. Inks may be commercial or self-produced and can include a wide selection of materials, including catalysts [131,132]. Furthermore, the expansion of nanomaterials in recent years has allowed the electrochemical properties of SPEs to consistently advance [133].

Though limited to flat substrates, there are many advantages to the screen-printing process. Designs are flexible; electrode area, thickness and composition are easy to control and adjust. Good reproducibility allows for statistically valid experimental results [132,134]. Given their bulk manufacture, SPEs can be produced cheaply and at high rates. Their simple operation negates the need for pretreatment or highly skilled personnel and allows for real-time, point-of-care testing [133]. These capabilities lend themselves well to the detection of *P. Aeruginosa,* commonly through recognition of PyoC [18,43,59,70,121–122].

2.1.1 Carbon SPEs

Various studies have utilised carbon WEs and CEs in conjunction with silver (Ag) REs to detect PyoC using square wave voltammetry (SWV) [18,43,70,121]. SWV techniques boast rapidity [121], although Cernat *et al.* also utilised CV for electrode characterization [70]. Sismaet *et al.* have successfully detected PyoC from clinical isolates taken from cystic fibrosis patients and HCAI patients, as well as fluid and biofilm samples from patients suffering from open wounds [18,43]. Their methods have proven potential for point-of-care *Pseudomonas* screening, with their PyoC probe displaying 71 % sensitivity and 57 % specificity when compared with 16S rRNA sequencing. Further work is required, however, to comprehend the clinical implications of false positive and false negative tests [43]. PyoC was detected by Cernat *et al.* in real biological samples with spiked analyte, using SPEs modified with an agar hydrogel and Au/Ag nanoalloy. They obtained a linear range of 0.12 – 25 μM, with a limit of detection (LOD) of 0.04 μM (SNR = 3). PyoC detection in whole blood was possible within 5 – 10 min of sample collection [70].

Screen printing also allows for innovations regarding print surfaces. Ciui *et al.* screen-printed electrodes onto nitrile disposable gloves to allow for simple operation and speed, with a detection time of approximately 4 min. Electrodes were highly sensitive (2.12 μA/μM), with an R^2 value of 0.935, and were stable over 14 days, with a relative standard deviation (RSD) value of 4.14 % (n = 5). Translating this innovative technology into a clinical setting will involve the integration of handheld, wireless instrumentation already commercially available [121].

2.1.2 Gold SPEs

Commercial screen-printed gold (Au) electrode chips (Au WE, Au RE, Ag CE) have also been used to detect PyoC through CV. These studies reported adequate biomarker detection, with relatively high sensitivity [59,122]. However, it is important to note that the use of Au can be limited by amalgam formation, which alters the structure at the electrode surface [135]. The necessary surface cleaning procedures can also be time consuming and involve hazardous chemicals, making them unsuited toward a clincal enviorment [136].

One significant advantage of gold-based electrodes however is the ability-two combine both electrochemical techniques with that of surface enhanced Raman spectroscopy (SERS), which can be performed on metallic surfaces such as gold and silver. Do *et al.* confirmed that PyoC detection was possible during early stages of infection through the combination of EC-SERS for the detection of other *P. Aeruginosa* bacterial biomarkers, thus indicating that it would equally be suited for PyoC detection for the *P. Aeruginosa* pathogen [122]. Alatraktchi *et al* obtained an R^2 value of 0.991 for PyoC quantification upon gold electrodes, with its detection in human saliva displaying a standard deviation of 2.5 % ± 1 % (n = 5) from the PyoC concentration added to samples. A detection window between 0.58 V and 0.82 V was identified as being free from the interference of other redox-active compounds naturally present within such matrices. Achieving a LOD of 2 μM through the negation of any interfering compounds [59].

2.1.3 Graphene SPEs

Graphene and graphite-based SPEs have also been utilised for PyoC and 2-AA [26], detection using CV and differential pulse voltammetry (DPV) [137–139]. These materials offer an advantage in terms of cost and potential range over other metallic surfaces such as gold or silver. Gandouzi *et al.* succeeded in detecting PyoC using surface-modified graphite SPEs [137,138]. Detection was achieved with linearity for a concentration range of 1 – 100 μML^{-1} (correlation coefficient, R^2 = 0.995, RSD = 4.916 %). The LOD was found to be 333.33 nML^{-1} [138]. Recovery tests were also performed for PyoC detection in real samples (tap water, human serum, saliva). Recoveries for 25 μM/L PyoC ranged from 98.41 % to 100.55 % for tap water; 98.83 % to 101.88 % for human serum and 96.5 % to 102.12 % for saliva, all of which lie within the acceptable tolerance range of analytical methods, indicating a significant reliability of the methodology. RSD values ranged from 1.9 % to 4.1 % [137–138]. All concentrations which lie within the μM to mM range of PyoC found within clincal samples. Metters *et al.* discussed the merits of SPEs for point-of-care electrochemical detection as well as their economic benefits. They were able to use carbon-based SPEs to detect 2-AA, with a detection limit of 7.6 mM. This study provided proof of concept that 2-AA can be detected with electrochemical techniques [139].

The utilization of SPEs for electrochemical sensing is a valuable area of research. Their capacity for cheap mass production, simple operation, and the fact that pre-treatment steps are not required, render them as a viable technology for future development [133]. The adaptability of SPEs currently allows for the detection of various analytes and will be the key to expanding their applications in future. For example, the incorporation of biomaterials within SPEs can greatly increase sensitivity and selectivity and is an area of research which is constantly expanding. The incorporation of unique materials will contribute significantly to the field, and broaden possible applications [131]. The electrochemical properties of SPEs are constantly improving, due to advances in nanomaterials; this is likewise an ongoing area of growth [133]. Also notable is the potential for SPEs to be incorporated into portable and miniaturized devices; given the enduring need for electronics miniaturization, it can be assumed that new applications will continue to arise within this field [131–132].

2.2. Paper based devices

Some studies have found success using paper-based sensors for PyoC detection [128,130]. Paper-based electrodes have several desirable attributes, including affordability [128,140], facile fabrication and modification, and ease of use. They are also disposable and biodegradable [128,130,141]. Alatraktchi *et al.* [130] used screen printing to deposit carbon ink onto photo paper for the fabrication of a 3-electrode setup. Square wave voltammetry (SWV) was utilised for the quantification of PyoC, producing an LOD of 95 nM and an R^2 vale of 0.993. Compared to a commercial ceramic-based sensor, it was noted that the paper-based SPEs exhibited a 2.3-fold enhanced performance. Manisha et al. [128] used computer numerical control (CNC) techniques to print paper electrodes using conductive carbon and Ag/AgCl ink. CNC is advantageous for large scale manufacture, due to high quality and uniformity, and has improved economy compared to screen printing, as substantially less ink is required. Printed electrodes were modified with carbon nanodots (CND), via the process depicted in Fig. 6 and gave an LOD of 154.47 nM with high selectivity.

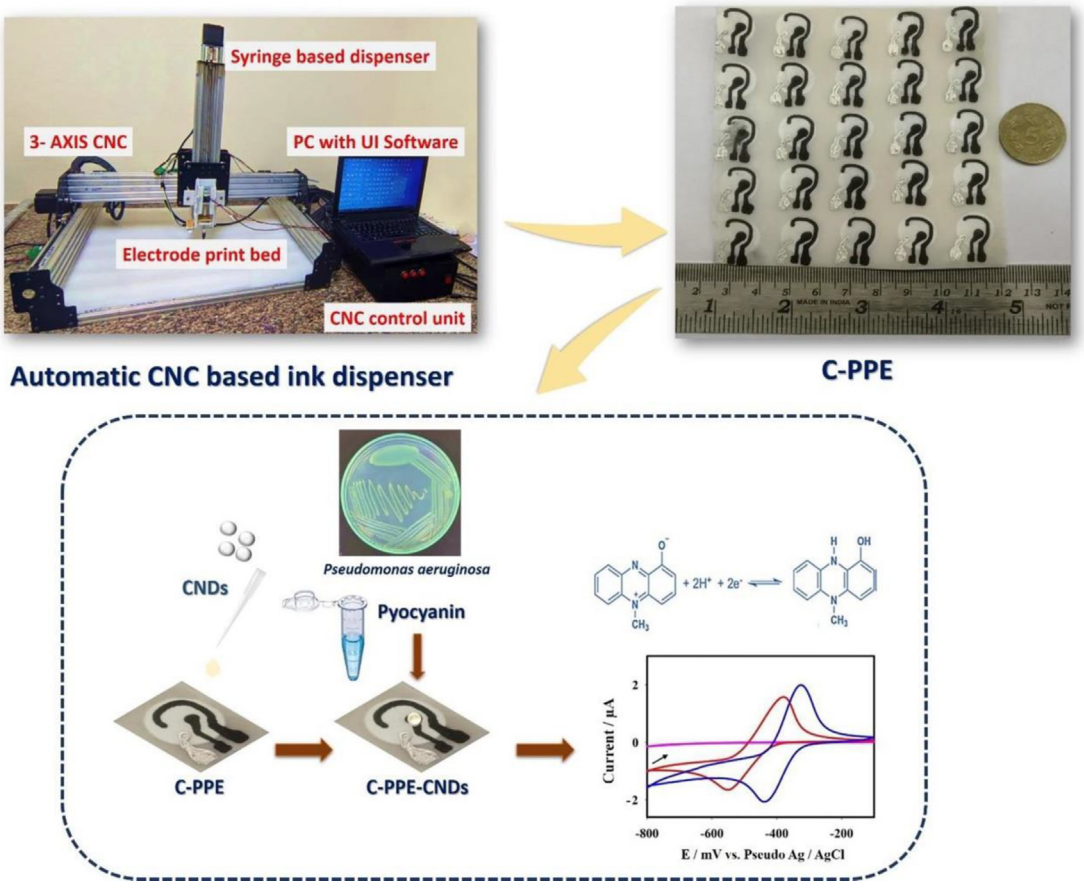

Fig. 6. Fabrication process for the production of the paper-based electrodes (C-PPE) including modification procedure with carbon nanodots and the resultant voltametric signal from PyoC. Reproduced from ref. 130 with permission from Elsevier copyright 2019.

Linearity was achieved for 4.45 µM to 25 µM PyoC, with an R^2 value of 0.99.

2.3 Arrays

The development of electrode arrays has increased in popularity within recent years, and are particularly suited toward POC devices, through their ability to easily incorporate multiplex detection allowing for a single specimen to be screened for a multitude of infections [125,142–146]. These are sensors, which contain at least two WEs [147], are utilised across a range of fields; including environmental, food and clinical analysis. Biosensors are one of the primary sensor types used in arrays, although they can contain multiple sensor types [148]. In the case of electrochemical sensor arrays (ESAs), multiple sensors allow for simultaneous detection of a number of different species [149]. The analytic capabilities of electrode arrays are considered competitive against the quantitative chromatographic separation techniques. Stefan *et al.* have summarised the primary electrochemical sensors used in sensor array construction [148]. The main aspects of array development are design, calibration, and convolution [150]. Design informs quality; especially regarding *in-vivo* clinical analysis [151]. In this sense, geometry and potentiostatic control are significant design factors. As the construction of ESAs has developed, it has become possible to glean chemical fingerprints for complex matrices [148]. The use of arrays is often preferred to chromatographic techniques, as there is no need for onerous sampling processes or expensive instrumentation. Their use increases rapidity of flow analysis [148] and generally, they offer valuable spatial and rapid temporal resolution [147]. ESAs are known to provide superior precision, particularly regarding clinical analysis [148]. However, high standards must be met to ensure satisfactory calibration, and it is necessary to record many measurements on known samples, prior to employment in point-of-care systems [150].

In a clinical setting, ESAs show promise as highly sensitive alternatives to PCR detection assays [152]. Sheybani *et al.* achieved bacterial detection levels of five orders of magnitude lower than comparable sensors, with an array which monitored local pH and bacterial cell attachment within wounds. This Au-based dual sensor array eliminated the need for biorecognition elements, external solutions, pre- and post- sample processing, and visual assessment of the wound. Arrays were stable over time and gave accurate, real-time results for initial testing within wound infection models [153]. The effectiveness of ESAs has also been illustrated within human clinical fluid samples. Liao *et al.* achieved species-specific detection of bacterial pathogens in uropathogens isolates and clinical urine specimens. The array consisted of sixteen sensors containing Au WE, CE and RE, with each WE containing a capture probe specific to a urinary pathogen, including a *P. Aeruginosa* probe. Capture probes acted to fix the target to the sensor, whilst detector probes allowed for target recognition on the sensor surface. Over 90 % of clinical microbiology library uropathogens were recognised by these probes. Results were acquired within 45 min from sample collection, without the need for labelling or target amplification. Notably, this was the first study to describe bacterial pathogen detection in human fluid samples using an ESA. Given the potential for miniaturisation, electrochemical sensors may well prove to be more cost effective and user friendly than current clinical sensors [152].

Baldrich *et al.* were able to detect N-(3-oxo)-dodecanoyl-L-homoserine lactone (oxo-C12-HSL), an acyl homoserine lactone (AHL) pro-

duced by *P. Aeruginosa*, in artificial saliva as well as spiked cultures and *P. Aeruginosa* supernatants, using Au microelectrode arrays. AHLs were detected indirectly, via beta-galactosidase (β-gal) activity, which is AHL-induced. CV scans resulted in detection limits as low as 2 pM, in 20 μL sample volumes. Measurements took seconds following a 2-hour assay [142]. Transparent carbon ultramicroelectrode arrays (*T*-CUAs) have been utilised in various studies to detect *P. Aeruginosa*-derived phenazine metabolites [143–145,146]. These arrays have excellent conductivity; they are inert, highly biocompatible and boast rapid response times, amplified current responses, high SNR and low LODs [125,145–147,154]. *T*-CUAs have been used by Simoska *et al.* to monitor PyoC, 5-methylphenazine-1-carboxylic acid (5-MCA), and 1-hydroxyphenazine (OHPHZ [143,144]). Elliott *et al.* successfully utilised *T*-CUAs to electrochemically detect PyoC, with LODs as low as 1.0 ± 0.3 μM for 1.54 *T*-CUA with an LDR of 1–100 μM.

The merits of sensor arrays are vast and can largely be attributed to their versatility. Utilising two or more electrodes makes it possible to detect multiple targets simultaneously, with each electrode individually addressable [147]. Individual sensors can be specifically modified, for example with nanoparticles (NPs) or biorecognition elements, to optimise their functionality [125]. Spatio-temporal analysis of analytes is also possible, as multiple sensors can be used for 'mapping' [147]. A broad range of electrochemical sensors may be utilised; particularly common are amperometric sensors, gas sensors, ion-selective and membrane electrodes [148].

However, conscientious array design is vital to sensor performance. Careful consideration of the target medium is necessary for addressing which matrix should be used. Polymer-based matrices are favoured over carbon-based, though they have relatively low construction reproducibility [148]. Where ultramicroelectrodes are utilised, often arrays will be designed to function in parallel to enhance SNR, since their ability to produce extremely low currents – in the picoamp (pA) range – may lead to noise limitations [145,155–158]. Despite this, their use enables higher current densities and low limits of detection [145].

2.4 Surface modification

Electrochemical sensors may be surface modified, to improve sensitivity and selectivity, a key component for any biomedical diagnostic platform [138]. Gold NPs (AuNPs) are often utilised for electrode surface modification and contribute to high sensitivity through signal amplification [123,137–139,159–160]. Elkhawaga *et al.* utilised ultrasensitive polyaniline (PANI)/Au NPs/indium tin oxide (ITO) mix to modified sensors for the rapid detection of PyoC in clinical isolates. A linear range was obtained from 1.9 μM to 238 μM, with a LOD of 500 nM. PyoC detection by SWV showed 100 % agreement with the molecular method regarding sensitivity and specificity, against other methods such as SPE and automated methods. High selectivity was also achieved for traces of PyoC in the presence of interferences such as vitamin C, uric acid, and glucose. Khalifa *et al.* utilised the same sensors for successful PyoC detection within corneal ulcer samples [123,124]. Zhang *et al.* used Super P (a highly conductive type of carbon black)/AuNPs treated Au electrodes to quantify *P. Aeruginosa*. The use of metal–organic framework (MoF) allowed for signal amplification, with the added advantage of a large surface area, as well as the ability to control its structure and aperture [161]. A zirconium series metal–organic framework (ZrMOF) has also been utilised, gifting the advantage of immobilise metal ions and biological ligands upon the electrode surface. Results were obtained in 120 min, with a linearity range of 10-10 [6] CFU/mL and LOD of 2 CFU/mL (SNR = 3) alongside good reproducibility and specificity, highlighting the potential advantages of MOFs for electrode surface modification [162].

The conjugation of an electrochemical active molecule (EAM) and a specific antibody upon redox-active AuNPs (raAuNPs) was utilised by Lee *et al.* This unique techqniue resulted in the enhancement of signals received by individual bacterium by six orders of magnitude at optimum conditions, since each AuNP contains thousands of EAMs. The self-assembled layer of EAMs presented good electrode coverage, which prevented direct solution contact; thus, electrode fouling was minimised [160]. Background noise from interferences was also reduced [163]. The resulting biosensor had high sensitivity, with a dynamic range of 10-10 [5] CFU/mL and a detection limit of 10 CFU/mL. As such, this sensor construction showed considerable potential as an electrochemical biosensor for pathogenic bacterial cell detection in blood plasma [160].

Chitosan gold NPs (CS/AuNP) and planar transparent macroelectrodes (*T*-Macro) were used by Elliott *et al.* to modify the surface of *T*-CUAs. Treatment with CS/AuNP elicited a LOD of 1.6 ± 0.2 μM and LDR of 1–100 μM; *T*-Macro modification increased sensitivity giving a LOD of 0.75 ± 0.09 μM and LDR of 0.75–25 μM. It was concluded that these parameters were sufficient for PyoC detection in a range of *in vitro* and *in vivo* cellular environments [125]. High sensitivity was achieved by Gandouzi *et al.*, who deposited a graphene-AuNP composite film upon graphite-based SPEs for utilisation in PyoC detection. Electrodes exhibited a linear range of 1–100 μM/L and a LOD of 0.33 μM/L and were stable long-term with good reproducibility. DPV scans produced a signal which was six times higher after surface modification [138]. AuNPs decorated graphene/graphite-modified SPEs were then used to detect PyoC with high sensitivity within tap water, human serum, and saliva. The sensor had linearity from 0.5 to 100 μM and a LOD of 66.90 nM (signal-to-noise ratio (SNR) = 3) when tested in PyoC solutions. In PyoC spiked saliva, serum and tap water, recoveries were observed between 96.75 %, 98.83 % and 100.55 % respectively, thus validifying practical applications for the sensor [137].

Successful wound monitoring was documented by Sheybani *et al.*, who used electrochemical sensor arrays to observe *P. Aeruginosa* growth. Arrays were based upon sensors containing an Au WE, Ag RE and Pt CE, and stability was improved with the addition of biocompatible polymeric coatings (chitosan and Nafion) which act to promote the attachment of bacterial cells and prevent non-specific cell and protein fouling. These advantages could allow for the long-term operation of in situ sensor arrays in areas of low resources [153].

Cernat *et al.* developed a thermosensitive polymer-based electrochemical sensor, modified with an Au/Ag nanoalloy, for the detection of PyoC. The sensor displayed a linear range between 0.12 and 25 μM and LOD of 0.04 μM (SNR = 3). Sensors were effective in spiked real samples and required no pre-treatment, except a dilution step. PyoC detection with high recovery in whole blood was possible within 5–10 min of sample collection [70].

Nanograss topography was investigated in a study by Alatraktchi *et al.*, with 200 nm Au deposited on top. Compared to a standard Au electrode, the addition of nanograss increases the electrode surface area by 3.9 times. This enables enhanced electron transfer gifting a lower LOD as a result of the increased sensitivity. A higher current is also permitted without the need to increase the WE footprint, which allows for an improved SNR and higher sensitivity. PyoC was detected in spiked hypertonic saline samples, with an R^2 value of 0.9901 and a LOD of 172 nM. When tested on airway samples from CF patients, the sensor identified *P. Aeruginosa* within 60 s, with no need for sample pre-treatment [120]. Krithiga *et al.* also modified the surface of GC electrodes to increase their surface area. Calcium cross-linked pectin-gold nanoparticles (CCLP-AuNPs) were deposited onto GC electrodes, with anti Ps drop-casted on top. A further layer of Au tagged anti rabbit horseradish peroxidase (IgG-HRP) was then added. The CCLP-AuNPs acted to increase the electrode's surface area, further immobilising the antibody, and thus improving response. The immunosensor was used to detect *P. Aeruginosa* in water, and displayed good sensitivity, with a detection limit of 9×10^2 CFU/mL, and high reproducibility. However, preparing the electrochemical immunosensor assay and fabricating the electrodes involved many steps, with the need for drying and incubation increasing production time [164].

The use of immunosensors to monitor water is appealing, since current methods such as PCR lack rapidity and are expensive, as well as requiring complex pre-treatment. Bekir *et al.* developed an immunosensor which utilised the immobilisation of purified polyclonal anti *P. Aeruginosa* antibodies on a poly(pyrrole-3-carboxylic acid)-modified SPCE. Immobilised antibodies are increasingly being integrated into biosensors for the recognition and capture of specific bacteria, offering unparalleled specificity [165–172]. The immunosensor was then successfully employed for the sensitive detection of *P. Aeruginosa* in groundwater solution [173].

Surface modification is an advantageous tool for the improvement of electrochemical electrode performance, in regard to both specificity and sensitivity. Metal NPs are often employed to augment electrochemical reactions, by means of their catalytic activity. Their superior conductivity facilitates improved communication between protein redox centres and the electrode surface. Better detection limits can also be achieved due to the increased surface area of NPs compared with bulk metal surfaces. Improved selectivity and detectability are possible, although this may involve the use of complex and costly equipment [160]. AuNPs have shown particular merit for surface modification, displaying a plethora of assets, including impressive biocompatibility, catalytic activity, and electron transfer rates [138,174]. Gandouzi *et al*. demonstrated the synergetic effect of utilising two nanostructures (Au and graphene), resulting in improved electrocatalytic efficiency for the electrochemical oxidation of PyoC, compared to bare SPEs [138]. Future work in this area will ultimately involve even further discoveries of viable surface modifiers, for optimal target analyte detection.

2.5 Biorecognition elements and aptasensors

Biorecognition elements are utilised to furnish a biosensor with analyte specificity, consequently increasing selectivity, sensitivity, and reproducibility, all aspects of which standard to improve POC device development. Careful consideration of which biorecognition element to use is necessary, since its specificity relies upon a strong affinity to the target analyte [175]. Naturally occurring elements, such as antibodies, enzymes (or, in the case of aptasensors, DNA or RNA aptamers) [176] may be used, as well as synthetic elements such as nanostructures [175]. The blood glucose biosensor is the current gold standard and has become a vital monitoring tool for those with diabetes [175,177]. Despite its simplicity, it is highly sensitive, selective, reproducible and cost efficient [175,178].

Amperometric detection of redox signals can be achieved if detector probes are coupled to oxidoreductase reporter enzymes [179,180]. Applying a fixed potential between the WE and RE, generates a current by enzyme-catalysed redox activity, which can then be measured and analysed [181–183]. Current amplitude is affected by how many target-probe-reporter enzyme complexes are affixed to the sensor. Again, electrochemical methods have an advantage over techniques such as PCR, as they can detect target nucleic acids directly in clinical specimens, since the initial step in the process is nucleic hybridisation and not enzyme-based target amplification [152].

Electrochemical DNA sensor structure comprises a recognition layer, which contains oligonucleotide probes, and an electrochemical signal transducer [152]. Often, they are formed using 'sandwich' hybridisation of target nucleic acids by capture and detector probes [184–187]. Liao *et al.* used capture probes to affix the bacterial 16S rRNA target to the surface of the sensor; detection was achieved by hybridisation to both the biotin-modified capture probe attached to the surface of the sensor, and to a second, fluorescein-modified detector probe [152]. Stem-loop structured probes were employed by Liu *et al.* in their fabrication of a DNA biosensor for *P. Aeruginosa* 16S ribosomal RNA (rRNA) detection. Probes were modified to include a thiol and a biotin and were immobilised onto an Au electrode. In the absence of the target, probes were 'closed', with the hybridisation of the target allowing them to 'open', triggering a reaction at the electrode surface between the biotin and streptavidin-horseradish peroxidase (HRP). Electrochemical techniques can then be employed for analyte detection. The biosensor performed well, boasting stability and selectivity, with a detection limit of 0.012 pg/μL. However, it must be noted that, in this case, sensor fabrication and hybridisation is a relatively lengthy process [188].

P. Aeruginosa is often employed for biofilm monitoring, as its electroactive phenazine products can be easily monitored [189]. ESAs have been utilised for the monitoring of *P. Aeruginosa* products [189,190], QS mechanisms [191] and biofilm formation at the electrode surface have all been monitored using voltammetric [192–194] and impedance [195,196] techniques. Robb *et al.* modified pyrolytic graphite (PG) electrodes with *P. Aeruginosa* PA01 to monitor the dispersion of PA01 biofilm, see Fig. 7 [198]. A prior electrochemical assay was utilised to observe antimicrobial peptide (AMP) exposure-induced alginate disruption and it was found that it was related to anti-biofilm activity [198]. This was improved upon by similarly immobilizing *P. Aeruginosa* PA01 onto the electrode and monitoring the response to anti-biofilm compound RA-13 [197].

P. Aeruginosa immobilisation improves simplicity of the assay, since electroactive phenazines (such as PyoC) are produced, which are easy to measure with electrochemical methods due to their redox acitivity [190,198–202]. Overall, the essay was shown to be capable of distinguishing anti-biofilm compound activity in real time [197].

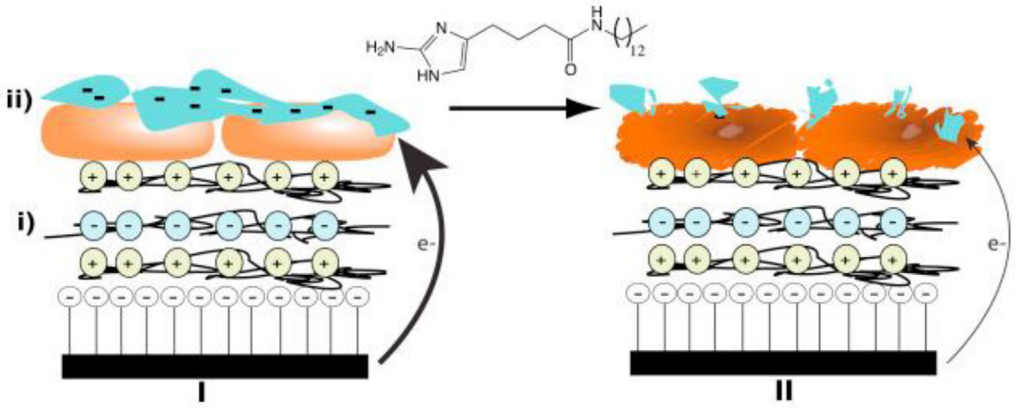

Fig. 7. Overview of the P. aeruginosa electrochemical assay. Formation of the electrode using (i) layer by layer cationic (yellow) and anionic (blue) polymers and (ii) P. aeruginosa PA01 (orange) that can produce biofilm (aqua) [197].

Roushani *et al*. describes the first impedimetric aptasensor for ultrasensitive *P. Aeruginosa* detection. After modifying the surface of a glassy carbon (GC) electrode with AuNPs, the NH_2-aptamer was then covalently attached [203]. Covalent bonding is achieved by the aptamer amino group to the AuNPs, which ensures attachment [193]. Electrode modification influences the intensity of the electrochemical signal and the biological molecule stabilisation. Use of AuNPs improved electrochemical signal due to an increase in surface area; electron transfer was also significantly accelerated. The aptasensor was tested in blood serum samples under optimum conditions, and results were confirmed by PCR. A linear range was achieved with a R^2 value of 0.9984 and inter-electrode repeatability with an RSD value of 4.75 %. The sensor also had the advantage of relatively lower cost and higher sensitivity when compared to other sensors. Thus, clinical diagnosis of *P. Aeruginosa* was deemed possible [203]. Aptamers can also be employed as mediators, as demonstrated by Das *et al*. in their fabrication of an innovative nanozyme sensor for *P. Aeruginosa* detection. The term 'nanozyme' describes a nanomaterial which exhibits characteristics of an enzyme [204]. The peroxidase-like nanozyme activity of AuNPs was inhibited by the adsorption of *F*23, an aptamer specific to *P. Aeruginosa*. Given the high affinity of *F*23 for *P. Aeruginosa*, the aptamer parts from the surface of the AuNPs in the presence of the pathogen. This re-enables the peroxidase-like activity of the AuNPs, resulting in the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB), which can then be electrochemically detected. Amperometry techniques including this apatsensor strategy saw detection limits down to 60.0 CFU/mL in water [205].

There are various advantages and disadvantages to each type of biorecognition element, which must be considered prior to sensor design. Natural biorecognition elements, such as antibodies and enzymes, boast good selectivity and reusability, but suffer when it comes to reproducibility [175]. New antibody discovery is also limited since the process is lengthy and costly [206,207]. Synthetic biorecognition elements have virtually the opposite characteristics. Molecularly imprinted polymers (MIPs) are known to have good reproducibility but lack selectivity. Pseudo-natural biorecognition elements also exist; they are a combination of natural subunits and synthetic supramolecular structures – for example nucleic acids and aptamers. Generally, these elements provide good sensitivity, typically high selectivity and reproducibility; though they are costly and can exhibit non-specific binding in some cases [175]. Pseudo-natural biorecognition elements have an extensive range of applications, with the exception of nucleic acid recognition elements, whose only optimal target are nucleic acids [208–211].

Biosensors are a constantly expanding area of research [175,183,178–181,207–212]. To enable their optimisation, it is essential that biorecognition elements are well understood; such as the invention of a comprehensive guide to biorecognition elements such as that suggested by Morales *et al.* that would extensively discuss selection criteria and biomarker characterisation. However, future work in this field will require the improvement of biosensor reusability and reproducibility of manufacture as well as expanding the range of biorecognition elements available to researchers [175].

3 Conclusion

P. Aeruginosa is a prevalent cause of nosocomial infection, with substantial morbidity and mortality rates in addition to displaying antibiotic resistance [12–20]. Current detection methods require substantial preparation and/or highly trained personnel and are unable to detect the pathogen in a timely manner [30,43,99,108–110]. A range of studies have spoken to utilising the electrochemically active virulence factors secreted by *P. Aeruginosa* for early detection, allowing for more effective treatment. Basic electrochemical sensor design utilising low cost and portable methods have demonstrated a key ability to apply electrochemical based devices for the rapid detection of *P. Aeruginosa* down to μM levels of its virulence factor PyoC. Although these systems represent the most basic of sensor design their benefits come in their ease of use and low manufacturing cost. However, this comes at the cost of their sensitivity. Recent advancements have however address this through the introduction of surface modification strategies and biological recognition elements providing the enhanced sensitivity and selectivity required, achieving detection limits in the sub nanomolar region. However, these are not without their own limitations, namely issues surrounding reproducibility of the sensors and additional cost of these more complex modification reagents. Yet research in this area has proven the efficacy of electrochemical detection, as well as its desirable sensitivity. It is fast becoming clear that this is a viable option for rapid diagnostics since these methods can be executed in seconds and eliminate the need for pre-treatment. Future research in this area will ultimately involve optimisation of detection methods, of which a combination of the methodologies discussed within are envisioned to achieve all desired criteria namely sensitivity and cost efficiency. While creation of portable testing equipment that could be utilised in a clinical environment is also necessary. Sensitive point-of-care testing would provide a significant advancement towards a reduction in morbidity and mortality, by allowing treatment before the pathogen is able to advance and develop resistance.

Data availability

No data was used for the research described in the article.

Declaration of Competing Interest

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.

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