Electrochemical Detection of Pathogenic Bacteria - Recent Strategies, Advances and Challenges

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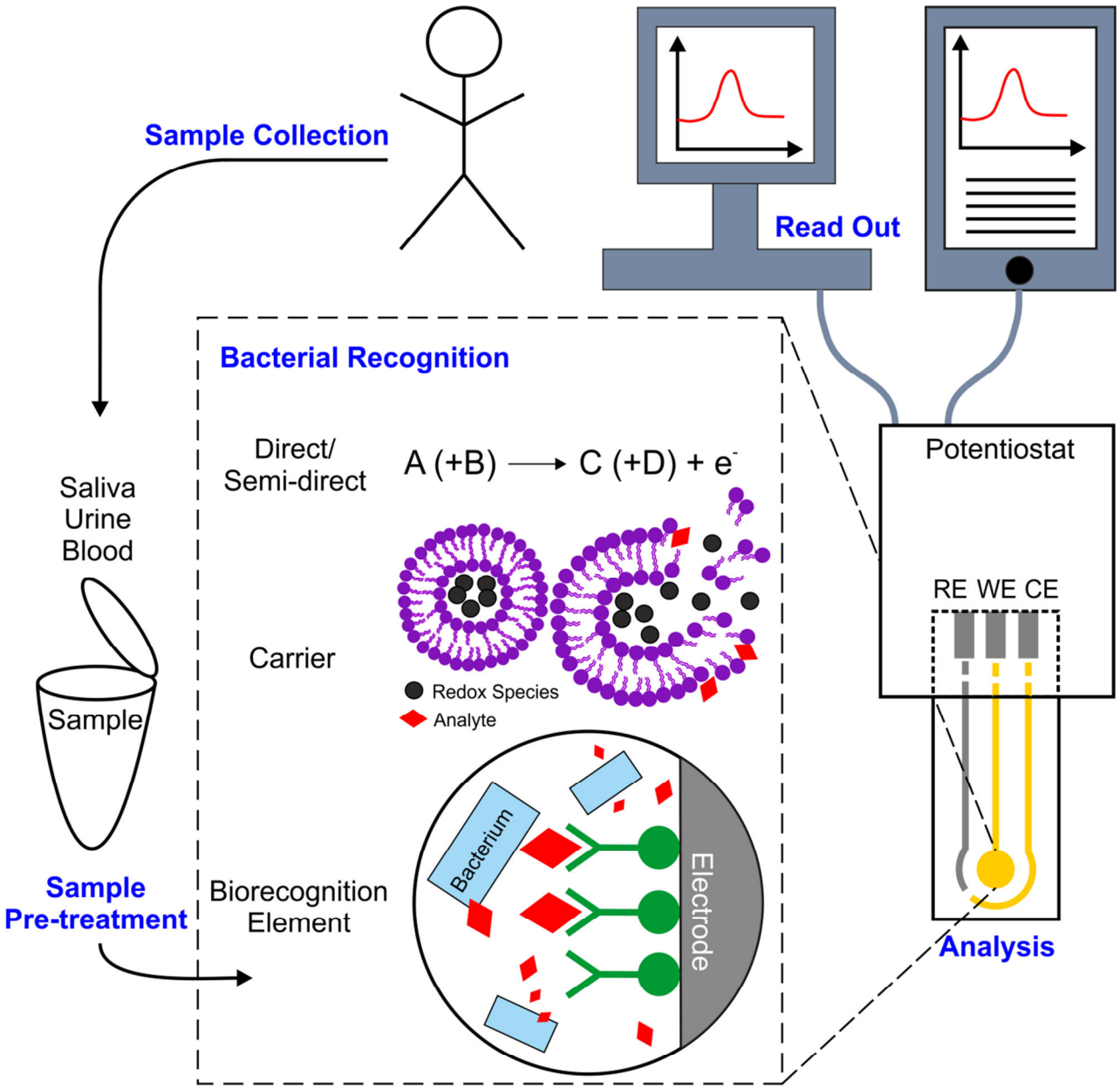

Abstract: Bacterial infections represent one of the leading causes of mortality worldwide, nevertheless the design and development of rapid, cost-efficient and reliable detection methods for pathogens remains challenging. In recent years, electrochemical sensing methods have gained increasing attention for the detection of pathogenic bacteria, due to their increasingly competitive sensitivity. However, combining sensitivity with cost efficiency, high selectivity and a facile working procedure in a portable device is difficult.

The presented review provides a summary of biosensing strategies for bacteria, published since 2015, by covering significant achievements towards custom-designed portable point of care devices. Herein, the direct chemical recognition of bacteria via enzyme activity or secretion products, as well as their detection at various electrode surfaces and materials, such as nanomaterials, indium tin oxide or paper-based immunosensors, is discussed. Furthermore, newly established hyphenated sensing principles, incorporated into lab-on-a-chip and microfluidic devices, are presented and remaining technical challenges and limitations are considered.

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1. Introduction

Research on biosensors for pathogens and infectious diseases is becoming a focus point of attention in medical research, as its importance becomes increasingly evident in health care, the environment, and food monitoring. Annually, ~340 million new infections related to sexually transmitted diseases alone, are recorded worldwide [1], and in general bacterial infections remain one of the leading causes of mortality.[2] As most infections occur through contaminated water, food, and bodily fluids, the spreading of diseases progresses rapidly and results in a great need to quickly identify pathogens to select most effective treatment strategies on an individual basis. As an example, about 53 million lives worldwide were saved through the diagnosis and treatment of tuberculosis between 2000 and 2016.[3] However, the rapid, cost-efficient and reliable detection of pathogens remains a challenge, as adequate recognition of infectious diseases still relies on lengthy conventional culturing methodologies and requires technical staff training.[4]

In recent years, electrode materials and bioelectrodes have emerged for the design of implantable and wearable sensors for *in vivo* analysis of electrolyte levels [5–7] and electrochemistry has been acknowledged as a successful tool for the detection of whole bacteria, bacterial byproducts and metabolites, as well as enzymes[8,9]. In contrast to the glucose sensor, which is based on the amperometric recognition of glucose in blood and generates global revenues in the order of billion dollars per year[10], bacterial biosensors are still not ready to enter the global market successfully as routine monitoring devices. To reach large scale commercialization of bacterial biosensors, the following requirements have to be fulfilled: cost efficiency, low detection limit, selectivity, a precision of about 5 to 7%, a short analysis time in the order of 5 to 10 minutes, no necessity of pre-enrichment methods for samples, portability to allow on-side monitoring, and no required skills to carry out the detection assay are all essential features demanded by the medical diagnostic industry.[8] In addition, disposable analytical substrates are desired to keep up with the increasing demand for test performance.

Previous recent reviews published on this topic covered fundamental sensing principles, case studies and challenges faced when aiming to develop portable point of care devices for on-site monitoring.[9,11,12] In 2015, Monzó *et al*. published a comprehensive overview of current advantages and disadvantages of various electrochemical sensing methods and concluded a slow penetration of biosensors into the global market due to limitations in sample preparation, analysis time and device sensitivity.[9] Distinct importance was attributed to the development of low-cost nanomaterials and synthetic

polymers, microarray platforms, as well as microfluidic devices, allowing an increased mass transport of the analyte towards the electrochemical interface. In 2016, Kokkinos *et al*. provided an in-depth review of electrochemical immunosensors, highlighting label and label-free immunosensors, magnetoimmunosensors, as well as capacitive and impedimetric sensors.[11] The authors reached the conclusion that exploiting characteristics of electrochemical sensors, such as the miniaturization of electrodes and instruments at a reasonable cost, the operation of sensing devices at low sample volume and its compatibility with clinical samples, will result in a suitable technology to meet current biochemical and chemical sensing demands in the field of medical diagnostics. An overview of clinical applications of sensors for the detection of physiologically important analytes, including bacteria, was published by Justino *et al*. in 2016.[12] By comparing the analytical performance of the sensors, the authors concluded that microfluidic and lab-on-a-chip devices reduce the consumption of costly reagents and shorten the experimental analysis time for the detection of bacteria significantly. Interestingly, in comparison with literature, antibody and epoxysilane-modified indium tin oxide (ITO) electrodes were reported as the most sensitive detection method, offering sensitivity as low as 1 culture forming unit (CFU) per milliliter.[13]

In this context, the presented review aims to provide an updated summary of biosensing strategies for the detection of pathogenic bacteria. Specific focus is laid on techniques already highlighted as most promising in previous reviews.[9,11,12] In addition to an update on these technologies, general advances and challenges in the development of biosensors are covered. In the following literature review, we first discuss sensors based on the chemical recognition of biological targets at electrodes (section 2.1). This includes principles and applications of promising detection strategies involving voltammetry, mediator-based detection, antibodies, and direct sensing of bacterial metabolites. Secondly, we discuss the bacterial recognition through various electrode surfaces and materials (section 2.2) by reviewing various electrochemical sensing probes, including carbon-nanotube-based surfaces, graphene-metal-structures and composites. Finally, and in contrast to previously published reviews, particular focus is laid on hyphenated sensing principles (section 2.3), such as flow injection biosensor systems, linked with electrochemical detection and chip devices are discussed. A comprehensive summary of current technical challenges and limitations is provided before conclusions and future perspectives are given.

2. Literature Review

This section provides an overview of recent publications in the field of electrochemical sensing for the detection of pathogenic bacteria. We first discuss electrochemical sensors, which are based on the chemical recognition of biological targets at an electrode, before reviewing the recognition of bacteria at different electrode surfaces, including metal nanostructures and composite materials. Furthermore, advantages of specialized electrochemical techniques, such as the nano-impact method and flow injection biosensor systems are presented, before advances in the development of hyphenated techniques for the sensitive and selective detection of bacteria are introduced and discussed. A concise summary of the in the following in detail discussed literature is provided in **table 1**.

2.1. Chemical Recognition of Biological Targets

Chemical recognition of biological targets refers to the detection of biological species through chemical interactions. This principle can be used to detect metabolites, DNA, or whole bacteria. The chemical recognition of biological targets can result in a straight forward electrochemical signal without the use of highly specialized material or instrumentation. However, reaching a satisfactory selectivity and sensibility by voltammetry is often not trivial. In the following section we provide an overview of recent literature, addressing the recognition of pathogenic bacteria based on voltammetry, mediator-based detection, antibody interactions and the direct sensing of bacterial metabolites.

The detection of the bacterial exotoxins has recently drawn attention due to its applicability in the health sector. The detection of the exotoxins rhamnolipid and delta toxin by electrochemistry was reported by Thet *et al*. [14] These exotoxins are secreted from *S. aureus* and *P. aeruginosa* and the presented detection principle might find application in smart wound dressing technologies.[15] The authors designed biomimetic lipid vesicles, containing the redox species potassium ferricyanide, which is released upon interaction of the toxins with the lipid bilayer and the consequent breakdown of the phospholipid vesicles. The enhanced electrochemical current response in the presence of the exotoxins results from the oxidation of the released potassium ferricyanide redox species. The authors successfully detected concentrations of toxins in the µmol range and concluded that the low cost, the selectivity and real-time electrochemical monitoring of the proposed sensor holds great potential for *in vivo* infection signalling.

In a second example, Elliott *et al*. [16] published the detection of pyocyanin, a virulence factor produced by *P. aeruginosa*. The electroactive nature of pyocyanin allows the electrochemical conversion of pyocyanin at transparent carbon ultramicroelectrode arrays (T-CUAs) by cyclic voltammetry, which is an electrochemical technique in which an applied potential is swept linearly between two limiting potentials, driving a chemical reaction at an electrode. The determined limits of detection of 1 to 1.6 µM fall within the range of *in vivo* cellular environments. Hence it is concluded that the proposed method represents a promising strategy for the application of T-CUAs for the quantitative study of biotoxins.

The use of thiol-chemistry to introduce a bio-recognition element for the specific detection of pathogens has been reported in the past [17] and remains a useful method in recent literature for the specific immobilization of bacteria for further analysis by electrochemistry.[18] Bekir *et al*. constructed self-assembled monolayers (SAMs) on gold electrodes and successfully detected *S. aureus* at the electrode using electrochemical impedance spectroscopy. An increase in the

charge transfer resistance ("off signal") can be a good indicator for the presence of bacteria on the electrode surface. However, careful attention needs to be paid to assure that the signal blockage is indeed due to target bacteria and not due to impurities or nonspecific binding events. For this reason, a true "on signal" is generally more desirable, as investigated by Kuss *et al*. [19], who reported the detection of *B. subtilis* and *E. coli* using the electroactive and colorimetric indicator N,N,N',N'-tetramethyl-para-phenylene-diamine (TMPD). In this study, living target bacteria are dropcasted onto gold macroelectrodes, before exposing the sensor to the radical cation $TMPD^{+\bullet}$. Bacteria expressing cytochrome c oxidase, a transmembrane protein complex that plays an important role as an electron acceptor in the respiratory electron transport chain, are able to oxidize TMPD to $TMPD^{+\bullet}$, which is converted back to TMPD at the macroelectrode, as shown in **figure 1**. In addition to the successful electrochemical pathogen recognition, the expression of cytochrome c oxidase was measured for the first time in anaerobic *E.* coli and was quantified and expressed as a turnover number. Although not yet specific, this principle is an excellent example for the direct detection of bacteria using a redox mediator instead of costly materials or instrumentation. In general, the detection of whole bacteria and avoiding sample pre-treatment remains challenging, as the levels of electroactive metabolites or by-products released from bacteria are low compared to their intracellular concentration.

It should be mentioned, although outside the scope of this review, that bacterial metabolites and secretion products are not just interesting targets for the direct detection of pathogens and their biomarkers are important in medical research [20], but recently have also been reported useful for the detection of environmental pollutants. [21]

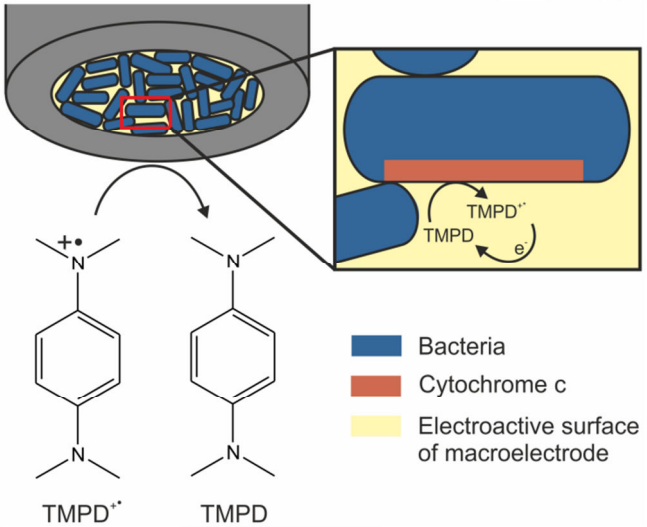

Figure 1. Schematic representation of the electrochemical detection of bacteria, expressing cytochrome c oxidase. The immobilization of bacteria results in the local oxidation of TMPD to $TMPD^{+\bullet}$. $TMPD^{+\bullet}$ can be converted to TMPD at the electroactive surface of the electrode, resulting in an increase in reduction current during cyclic voltammetry or chronoamperometry. Reprinted from [19], published by The Royal Society of Chemistry.

2.2. Bacterial Recognition through various Electrode Surfaces and Materials

2.2.1. Materials

In the last 10 years, enormous advances in nanomaterials and sensing approaches, such as the use of graphene [22,23], CNTs [24,25], immunomagnetic nanoparticles or beads [26], screen printed electrodes (SPEs) [27], paper-based [24] and inkjet-printed platforms [28], as well as interdigitated array microelectrodes [29] have been achieved to improve the specificity and sensitivity of biosensors.[30] Herein, only electrochemically relevant materials will be discussed to illustrate how improve the sensitivity, specificity and automation of the pathogen biosensors can be improved. Interested readers are encouraged to read comprehensive reviews, which provide previous detailed literature surveys and a deep description of some of the employed techniques and methods below.[30–32]

2.2.1.1. Carbon Nanomaterials

The integration of nanomaterials into biosensors has gained great interest due to their ability to improve sensitivity and selectivity. Carbon nanotubes have been integrated into electrochemical sensors, primarily with the aim of improving electron transfer rates and to increase the working surface area.[33,34] However, it is important for the applicability of this material to practical sensing tools to keep the fabrication procedure simple and straightforward. This requirement becomes obvious in recent literature. Tahir *et al*. report a multiwalled carbon nanotube (MWCNTs) based zinc nanocomposite DNA platform, which showed high specificity towards recognition of the plant pathogen, chili leaf curl betasatellite.[35] Although the actual electrochemical recognition of complementary DNA using the ferrocyanide/ ferricyanide redox couple is straight forward, the elaborative preparation procedure, including the preparation of MWCNT-zinc nanocomposite, polymerase chain reaction, electrophoresis, snap cooling incubation after DNA amplification for 3 h, dropcasting and washing procedures, makes this approach less efficient, despite the reported three times greater specificity for complementary DNA than for non-complementary DNA. In fact, such a high specificity, as reported by Tahir *et al*.[35], is not always desirable when investigators are in the process of tackling the goal of the simultaneous detection of multiple pathogens in physiological samples. In conventional methods, a high number of individual tests are required to detect multiple types of bacteria in fluid samples, making procedures costly and time intense. Hence, the development of array systems is currently explored in recent research. An example for the efficient simultaneous detection of pathogens is the research by Yamada *et al*., which is based on a multi-junction array system. The authors report a single step bacterial detection method, using a single walled carbon nanotube- (SWCNT) based multi-junction sensor.[36] Four SWCNT coated wires were placed and soldered onto each disposable sensor chip device, to create a 2x2 junction array. Bacteria specific antibodies were immobilized

onto each junction, created by the SWCNT coated wires. A change in current was observed in the presence of *E. coli* (K-12) and *S. aureus* cultures, indicating their binding to the sensor in microbial cocktail samples, and a limit of detection of 10^2 CFU mL^{-1} was achieved. This procedure offers a relatively short analysis time of 10 to 15 min after the substrate modifications of the sensor chips are completed and the authors concluded that the detection limit might be improved with the use of bacterial cell concentration methods.

2.2.1.2. Indium-Tin Oxide

An alternative strategy for electrode substrate modifications is the use of indium-tin oxide (ITO), which is considered an appropriate material due to its optical transparency, high electrical conductivity, wide electrochemical working window, excellent substrate adhesion and stable physical properties.[37] Therefore, ITO has been used in the past as substrate for the immobilization of antibodies in electrochemical impedance sensors.[13,38]

In a very recent approach, ITO was employed to increase the electroactive surface area and introduce a nanocolumnar structure to the surface of an electrochemical impedimetric sensor.[39] In the work of Lin *et al*. ITO is used as a matrix to immobilize toll-like receptor proteins (TLRs), known as biorecognition molecules. This meso-porous ITO was fabricated by glancing angle deposition (GLAD) method, and as a result, two different pathogen-associated molecular patterns (PAMPs) could be successfully recognized: lipopolysaccharide from *E. coli* (O157:H7) and flagellin from *S. typhimurium*. By exposure of the sensor to PAMPs, a change in the charge transfer resistance was measured. This GLAD ITO-based sensor showed superior performance compared to the planar ITO-based sensor: An 83 times lower detection limit (2-3 ng mL^{-1}) and a 30 times higher sensitivity. Moreover, the incubation time of 10 minutes for this sensor was compared favorably to previous EIS sensors.[40,41] The authors concluded that combining of the GLAD ITO-based sensor with a microfluidic system could be a promising approach towards an automatic device.

2.2.1.3. Paper-based Immunosensors

Nanomaterials have been proven useful for small scale sensors, however, for large scale implementation of biosensors the use of inexpensive materials such as paper or plastic is more desirable. Therefore, paper-based immunosensors are interesting alternatives to metal or ITO materials for pathogen detection.[42] Paper-based sensors have several advantages such as biocompatibility, easiness to pattern, flexibility, lightness and low in costs.[43] In recent years, paper-based electrochemical biosensors have attracted increasing attention for the detection of bacteria.[24,42,44,45] In general, patterning of papers can be achieved by different methods: wax printing, inkjet printing, screen printing, photolithography, stamping and cutting.[24]

A disposable, rapid, cost-efficient and label-free detection method was established by Liu *et al*. [44], designing a paper-based bipolar electrode electrochemiluminescence (pBPE-ECL) analysis system for the detection of *Listeria monocytogenes*. For the sensor chip, a hydrophilic channel was produced by wax-screen printing and the BPE and driving electrodes were fabricated on the hydrophilic channel by a screen printing technique with carbon ink. The "light-switch" molecule $[Ru(phen)_2dppz]^{2+}$ (phen = 1,10-phenanthroline; dppz = dipyridophenazine) intercalates into the base pairs of fabricated dsDNA-PCR amplification products, whereby these constructs are further introduced to the pBPE-ECL sensor in addition to the co-reactant tripropylamine for electrochemical analysis, using a photomultiplier tube. This procedure resulted in the specific detection of *L. monocytogenes* until a genomic DNA concentration of 10 copies/µL. This study represents a great alternative to conventional methods, as costs, associated with nanomaterials, can be avoided. The necessity of DNA amplification makes this strategy less time efficient; however, with a simple addition step and low volume of sample suspension, it demonstrates the usefulness of paper-based biosensors.

One of the major advantages of paper-based immunosensors is the possibility of working with transparent platforms, allowing the comparison between electrochemical sensing and colorimetric assays. Adkins et al. successfully detected enzyme activity in inoculated food and water samples containing *E. coli* and *Enterococcus ssp*. using stencil-printed carbon electodes (SPCEs), shown in **figure 2**.[45] The *β*-galactosidase and *β*-glucuronidase production by *E.coli*, as well as *β*-glucosidase expression by *Enterococcus ssp*. is detected colorimetrically using a simple cardboard box and smart phone.

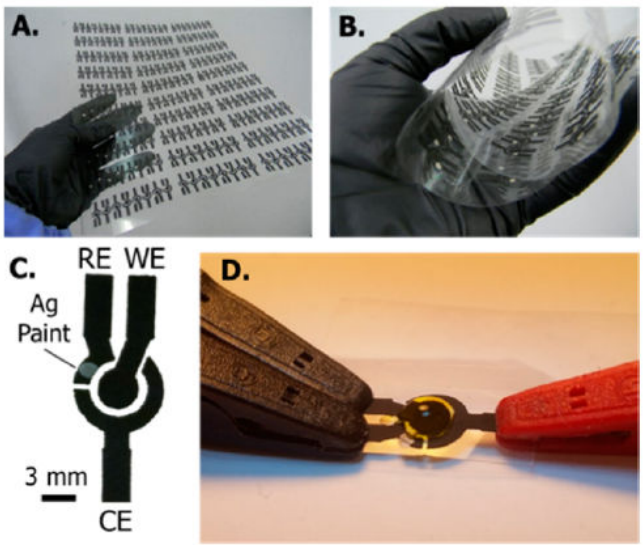

Figure 2. SPCEs on transparency film shown as a (A) printed sheet that is (B) flexible. (C) A single printed electrode image (with background removed for visualization) showing working (WE), silver paint reference (RE), and counter (CE) electrode geometries and connections. (D) Final device image with 30 µL of solution contained within the central well and connected to potentiostat leads. Reprinted with permission from [45]. Copyright 2017 American Chemical Society.

Alternatively, these enzymes can be oxidized and thereby recognized electrochemically. Concentrations as low as 10 CFU ml^{-1} could be detected and the authors concluded that while electrochemistry did not decrease the overall analysis time, it compared favorably with the colorimetric test in terms of detection limit. Although rather time intense (4 to 12 h), this electrochemical detection method is a paradigm of an inexpensive and disposable sensor.

In recent literature, Bhardwaj *et al*. demonstrated a paper-based electrochemical immunosensor for the label-free detection of *S. aureus*, using antibodies, covalently conjugated to SWCNTs, [24] The authors used wax printing method to create hydrophobic barriers on a Whatman paper. Interestingly, after the conjugation of SWCNTs to the paper, the covalent linking of antibodies, specific to *S. aureus* to SWCNTs resulted in a fabrication process of approximately 2 h, minimizing the damage of the hydrophobic patterns during fabrication and increasing the stability of the sensor for over a month. During the tracking of current alterations upon antibody-antigen complex formation in spiked milk samples, the sensor demonstrated an overall analysis time of 30 minutes and a limit of detection of 13 CFU mL^{-1}. However, the drawback of this combination of paper-based and nanomaterial-based methodology is evidently its insufficient selectivity, as non-specific bacteria resulted in more than 50% of current signal compared to the specific response by *S. aureus*.

2.2.2. Techniques

A number of electrochemical techniques can be used for detection and quantification in electroanalytical chemistry.[46] In biosensing recent years have seen particular developments in impedance based measurements and the development of impact electrochemistry.

2.2.2.1. Nanomaterial-based Impedimetric Biosensors

Electrochemical impedance spectroscopy (EIS) has been used in various applications including detection of pathogens. In EIS sensing, the electrical impedance in alternating current at an electrode/electrolyte interface is recorded while a sinusoidal direct current voltage is applied at certain or a range of frequencies. Once the target bacteria bind to the recognition probe, the impedance at the interface changes and consequently the target bacteria are quantified based on this change.[47] The bio-recognition element can thereby be an antibody [48], aptamer [47], RNA [49] or polymer.[50] Most recently, the integration of nanotubes [51,52] and nanoparticles in EIS biosensors has been reported previously.[53–55] As an example, a novel and multifunctional sensor for the simultaneous detection, elimination and inactivation of bacteria was reported by Yang *et al*.[55] This sensor is based on silver nanoparticles (AgNPs) decorated zinc oxide nanorod arrays (**figure 3**). This platform was functionalized with vancomycin for specific recognition of *S. aureus*. Notably, the sensor showed 50% bacterial elimination efficiency at low concentrations and demonstrated excellent antibacterial activity (99.99%), which could be due to a synergistic germicidal effect between the antibacterial AgNPs and vancomycin.

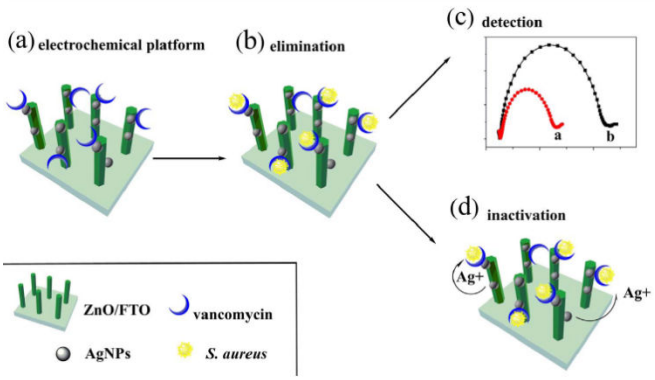

Figure 3. Schematic representation of an electrochemical platform, based on impedance spectroscopy for the simultaneous detection, elimination, and inactivation of *S. aureus*. Reprinted from [55] with permission from Elsevier.

2.2.2.2. Nano-impact Method

A powerful new technique for the detection of single biological entities is the “Nano-impact method”. The principle of this technique was demonstrated in the detection and characterization of silver nanoparticles in solution and is based on the Faradaic charge transfer, following the collision of silver nanoparticles with an electrode.[56] The movement of silver nanoparticle in solution is governed by diffusional Brownian motion. When a particle suspension is brought into contact with a microelectrode, held under an oxidizing potential (exceeding that of the standard potential of silver), the collision of single particles with the electrode results in a short current burst (or “spike”), as a result of the silver oxidation. Sepunaru *et al*. employed this principle to detect single *E. coli* bacteria, labeled with silver nanoparticles.[57] In this study, the movement of bacteria in solution following Brownian motion enables the approximation of collision frequency. In accordance with theory, at a cell molarity of ~0.3 pM, the authors recorded bacterial impacts on average every 4 seconds, and calculated a total charge transfer per impact of $1.2 \pm 0.5 \times 10^{-12}$ C. This corresponds to an attachment of about 295 ± 125 silver nanoparticles per single *E. coli*. Given the concentration dependency of the impact frequency, information about the concentration of pathogenic bacteria in a sample solution can be determined electrochemically. Interestingly, although outside the scope of this review, the nano-impact method was also utilized for the concentration determination of red blood cells in aqueous solutions [58], as well as the detection of single virus entities, tagged with silver nanoparticles.[59] These examples present the nano-impact method as a promising new approach towards the detection of single biological entities. The use of nanoparticles, however, is less desirable, as it increases analysis time and costs of the overall procedure.

A label-free method, based on the nano-impact principle, also called single-particle collision method, was established by Lee *at al*.[60] In this study, the authors make use of the redox couple ferrocyanide/ferricyanide redox couple, which is oxidized

at a carbon fiber microelectrode. As shown in figure 4, single *E. coli* bacteria in solution are detected upon impact at the microelectrode, as a result of the physical diffusion blockage of the mediator towards the electrode. Consequently, the recorded electrochemical current decreases and the impact frequency can be monitored, in dependence on the bacteria concentration. The advantage of the label-free approach is clearly that it might be easier to be transferred to a variety of pathogenic bacteria, in contrast to the necessary silver particle tagging during the method presented by Sepunaru *et al.*[57]

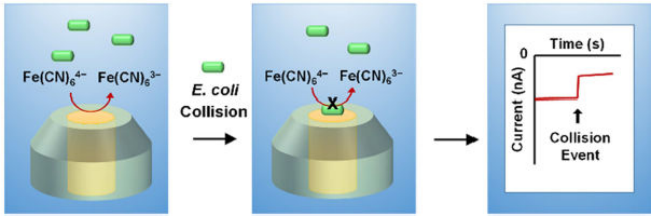

Figure 4. Schematic diagram of E. coli detection by collision event on a UME. Reprinted from [60] with permission from natureresearch. This work is licensed under a Creative Commons Attribution 4.0 International License.

2.3. Hyphenated Sensing Principles: Towards custom-designed portable devices

Aside from commonly used electrochemical recordings using voltammetry or chronoamperometry, specialized and hyphenated techniques have emerged, providing novel strategies and starting-points for pathogen detection. The most recent and remarkable achievements are presented in the following.

2.3.1. Lab-on-a-chip

Lab-on-a-chip (LOC) systems combine multiple functions of a laboratory in a single device and hence provide a marketable approach with the potential for on-site analysis of pathogens at low cost.[61] As an example that demonstrates the principle of LOC devices, a collaboration of seven Fraunhofer Institutes in Germany resulted in the development of a fully automated LOC cartridge for the analysis of nucleic acids and protein markers.[62] The electrochemical chip (16 electrodes with a diameter of 350 µm) was modified with different immobilized capture molecules, so that different targets can be selectively detected. Schumacher *et al.* proposed the configuration of the cartridge to work with a fluorescence biosensor or silicon based electrochemical biosensor. In this example, using the optical transducer, the simultaneous detection of the C-reactive protein and the prostate-specific antigen was achieved. The main advantages of this cartridge are the high integration of several steps (e.g. pumping, heating, cooling, reagent reservoirs and assays), use of common assay types and the possibility for mass production.

The application of LOC chip sensors for the detection of pathogenic foodborne bacteria *S. aureus* and *L. monocytogenes* has been reported recently.[63] This LOC, designed by Primiceri *et al.*, is based on electrochemical impedance spectroscopy, and was able to detect these bacteria at concentrations as low as 1.2 (*S. aureus*) and 5 CFU mL^{-1} (*L. monocytogenes*). However, further development is required to integrate the chip with a sample pretreatment unit in order to implement this device for on-field analysis. In other examples, circuit LOC chips consisted of up to 100 working electrodes with 30 off-chip contacts and 5 liquid channels were described.[29,64] These systems revealed high sensitivity (1 CFU mL^{-1}) for *E. coli* and *S. aureus* [29] and require short analysis time (2-5 minutes).[64] In addition, space resolved information beyond pathogen detection, such as colony imaging, are obtained.

Most recently, an LOC device based on suspended carbon nanowires was presented.[65] The carbon nanowires were fabricated in a controlled manner using electrospinning and photolithography techniques, as shown in figure 5. This platform was then integrated with a microfluidic system to build the LOC device. The performance of this chemiresistive bacterial sensor was examined for label-free detection of *S. typhimuruim* bacteria using amine-ended aptamer, which was immobilized on the nanowires via a carbodiimide crosslinker. The changes in conductivity of the nanowires upon binding of bacteria to the nanowire were measured from the current-voltage curves. The device revealed high specificity and sensitivity for detection of *S. typhimuruim* with a detection limit of 10 CFU mL^{-1} and an analysis time of 5 min. Notably, this limit is much lower than conventional methods, such as PCR[66] and even other recent reported lab-on-disc devices (2.7×10^4 CFU mL^{-1}).[67] Moreover, the system requires only 5 µL sample volume, which compares favorably to other devices (12.5 µL).[67] The authors concluded that by employing other specific aptamers, this sensor may also be used for the detection of different target analytes such as antibodies or DNA.

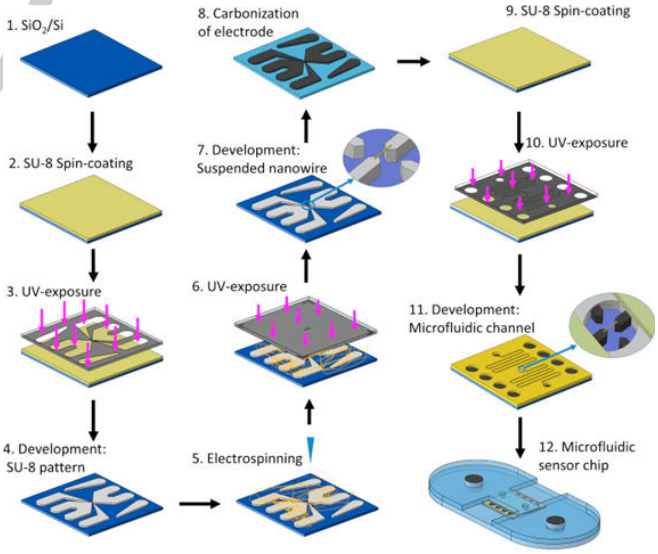

Figure 5. Schematic illustration of the fabrication steps of the carbon nanowire LOC biosensor using electrospinning and photolithography. Reprinted from [65] with permission from Elsevier.

2.3.2. Microfluidic Devices

As mentioned before in section 2.2., aside from achieving low detection limits, the simultaneous detection of multiple pathogens is currently a challenging topic in the field of biosensing. An exciting approach for such multiplexed detections is the use of microfluidic systems. Tian *et al*. have presented a novel microfluidic system integrated with nanoporous membrane for detection of multiple types of foodborne bacteria.[68] The integration of a polyethylene glycol (PEG) based microchip with a functionalized alumina membrane enabled the sensitive and simultaneous detection of *E. coli* (O157:H7) and *S. aureus* from mixed samples without labelling. As shown in **figure 6a**, in this device, two pieces of the membrane were immobilized with bacteria specific antibodies. The sensing mechanism is based on changing the electrical impedance across the membrane before and after the bacteria capture. Thereby, the bacteria block some of the nanopores, which lead to an increase in impedance. This interesting concept achieved a detection limit of 10^2 CFU mL^{-1}, and showed a specificity for the target bacteria with a minimum cross-reactivity of about 5% to non-target bacteria. This capture/sensing principle was utilized and advanced by Ye *et al*. to monitor the bacterial response to antibiotics. The authors modified an alumina membrane with graphene quantum dots (GQDs) to increase the surface-to-volume ratio.[69] As a result, the detection of antibiotic response can be monitored within 30 minutes with an approximate detection limit of 1 pM. Therefore, this sensor has potential application in early disease diagnosis.

Most recently, the combination of all advantages provided by microfluidic systems, together with electrode arrays in a fully automated device was achieved by Altintas *et al*.[70] In this publication, the authors provided the design of a portable microfluidic-based electrochemical sensing device (MiSens) for real time detection of waterborne pathogens. This custom-designed biosensor realized the integration of a microfluidic system, an electrode array and the electrochemical unit in an all-in-one portable device (**figure 6c**). In this device, the biosensing chip is composed of 8 sets of gold electrode arrays, whereby each set consists of 3 working electrodes. The chip was docked into the system to constitute the fluidic and electronic connections to form a microfluidic channel (7 µL). In this system, a sandwich immunoassay was used: *E. coli* bacteria were captured in between a surface antibody, which was immobilized onto the chip sensor, and a horse radish peroxidase (HRP) labelled detection antibody, as shown schematically in **figure 6b**. The principle of sensing was based on antibody and enzyme amplification. To further improve the sensitivity of the proposed method, Au nanoparticles were added to the sensing chip in order to facilitate the adsorption of the detection antibody. This modification led to a detection limit of 50 CFU mL^{-1}. Interestingly, this device showed high specificity as the binding to non-specific bacteria (*Shigella*, *Salmonella spp*., *Salmonella typhimurium* and *Staphylococcus aureus*) in the presence of an *E. coli* antibody was reported to be less than 13%.

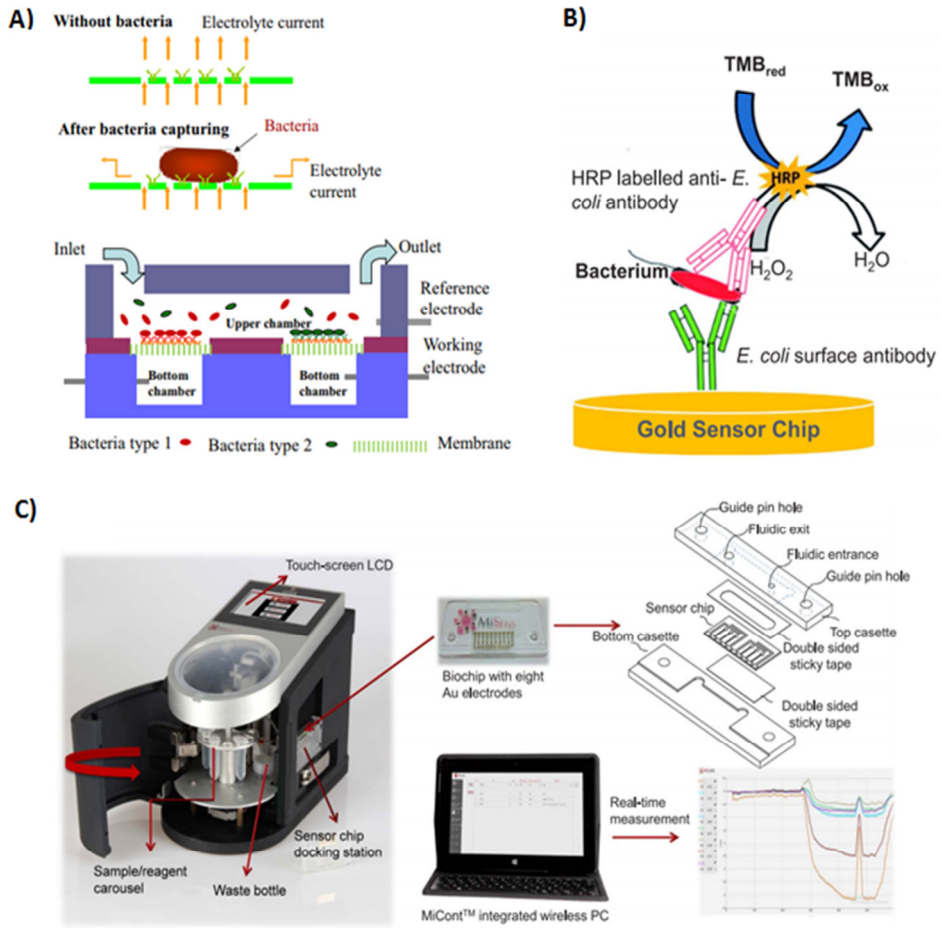

Figure 6. (A) Sensing mechanism of nanoporous alumina membrane (upper panel) and illustration of the simultaneous detection of two types of bacteria using microfluidic device integrated with the nanoporous membranes (lower panel). Reprinted from [68] with permission from Elsevier. (B) Sensing mechanism of an antibody-bacterium-antibody-HRP sandwich immunoassay. Reprinted from [70] with permission from Elsevier. (C) Schematic of a custom-designed fully automated microfluidic-based electrochemical sensor (MiSens) device, image also taken from [70].

Table 1. Summary of published material reviewed in detail in this report.

Target Bacteria	Sensing Strategy	Material	LOD achieved	Analysis time	Pre-Treatment	Ref
S. aureus *P. aeruginosa*	Detection of bacterial exotoxins using potassium ferricyanide, released from lipid vesicles in presence of toxins	Biomimetic lipid vesicles	11 and 2.9 μM	1 h	Supernatant preparation from bacteria	[14]
P. aeruginosa	Electrochemical conversion of pyocyanin	Polystyrene spheres	1 to 1.6 μM	Not specified	--	[16]
S. aureus	Thiol chemistry to immobilize bacteria via antibodies, EIS	--	10 CFU mL^{-1}	Not specified	Antibody immobilization, bacteria exposure to sensor	[18]
E. coli *B. subtilis*	Electrochemical reduction of $TMPD^{+\bullet}$ to TMPD, which is oxidized by bacteria (voltammetry)	--	5x 10^6 bacteria	5 min	--	[19]
S. aureus	SWCNT conjugation to paper, antibody immobilization, DPV	Paper, SWCNTs	13 CFU mL^{-1}	30 min	Antibody immobilization, bacteria exposure to sensor	[24]
E. coli *S. aureus*	Circuit LOC chips, cyclic voltammetry in ferrocyanide	--	1 CFU mL^{-1}	1 to 2 h	Lysis of bacteria, chip functionalization, sensor exposure to lysate	[29]
Chili leaf curl betasatellite	MWCNT-Zn-DNA platform, ferrocyanide/ ferricyanide redox couple, signal reduction in the presence of complementary DNA	MWCNTs	5 mM	Several hours	DNA amplification, drop casting	[35]
E. coli *S. aureus*	2x2 junction array, constructed from SWCNT coated wires, antibodies immobilization, current change observed in the presence of bacteria	SWCNTs	10^2 CFU mL^{-1}	10 to 15 min	Bacteria exposure to sensor	[36]
S. aureus	Silver nanoparticle decorated zinc oxide nanorod arrays, platform functionalized with vancomycin, EIS	AgNPs ZnO nanorods	330 CFU mL^{-1}	Not specified	Bacteria exposure to sensor	[55]
E.coli *S. typhimurium*	ITO as antibody substrate, EIS	ITO	2-3 ng mL-1	10 min	Bacteria exposure to sensor	[39]
E. coli	Nano-impact method	AgNPs	0.3 pM	10 min	Silver nanoparticle tagging of E. coli	[57]
E.coli	Diffusion blockage of redox mediator species towards carbon fiber microelectrode	--	1 bacterium	Not specified	--	[60]
L. monocytogenes	DNA intercalation with $[Ru(phen)_2dppz]^{2+}$, ECL	Paper	10 copies/μL of DNA	30 min	DNA amplification, bacteria exposure to sensor	[44]
E.coli *Enterococcus spp.*	Oxidation of bacterial enzymes β-gal, β-glucur and β-gluco using square wave voltammetry	Paper	10 CFU mL^{-1}	4 to 12 h	Sample mixing with media, incubation, centrifuging, sonication	[45]
S. aureus *L. monocytogenes*	Microfluidic LOC platform with electrochemical impedance sensors, bacteria recognition via antibodies	--	1.2 CFU mL^{-1} (S. aureus) 5 CFU mL^{-1} (L.	Not specified	Antibody immobilization, bacteria exposure to sensor	[63]

			monocytogenes)			
P. aeruginosa	Circuit LOC chip, containing thin film gold electrodes, square wave voltammetry	--	2.6 µM for metabolite phenazines	2 to 5 min	Phenazines extraction from agar	[64]
S. typhimurium	Microfluidic LOC chip, based on carbon nanowire	carbon nanowires	10 CFU mL^{-1}	5 min assay	--	[65]
E. coli *S. aureus*	Microfluidic device with integrated nanoporous membranes, bacteria captured by antibodies, EIS	Nanoporous alumina membrane	10^2 CFU mL^{-1}	> 24 h	Antibody immobilization, bacteria exposure to sensor	[68]
S. typhimurium	Microfluidic device with integrated nanoporous membranes, bacteria captured by antibody- GQDs conjugation, EIS	GQDs	1 pM	24 h to bind bacteria 30 min to detect antibiotics	Antibody-GQDs-conjugation, antibody-GQDs immobilization, bacteria exposure to sensor	[69]
E. coli	All-in-one device (microfluidic system, electrode array, electrochemical unit), bacteria captured by antibodies, amperometric measurements	AuNPs	50 CFU mL^{-1}	Not specified	Antibody immobilization, bacteria exposure to sensor	[70]

SWCNTs: single-walled carbon nanotubes; MWCNTs: multi-walled carbon nanotubes EIS: electrochemical impedance spectroscopy; DPV: differential pulse voltammetry; AuNPs: gold nanoparticles; AgNPs: silver nanoparticles; ITO: indium tin oxide; ECL: electrochemiluminescence; LOC: lab-on-a-chip; TMPD: N,N,N',N'-tetramethyl-para-phenylene-diamine; GQDs: graphene-quantum-dots

3. Conclusions and Perspectives

The ongoing and growing research towards the development of functional biosensors for the detection of bacterial pathogens demonstrates the significance and urgent need of such devices. Although numerous reports on the detection of pathogens using electrochemical sensors have been published, a product for real sample applications has still not entered the commercial market. Particular challenges include a sufficiently low detection limit, high selectivity, cost efficiency, short analysis, no or minimal pre-enrichment methods for samples, no required skills to carry out the detection, and the portability of the sensor to allow on-side monitoring. Ideally, all of the above aspects will be realized in a fully functional commercial product in the future, and the present review outlines great progress that has been made in recent years, regarding particular features, such as sensitivity and selectivity.

One of the key advantages of electrochemical sensing technology is its sensitivity and hence detection limits as low as one or very few culture forming units (CFU) have been achieved.[18,24,29,45,60,63,65,70] However, it has to be questioned if detecting infections at such low bacterial counts will truly be beneficial. It has recently been reported [71], with the example of urinary tract infections (UTIs), that diagnosing patients on the basis of CFU of less than 10^5 per mL, resulted in treatment of non–clinically significant UTIs and inappropriate use of antibiotics. Of course the threshold for clinical significance might vary with individual pathogens, but in general we conclude, given the already excellent performance of current electrochemical sensing methods, that less focus should be laid in reaching detection levels below 10^5 CFU mL^{-1}, but rather on other aspects of sensing devices, as discussed below.

The integration of nanomaterials in biosensors has provided significant improvements in terms of selectivity and analysis time of biosensing methods.[24,36,55,57,65,69] Considering the type of nanomaterial for sensor applications is crucial, as most of these materials are rather costly or require lengthy synthesis procedures. Furthermore, the possible release of nanoparticles in the environment and associated risks has been reported [72] and should be taken into consideration. Current research in nanotechnology is growing, and thus further development of smart nanomaterials with useful functions at an efficient cost level is expected for the future. In this context, the presented report has discussed examples for the recent development of paper or plastic-based materials, which often also offer a facile procedure of handling.[24,44,45]

Straightforward sensing principles are crucial for the applicability of biosensors in routine monitoring, as extensive training of staff needs to be avoided. Sensors based on nanomaterials, or ideally, the direct measurement of bacterial metabolites and secretion products, represent thereby desirable approaches.[14,16,19] These methods offer pathogen detection via an "on" signal, rather than an "off" signal, as obtained during

impedance measurements and hence are less prone to cause false positive results. The integration of such direct or semi-direct bacterial detection methods into portable devices would represent a major advance in the field of biosensor development. The design of universal sensors, which are able to detect foodborne and waterborne pathogens has been discussed [36,55,62,68], and although multiple types of bacteria can be recognized simultaneously, unfortunately, these devices still require complex assembly and costly components. The progress towards the integration of all sensing units as demonstrated in a fully automated device by Altintas *et al*. [70] is remarkable, however, this particularly complex example, consisting of microelectrode arrays, a microfluidic system, antibody immobilization and the use of nanoparticles, shows that the cost-efficient commercialization of a small easy to construct and handle, portable and compact device for on-site rapid disease diagnosis is still challenging. Therefore, future progress can certainly benefit from collaborations between research areas, such as engineering, material science, biology, chemistry and medicine, but also from engagements between academia, industry and the health care system to develop a consumer friendly product, addressing important issues such as material development, sample preparation, long-term stability study, clinical approval, real sample applications, design engineering and marketing.

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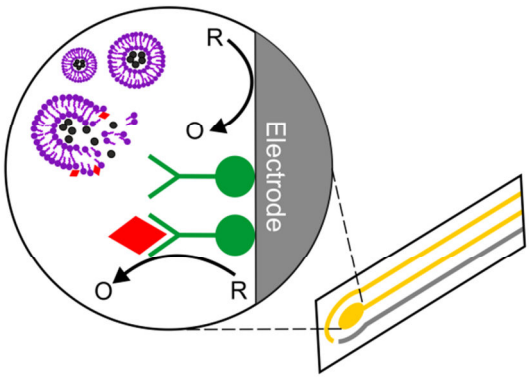
Entry for the Table of Contents

FOCUS REVIEW

Bacterial infections represent one of the leading causes of mortality worldwide and the development of rapid, cost-efficient and reliable detection methods for pathogens remains challenging. The presented review provides a summary of recent biosensing strategies, by covering direct sensing methods as well as procedures, using various bio-recognition elements towards custom-designed portable point of care devices.



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