Bacteriophage-based biosensors: detection of bacteria and beyond

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Abstract

Pathogenic infections cause tremendous health threats and socioeconomic burdens worldwide. Conventional approaches for bacteria detection are laborious, costful, require particular devices. Usually highly qualified personel is also nessesary. Sensitive, selective, inexpensive, quick, and user-friendly biosensors are in urgent demand to prevent and detect bacterial infections in many fields, e.g., healthcare, food industry, or terrorism prevention. Among biorecognition elements utilized in biosensors, bacteriophages are highly promising due to their numerous advantages, such as host specificity, cheap and simple production, resistance to external factors, and ease of immobilization. Here we reviewed currently used methods for bacteria detection, pointing their advantages and disadvantages. We paid particular attention to bacteriophage-based methods, including phage-based sensors and phage display method.

Keywords: bacteria, bacteriophage, biosensor, detection, applications

Introduction

Although quality of living is constantly improving through technological progress, bacterial infections remain a major problem in the modern world. Approximately 13% of the deaths are related to bacterial diseases [1]. Furthermore, bacteria are also involved in specific types of cancers [2] and various metabolic disorders, including obesity, which affects 39% of adults [3].

Bacteria are a significant threat for children or elders and developing countries, where respiratory diseases such as tuberculosis cause millions of deaths [4]. A major source of bacterial infections is food and water poisoning, causing 1.8 million casualties worldwide in 2005 [5]. In a publication from 2009, it was shown that only in the USA number of infections and illnesses originating in food reached 76 million. Among them, 325 000 were admitted to hospitals, and 5 200

died [6]. There are also socioeconomic costs related to outbreaks of an epidemic caused by food hazards. The report from 2011 showed 9.4 million episodes of foodborne illnesses in the USA [7]. 3 816 people got sick, and 54 died due to *Escherichia coli* O104:H4 outbreak in Europe in 2011 [8]. The total financial burden was estimated to reach 3 billion Euros. In 2015, the Center for Disease Control and Prevention (CDC) reported 15 202 foodborne infected patients, 950 hospitalizations, and 15 deaths [9]. World Bank study conducted in 2018 in low- and middle-income countries estimated cost of food-born illnesses at 110 billion USD and treatments cost at 15 billion USD annually [10].

Another major cause of bacterial infections is hospitals. According to a World Health Organization report from 2011, 4.1 million patients are affected by healthcare-related illnesses each year in Europe [11]. Furthermore, only in the USA, nosocomial infections cause 100 000 deaths each year [12]. Due to the impact of the COVID-19 pandemic, the number of hospital-acquired infections (HAI; central-line-associated bloodstream infections, ventilator-associated events, and laboratory-identified methicillin-resistant *Staphylococcus aureus* bacteremia) increased by 34 - 47 % in 2020 compared to the number of cases that occurred in 2019 [13]. According to the report published by Quince Market Insights in July 2021, the HAI market reached over 12 billion USD in 2020 [14].

The appearance of multidrug-resistant bacteria strains makes this problem even more urgent. The knowledge about antibiotic resistance mechanisms is still unsatisfying, and our main weapon against bacteria lost its potential [15]. Due to the lack of enough funding in antibiotic development and the uncontrollable use of antibiotics, the danger of antibiotic resistance is increasing radically. The CDC's latest estimation of death and infection in the US conducted in 2018 suggested that more than 2.8 million antibiotic-resistant infections occur each year, and more than 35 000 people die because of it [16]. In 2015, antibiotic-resistant Gram-negative pathogens caused losses estimated at 287 million EUR [17]. The cost of critical measures for antimicrobial resistance containment is estimated to be 9 billion USD globally [18]. Research conducted by the World Bank Group estimated that the global economic cost of antibiotic resistance will range between 1.0 – 3.4 trillion USD in 2030, which is 1.1 – 3.8% of global GDP [19].

Cost for biodefense and prevention from threats of biological warfare and bioterrorism also cause enormous expenses. For instance, statistics from 1997 indicated that the cost of prevention from brucellosis was estimated to be around $477.7 million per 100 000 persons exposed and anthrax was $26.2 billion per 100 000 people exposed [20]. These costs have multiplied over the last 2 decades [21].

Therefore finding an efficient way to overcome problems caused by pathogens is paramount. There is also a need for a rapid and specific method to detect and recognize bacteria. Most methods that are currently in use rely on culturing, biochemical tests, or molecular protocols (e.g., PCR, *polymerase chain reaction*, amplification). Although these approaches are useful, there is still no method allowing to combine short time of the analysis and very low detection limit (e.g., 1 CFU (colony forming unit)/mL), even at the expense of the cost of analysis.

Culturing methods

The bacteria identification based on cultivation aims to get pure culture from repeated collection and seeding of an isolated colony. Commonly applied isolation techniques involve seeding by dewatering, deep seeding in solid media, and liquid dilution. General-purpose agar-based media is commonly used to cultivate various pathogens, but some bacteria require more specific culture media for more accurate identification. For instance, "differential" culture media relies upon the metabolic difference of the pathogens by using a biochemical indicator system as well as a pH indicator to detect them. "Selective" culture media has antimicrobials that inhibit the commensal flora from increasing the growth of certain bacteria of interest [22].

Chromogenic media is frequently used as a microorganism identification method since it is cheap and straightforward. This technique is based on the reaction of the medium with the released metabolites. The chromogenic media method requires culturing of bacteria samples, using appropriate broth or agar media enriched with colorless or fluorescence chromogenic enzyme substrates. The substrates are then colorized by the bacterial enzymes [22]. Media is often supplemented with chromogenic substrates, aiming at glycosidases, such as β-galactosidase or β-glucosidase produced by the target bacteria, and combined with carefully chosen antibacterial agents to hinder non-target bacteria [23]. The chromogenic media method is commonly employed in clinical laboratories since it requires a small workload and increases the chances of identification due to colored colonies, especially when multiple species are present in the sample.

Commercially available biochemical tests are frequently used after isolation to identify genus and species levels. Commercial kits such as Analytical Profile Index (API) kits can be applied to carry out the inoculation and reading of biochemical panels manually, so do automated tests such as the BD Phoenix or the Vitek 2. These systems can identify bacteria in 2 to 3 hours and execute automated antimicrobial susceptibility testing [24].

Even though these methods cost less and provide quantitative and qualitative information about the bacteria, they require a lot of work and time for media preparation, dilution, plating, incubation, counting, isolation, and characterization. The main disadvantage of the chromogenic media method is that this method is usually time-consuming and require up to a few to days obtain the results [25]. Also, in some cases it requires additional examination using other analytical, often instrumental, methods. At times, biochemical properties inaccurately indicate the genomics of a given species [26], and results can be false positives considering similar species [27].

Molecular methods

Molecular methods present multiple tools and techniques for bacteria characterization, detection, and identification [28]. They brought remarkable insights by detecting previously unidentified bacteria, classifying uncultivable bacteria, and allowing the metagenomics study of diverse bacterial communities on a large scale. Most molecular techniques for bacteria detection and identification are based on DNA

analysis, extending from rather simple DNA amplification-based methods, such as polymerase chain reaction (PCR), real-time PCR, random amplification of polymorphic DNA PCR (RAPD-PCR) to more intricate approaches that rely upon restriction fragment analysis, targeted gene, and whole-genome sequencing [29]. Molecular methods can be classified as amplification methods (PCR, quantitative real-time PCR (qPCR), and reverse transcription PCR (RT-PCR)), DNA microarrays, hybridization-based detection methods (FISH), and whole-genome sequencing (WGS). These methods are culture-independent and enable bacteria identification at the genus level. It is crucial to understand the basic operating principles of each method, as well as their uses and limitations [28].

Gene amplification and target gene sequencing is an effective method for bacteria identification. Over the past years, PCR amplification and gene sequencing have been utilized for detecting and identifying bacteria from colonies. Gene sequencing is a more objective method of bacteria identification, which does not regard fastidious growth or cell viability. This method provides reliable results and enables an increase in the diversity of bacterial taxa [30]. Amplification methods provide a relatively quick results, but there is a risk of cross-contamination associated with their sensitivity.

The 16S ribosomal RNA (16S rRNA) gene, the 26S rRNA gene, or particular genes encoding bacterial toxins are sequenced to detect bacteria. The 16S rRNA, a 1500 base pair gene common to all bacteria, is the most frequently utilized gene target for bacterial identification due to its high specificity to each specie [31]. Real-time PCR is qualitative, more sensitive, and accurate compared to conventional PCR techniques. qPCR with fluorescence intensity enables the analysis of DNA amplification in real-time and doesn't require any post-PCR detection, which explains its broad usage in clinical and research fields. For instance, real-time-based 16S rRNA PCR was applied to identify and quantify microorganisms in chronic wound tissue and saliva sample [32]. Quantitative real-time PCR (qPCR) and reverse transcription real-time PCR (RT-qPCR), and other amplification methods were used to identify foodborne pathogens, such as *Listeria monocytogenes*, *E. coli* O157:H7, *S. aureus*, *Campylobacter jejuni*, *Salmonella* spp., and *Shigella* spp. [33]. Random amplification of polymorphic DNA (RAPD), on the other hand, uses short primers with random sequences that result in the amplification of arbitrary, repetitive regions of template DNA. Since the short primers for RAPD-PCR are intended to bind randomly to the template, this method does not oblige any prior information of the target genome sequence. RAPD-PCR can be utilized not only to detect bacterial genetic variability but also to discover and detect unidentified microorganisms [25].

Microarray is an ordered assemblage of samples (DNA, RNA, protein, tissue) that can be probed with target molecules to generate gene expression or diagnostic information. Microarray analysis can simultaneously detect and characterize numerous bacteria. Several microarray methodologies are available for application, such as printed and *in situ* - synthesized microarrays, electronic and suspension bead microarrays, and high-density bead arrays. Generally, the ssDNA sequence is synthesized and immobilized as discrete features or spots on the microarray surface. The "unknown" target sequence of interest is fluorescently labeled and then hybridized to the probe microarray. Hybridization between the

immobilized probe and the labeled target enhances the fluorescence intensity. The fluorescence scanner measures the intensity, and the collected data is analyzed further [34].

Fluorescence *in situ* hybridization (FISH) is considered a less time-consuming and reliable cytogenetic technique for bacteria detection and identification at the genus or species level. The principle of the FISH method relies upon the binding of short (18-25 base pair), fluorescence-labeled target-specific DNA or nucleic-acid mimicking peptide-nucleic-acid (PNA) probes to the ribosomal RNA with subsequent analysis under the fluorescence microscope. The FISH analysis offers information on spatial resolution, morphology, identification, and fast differentiation of bacteria from a mixed-species solution. The method offers rapid and reliable detection at the genus and species level, minimal technical equipment necessity, and cost-effectiveness. The main drawbacks are a need for specifically targeted investigation, trained and experienced personnel, and lower sensitivity than PCR [35].

Whole-genome sequencing (WGS) is becoming a highly applicable technique that provides rapid detection and identification of bacteria, viruses, and fungi due to advancements in sequencing technologies [36]. WGS technologies permit valuable data about difficult-to-grow pathogens and drug resistance, bacteria's evolution and spread, possible virulence factors, candidate drug complexes, and a deep understanding of infection mechanisms. WGS technologies can compete with standard methods in speed, specificity, expense, and monitoring/investigating outbreaks of infectious diseases. Currently, WGS is commonly used in addition to real-time diagnostics in medical laboratories. Apart from detecting, identifying, and characterizing bacteria, WGS is applied to design diagnostic tools, assess multidrug resistance, examine and track the emergence of pathogens in hospital environments [1].

Probes for bacteria detection

Probes techniques such as Southern blot, Northern blot, and Western blot are relatively old yet not overused methods for detection. Southern blot was developed based on Southern sequencing, which was the first used DNA sequencing technique. This sequencing method relies on isolating the DNA from the 'target' sample, amplification reaction using specific primers with controlled termination of amplification by dehydrogenated nucleoside triphosphates, agarose gel electrophoresis, and gel visualization by the usage of ethidium bromide [37]. Then, protocols were modified to detect specific DNA sequences in DNA samples. At first, the DNA sample is cut by restriction nucleases. DNA fragments are separated by size through agarose gel electrophoresis, then transferred to nitrocellulose membrane and crosslinked the membrane via exposure to ultraviolet radiation. The critical step in Southern blotting is exposing the crosslinked membrane to a hybridization probe – a single-stranded DNA fragment complementary to the sequence of interest, usually tagged with a fluorescent dye or radioactive marker. After hybridization, membranes are blocked, washed, and then visualized [38]. The main advantage of Southern blot is that it detects unculturable, usually environmental, bacteria [39].

Northern blot is commonly used to analyze the gene expression by detecting RNA in the sample. In principle, it is similar to Southern blotting, but electrophoresis gels have to contain formaldehyde to

limit RNA secondary structure. Probes are complementary to the RNA sequence of interest. Still, they can be DNA, RNA, or oligonucleotides, usually labeled with radioactive isotopes, but chemiluminescence probes are becoming more and more common in use [40]. Northern blot is not directly used for bacteria detection. Still, it allows detection of some particular bacterial small RNAs (sRNAs) in total RNA extract [41], which makes a fine way for examining gene expression. Its drawback is the impermanence of the analytical material, for it is tough to avoid RNase contamination.

Western blot, also known as the protein immunoblot, allows for the detection of specific proteins. In this method, proteins are separated by size via electrophoresis, usually in polyacrylamide gel, then transferred on the membrane and blocked. A protein of interest is targeted by incubation with a primary antibody. Then a secondary antibody targets the primary one. The secondary antibody is visualized through colorimetric, chemiluminescence, immunofluorescence, or radioactivity assays, indirectly detecting a target protein [42]. Because bacteria produce species-specific proteins, such as toxins, it is possible to detect and recognize them with Western blotting protocol [43]. The main advantage of this technique is its simplicity and unambiguity of the results. Unfortunately, the analysis may require about a week to complete, making it an extremely lengthy procedure. Also, analyzed proteins tend to form complexes. This phenomenon may cause the antibody-binding site to become unavailable, or even worse – the protein complex may be visualized and mistakenly recognized as an additional target protein [42].

Microscopic methods

The optical microscope is a fundamental detection device for bacteria identification. Obtained images allow determining the shape, following motion, and categorizing species by their morphological contrast [44]. However, only using microscopy for bacteria detection is not enough. In natural samples, smaller cells can be missed due to the density of larger cells. Distinguishing cells from other objects or living cells from dead cells can also be challenging [25]. Another major disadvantage of microscopy is that none of them displays the microorganisms' phylogenetic diversity.

In most cases, microscopic methods are used with fluorescent dyes due to more specific visualization and uncomplicated performance. Dyes such as DAPI (4’,6-diamidino-2-phenylindole), acridine orange, SYBR® Green I bind to the DNA of the bacteria and fluoresce after ultraviolet (UV) exposure arraying (DAPI absorption at 400 nm, acridine orange absorption maximum 500 nm, SYBR Green I maximum absorption 497 nm) making the bacteria detectable [25]. Flow cytometry can also be applied for detecting individual cells. This method enables the possibility to count and evaluate individual cells' size, shape, and features. Cells are suspended in a fluid flow and passed through a detector, collecting fluorescence or scattered light. Clausen et al. used a label-free technique of electrical impedance flow cytometry to distinguish Gram-negative from Gram-positive bacteria successfully and accurately determined the concentration of the bacteria solution [45].

Spectroscopic methods

Spectroscopy is the study of matter and its interactions with electromagnetic radiation. Spectroscopic techniques are used in nearly all technical areas of science and technology for quantitative and qualitative analyses [28]. This multivariate, reproducible methodology is used to solve numerous analytical problems due to its non-destructive, simple, and precise approach, enabling broad amounts of information acquired in a single measurement [46]. Spectroscopic techniques vary based on the examined species (molecular or atomic spectroscopy), the type of radiation–matter interaction to be monitored (absorption, emission, or scattering), as well as the used range of the electromagnetic spectrum. Spectroscopic methods require a combination of spectral pre-processing and different chemometric techniques to quantitively analyze and differentiate bacteria.

One of the latest developments in applying new spectroscopic techniques is the Fourier transform infrared spectroscopy (FTIR), an adjustable, rapid, non-invasive, and effortlessly operated method [46]. This analytical method is a chemical and label-free approach that provides a comprehensive interpretation of the chemical compounds and the physical state of the whole sample in which numerous biomolecules can be examined. It is possible to acquire precise, thorough information about nucleic acids, carbohydrates, lipids, and proteins only in one measurement with a small sample volume [47]. Also, FTIR enables an efficient biochemical characterization of sophisticated biological systems, such as intact cells, tissues, and even whole-model organisms. FTIR application to examine microorganisms leads to quite a complicated spectrum with the principal compounds' overlapping absorption bands. Hence, a proper multivariate statistical analysis is required to extract only the essential materials from spectra [25]. The major advantage of the FTIR method is the capacity to examine numerous compounds at once. Also, this method doesn't require cell lysis to evaluate the biomolecules and is considered eco-friendly since toxic compounds are not used. FTIR can be applied for real-time process monitoring besides the achievement of high-throughput screenings [47]. However, for the analysis of microbial diversity, much more applicable are the near-infrared (NIR), the mid-infrared (MIR), and the far-infrared radiation [48].

In the near-infrared (NIR) spectral region was shown in the food microbiology industry via effective detection and identification of *Lactococcus lactis*, *Listeria innocua*, *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Pseudomonas mendocina* on chicken breast muscle inoculated with a pure bacterial suspension [49]. The major drawback of using NIR spectroscopy in food or microbiological analyses is the samples' sensitivity to temperature shifts or the occurrence of photodegradation triggered by the light sources. Furthermore, the infrared (IR) signal is frequently dominated by water, an essential part of culture media and food products [28].

Raman spectroscopy is another popular spectroscopic method recognized for its non-invasive and rapid identification and characterization of bacteria. It is based on the inelastic scattering of monochromatic light. Inelastic scattering implies the shifts of photon frequency in monochromatic light upon contacting the sample. The sample absorbs light photons and then reemits. The reemitted photons'

frequency is altered compared with the original monochromatic frequency (the Raman effect). This shift provides information about molecules' vibrational, rotational, and other low-frequency transitions to create a structural fingerprint. The obtained structural fingerprint is then utilized to distinguish microorganisms since this method is capable to accurately differentiate between species and strains in a small amount of time [28]. Additionally, the Raman signal is not affected by water, but fluorescence signals can give high background because of amino acids and nucleic acids [28]. Even though Raman spectroscopy has high specificity, it has inadequate sensitivity.

Surface-enhanced Raman spectroscopy (SERS) enables greater sensitivity in detecting low concentration analytes by intensifying electromagnetic fields created from the excitation of localized surface plasmons. Comparing to the standard Raman, a signal can be boosted from 10^3 to 10^6 times using SERS [50]. Bacteria detection using SERS can be carried out in one of the two following ways: the direct detection - the intrinsic vibrational fingerprint of bacteria; the indirect detection - a nanotag used as a quantitative reporter. SERS signal relies upon the active substrate's material since each substrate has unique enhancement effects on the samples. The shape and size of the nanoparticles, the active substrate's material, distance, and the number of probes adsorbed on the active substrate affect the signal [28]. Wei et al. successfully detected and identified *E. coli*, *S. aureus*, and *Salmonella* spp. using SERS coupled with silver colloidal nanoparticles. The distinctive differences of each pathogen were observed in the SERS spectral data, and a short time was required for the assay [51].

Chromatographic methods

Mass spectrometry (MS) based techniques are recognized as a microbial typing tool because of their rapidity, low expense, ease of use, and effectiveness to all kinds of bacteria, archaea, and fungi. Mass spectrometry can be associated with multiple ionization and separation methods, such as gas chromatography (GC) [52], matrix-assisted laser desorption ionization time-of-flight mode (MALDI-TOF) [53], electromigration techniques [54], and electrospray ionization (ESI) [55].

Liquid chromatography (LC) combined with MS (LC-MS) transformed the analytical determination of metabolome, thus, enabled bacteria identification [56]. In LC-MS, the temperatures are relatively low, and the sample's volatility is not obligatory, which simplifies the preparation process of the sample and decreases the costs. Samples are introduced into the solvent then separated within the column with the stationary phase. Subsequently, the column's eluent moves through a flow cell in a spectrometer for non-destructive recognition of compounds with spectrometric structures [57]. LC depends on the gravity force to move the mobile phase across the column, but for HPLC, pressures reach 50-350 bars. Moreover, it can be utilized at higher temperatures (high-temperature liquid chromatography) or in monolithic columns [58].

MALDI-TOF MS is the new generation tool that is widely used for rapid bacterial identification and classification in most advanced clinical laboratories. It relies upon the microbial cells' ionization with short laser pulses and accelerates the particles in a vacuum system *via* an electric field [59].

A molecular fingerprint in the form of a spectra profile is acquired that is unique for each bacteria. The spectrum is then compared with an existing database [53]. Nowadays, MALDI-TOF MS is commonly used with culture methods to verify the identification of pathogens, which are amplified by selective culture and make quick and enables species-level detection within minutes [22].

Electrokinetic separation methods

Capillary electrophoresis (CE)–MS merges the separation process of electrophoresis with MS detection. Comparing to GC and LC, it provides more efficient separation, faster analysis, allows for small volumes of sample required, inexpensive reagents, and separation of cations, anions, and uncharged molecules in one run. This method is applied to examine the metabolome of various bacteria, in which results were intriguing in the detection and quantification of numerous metabolite classes [60]. CE lacks sensitivity due to the small sample volumes. At the same time, combined with MS, it has a limited number of accessible commercial libraries, and last but not least, decreased retention time reproducibility.

Researchers combined CE with capillary isoelectric focusing (CIEF) to isolate and identify bacteria species with different sizes and shapes [61]. This experiment showed that intact biological cells could be successfully isolated *via* methods typically limited to macromolecules. Another combination is CE fused with fluorescence that can be utilized to monitor the separation process, operational conditions, and microbial dynamics regarding cell aggregation [62]. The primary benefit of these methods is the capacity to control parameters (size, shapes, and charges) for isolation and detection.

Electrical field-flow fractionation (EIFFF) is an alternative method. It depends on the separation of sample components in a channel because the various electrical fields result in a distinct layer of each component. Two main walls of the channel are utilized by the EIFFF device to generate a difference in the potential, which allows for the separation of charges [63].

Biosensors

Biosensors appear as the most promising devices for the detection of microorganisms. Biosensor-based methods are perceived to have great potential for further development [64, 65]. According to IUPAC, "*a biosensor is a device which uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compound usually by electrical, thermal or optical signals*". Antibodies, enzymes, and nucleic acids are commonly utilized as bio-receptors [66].

Biosensors are divided into physical and chemical biosensors depending on the transducer used to detect the target analyte. Physical biosensors sense shifts in mass, resonance frequency, refractive index, fluorescence and are further categorized as optical and mechanical biosensors. Optical biosensors measure the analyte by its interaction with photons, such as fluorescence or phosphorescence emissions. Optical biosensors are divided into labeled and label-free. Mechanical biosensors detect the analytes by examining the shift in mass during the recognition stage. These sensors have several beneficial

characteristics, such as no sample preparation step and label-free detection comparing to other sensors. The most frequently employed mechanical biosensors are quartz crystal microbalance or cantilever technology [67].

Chemical biosensors detect the shifts in the chemical reactions during the interactions between the analytes and biorecognition elements. Chemical biosensors are further classified into electrochemical and biochemical sensors. Electrochemical biosensors analyze the differences in electrical properties, such as current, potential, or impedance at the electrode surface during the binding step. Based on the detection technique, electrochemical biosensors are categorized into labeled and label-free. Labels, such as enzymes, metal particles, or nanoparticles, are employed to target the analytes in labeled biosensors. In label-free biosensors, the attachment of biomolecules to the surface of the electrode cause shifts in electrical parameters. Electrochemical biosensors are categorized into amperometric, potentiometric, voltammetric, conductometric, and impedimetric [68].

Analytes in biosensors range from ions and molecules, through nucleic acids and proteins, up to the whole viruses and bacteria. Biosensors can detect bacteria by targeting bacterial components, such as DNA, RNA, intracellular proteins, exotoxins. This method requires sample processing and additional reagents, which raises costs and time. An alternative method to detect bacteria is to target whole bacteria cells. This direct method does not require additional reagents, which is more suitable for quick and inexpensive point of care testing. For the whole bacteria detection, impedimetric and optical methods are frequently applied [69].

Even though biosensors are rapid and specific, they are not consistently applied in bacteria detection due to cost, limit of detection, complex matrix, and difficulty in detecting more than one bacteria simultaneously [65]. Primarily, much depends on the chosen type of bioreceptor element that can be more or less sensitive to contaminants [70].

Bacteriophage-based methods

Bacteriophages

Bacteriophages are viruses that attach to particular bacterial receptor proteins to infect the host cells. Most known bacteriophages belong to *Caudovirales*, whose representatives are characterized by dsDNA genome and icosahedral, tailed capsid with the fibers attached to the tail [71]. The size of the virion is usually about 50 – 200 nm. However, some filamentous phages (e.g., M13) may reach even 400 nm length [72]. Recently, even bigger bacteriophages were discovered from marine water.

Based on their life cycles and means of propagation, phages are classified into two categories. Lysogenic (temperate or reductive) phages fuse their genetic materials into the bacterial genome and are inherited by daughter cells during binary fusion. Lytic (virulent or productive) phages undergo four steps process during infection: 1) binding to the receptors (protein or sugar moieties) of the bacterium due to host specificity properties; 2) injection of genomic materials into the cytoplasm of the bacteria; 3) viral replication *via* bacterial transcription, translation, and replication; 4) newly assembled phages leave the

cell through bacterial lysis with the help of cholins and endolysins proteins, causing the death of the host cell. This process is the basis of phage therapy for targeting pathogens [73].

Bacteriophages are gaining recognition as a promising recognition element in the area of rapid detection of bacteria. Bacteriophages demonstrate advantageous qualities such as excellent specificity, robustness, toughness, and cheap preparation, making them popular biorecognition elements in biosensors and other assays for bacteria detection [15, 74]. The most crucial advantages of phage-based methods for bacteria detection are:

- Phages are ubiquitous and highly specific to bacteria [75] but cause no major threat to humans [76].
- Because of being „molecular parasites" [77], phages need to infect a viable host to multiplicate by using its transcriptional machinery. This fact allows us to distinguish between living and dead bacteria, which is usually a significant issue for bacteria detection protocols. However, it may be phages absorb on the surface of the dead cell [78].
- Phages can self-amplify, which makes their "production" simpler and cheaper than, e.g., antibodies,
- Phages targeting particular bacterial species may be isolated from various environments, such as hospital sewage water [79], environmental water or sewage samples [80–82], or the soil [83]. The isolation process is quick and cheap, can be provided in every biological laboratory, without even identifying the isolates.
- Phages display more shelf life due to their resistant nature to external factors, which decreases the environmental limitations and allows regeneration of the sensor surface [84].
- Finally, phages are biological entities that evolve. This allows them to compete in the arms race with bacteria [85] and overcome developing resistance mechanisms. For instance, not long after discovering CRISPR [86], anti-CRISPR mechanisms were also found [87].

Phage-based biosensors rely on two different approaches. The first one is the generation of the analytical signal upon the capturing of bacteria. These are usually surface-sensing elements or phage-based probes. Their main advantage is the speed of the analysis, yet a single event is difficult to detect. While being one of the major problems, this fact is responsible for relatively high detection limits (LOD). The sensitivity of these types of biosensors can be improved by using phage-based bioconjugates, layered sensors, and methods utilizing parts of phages without additional pre-incubation steps. This might be done by developments in biorecognition elements themselves (e.g., by ordering of phages within sensing layers) or by utilizing ultrasensitive transducers (e.g., optoelectronic-based).

Alternative designs are based on the infection of the target bacteria, which generates the measurable signal by affecting the cell's metabolism (the release of progeny virions, products of reporter genes, or metabolites). These methods already showed some ultra-sensitivity, but they are lengthy due as they rely strongly on the metabolism of the bacteria. Schemes showing the phage-based approaches for bacteria detection are shown in **Figure 1**.

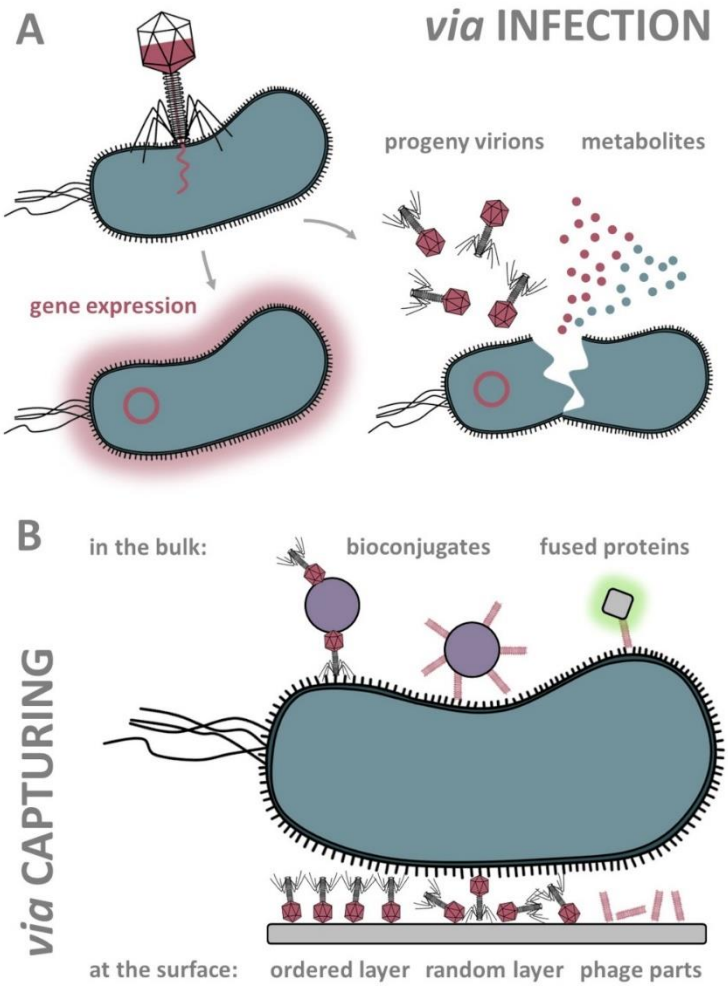

Figure 1. Designs of most commonly used phage-based biosensors. Bacteria detection by depositing phages on sensor surface or phage-based probes is rapid, but signal amplitude is usually low. On the contrary, infecting bacteria and using its molecular mechanism increases sensitivity by the generation of progeny virions, introduction reporter genes, or releasing bacterial metabolites due to lysis, but the process is time-consuming. The figure was inspired by [15] based on Creative Common CC BY 4.0 license.

Methods targeting bacterial metabolites

Upon completion of the lytic cycle, the content of the bacterial cell, including essential biomarkers, is released. ATP, adenylate kinase, bacterial β-D-galactosidase, and α- and β-glucosidase can be used as bacterial cytoplasmic markers in phage-mediated bacterial detection. The application of phages increases the specificity and allows for the recognition on species-level. A research was conducted using a T7 phage-based biosensor platform based on the detection of β-galactopyranosidase released during the lysis of bacterial cells. β-galactopyranosidase catalyzed the cleavage of the substrate resorufin β-D-galactopyranoside. The reaction resulted in resorufin formation, in which fluorescence was detected. The limit of detection (LOD) was about 10 CFU/mL of *E. coli* within 8 hours [88]. He et al. showed *P. aeruginosa* detection setup combining magnetoseparation, phage amplification, and sensing intracellular adenosine triphosphate (ATP). The release of ATP accompanies the cell lysis caused by the release of progeny virions. First, the PAP1 phage was conjugated with magnetic beads. Luciferase -

adenosine triphosphate bioluminescence system was used to indirectly determine the concentration of *P. aeruginosa*, based on the concentration of released ATP. Reported LOD was 2×10^2 CFU/mL obtained within 2 hours [89].

A further step for developing such an approach was to introduce genetically modified phages to obtain both sensing and signal-generating elements. Reporter phages are genetically modified phages used as gene importers to inject a specific gene into the bacteria's genome. The most suitable genes are fluorescent coding proteins or other easy-to-detect products expressed inside the host cells during the infection. Genes, such as bacterial *lux* [90] or firefly *luc* [91] gene injection produce bioluminescence, *inaW* gene-ice nucleation [92], *lacZ* gene-β-galactosidase [93], and I*gfp* gene [94] were used as a reporter for various pathogens detection.

A review published by Pizarro-Bauerle and Ando [95] presented the current state of the art of engineered bacteriophages' practical applications. This report summarized examples of the utilization of genetically modified bacteriophages in the fields of medicine (including phage therapies), animal industry, agriculture, biocontrol, and genetic engineering tools. Here, we present the most significant reports consisting of biosensing using genetically modified phages.

A report from 2000 by Irwin et al. described the usage of *Salmonella*-targeting bacteriophage encoding ice nucleation protein (INP) to infect the bacteria. After the supercooling with a phase-sensitive dye, the quantitative analysis of bacteria solutions was conducted. This allowed for the detection of *Salmonella* spp. with a minimum detectable level of about 2 CFU/mL within 3 hours [96].

Wisuthiphaet and coworkers showed the application of T7-ALP phage carrying the gene of alkaline phosphatase. The detection of *E. coli* BL21 bacteria was provided *via* fluorescence imaging of ELF-97 alkaline phosphatase substrate used to stain the bacteria left on the filter. The LOD was around 10^2 bacteria per gram of model beverage and the time of analysis was about 6 hours [97]. Another recent publication by Wisuthiphaet et al. described a rapid colorimetric pathogen detection method in a food matrix. T7 phage engineered with *phoA* gene was used to detect *E. coli* in coconut water and spinach leaves. The method consisted of phage-induced expression of an exogenous enzyme, alkaline phosphatase, specific colorimetric substrate, and filtration. Results showed that 10 CFU/mL of *E. coli* were detected from coconut water and 10^2 CFU/mL from spinach leaves within 5 hours [98].

A paper by Nugen et al. reported detection of *E. coli* with T7 containing NanoLuc luciferase expression cassette. This method requires the addition of luciferin NanoGlo substrate to detect the chemiluminescent signal. Modified phages were prepared by synthetic biology approach [99] - PCR fragments and *in vitro* DNA assembly were used. This protocol provided relatively fast and straightforward preparation of modified phages. The LOD was about 5×10^2 CFU/mL after 2 hours of incubation. Later on, the same research group proposed an approach to develop a new, sufficient protocol for analysis of drinking water against generic *E. coli* (according to the US standards, there can be no 'coliforms' in 100 mL of drinking water). 100 mL of water sample was filtered on the cellulose filter. After 8 hours of incubation, two modified T7 phages carrying a reporter gene (luciferase or

alkaline phosphatase) were added. In both cases, reporter genes were fused with genes encoding cellulose–specific carbohydrate-binding modules (CBM). After 1.5 hours of incubation, enzymatic substrates were added to visualize the colonies. The overall time of this procedure was about 10 hours, making it significantly faster than the plating method (24 h) and providing the most satisfying limit of detection of 1 CFU/100 mL [100].

To make the analysis time shorter, the same group proposed a combination of their previous research – detecting *E. coli* by T7 phage with the NanoLuc reporter gene fused to the CBM. The water sample was supplemented with growth media. After 60 minutes, genetically modified phages and microcellulose were added, the mixture was incubated for 90 minutes to allow the expression of NanoLuc-CBM. The target protein got bounded to cellulose and centrifuged. The luminescence was measured after the addition of the NanoGlo substrate to the sample. The final LOD was about 10 CFU/mL [101]. According to Wisuthiphaet, these detection limits can be acquired only in simple matrices. In complex matrices, the background signals may cover the signals from bacteria detection [97]. Eventually, Nugen's group reported a syringe-based biosensor using the same engineered T7 phage containing the NanoLuc-CBM cassette. This time the LOD was about 20 CFU of *E. coli* in 100 mL of drinking water within the time of the analysis of 5 hours [102].

Some systems do not require any external substrates. Vinay and coworkers proposed the detection of *E. coli* and *S. enterica* ser. Typhimurium using HK620 and P22 phages with introduced *gfp* gene. As a method of detecting, the flow cytometry approach was used. Acquired LOD was about 10 cells/mL in seawater after 1 hour of incubation [103]. Following research focused on the genetic modification of phages HK620 and HK97 to express the entire lux operon - *lux*AB genes coding luciferase and *lux*CDE coding fatty acids reductases. Although the reported LOD wasn't satisfying (10^4 bacteria/mL), incorporating the *lux*CDABE cassette into the COMBITOX instrument was successful. When upgraded, this instrument may become a useful tool for accommodating several bio-detector systems to detect bacteria, toxins, and heavy metals [104]. A report published by Kim et al. presented quite a different approach for utilization of *lux*CDABE operon. Their phage-of-choice was phiV10 phage, targeting *E. coli*. The sensor detected *E. coli* O157:H7 with LOD of around 1 CFU/mL in a pure culture within 40 minutes after 5 hours of pre-incubation. Artificially contaminated romaine lettuce, apple juice, and ground beef were spiked with bacteria, and phiV10lux allowed detection limits of around 10 CFU/cm^2, 13 CFU/mL, and 17 CFU/g, respectively [105]. In 2016 Wu et al. fused the tetracysteine (TC)-tag with small outer capsid protein of the wild-type PP01 bacteriophage and used them to infect *E. coli* O157:H7 host cells. Then the progeny PP01-TC phages were fluorescently labeled, and a flow cytometry procedure was used to measure the fluorescence. The LOD in the complex fluid (apple juice) provided the LOD of 1 CFU/mL within 1 hour [106]. Recently, the same research group developed a rapid, sensitive, and multiplex detection method targeting *E. coli* O157:H7, *S. typhimurium*, and *P. aeruginosa* using dual-modified M13KE phage. The M13KE phage-displayed the targeting

peptide on the minor coat protein pIII and the streptavidin-binding peptide on the major coat protein pVIII. The LOD of this method was 10^2 cells/mL in 40 mL of sample volume *via* flow cytometry [107].

Wang et al. proposed the electrochemical detection of *E. coli* using T7 phage expressing the *lacZ* gene encoding β-galactosidase. The substrate was 4-aminophenyl-β-galactopyranoside (4-APG). 4-APG forms an electroactive product when cut by β-galactosidases. This product was detected differential pulse voltammetry. The detection limit was in the range of 10^2 CFU/mL within 7 hours [108].

FASTPlaqueTB assay for detecting *Mycobacterium tuberculosis* in sputum uses the lytic virulence of the phages as a "sensor". Phage particles containing a luciferase gene are commonly used as reporters due to the highly sensitive detection of the bioluminescent signal luciferase generates. In addition, green fluorescent protein (GFP) and several other reporter genes are considered suitable. GFPs retain major advantages, such as high stability, low toxicity, and the fact that fluorescence is triggered by excitation light, eliminating the additional substrate as required for luciferases [109].

Detection of progeny virions

Bacteriophages offer a "built-in" amplification system – after the infection of the host cell and multiplication using its translational machinery, progeny virions destroy the host cell and are released. These progeny virions can be used for the detection of bacteria. Such amplification improves the detection limit, for much more objects can be detected.

To provide a faster time of analysis and sensitivity, phage amplification methods for detecting progeny virions are usually combined with the PCR technique. Luo et al. proposed the detection of *Acinetobacter baumannii* in serum using p53 phages acquiring the LOD in the range of 10^2 CFU/mL within 4 hours [110]. Later, the same group used qPCR combined p53 phage recognition of *A. baumannii* LB8 isolated from sputum samples. They designed the primer pairs to recognize the phage or the bacteria. It allowed for a detection limit of around 1 CFU/mL within 6 hours [111]. Garrido-Maestu et al. reported the detection of 8 CFU of *S. enterica* ser. Enteritidis in 25 g of chicken meat samples within 10 hours [112]. Sergueev reported the detection of zoonotic bacteria *Brucella abortus* in mixed cultures and blood samples with the LOD of around 1 CFU/mL within 72 hours [113]. One of the most spectacular examples was published by Anany et al. [114], who developed a phage-based paper dipstick biosensor to detect foodborne pathogens directly in food matrices. Authors used piezoelectric inkjet printing to prepare phage-based bioactive papers, on which bacteria were first actively lysed. This protocol provided the detection with the limit of 10 to 50 CFU/mL in the number of various samples with a total assay time of 8 hours. This required a combination of the phage-based method with qPCR.

Mido et al. proposed combining phage amplification with immunoassay protocol. Progeny MS2 phages coupled with specific antibodies were immobilized on the surface of magnetic beads. Upon the addition of the detector antibody, binding to MS2, the fluorescence was measured. A fluorescence-based method allowed for detection after 3 hours of incubation with the LOD of around 10^2 cells/mL [115].

The most archaic but also the simplest method of detection of progeny virions are titration using the plaque counting method. Phages are deposited onto the agar plate inoculated with bacteria. Bacteria get lysed where virions are presented, which is visible as the holes in the bacterial layer. These holes are called plaques, and they mark the number of phages in the stock solution. In the research by Said et al., this approach was used to monitor the activity of a foodborne and waterborne pathogenic bacterium, *Salmonella typhi*, under starvation conditions. The phage infectivity rate was much more suitable than the traditional plate culturing technique. It allowed for detecting active bacteria that are not detectable by conventional methods, i.e., VBNC (viable but non-culturable) cells [116]. In 2006 Ulitzur and Ulitzur reported the usage of mutant phages (mutants that cannot form plaques at concentrations lower than their reversion rate and temperature-sensitive mutants) as a method for bacteria detection and determination of their antibiotic susceptibility. The method is based on plaque formation as the endpoint of the phage lytic cycle. The detection limit was 1 to 10 living *Salmonella* or *E. coli* O157:H7 cells after 3 to 5 hours [117]. Jassim and Griffiths reported an interesting *P. aeruginosa* detection method using Pseudomonas Phage NCIBM 10116 for standard plague counting method combined with live/dead fluorescent measurement. This resulted in the highly specific analysis is a reasonable period (1 cell/mL within 4 h) that allows monitoring viable cell numbers [118].

Utilization of whole virions for bacteria detection for biosensors

There are disadvantages to approaches depending on the completion of the phage lytic cycle. First, such methods require choosing only the virulent phages. Secondly, progeny phages are usually not produced if a prophage is already incorporated in the host's genetic material. Finally, bacterial phage-infections-preventing mechanisms, such as CRISPR-Cas, influence the process [86]. Also, methods relying on genetically modified phages have some drawbacks that need to be considered. Genetic modification demands extensive technical and biological knowledge, which makes reporter phage-based assays complicated. The modification process and its optimization need to be repeated for each new target bacteria. Furthermore, modified phages are often less infectious [119]. Finally, the release of genetically modified phages into the environment could cause unpredictable effects on the biosphere [120].

All this resulted in developing phage-based methods for bacteria detection, which generates an analytical signal upon capturing target cells. This can be done both in bulk via bioconjugates (cf. following section) or at the surface (**Figure 1**).

The transducer is an element of the sensor, which generates a measurable signal upon capturing target bacteria. The contact between the target analyte (here bacteria) and the surface is necessary for many analytical techniques, e.g., SERS, microbalance-based, magnetoelastic-based, or electrochemical methods. Phages immobilized at the surface must maintain their infectivity, binding affinity, and selectivity. Physical adsorption, covalent bonding, specific interactions, and entrapment in polymer matrices are the most commonly utilized techniques for immobilization. Physical adsorption is the

fastest and simplest way, but there is the risk of desorption and low surface coverage. Hence, the covalent bonding method is more frequently used for phage immobilization.

Richter et al. and Zhou et al. deposited phages on conductive support in a controlled approach. Experiments were carried out based on the surface charge and dipole moment of phage particles. The negatively charged head of the phage was attached to the modified support by applying an electric field, while the positively charged tail was aligned towards the bacteria. An alternating electric field combined with chemical surface modification was used to immobilize T4 phages on a gold surface, oriented with their tail towards *E. coli* bacteria. Chemical attachment of bacteriophage onto the biosensor surface considerably increases the overall detection's stability and performance [109, 121–123].

Recently the number of reports on electrochemical approaches for bacteria detection increases rapidly [79, 108, 123–128]. Electrochemical methods offer satisfying sensitivity, low-cost analysis, a vast field of possibilities for miniaturization. Moreover, the signal in the form of an electric current or voltage is easy to process. Here we present a couple of the latest reports on bacteriophage-based electrochemical methods for bacteria detections [129–131].

Sedki et al. described utilization of M13 phage immobilized on the electrodes combined with electrochemical impedance spectroscopy to target coliforms. The LOD of around 14 CFU/mL within 30 minutes [127]. This research presents a single phage balance high specific with a wide range of hosts - multiple strains of *E. coli* can be detected, while there is no response to non-*E. coli* bacteria. Niyomdecha et al. used M13 phage displaying *Salmonella*-specific peptide immobilized on the electrode and applied it into a capacitive flow injection system. Their sensor provided measurements with sensitivity ranging from $2x10^2$ – $1x10^7$ CFU/mL within 40 minutes. The sensor was reusable up to 40 times, thanks to the alkaline eluting solution [132]. Recently the review of available M13 phage-based biosensors was published [133].

Yue et al. reported a label-free electrochemiluminescent (ECL) biosensor capable of detecting *P. aeruginosa* with LOD of 56 CFU/mL within 30 minutes [134]. As the sensor, they used a glassy carbon electrode made of carboxyl graphene-PaP1 phage composite, and the luminol was a source of chemiluminescence.

Xu et al. detected viable bacteria by chemically immobilizing T4 bacteriophages on the surface of the extended gate connected to a metal oxide semiconductor field-effect transistor (MOSFET) device. The obtained LOD was 14 ± 3 CFU/mL with a wide range of detection ranging from 10^2 - 10^8 CFU/mL within 35 minutes.

Another approach is magnetoelastic sensors. They are usually ribbon-like strips of amorphous ferromagnetic alloys that vibrate under magnetic excitation. Mechanical vibrations generate secondary magnetic flux that can be detected remotely. The amplitude of these vibrations changes when the analyte is deposited on the sensing surface. The first sensor prepared according to this protocol, targeting methicillin-resistant *S. aureus* (MRSA) strain, reached the limit of detection of 3×10^3 CFU/mL within 30 minutes [135]. In 2017 the same research group confirmed their sensor was detecting MRSA strain

even in the presence of other competing bacteria [136]. Chen et al. [137] and Mack et al. [138] described the detection of *S. enterica* and *S. typhimurium* at the surface of chicken and lettuce, respectively.

Recently, Halkare et al. developed a new method for *E. coli* B40 bacteria label-free detection using T4 phages as biorecognition elements on a plasmonic fiber-optic platform. The novelty of this method relies upon capturing the analyte before subjecting the sensing layer to bacteriophages. Application of this method resulted in detection concentration of 10^3 -10^7 CFU/mL in environmental matrices within less than 4.5 hours with high specificity to only *E. coli* B40 [139].

Srivastava's group proposed to use bacteriophages as a sensing layer in SERS-based sensors. T4 phages were immobilized along with thin silver films on a silicon platform, reported limit of detection of *E. coli* was 1.5×10^2 CFU/mL [140]. Rippa et al. [141] immobilized bacteriophages on the surface of a substrate made of plasmonic nanocavities. The same group presented the meta structures functionalized with *Tbilisi* bacteriophages for *Brucella*'s SERS-based detection with the measurement time of 1 hour. With the sensitivity on the single-cell level, authors were able to detect bacteria in a suspension of concentration higher than 10^4 CFU/mL [142]. Lai et al. obtained a similar LOD by providing analysis using gamma phages targeting *Bacillus* spp., by using Principal Component Analysis (PCA) to process obtained SERS spectra [143].

Phage-based bioconjugates

In the case of detection of small molecules, even low concentrations translate to a reasonably large number of objects to be detected (e.g., for picomolar concentration, the number of molecules is around 10^8 per mL). The situation looks quite different for bacteria when the goal is to detect single cells in relatively large volumes (with the goal of 1 CFU/mL). Such a low concentration of bacteria causes a small number of detection events. The other problem is the relatively low probability of the attachment of bacteria to the sensing surface covered with immobilized phages and long search time.

Moving from the detection at the surface towards the bulk solves these issues. Bioconjugates offer shorter search time, more capturing events, and a broader range of analytical techniques for signal acquisition. The application of P9b phage conjugated with the gold nanoparticles to bind *P. aeruginosa* for SERS detection was reported in 2020 [144]. Another protocol proposes the usage of gold nanoparticles to prepare a colorimetric sensor. Gold nanoparticles solution changes its color during the aggregation. Peng and Chen used chemically and genetically modified M13 phages with exposed SH groups and displayed receptors against target bacteria (two strains of *E. coli*, *P. aeruginosa*, *Vibrio cholerae*, and two strains of the plant pathogen *Xanthomonas campestris*). M13 phages were added to the sample to bind the target bacteria, then centrifuged. The pellet was resuspended in a buffer containing gold nanoparticles (AuNPs), which were attached to SH groups at the surface of virions. The presence of phages in the pellet resulted in the change of color of gold nanoparticles solution and confirmed the presence of target bacteria in the sample. The detection limit reached around 10^2 cells (in 1 mL of the sample) within a 30 minutes procedure [145]. SiO_2@AuNP nanoparticles were also used to get

bacteriophages immobilized on their surface. Such conjugates were analyzed by darkfield microscopic detection. Conjugates attached to the target cells and resulted in aggregation and strong light scattering. The authors reported a detection limit of *S. aureus* of around 8×10^4 CFU/mL in up to 20 minutes [146].

Janczuk et al. proposed magnetic and fluorescent particles to create phage-based bioconjugates used as flow cytometry probes. For this purpose, T4 phage-based bioconjugates were used. Reported LOD of *E.coli* was in the range of 10^4 CFU/mL, and the incubation time was 15 minutes [147]. There were also attempts to use magnetic particles with immobilized bacteriophages for isolation and separation protocols [148], which can be combined with the detection of bacteria. Yan et al. combined bacteriophages deposited on the magnetic particles with immunoassay, targeting *S. aureus* in complex fluid (apple juice). This approach resulted in a LOD of around 9×10^3 CFU/mL within 90 minutes without any pre-enrichment [80].

Bhardwaj and coworkers used metal-organic frameworks (MOF) crystallites as phage carriers. They combined IRMOF-3 ($Zn_4O(NH_2\text{-}BDC)_3$) (BDC = benzene-1,4-dicarboxylic acid) with lytic bacteriophage as a fluorescence probe. Bacteria concealed MOF particles while binding, which caused the restriction of the excitation energy which reached MOF particles. This was observed as a loss in the fluorescence intensity directly correlated with an increasing number of bacteria. The reported LOD of *Staphylococcus arlettae* was around 10^2 CFU/mL [81]. The following research used NH_2-MIL-53(Fe), a substance similar to IRMOF-3. Similar to previous research, the fluorescence of the probe decreased with an increase in the bacteria concentration. The LOD of *S. aureus* was 31 CFU/mL, and the assay time was around 20 minutes [82].

Li et al. [126] proposed complex organic-inorganic particles supporting the cascade of three electrochemical reactions. $Cu_3(PO_4)_2$ nanoflowers were first loaded with glucose oxidase, horseradish peroxidase, and thionine. Afterward, gold nanoparticles were incorporated and T4 phages were bond *via* gold nanoparticles. Bacteria were immobilized at the surface of the electrode by antibodies or antimicrobial peptide magainin I. Loaded nanoflowers were binding to bacterial cells *via* phages. As glucose oxidase, horseradish peroxidase, and thionine appeared in the vicinity of the electrodes, the electrochemical reactions occurred, which eventually resulted in signal generation. Change in the generated signal was measured by differential pulse voltammetry. Achieved LOD was in the range of 1 CFU/mL within 140 minutes.

Farooq et al. developed a highly efficient sensing surface integrating bacterial cellulose and carboxylated multiwalled carbon nanotubes (c-MWCNTs) with immobilized phages targeting *S. aureus*. They achieved the LOD of 3 – 5 CFU/mL within 30 minutes, which is the best balance between LOD and time for now-on, and were able to distinguish living and dead bacterial cells [79].

Parts of virions as sensing elements

There are particular issues with whole-phage biosensors as sensing elements. First, the size of the virions marks the limits of miniaturization. For instance, magnetic particles conjugated to virions need to be in

a sub-micrometer scale for efficient magnetophoretic separation. Moreover, in many analytical techniques, such as surface plasmon resonance, binding needs to occur within the proper distance from the transducer. Analytical signals can be hindered due to the size of phages that create a distance that is too long to be examined. Finally, most bacteriophages ultimately cause lysis of the bacterial cells, making prolonged analysis almost impossible. The first bound bacterial cell may be already lysed before the end of the procedure.

These problems can be solved by using only parts of the virions that take part in bacteria capturing to prepare biosensors. He et al. used recombinant tail fiber protein (P069) expressed in *E. coli* to detect *P. aeruginosa* in two approaches. P069 protein was conjugated to magnetic beads. Then beads were added to target the bacteria in the sample. After the incubation, target bacteria were magnetically separated, washed, and the cells were lysed. Bacteria were detected indirectly, based on the concentration of released ATP, and evaluated by using the bioluminescence method. The second approach was based on P069 combined with the fluorescent marker, deposited onto the solid substrate. After capturing bacteria, fluorescently labeled P069 was added. The resulting LOD was 6.7×10^2 CFU/mL and 1.7×10^2 CFU/mL for bioluminescent and fluorescent methods, respectively, within 60 to 80 minutes [149].

Wang et al. presented bacteriophage cell-binding domain (CBD) fused with GFP for a broad-spectrum recognition of MRSA strain [150]. Target cells were separated by magnetic beads conjugated with CBD and incubated. The analysis was provided with flow cytometry protocol. The procedure resulted in a LOD of around 40 CFU/mL, and a time of analysis of about 1 hour. Gomez-Torres' research team used CBD-GFP protein and compared it with GFP-CTP1L - bacteriophage endolysin active against *Clostridium tyrobutyricum* [151]. The method was sensitive enough to detect 17 of 20 examined *Clostridium* strains, including the clostridial spores.

A report by Liu et al. described the usage of bovine serum albumin-templated Co_3O_4 magnetic nanozyme (Co_3O_4 MNE) conjugated to *S. aureus*-specific fusion-pVIII (Co_3O_4 MNE@fusion-pVIII) [152]. Target bacteria were detected with a limit of around 8 CFU/mL.

Recently, Cunha et al. developed a method that combined the sensitivity magnetoresistive sensors, the portability of a lab-on-chip platform, and the specificity of phage receptor binding proteins as probes for the rapid and multiplex detection. This method used protein *gp18*, a phage RBP, to detect *E. faecalis* I809 and protein *gp109* for detecting *S. aureus* Sa12 with LOD of 10 CFU/mL in less than 2 hours [153].

Braun et al. created an exciting approach for detecting pathogens by developing a single-tube centrifugation assay that simplifies the rapid analysis of suspect colonies. Two enzyme-linked phage RBP assay (ELPRA) were used to detect and identify vegetative cells of *B. anthracis*. Counting from the moment of collection of the colony, this assay can be completed within less than 30 minutes. The assay for now-on is rather qualitative than quantitative – allows to distinguish if *B. anthracis* spores were present in the sample [154].

Table 1. Summary of recent developments (focusing on reports from 2018 to 2021) in phage-based sensors for bacteria detection. This is an updated version of the table published in [15] and was adapted based on the Creative Common CC BY 4.0 license.

Bioreceptor	Bacteria	Method	LOD	Time	Comments	Ref.
Phages at the surface						
T4 phage	*Escherichia coli* B	differential pulse voltammetry	14 ± 5 CFU/mL	20 min	virions properly oriented in the alternating electric field on the micro-electrochemical sensor	[128]
lytic phage isolated from the hospital sewage water	*Staphylococcus aureus* CCTCC AB2013186	differential pulse voltammetry	3 CFU/mL in PBS 5 CFU/mL in milk	30 min	the best balance between LOD and time of analysis reported to date	[79]
M13 phage	*E. coli* XL1-Blue and K12 strains	electrochemical impedance spectroscopy	14 CFU/mL	30 min of incubation	virions chemisorbed on glassy carbon electrode decorated with gold nanoparticles	[127]
M13 displaying NRPDSAQFWLH HGGGSC	*Salmonella spp.*	the capacitive flow injection system	2×10^2 CFU/mL	40 min	reusable (up to 40 times) biosensors; virions immobilized on a polytyramine/gold surface	[132]
T4 phage	*E. coli* B	differential pulse voltammetry	14 ± 3 CFU/mL	35 min	T4 bacteriophages were chemically immobilized on the surface of the extended gate connected to a MOSFET device	[155]
T4 phage	*E. coli* B40	optical	$10^3 – 10^7$ CFU/mL	less than 4.5 h	plasmonic fiber-optic sensor could specifically detect target bacteria from a mixture	[139]
Bacteriophage based bioconjugates						
isolated from local bakery in India	*S. aureus*	fluorescence quenching	31 CFU/mL	20 min of incubation	NH_2-MIL-53(Fe) was used	[82]
T4 phage	*E. coli* ATCC 11303	differential pulse voltammetry	1 CFU/mL	140 min	$Cu_3(PO_4)_2$ nanoflowers loaded with glucose oxidase, horseradish peroxidase, thionine, and gold nanoparticles were used as the electrochemical signal amplification system	[126]

P9b displaying specific peptide (QRKLAAKLT)	*P. aeruginosa* ATCC 27853	SERS	NA	around 2h	gold nanoparticles were used	[144]
chemically modified and genetically engineered M13	*E. coli* (2 strains), *P. aeruginosa, Vibrio cholerae, Xanthomonas campestris* (2 strains)	colorimetric sensor	60 to 10^2 cell/mL	30 min	gold nanoparticles were used	[145]
S13` phage	*S. aureus* SA27	dark field microscopy	8×10^4 CFU/mL	15 – 20 min	virions were oriented according to charge driven assembly on the surface of core−shell nanoparticles	[146]
Genetically modified phages						
T7-ALP phage	*E. coli* BL21	fluorescence imaging and image analysis	around 10^2 bacteria per g	6 h	fluorescent substrate for alkaline phosphatase activity was added; detection in model beverage	[97]
NRGp6 (T7 with NanoLuc)	*E. coli* BL21	spectroscopic detection	5×10^2 CFU/mL	2 h of incubation	NanoGlo substrate was added	[99]
T7 with luciferase or an alkaline phosphatase fused with CBM	*E. coli*	visualization of colonies	1 CFU/100 mL	10 h	filtration based method; enzymatic substrate was added	[100]
T7 phage with NanoLuc-CBM	*E. coli*	luminescence of cellulose bound fused proteins	<10 CFU/mL	2.5 h	NanoGlo substrate was added	[101]
T7 phage with NanoLuc-CBM	*E. coli* ECOR13	luminescence	20 CFU/100 mL	5 h	NanoGlo substrate was added	[102]
dual modified M13KE phage	*E. coli* O157:H7 *Salmonella Typhimurium* *P. aeruginosa*	flow cytometry	10^2 cells/mL	1 h of incubation	the cocktail containing a mixture of three dual-modified phages allows multiplexed detection of their three target bacteria with a good linear dynamic range	[107]
T7 with a *phoA* gene	*E. coli*	colorimetry	10 CFU/mL coconut water 10^2 CFU/mL spinach leaves	5 h	Combination of bacteriophage-induced expression of an exogenous enzyme, alkaline phosphatase, and specific colorimetric substrate	[98]

A511::luxAB	*Listeria monocytogenes* *Listeria innocua* *Listeria ivanovii*	magnetic separation combined with fluorescence	around 10^2 cells/mL	6 h	magnetic beads with cell wall-binding domains from *Listeria* phage endolysins were used for magnetic separation	[156]
Phage amplification						
p53 phage	*Acinetobacter baumannii*	qPCR	10^2 CFU/mL in serum	4 h	15 various clinical isolates were studied	[110]
vB_SenS_PVP-SE2 phage	*Salmonella Enteritidis*	qPCR	8 CFU/25 g chicken	10 h		[112]
rV5 phage	*E. coli* O157:H7	qPCR, phages printed onto paper strips using modified inkjet	10 – 50 CFU/mL	8 h	in spinach and broth	[114]
AG2A phage	*E. coli* O45:H2				in ground beef	
CGG4-1 phage	*Salmonella* Newport				in chicken samples	
MS2 phage	*E. coli* C-3000	bead-based sandwich-type immunoassay	10^2 cells/mL	3 h		[115]
Detection of bacterial metabolites						
T7 phage	*E. coli* BL21	fluorescence	10 CFU/mL in spinach wash	8 h	resorufin β -D-galactopyranoside was added after lysis	[88]
Phage fragments						
pVIII protein	*S. aureus*	magnetophoretic chromatography combined with colorimetric readout due to activity of nanozyme	8 CFU/mL	NA	magnetic nanozyme Co_3O_4 MNE@fusion-pVIII	[152]
cell-binding domain (CBD)	methicillin-resistant *S. aureus* (6 strains)	flow cytometry	40 CFU/mL	Around 1 h (2x 30 min incubation + washing)	The CBD-GFP fusion protein was used, broad host recognition due to CBD; no lysis	[150]

bacteriophage endolysin CTP1L	*Clostridium tyrobutyricum* (17 strains)	fluorescence microscopy	3 spores per g of cheese	around 35 min + washing	GFP-CTP1L and GFP-CBD were used; also bind to clostridial spores	[151]
fiber protein (P069)	*P. aeruginosa* (4 strains)	bioluminescence	6.7×10^2 CFU/mL	around 60 min	two very different detection approaches. BL based on magnetic beads, FL on the interactions with modified surface	[149]
		fluorescence	1.7×10^2 CFU/mL	around 80 min		
*gp*18 protein (RBP) *gp*109 protein (RBP)	*Enterococcus faecalis* I809 *S. aureus* Sa12	differential pulse voltammetry	10 CFU/mL	Less than 2 h	sensitive magnetoresistive sensors, portable lab-on-chip platform, specific phage receptor binding proteins were used for and multiplex detection	[153]
two enzyme-linked phage RBP assays (ELPRA)	*Bacillus anthracis*	colony lift and blot ELPRA	NA	NA	developed a single-tube centrifugation assay simplifying the rapid analysis of suspect colonies	[154]
gp18 protein (RBP) gp109 protein (RBP)	*Enterococcus faecalis* I809 *S. aureus* Sa12	spectrofluorometry	1 – 5 CFU/mL	Less than 1.5 h after 15 h of enrichment	fusing RBP to fluorescent proteins and combining them with a spectrofluorometer	[157]
cell-binding domain (CBD)	*S. aureus*	flow cytometry	1 – 5 CFU/mL	16 h of enrichment	amidase (AMI), SH3 and amidase+SH3 (AMI_SH3) were cloned fused with GFP	[158]
Best performing phage-based methods reported before 2018 [74]						
Lambda phage	*E. coli*	amperometric	1 CFU/100 mL	6 – 8 h	detection of metabolites	[124]
P22 phage	*Salmonella*	colorimetric	1 CFU/24 mL	6 h	phagomagnetic separation of bacteria labeled with antibodies conjugated with horseradish peroxide	[159]
AR1 phage	*E. coli*	plaque count method	1 CFU/mL	3 h	phage amplification	[117]
PP01 phage	*E. coli*	fluorescence	1 CFU/mL	3 h	genetically modified phages	[106]
M13 phage	*E. coli*	amperometric	1 CFU/mL	3 h	detection of metabolites	[125]
HK620 phage P22 phage	*E. coli* *Salmonella*	flow cytometry	10 CFU/mL	1 h	genetically modified phages	[103]
T7 phage	*E. coli*	flow cytometry	10 CFU/mL	1 h	conjugates of biotinylated phages and streptavidin bound quantum dots	[160]

Phage display method

Another aspect of phage-based recognition is a method developed by George P. Smith in the 80s, known as *phage display* [161]. This method allows using phages as universal recognition elements (not only for bacteria detection) instead of antibodies. The usage of antibodies is relatively expensive because of their preparation, and very often, the specificity of these sensors isn't satisfying enough. G. P. Smith was the first one to obtain phages displaying specific peptides on the surface [162].

Filamentous phages (M13, f1, or fd) are usually used in phage display [163], with several examples of using icosahedral phages (e.g., T4 or T7). Several types of filamentous phage-based phage display can be distinguished. Their classification is based on the surface protein used, i.e., pIII or pVIII [164]. These proteins were chosen because of their location in the virion and presence in a couple of many copies. pIII protein is located in the distal part of the virion in the number of copies of 3 to 5, while pVIII is present in about 2 700 copies and is a major protein building viral capsid. Also, both of these proteins have an N-terminal signal sequence, so a foreign peptide sequence can be placed between the signal peptide and actual pIII/pVIII protein, forming the transcriptional fusion [164].

The purpose of the phage display is to obtain a library of bacteriophages expressing various peptides. A library is defined as a heterogeneous mixture of phage clones carrying different genetic inserts [162]. First, a surface needs to be covered with objects to be detected. Then, bacteriophages with inserted sequences of random oligopeptides or proteins are incubated with their target. Once the incubation is over, unbound/unspecific virions are washed off. At the same time, bound phages are eluted and amplified [164]. The scheme of this procedure is shown in **Figure 2**.

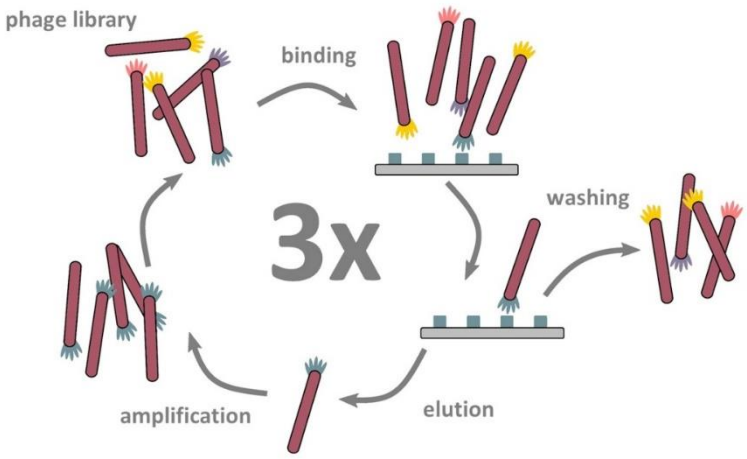

Figure 2. Schematic presentation of biopanning of phage display library. Adapted from the ref. [165] on the Creative Common CC BY 4.0 license.

This approach made a pathway to the alternative for using antibodies, so-called phage antibodies – phages with a domain of chosen oligopeptide or protein displayed. Because obtaining phage displaying molecule is a routine procedure, within a short time, it is possible to distribute a library of virions expressing numerous types of antibodies, making the research quicker and cheaper [166].

Phage display is also a solution for one of the greatest limitations of phage therapy – bacteria getting resistant to phage infections [167]. When treated with the same bacteriophage, bacteria strain would eventually become resistant to infections by this particular phage to improve fitness. A phage requires a virulence factor, which is a surface receptor, lipopolysaccharide, pili, or secretion system for a successful infection [168]. Phage display provides the selection of virions able to infect phage-resistant bacteria. Moreover, the correlation between phage and antibiotic sensitivity was observed. This phenomenon involves two strategies for fighting multi-drug resistant bacteria strains – directly killing them by bacteriophages or using bacteriophages to make bacteria antibiotic-sensitive again [73].

Phage display formerly designed for molecular biology, once improved became an application, e.g., allowed to find new antibody reagents for blood cell subpopulation discrimination. Targeted therapeutics and reagents for *in vivo* imaging are being developed. Among the first obtained antibodies against red blood antigens used for hemagglutination assays were anti-ABO, anti-Rh, and anti-Kell antibodies [169]. Antibodies against the cluster of differentiation (CD), AITP, GPIa, GPIII antigens, or 11-dehydro-thromboxane B2 cloning factor) were obtained. Other applications are diagnostic of immune diseases – antiTNFα or anti-CD52 antibodies, neurological disorders, tissue homing and anti-angiogenic strategies, and molecular imaging and tumor targeting. Tumour targeting agents were already developed for B-cell lymphoma, cervical, colon, gastric, breast, lung, glioblastoma, hepatic, prostate, neuroblastoma, and thyroid cancers [170].

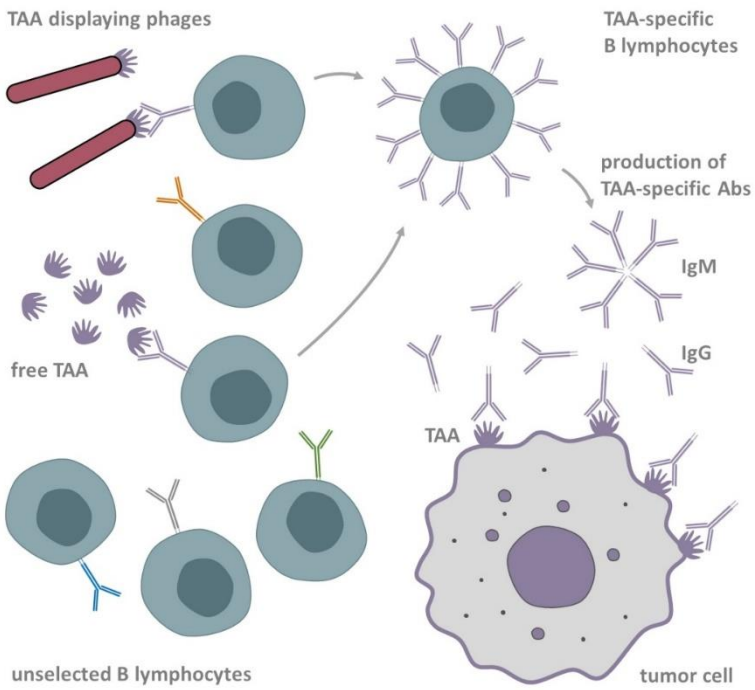

Figure 3. Illustration of humoral response to tumor cells initiated by using tumor-associated antigens (TAA) - displaying bacteriophages. Phages displaying TAA are considered anti-cancer vaccines. The figure is inspired by [171] based on the Creative Common CC BY 4.0 license.

Also, modified bacteriophages were used as the nanocarriers for tumor-associated antigens (TAAs) or TAA-mimic molecules. The importance of this application is that, in general, tumors produce immunosuppressing factors that inhibit the immunological response. TAAs and TAA-mimic molecules are presented to immune system agents, first by exposure to the MHC (major histocompatibility complex), then through CD4+ and CD8+ T lymphocytes to B lymphocytes to induct cytotoxic response *via* production of TAA-specific antibodies [171]. TAA-displaying phages are sometimes considered anti-cancer vaccines (**Figure 3**). It is also possible to modulate the activity of immune cells, CD11c/CD18 (integrin $\alpha_X\beta_2$) from the surface of APC (antigen-presenting cells) were fused with 12xhistidine tag and crosslinked to liposomes, creating the artificial tumor cells. By treating patients that way resulted in regression of primary cancer [171].

Phage-based detection of other analytes

Bacteriophages can also be used to detect analytes other than bacteria, e.g., ions [172] or organic compounds [173]. Kim et al. [174] showed the applications of bacteriophages for the detection of medical chemicals. Three variants of M13 phage were used: a wild-type virus and two displaying specific peptides changing the hydrophobic/hydrophilic balance of virions. A color pattern made an additional value for the determination of the response. By using this colorimetric method, it is also possible to detect volatile organic chemicals and endocrine-disrupting chemicals [173]. A review done by Armon and Kott summarizes the usage of bacteriophages as indicators of pollution [175]. Many bacteriophages have the survival time related to the survival time of human viruses. Therefore they might be used to estimate the index of viral pollution. Coliphages might also be used for the detection of airborne viruses and enteric viruses. Moreover, some phages are also indicators of water quality.

Summary

Critical parameters of sensors for bacteria detection are the time of analysis, the limit of detection (LOD), sensitivity, and specificity. The matter of cost, portability, and ease of use are crucial for the applicability of each solution.

The important milestone to be achieved in bacteria detection is to develop methods allowing for the LOD below 10 CFU/mL achieved in under one hour and in complex samples [15]. This is because 10 CFU/mL in the blood is a mark of sepsis in neonates [176]. In such cases, medical professionals are willing to wait only one hour before the administration of wide-spectrum antibiotics [177]. The following challenge will be to lower the detection limit to below 1 CFU/100 mL within a one-shift period (around 8 h). This is crucial for online analysis of drinking water [102]. The best-performing phage-based sensors were summarized in **Table 1**.

There is still no method bringing together a short time of analysis and low detection limit, even at the expense of costs. The conventional detection method depends on culturing and isolation of the target bacteria, followed by biochemical confirmation. Such an approach is straightforward, cheap, and

reliable but requires trained operators, laboratory space, and up to a few days to obtain reliable results. Thus, new detection techniques are being introduced. New molecular and instrumental techniques are advertised as extremely sensitive (e.g., PCR), label-free (e.g., SERS-based methods), or quick and easy to do (e.g., microscopic methods). But neither is universal, and each has some significant drawbacks, limiting its applicability. Some require expensive and sophisticated equipment, skilled personnel and are costly. Nucleic acid-based and MS are prone to give false-positive results in the case of dead bacteria. PCR is very sensitive to the proper design of preselected genetic probes to pair with target bacteria - mutants might escape appropriate identification. The availability of specific antigens limits immune-based tests. Therefore, methods based on biosensors gain recognition as a feasible alternative [66]. Among them, bacteriophage-based methods seem one of the most promising. However, the main challenge in front of researchers working in the field is to bring phage-based methods to the market. To our best knowledge, there is only one product already available - the Sample6 DETECT HT System (Microbiologique). It is not only the matter of LOD (1 CFU per 100 mL was reported already 18 years ago [124]) or time of analysis (LOD of 3 – 5 CFU/mL within 30 minutes was achieved in 2020 [79]). Scientists need to focus also on other factors, i.e., repeatability, stability, portability, ease of use, selectivity, price, and ease of shipping. These parameters are not necessary to produce scientific publications but are crucial to solve the socioeconomic problem of bacterial infections.

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