

Review

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Review

Aptamer-Based Biosensors for Bacterial Detection

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Abstract: The rapid and sensitive detection of pathogenic bacteria is becoming increasingly important for the timely prevention of contamination and treatment of infections. Biosensors based on nucleic acid aptamers integrated with optical, electrochemical, and mass-sensitive analytical techniques have garnered intense interest for their versatility, cost-efficiency, and ability to exhibit high affinity and specificity in binding bacterial biomarkers, toxins, and whole cells. This review emphasizes the development of aptamers, their structural characterization, and chemical modifications enabling optimized recognition properties and enhanced stability in complex biological matrices. Furthermore, recent examples of apta-sensors for the detection of bacterial cells, biomarkers, and toxins are discussed. Finally, we explore barriers and develop perspectives in applying aptamer-based bacterial detection.

Keywords: detection; biosensors; aptamers; bacterial pathogens; food security

1. Introduction

Pathogenic microorganisms have been a relentless challenge to human health and well-being throughout history. They encompass a wide range of bacteria, viruses, fungi, and parasites that can be transmitted through food, water, soil, and air or contact with infected individuals or animals. A survey for pathogens is one of the most important elements in tracing infectious diseases, determining the distribution of agents according to sites, and preventing their spreading. Among bacterial pathogens of particular concern are those resistant to antibacterial drugs commonly used to treat infections caused such as pathogenic strains designed by the acronym ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*). Multidrug-resistant bacterial strains cause the majority of nosocomial and community-acquired infections worldwide [1,2]. In addition, more than 200 identified bacterial pathogens are associated with foodborne illnesses that can occur through either foodborne infection (when ingested bacteria colonize and multiply in the human host) or foodborne intoxication (resulting from consuming food containing bacterial toxins). The most widespread foodborne and waterborne bacteria are *Escherichia coli* O157:H7, *Salmonella* spp., *Clostridium perfringens*, *Cronobacter sakazakii*, *Bacillus cereus*, *Campylobacter jejuni*, *S. aureus*, *Listeria monocytogenes*, *Shigella* spp., *Clostridium botulinum*, *Vibrio* spp. and *Yersinia enterocoli* [3,4]. Some foodborne bacteria, such as *L. monocytogenes*, pose a significant risk due to their high mortality rates, while others, such as *Salmonella* and *C. jejuni*, present high-frequency occurrence. In addition, some pathogenic bacteria are spore-forming (e.g. *Clostridium botulinum*, *Bacillus cereus*, *Clostridium perfringens*, *Clostridioides difficile*) [5–7]. In their sporulated form, bacteria are highly heat-resistant and survive most of the disinfection procedures used by the food industry or hospitals. Also, some bacterial toxins, like staphylococcal enterotoxin B (SEB) and cereulide are resistant to heat and may persist in food products for a long period. In 2019, it was estimated that 7.7 million deaths were caused by pathogenic bacteria [8].

Bacterial detection holds practical importance in the pharmaceutical industry, environmental control, and food safety. Foodborne infections and intoxication resulting from contaminated beverages or food are considered serious problems and have become more prevalent nowadays. In

2015, the World Health Organization (WHO) estimated that there were 600 million cases of foodborne illnesses, representing 11.6% of the world population and resulting in no fewer than 420,000 deaths. However, it is important to note that these data probably do not reflect the full scale of the problem. For instance, foodborne microorganisms can induce severe symptoms, such as putrefactive diarrhea, vomiting, abdominal cramps or fever and cause also debilitating diseases like meningitis in young children [9,10]. In the interest of public health and consumer protection, it is evident that there is a need to concentrate efforts on developing new methods for production control, including new detection techniques.

To reduce risks and control disease progression, diverse methods have been developed for pathogen detection including culture methods and molecular methods such as those based on nucleic acid Amplification (e.g. Polymerase Chain Reaction (PCR)) [11–13], and Enzyme-Linked ImmunoSorbent Assay (ELISA) [14]. Although they are the most frequently used methods for pathogen detection, they show many disadvantages to enable efficient health security and sanitary control. Methods based on cultivation are time-consuming, as they require several steps for bacterial enrichment, isolation, and identification. In addition, they can be performed only by qualified personnel and within a laboratory setting. Molecular methods are less time-consuming, but they also require a pre-enrichment step to increase the number of target bacterial cells and to prevent detection of dead bacteria. In addition, molecular methods can give false negative results because they rely on enzymes (DNA-polymerase) or antibodies that can be inhibited by some molecules or ions in food or biological samples [15].

The development and improvement of new diagnostic methods play a pivotal role in managing and mitigating the risks associated with pathogens. Fortunately, many new effective analytical techniques for rapid and sensitive detection of bacterial pathogens directly in complex matrices are under development. In 2003, the WHO established some criteria to ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment-free, Delivered) an access to high-quality diagnostic devices that can be used across all levels of the healthcare system. Recently, two additional criteria, RE (Real-time and ease of specimen collection and environmental friendliness) were added creating the new acronym of REASSURED devices [16]. Biosensors as portable tools providing rapid, sensitive, and efficient detection of pathogens can reach the REASSURED criteria. Biosensors are analytical devices that integrate a bioreceptor, which assures the recognition of the target, to a transducer that converts the recognition event in a measurable signal enabling the detection of chemical or biological analytes [17]. Among recognition elements, aptamers, small artificial single-stranded DNA or RNA molecules (usually 20-100 nucleotides long) have emerged as specific sensing elements.

Aptamers have usually no biological function but exhibit a high affinity for a specific target, similar to the natural interactions observed between antigens and antibodies. Aptamers possess several advantages over expensive antibodies and stand out as strong candidates to replace them. They possess the ability to selectively recognize the desired target with high affinity and specificity, and they can be easily customized to accommodate changes [18–20]. Due to such properties, aptamers have gathered signification attention in recent years for their potential to revolutionize various fields, including diagnostics, therapeutics, as illustrated in Figure 1. The utilization of aptamers as recognition elements in biosensors offers several advantages for bacterial detection [21]. The primary advantages of aptamers include their bio- an chemical stability in complex matrices, ease of production due to their small size, and their low immunogenicity [10,22,23]. In addition, these small nucleic acid molecules has a relatively low cost of synthesis compared to antibodies [24]. Nonetheless, their binding affinity is comparable to that of the monoclonal antibodies. Moreover, various chemical modifications of aptamers can enhance both their stability and affinity to bind a specific target [25,26]. Recent years have seen an exponential increase in the development of aptamer-based systems for the monitoring, detection, and identification of pathogenic microorganisms. Figure 2 shows articles published for “aptamers” and “aptamers for bacteria”, along with future trends according to their publication rate in the last twenty years.

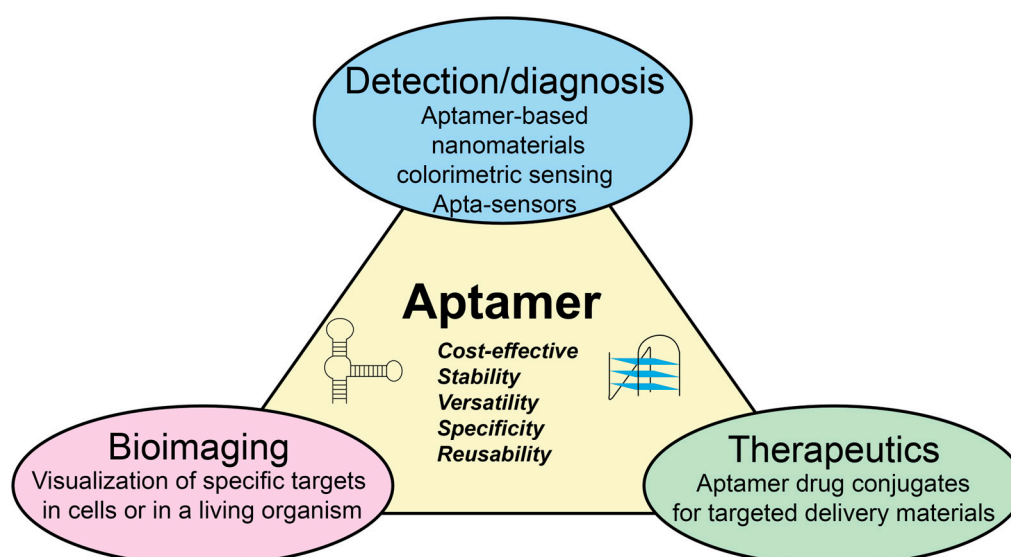


Figure 1. Major aptamer characteristics and applications.

In this review, we present the latest advancements in aptamers for bacterial detection. First, we provide a short overview of the strategies for selecting aptamers targeting pathogenic bacteria. Second, we present their kinetics, thermodynamics, and structural characterization. Third, we focus on recent progress (2020–2023) and strategies utilizing aptamers for the detection of bacterial cells, biomarkers, and toxins. Alongside aptasensors for bacterial screening in food and clinical samples, we also highlight some aptamers developed for fundamental microbiological studies. Furthermore, we discuss the principal aspect of aptamers' optimization. This involves a discussion of methodologies and techniques focusing on both kinetic and structural characterization, as well as chemical modifications ultimately enhancing the overall efficiency and functionality of aptamers for improved bacterial detection. It is noteworthy that some aspects of aptamer applications for bacterial detection, namely aptamer identification, optical and electrochemical transducer technologies used in aptasensors, the nanomaterials for aptasensors, and aptamer selection, have been comprehensively described in some recent reviews [27–32]. Finally, barriers and developing perspectives in the application of aptamer-based bacterial detection are analyzed.

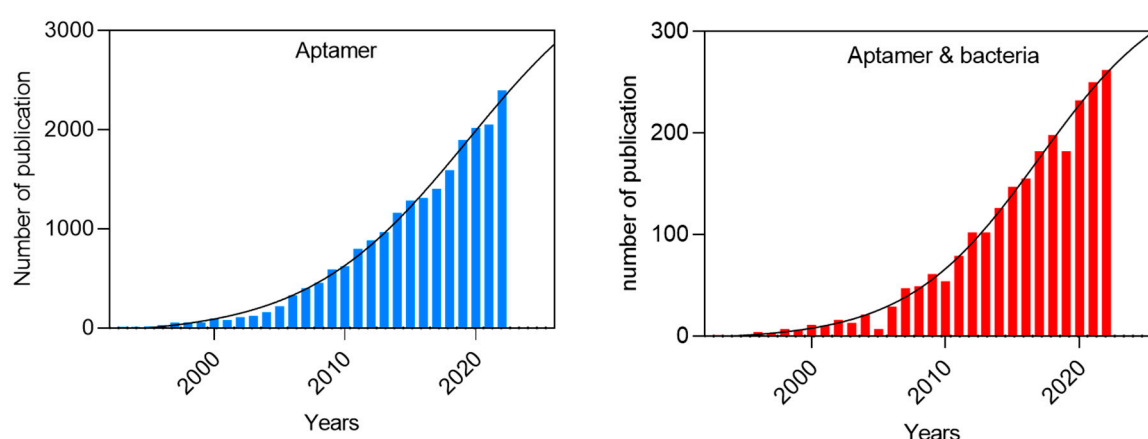


Figure 2. Aptamer development and their application in bacteriology in the literature in the period of 1990–2021. Values were obtained by searching “aptamer” and “aptamer & bacteria” keywords in Scopus. Trends obtained by fitting a tendency curve and projecting it for the next 4 years.

2. Aptamer Selection Strategies for Bacterial Pathogen Detection

Aptamers, first developed in the early 1990s by two independent teams, Ellington and Szostak [33] and Tuerk and Gold [34], refer to distinct nucleic acid sequences with selective and specific binding to a target. These sequences are selected from a large pool of nucleic acids, using an *in vitro* selection technique known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [35,36]. SELEX is a series of selection and amplification steps in which a large pool of nucleic acid molecules binds to desired target molecules under specific conditions, including temperature and salt concentration. The molecules that bind to the target are isolated from those that remain unbound, and then they are amplified to generate a newly enriched population. This cycle is repeated until a small population of highly specific molecules is obtained. The SELEX technique has undergone numerous modifications and improvements over time, making it more efficient, reliable, and affordable for the identification of aptamers [37,38]. Conventional SELEX methods have been extensively reviewed [39,40].

In the case of bacterial pathogen detection using aptamers, it can be achieved indirectly, where aptamers are designed against secretory molecules like toxins. A direct approach employs aptamers selected against either a specific molecule of bacteria or the whole-cell bacteria.

Indirect detection of bacteria is important for food, medical, and environmental safety. Indeed, some bacterial toxins can survive the pasteurization process and have a high degree of resistance to high temperatures (<100 °C), making them indicative of the past or current presence of bacteria. For instance, SELEX methods against toxins have been implemented, and aptamers have been discovered to identify toxins of *S. aureus*, *Vibrio cholerae*, and *C. botulinum*, named enterotoxins, cholera toxin, and botulinum toxins, respectively [41–46].

Direct detection of bacteria using aptamer selected against surface epitopes such as membrane and cell wall proteins or peptidoglycan has been developed. For instance, RNA aptamers have been reported to target *S. aureus* teichoic acid [47], a component of the cell wall of the majority of Gram-positive bacteria. Additionally, peptidoglycan-specific aptamers for the detection have also been developed [48]. More recently, aptamers that specifically target *isdA*, a crucial surface protein for *S. aureus* survival and colonization, have been successfully generated. Over time, this protein is gradually becoming a key marker for the detection of this bacterium [49,50].

Aptamers can also be selected to directly detect a specific bacterium by using the whole cells or spores as a target. This approach is termed cell-based aptamer selection or Cell-SELEX [51,52]. It was first introduced in 1998 using Human red blood cells by Morris *et al.* to generate aptamers capable of selectively binding to unidentified molecular biomarkers present on the surface of live cells [53].

Bacterial cell surfaces present a multitude of molecules, particularly proteins, which can serve as potential targets for aptamers. Many aptamers that selectively bind Gram-negative and Gram-positive bacteria such as *E. coli* [54,55], *C. jejuni* [56,57], *Streptococcus pyogenes* [58], *S. aureus* [59–62], *B. cereus* [63], *Salmonella Typhimurium* [64], or *Listeria* spp.[65], can be found in the literature. The Cell-SELEX was shown to be effective in generating specific aptamers against a particular bacterium or even a particular clone. Similarly, bacterial spores were used to develop specific aptamers through the Cell-SELEX procedure. In its sporulated form bacteria exhibit higher resistance to various stress factors such as UV radiation and heat compared to the corresponding growing cells [66,67]. The conventional microbiological methods to detect bacterial spores have lengthier procedures compared to those for vegetative cell detection. This is because spores are first subjected to germination, and then their vegetative cells are characterized. Therefore, conventional detection methods such as PCR or immunological assays, are not adapted to fully ensure safety. In contrast, aptamers that bind to the surface of bacterial spores offer a potential for rapid and direct detection. Particularly, aptamers have been developed and used in different aptasensors for the detection of spores from *Bacillus* species, which are ubiquitously present in air, water, and soil [68–70].

The procedure of Cell-SELEX does not require any purification or prior knowledge of a specific targeted surface epitope. Even more significant is that proteins on the cell surface represent a more physiological and natural folding condition than purified proteins. However, the Cell-SELEX might be difficult to perform, because the bacteria cell surface is negatively charged, leading to DNA

repulsion from the cell surface [71]. Additionally, the process can introduce selection biases. For instance, dead cells in a suspension can result in a non-specific binding, strongly influencing the selection. Another challenge lies in identifying the specific target(s) of the selected aptamers. Much of the literature using Cell-SELEX lacks a comprehensive understanding of the targets.

Alternative *in silico* strategies have been developed in the last decades. These methods are based on a computational screening method of a large library of aptamer sequences to fit modeled target structures. These techniques require 3D structure predictions, through docking methodologies using software such as HADDOCK, AutoDock, Xdock, enabling the study of aptamer folding and selection of structural patterns responsible for the aptamer/target interaction based on predicted affinity and Gibbs free energy (ΔG) [72–74]. The *in silico* method has been successfully conducted to select aptamers for bacterial surface proteins [75–77]. Another example is a recently employed computational screening method to select antimicrobial aptamers against the pathogen *Pseudomonas aeruginosa* [78]. *In silico* strategy is a powerful and complementary tool to SELEX, for developing new aptamers against pathogenic bacteria. As a computational-based approach, it offers timesaving and cost-effectiveness compared to SELEX. Nevertheless, *in silico* methods still require knowledge of aptamers' tridimensional (3D) structure and *in vitro* testing to confirm the binding interaction between selected aptamers and the targets.

3. Aptamer characterization: affinity, stability, and structure

Aptamers are typically short, single-stranded nucleic acids, either DNA or RNA molecules, that can fold into various 3D structures. Nucleic acids are chemically and structurally stable. Both share similar functionalities and have their advantages: RNA aptamers offer more diverse and complex 3D structures that can enhance binding specificity, and DNA is known for its stability and lower production costs [19]. They can recognize and bind their target with high specificity and affinity in the nano- and picomolar range. The structural conformation of an aptamer depends not only on its base composition but also on its environment including pH and salt type, ionic force. Those parameters have a significant influence on the formation and stability of specific conformations of aptamers, they are particularly true for intercalated motif (I-motif) or G-quadruplex structures [79–82].

The initial pool of nucleic acids used in SELEX contains a random nucleotide region flanked by two constant regions, the primers, that serve for the amplification in the SELEX process to enrich the sequences that bind to the target molecule. The primers are usually considered nonessential nucleotides and are not involved in the folding process, nor are they used for recognizing the target [83]. One study tried to explore the influence of the random-region length on aptamers, but they found no significant correlation between length and aptamer affinity [84]. However, a longer random region enhances the complexity of secondary and tertiary structures, while a shorter one provides better coverage and gives production advantages. [85]. One or a few sequences are identified at the end of the SELEX process. It is then necessary to determine its kinetics parameters, such as binding affinity (K_d).

Different methods have been used to evaluate the K_d , relying on either label-based and label-free techniques [86]. The label-based approaches, fluorescent or radioactive labels, lower the detection limit in the experiments, resulting in a more accurate determination of the K_d . Label-free methods, such as surface-based detection methods, are often used. One of the most commonly used methods is Surface plasmon resonance (SPR), an optical method to detect molecular binding interactions between one mobile and one fixed molecule. SPR is used for evaluating different kinetic parameters, such as the association and dissociation rate constant and the dissociation/association constant [87].

Those traditional methods are adapted to evaluate the binding affinity for an aptamer when the target is known, such as proteins or toxins that can be isolated or produced in a purified form. However, in the cell-based SELEX method, the target(s) for the aptamer is unknown. This, in turn, complicates the measurement of kinetics parameters because the number of targets and their concentration are unknown. This uncertainty makes it challenging to measure binding affinity accurately. On top of that, working with whole cells is technically challenging and requires complex

protocols in terms of detection sensitivity and apparatus systems, such as microfluidics. Several studies have estimated the K_d using Fluorescence-Activated Cell Sorting (FACS) methods [49,88–90]. FACS offers the advantage of comparing different aptamer candidates for a whole cell biomarker. Although assessing the kinetics parameters is essential, the aptamer's stability plays a crucial role in determining its overall suitability. Comprehensive descriptions of aptamers, including details on thermal stability, topology, and structure, are essential for their effective utilization in various applications and for ensuring their effectiveness in real-life scenarios.

The stability of nucleic acid structures can be evaluated through thermal denaturation experiments using either UV-CD absorption techniques or fluorescence techniques such as FRET [91–93]. Isothermal titration calorimetry (ITC) is used to derive thermodynamic parameters and stoichiometry of the aptamer/target interactions. Several factors can influence the melting temperature of an aptamer, affecting its stability, such as the base composition, length, structures, ionic strength, and pH [94–98]. Moreover, the potential conformational change of aptamers upon binding with their targets are rarely explored and deserve better investigations, [99]. Resolving the high-resolution or even the topology of an aptamer could lead to a better understanding of target specificity and pave the way for further optimization of stability and specificity. It was shown that aptamers could adopt various tertiary structures that can be classified into three categories: Structures containing duplexes, loops, and bulges (e.g. Hairpins, pseudoknots, triplex), G-quadruplexes, and I-motif (Figure 3). The aptamer structure may include loops and bulge regions (Figure 3). They play a crucial role in their structure and function, influencing their binding affinity and specificity [100–102].

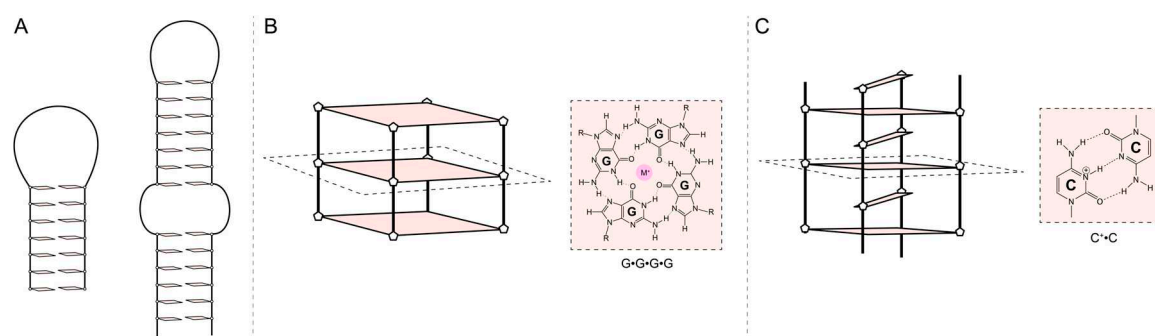


Figure 3. Schematic of non-canonical aptamer structures: hairpins and stem-loop (A), G-quadruplex (B), and I-motif (C).

Structures containing a duplex stem with different loops and bulges are very diverse (loop length number and composition can vary), ranging from simple hairpins to pseudoknots (Figure 3A), all resulting in a spectrum of distinct conformations that can be used to bind a target selectively. G-quadruplexes are four-stranded non-canonical nucleic acid structures that can form under physiological conditions [103]. G-quadruplex structures are stacked G-tetrads formed by four hydrogen-bonded guanines stabilized by cations (Figure 3B). G-quadruplex structures are diverse in terms of strand orientations, loop length, and composition and have high structural variability, including interesting hybrid duplex-quadruplex structures [104]. They have shown promise in the bacterial detection [105]. The intercalated motif (I-motif) is a structure involving a hemiprotonated C-C⁺ base pair stabilized under acidic pH conditions [106] (Figure 3C). To our knowledge, no aptamer has been shown to form an I-motif, and none have been developed in the bacterial detection context. This may be explained by the lack of structural studies on the aptamers field, although several aptamers have C-rich sequences [61]. Aptamers forming an I-motif for bacterial detection are an area of potential future research. Prediction software has been overwhelmingly used to predict the secondary structure of an aptamer. However, those predictions consider simple rules of base pairing and free energy and fail to predict other secondary structures such as G-quadruplex or I-motif [107–109].

To have insight into the structure adopted by an aptamer, experimental data are required. Different experimental methods can be used to gain insight into the folding of aptamers. Shape-type experiments, which chemically probe unpaired or paired base pairs are used to determine the secondary structure of nucleic acids. These experiments are convenient for long sequences. Spectroscopic methods such as UV, circular dichroism, or fluorescence can be used to obtain information on the folding topology of nucleic acid [110]. For example, CD is widely used for G-quadruplex or I-motif to gain insights into the G-quadruplex topology [111]. Those relatively easy methods can be used to look at the dependence of the secondary structure, such as the ion type and concentration, pH, and buffer composition. This is particularly true for I-motif [112,113], and G-quadruplexes [114,115]. This information is crucial for establishing a protocol that ensures the correct folding and the monitoring of their stability over time. Three main experimental techniques have been used to resolve the structure of aptamers free or in complex with their target: Nuclear Magnetic Resonance (NMR), X-ray crystallography, and Cryogenic electron microscopy (cryo-EM). All the methods can give rise to high-resolution structures (see [116] for a comprehensive analysis of aptamers structure). NMR uniquely investigates nucleic acid conformational states and folding through simple 1D experiments. It monitors structures like I-motifs, G-quadruplexes, or hairpins, specifically through the imino-proton chemical shift [117]. Those methods provide detailed information and are becoming crucial for understanding the aptamer-binding mode. One notable example is an RNA aptamer's three-dimensional resolution that can recognize the bacterial protein Hfq of *Bacillus subtilis* [118]. Recently, there has been a growing interest in solving these aptamers' complex structures [116,119,120].

Aptamers' chemical modifications and optimizations are increasingly explored, serving as a crucial strategy to enhance binding affinity, thermal stability, and protection against nucleases. These properties directly influence the overall suitability of aptamers for biosensing applications. One significant disadvantage of aptamers as oligonucleotides is their high sensitivity to nuclease degradation, which affects detection in biological samples, especially in serum [121]. Temperature fluctuations can also impact aptamer binding capacity and susceptibility to enzymatic degradation by nucleases. To overcome these limitations, various chemical strategies have been devised to thermally stabilize, protect against degradation, and enhance the binding affinity of the active conformation of an aptamer.

Chemical modifications to the base, phosphate group, or sugar unit have been used to enhance the aptamer suitability [122–124]. For instance, incorporation of non-natural phosphorothioate into the aptamer backbone structure or modification of the 5'-end or sugar structure of strands were shown to slow down the hydrolysis rate by nucleases present in biological fluids while retaining their activity [125–128]. Another approach to avoiding the problems related to aptamer degradation is by incorporation of unnatural base pairs (such as 5-methyl-isocytidine) or utilization of L-DNA to make strands highly resistant to nuclease attacks [129]. The majority of such chemical modifications aim to improve the stability of nucleic acid structures that are highly vulnerable to nuclease digestion, particularly in single-stranded segments and internal nicks.

Aptamer can acquire a relative nuclease resistance when covalently linked to a protective group such as protein. For instance, DNA-type thrombin binding aptamer was shown to resist a variety of serum nucleases when it was tightly bound to its target protein [130]. Moreover, different protective coatings of DNA strands increase their nuclease resistance. Lacroix et al increased stability of the DNA strand by its coating with human serum albumin and DNA dendrites [131]. In another study, an oligolysine-PEG (polyethylene glycol) coating was shown to protect DNA structure from low-salt denaturation and nuclease degradation [132]. The stability can also be improved by ligation of strand termini or by the introduction of terminal functional groups, such as hexanediol or hexaethylene glycol, crosslinking of nucleic strands by click chemistry or through the formation of a thymidine dimer using ultraviolet irradiation [133,134]. These chemical modifications eliminate internal nicks once they are constructed.

In a different approach, El-Khoury and Damha used a chemical end-ligation to stabilize an intramolecular I-motif at both acidic and neutral pH using deoxy-2-fluoroarabinocytidine [135]. This method allows for the use of I-motif on a large scale for in vitro applications such as detection.

Finally, the effect of nucleases on the aptamer structure can also be minimized by applying conditions to denature nucleases or by using nuclease inhibitors. For instance, the detection in serum can be performed after serum heat pretreatment at 75°C for 10 min, thus rendering nucleases inactive [136].

4. Bacterial aptasensors

To advance the development of diagnostic tests to detect pathogenic bacteria, significant focus has been directed toward biosensors paired with aptamers, named aptasensors. They are devices that incorporate one or several aptamers and a signal transducer that can be electrochemical, optical, or mass-sensitive. Infectious bacterial agents differ in numerous aspects, comprising their virulence factors, contagion, and transmission. All these factors and molecules playing a role in the bacterial life cycle can be targeted by aptamers to enable specific and efficient bacterial detection and identification using aptasensors. Many innovative aptasensors have been developed recently and can be divided into aptasensors targeting whole bacterial cells, bacterial toxins, and specific biomarkers. Table 1 summarizes recently reported aptamer-based biosensors with various detection methods and their characteristics. It illustrates the remarkable performances of aptasensors for bacterial detection.

Table 1. Aptamer-based biosensors for bacterial detection. Aptamer sequences are provided in Supplementary data.

Bacterium	Aptamer	DNA or RNA	Target	K _d (nM)	Detection method	LOD	Linear range (CFU/mL)	Ref
<i>S. aureus</i>	SA20 SA23 SA34 SA31 SA43	DNA	Whole cell	70.86±39.22 61.50±22.43 72.42±35.23 82.86±33.20 210.7±135.9	Fluorescence	/	/	[59]
<i>S. aureus</i>	T1 T2 T3 A14	RNA & DNA	IsdA	2.2 ± 0.5 1.0 ± 0.3 0.7 ± 0.4 4 ± 2	FRET-switch	113 pM 17 pM 11 pM 485 pM	/	[50]
<i>S. aureus</i>	H1	DNA	/	/	Piezoelectric sensor	41 CFU/mL	4.1 × 10 ¹ to 4.1 × 10 ⁵	[137]
<i>S. aureus</i>	H1 H2 cApt	DNA	/	/	Fluorescence	10 ¹ CFU/mL	10 ² -10 ⁶	[138]
<i>S. aureus</i>	Apt1 Apt2	DNA	Whole cell	35 129	Colorimetric	7,5 – 8,4 * 10 ⁴ CFU/mL	10 ⁴ - 10 ⁸	[139,140]
<i>S. aureus</i>	SH-Apt ₂	DNA	Whole cell	210.7	Surface-enhanced Raman scattering (SERS)	/	/	[55,141]
<i>S. aureus</i>	APT ^{seb1}	DNA	Staphylococcal Enterotoxin B (SEB)	/	SDS-Page	/	/	[142]
<i>S. aureus</i>	G1 #2 #18	RNA	Teichoic acid	/	SDS-Page	/	/	[47]
<i>S. aureus</i>	AT-27 AT-33 AT-36 AT-49	DNA	α-toxin	/	Neutralization	/	/	[143]
<i>S. aureus</i>	H1 H2	DNA	Whole cell	/	Fluorescence	4-8 CFU/mL	45–4.5 × 10 ⁶	[144]

<i>S. aureus</i>	Antibac1&2	DNA	Peptidoglycan	415 + 0.047 1261 + 0.280	Photoelectrochemical sensor	82 pg/mL	/	[48,145]
<i>E. coli</i>	SH-Apt ₁	DNA	Whole cell	25,2	Surface-enhanced Raman scattering (SERS)	/	/	[55,123]
<i>E.coli</i>	/	DNA	Whole cell	/	Optical	45 CFU/mL	10 ² -10e ⁸	[146]
<i>E. coli</i>	GN6 GN12	DNA	Outer membrane vesicles (OMV)	29,94 20.36 59.70 24.80 38,98 53.83	Enzyme-linked aptamer assay (ELAA)	/	/	[147]
<i>E. coli</i>	6-3 8-1 8-7 8-8 8-12 8-13 8-19 8-35	RNA	Heme	188 309 256 371 445 425	Functional assay	/	/	[148]
<i>E. coli</i>	ECA I ECA II	DNA	Outer membrane proteins (OMP)	/	Electrochemistry	/	1*10 ⁻⁷ –2*10 ⁻⁶ M	[149,150]
<i>E. coli</i>	/	DNA	Whole cell	/	Plasmonic sensor	0.05 CFU/mL	0.1-10 ⁴	[151]
<i>E. coli</i>	Stx1 stx2	DNA	Shiga toxin Viz, stx1 & stx2	47 pM 29 pM	/	44,5 pg/ml 41,3 pg/mg	50 pg/ml – 100 ng/mg	[152]
<i>P. aeruginosa</i>	F23	DNA	Whole cell	17.27 ± 5.00	colorimetric	10 ⁴ CFU/mL	/	[153]
<i>B. cereus</i>	/	DNA	Whole cell	/	Fluorescence	22 CFU/mL	49-49*10 ⁶	[154]
<i>B. cereus</i>	/	DNA	Whole Cell	/	Fluorescence	4 CFU/mL	20-2*10 ⁸	[155]
<i>B. cereus</i>	B15 B16	DNA	Whole cell	16,13 20.67	Electrochemical sensor	10 CFU/mL	/	[89]
<i>B. cereus</i>	13-18 13-24	DNA	Whole cell	22.75 36.72	Microfluidic chip	9.27 CFU/mL	/	[156]
<i>Acinetobacter baumannii</i>	AB K2	DNA	Whole cell	5.377 6.8	SERS	10 CFU/mL	10–10 ⁵	[157]

<i>Acinetobacter baumannii</i>	/	DNA	Whole cell	/	Electrochemical sensor	150 CFU/mL	1*10 ³ –1.0*10 ⁸	[158]
<i>Klebsiella pneumoniae</i>	K2	DNA	Whole cell	/	SERS	10 CFU/mL	10–10 ⁵	[157]
<i>Leptospira interrogans</i>	LAP3	DNA	Outer Membrane protein	133,13	Colorimetric	57 CFU/mL	60–6*10 ⁵	[159]
<i>Salmonella</i>	Multi-apt	DNA	Multi	11.72	Fluorescence	7 CFU/mL	10-10 ⁷	[160]
<i>Yersinia enterocolitica</i>	/	DNA	Whole cell	/	Fluorescence	3 CFU/mL	10–10 ⁹	[161]
<i>Bacillus cytotoxicus</i>	BAS6R	DNA	Spore	/	Colorimetric	10 ² – 10 ⁴ CFU/mL	10 ³ - 10 ⁴	[29]
<i>Vibrio cholerae</i>	CT916		Cholera toxin (CT)	48,5	ELAA	2,1 – 2,4 ng/ml	0-10 ng/mL	[43]
<i>Clostridium difficile</i>	No name	DNA G-quadruplex	Toxin A (TOA)		Electrochemistry	1 nM	0-200 ng/mL	[162]
<i>S. typhimurium</i>	H2	DNA	Whole cell	/	Fluorescence	4-8 CFU/mL	36–3.6 × 10 ⁶	[144]
<i>S. Typhimurium</i>	Apt S.T	DNA	Whole cell	10	Fluorescence	30 CFU/mL	10 ² -10 ⁶	[163]
<i>Vibrio parahaemolyticus</i>	Apt VP	DNA	Whole cell	16,88	Fluorescence	10 CFU/mL	10 ² -10 ⁶	[163]

4.1. Detection of bacterial cell

There are significant practical advantages in developing aptasensors for detection of whole bacterial cells. The diversity of biomarkers and their abundance on a cell surface provide the potential for higher sensitivity than targeting a single protein expressed at a low level. In addition, targeting whole cells facilitates the overall detection procedure since the sample preparation prior to analysis is highly simplified [164]. In most traditional and molecular methods for bacterial detection, sample preparation is the most expensive and the most time-consuming step. Finally, the selection of specific aptamers is also facilitated because there is no need for the lengthy process of purifying a specific target biomarker [165]. However, bacterial surfaces can vary in structure and composition depending on the environmental condition. This bacterial adaptation to external conditions may modify their surface molecule composition, which influences the reliability of diagnostic tests. This problem can be overcome by the utilization of a mixture of aptamers that target different surface epitopes of the target bacterium [166,167].

One recent development of bacterial electrochemical aptasensor was designed for selective and ultrasensitive detection of *S. aureus* at the single-cell level using a sandwich assay [168]. The aptasensor relied on dual recognition through a bacteria-imprinted polymer film (BIF) and an aptamer (Figure 4). The BIF, which served as a capture probe, was fabricated on a glass carbon electrode, whereas the aptamer, which served as a detection probe, was functionalized with 6-Ferrocenyl-hexanediol on gold nanoparticles (AuNPs). Aptamer was produced with a thiol on its 5' to allow its covalent binding to AuNPs. After anchoring *S. aureus* on the BIF-modified electrode, AuNPs-aptamers were introduced to bind the captured bacteria, leading to an amplified current signal. Obtained ultrasensitive detection enabled sensing of a single *S. aureus* cell in a buffer. The limit of detection (LoD) of the aptasensor was 10 CFU/mL when used in complex, lipid-rich media like milk. This high sensitivity suggests the sensor's applicability in food safety and prevention.

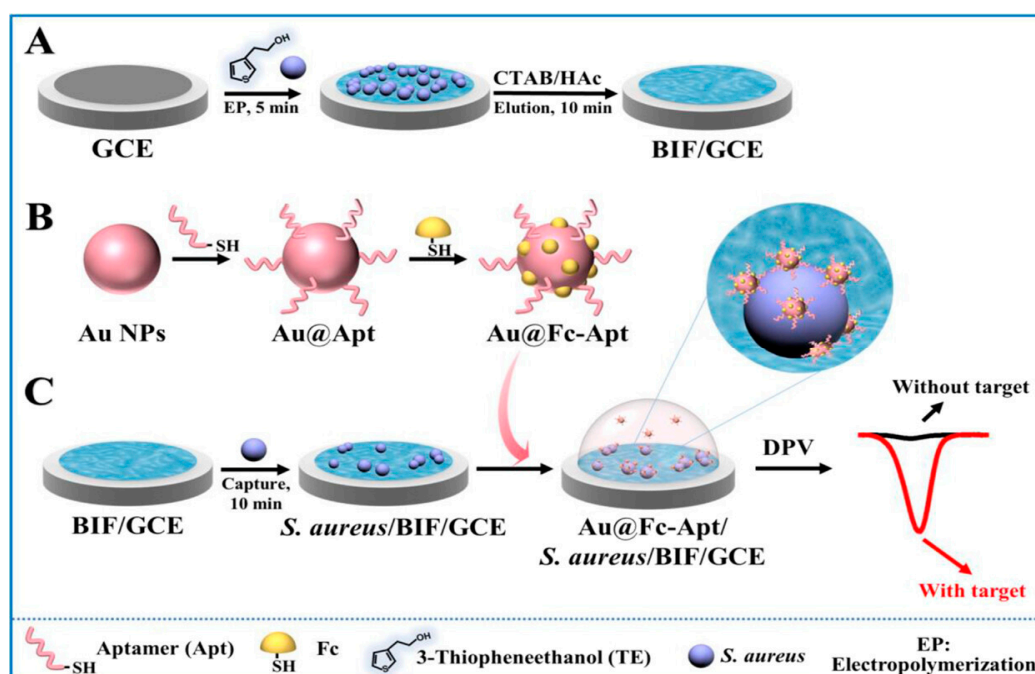


Figure 4. Schematic illustration presenting (A) Preparation of the BIF-modified glassy carbon electrode and (B) Preparation of aptamer carrying signal nanoprobe; and (C) Dual BIF-aptamer based electrochemical sandwich detection of *S. aureus* cells. Reprinted with permission from [168]. Copyright 2023. American Chemical Society.

In another recent example, Ding et al. [169] developed a novel ratiometric dual-signal electrochemical aptasensor for pathogenic bacteria at the single-cell level. This sensor was based on

the aptamer recognition-induced Rolling Circle Amplification/G-quadruplex strategy [170–172]. G-quadruplex can interact with various signaling molecules, including electrochemical signal redox indicator methylene blue (MB). In this study, the signal amplification was achieved through the utilization of two probes—one being an aptamer modified with ferrocene for the target bacteria and the other being the primer sequence for rolling circle amplification (RCA) anchored to the gold electrode with a sulfhydryl probe (Figure 5). These two probes can form a complex disrupted by the presence of the target bacteria *S. aureus*. Consequently, when contaminated samples were tested, only the RCA probe remained on the gold surface. To stimulate the RCA reaction, a C-base-rich circular template was introduced to the reaction mixture, generating a multitude of G-base-rich nucleic acid sequences that formed RCA products into a G-quadruplex. Upon the addition of MB, there was a substantial cascade amplification of electrochemical signals due to its strong affinity of the G-quadruplex [173].

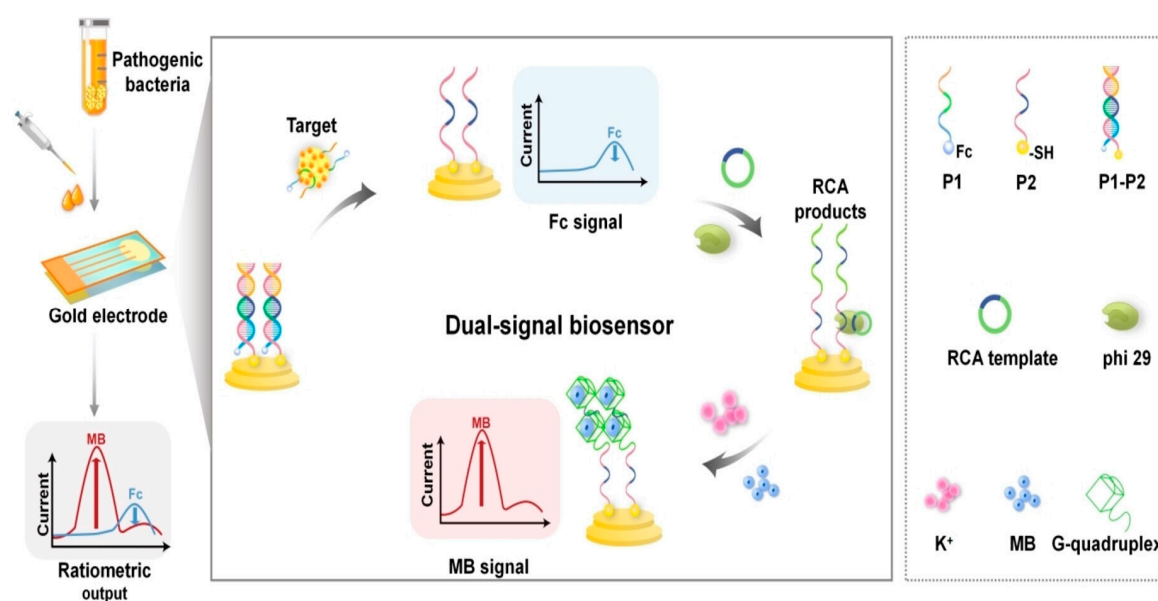


Figure 5. Schematic illustration presenting ratiometric dual-signal electrochemical biosensor for ultrasensitive detection of pathogenic bacteria based on aptamer recognition-induced rolling circle amplification (RCA)/G-quadruplex strategy. Adapted with permission from [169].

Bacterial spores remain the major concern in food industry and hospitals since they are remarkably resistance to high temperatures, chemical agents, radiation, and harsh physical conditions. In sporulated form bacteria exhibit resilience to unfavorable environmental conditions, making them the most resistant life forms known to date. Typically, they can withstand temperatures 40-45 °C higher than their corresponding vegetative cells. Additionally, spores can enter dormancy and survive in a wet state for thousands of years. Furthermore, spores are regarded as potential biological weapons. Indeed, *Bacillus anthracis* was initially used during the First World War. The dispersal of spores in the ambient air can result in the development of the respiratory form of anthrax. Due to these unique characteristics, for biosecurity and food safety, researchers have developed aptasensors specifically designed for the detection of target bacteria in their sporulated form [5,68,174]. Recently, Rizzotto et al. developed a colorimetric assay for the detection of *Bacillus cytotoxicus* spores in food [29]. *B. cytotoxicus* belongs to a large group of bacteria commonly named the *Bacillus cereus* group [6,167]. The group is constituted of different species some of them are human pathogen (such as *B. anthracis*, *B. cereus sensu stricto* or *B. cytotoxicus*) but others are harmless (such as *B. thuringiensis*, *B. weihenstephanensis* and *B. mycoides*). The detection test relied on the spore-enhanced peroxidase-like catalytic activity of gold nanoparticles (Figure 6). The test was performed in a simple microtube containing AuNPs and magnetic beads (MP) both conjugated with a specific aptamer BAS6R as a recognition element for *B. cytotoxicus* spores. The presence of spores was detected with

the naked eye due to the change in color in the solution (blue to dark blue) upon oxidation of tetramethylbenzidine (TMB) with H_2O_2 . This method has the advantage of the aptamer binding to an unknown epitope on the spore surface, eliminating the need to extract, purify, and amplify a specific biomarker. However, BAS6R was shown to bind to spores of different *B. cereus* species. It cannot thus distinguish between pathogenic and non-pathogenic strains of this group of bacteria.

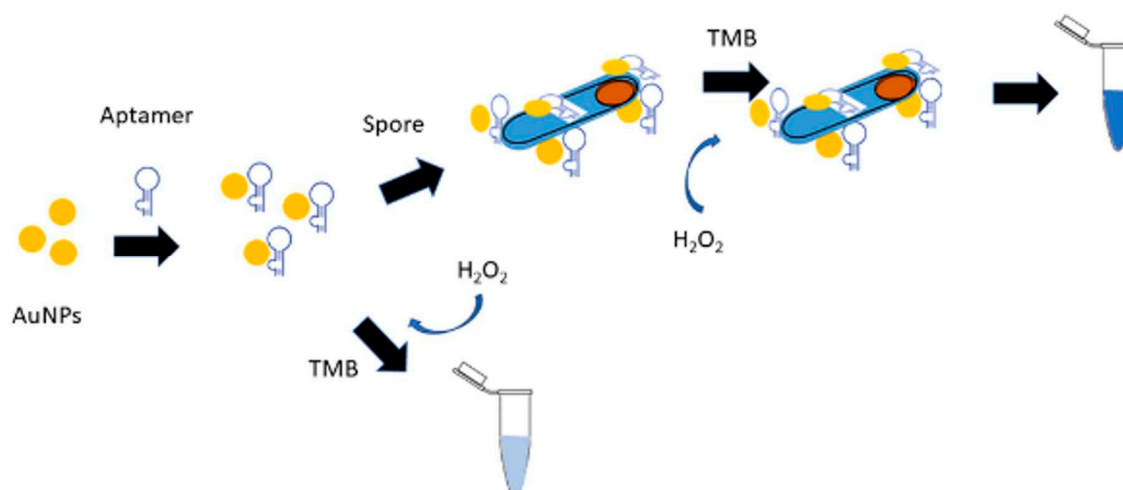


Figure 6. Schematic illustration presenting colorimetric biosensor for detection of *B. cytotoxicus* spores based on spore-enhanced peroxidase-like catalytic activity of AuNPs. Adapted with permission from [29].

In another study, Zhou et al, developed a dual-aptamer microfluidic chip for detection of both *B. cereus* vegetative cells and spores [156]. To improve the selectivity and sensitivity of detection, aptamers selected through the Cell-SELEX were additionally, step-by-step, tailored and optimized using molecular docking. Based on this dumbbell aptamer design, two best aptamers were obtained and integrated into a microfluidic chip biosensor. These two aptamers had repeated G-based both with a high affinity to bind the polar amino-acids in the α -helix of epiprotein of *B. cereus*. The biosensor applied directly in food, such as milk and rice, provided a complete analysis within 1 hour and showed a LoD of only 9.27 CFU/mL.

4.2. Bacterial toxin detection

Detecting bacteria is not always sufficient. Many bacterial pathogens such as *S. aureus*, *E. coli*, *C. botulinum*, *C. perfringens*, *C. difficile*, *B. cereus*, *C. jejuni*, *L. monocytogenes*, *Yersinia enterocolitica*, and *Salmonella* spp. produce toxins [175–183]. Ingested bacterial toxins cause various effects, ranging from disrupting cell membranes and causing cell death to interfering with the host immune response. They play a crucial role in the pathogenicity of many bacterial species, contributing to the symptoms and severity of bacterial infections [184]. For instance, *C. botulinum* toxins can cause botulism, a severe illness characterized by muscle paralysis, general weakness and potentially fatal respiratory failure. *S. aureus* enterotoxins in food can lead to symptoms such as nausea, vomiting, huge diarrhea. As already mentioned, these toxins are thermostable and can hardly be eliminated by food treatment or preparation like cooking, frying or freezing that eliminate bacterial cells. Moreover, toxins can go undetected during classic method pathogen detection. Usually when strains are isolated, the presence of toxins is tested by PCR amplification of genes that encode them. This additionally step prolongate the procedure and cannot be performed in the field. In addition, the presence of gene does not necessary yield to the toxin production. There is, thus, a need to directly detect the presence of bacterial toxins in food and biological samples.

An electrochemical aptasensors was designed for the detection of staphylococcal enterotoxin B (SEB), [100] a potent bacterial toxin responsible for severe food poisoning [185,186]. The sensor utilized a commercial carbon screen-printed electrode modified with reduce graphene oxide (rGO) and gold nano-urchins (AuNUs). The electrode was further functionalized with a single-stranded DNA probe, followed by the attachment of a specific aptamer. Upon encountering SEB molecules, the aptamer detached from the electrode surface. Following this, an electrochemical signal of the redox probe, hematoxylin, decreased significantly (Figure 7). The aptasensor analytical performance was validated with food samples, serving as simulated real samples. This tool surpasses a commercial ELISA kit for SEB detection. By employing this method, a promising application of the aptasensor was demonstrated for food sample screening.

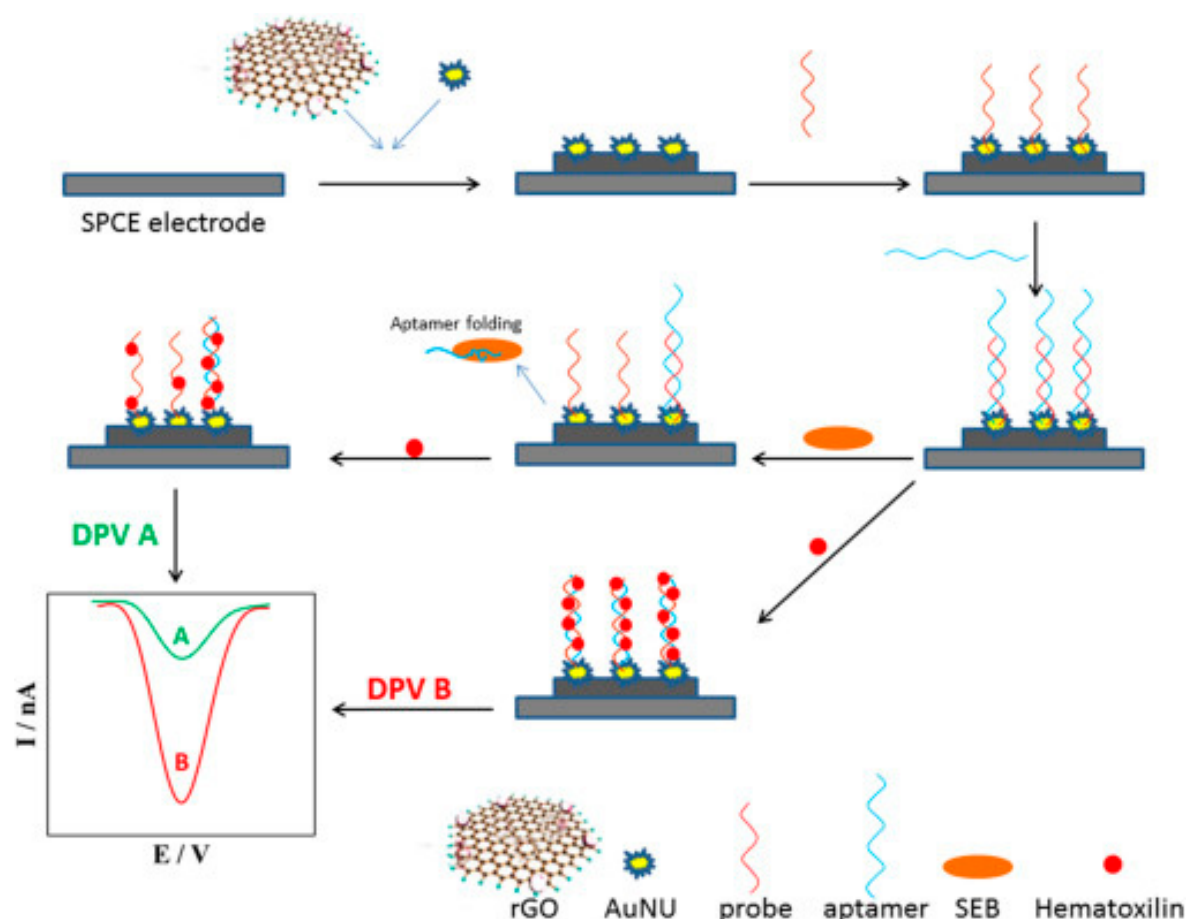


Figure 7. Schematic illustration of fabrication process of SEB aptasensor using reduced graphene oxide and gold nano-urchins. Adapted with permission from [185].

To assess food safety, a multiplex aptasensors were developed for the detection of different targets in a food sample. Jin and al. proposed a lateral-flow strips (LFT) aptasensor for *Salmonella*, Hg^{2+} -ions and ochratoxin A (OTA) using an upconversion nanoparticles (UCNP) as a signal source and a smartphone equipped with a CCD camera for the detection [187]. This approach ensures a fast and portable detection process. As illustrated in Figure 8 three different aptamers were used in the sensor, each grafted on the surface of different color UCNP and hybridized with their complementary DNA (cDNA) fixed on the nitrocellulose membrane. In the presence of bacteria, ions, and small molecules, the three aptamers preferentially bond to their respective targets. Consequently, the liberated cDNA decreases the fluorescence from the UCNP. It was demonstrated that this device had no non-specific or cross-reactivity due to the separate color channels providing detection signal. This feature was crucial to enable multiplexing. The method of detection holds promising prospects for the simultaneous detection of multiple targets in food samples for enhanced security.

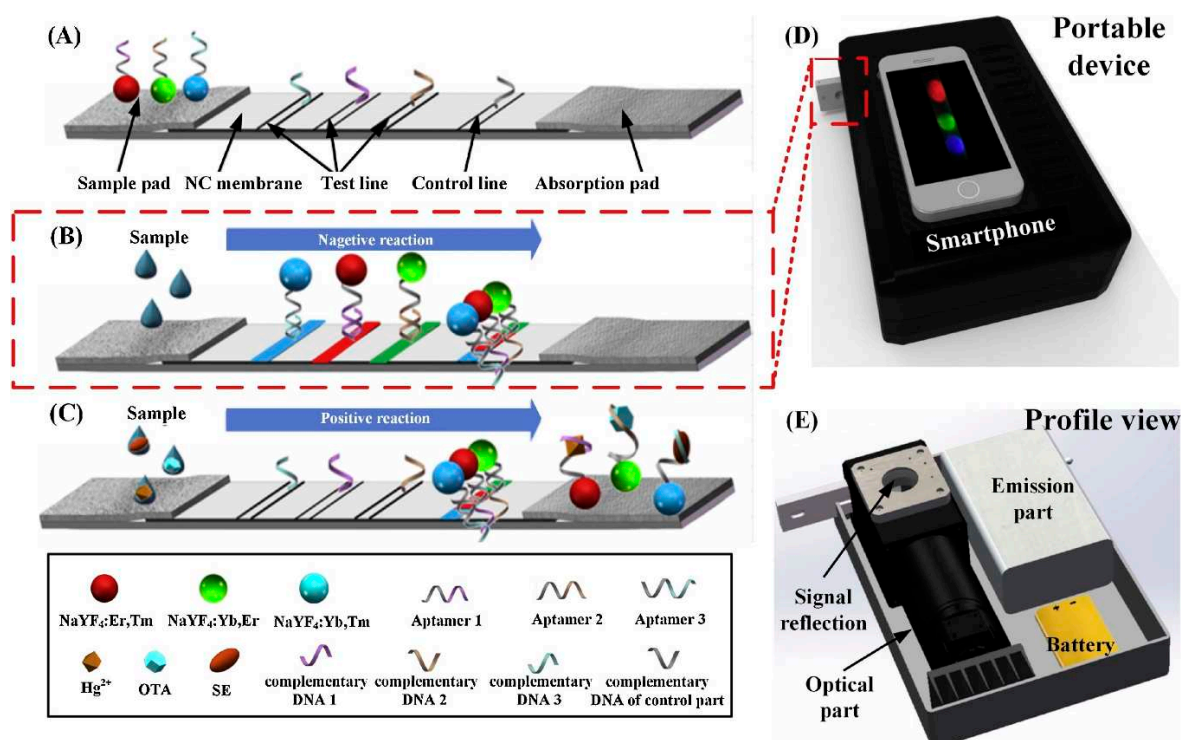


Figure 8. Schematic illustration of lateral flow assay based on 3 different aptamers for simultaneous multiple targets detection. Adapted with permission from [187].

4.3. Bacterial biomarker detection

A bacterial biological marker or bacterial biomarker can be defined as a specific characteristic that is measured as an indicator of bacterial presence, their biological processes, or responses to an external exposure or intervention, including therapeutic interventions. Detection of biomarkers enables various applications, such as monitoring of infection and therapy efficiency, risk estimation, diagnosis, or estimation of bacterial adaptation to environmental signals. Numerous aptamers have been developed to target a wide array of bacterial biomarkers although targeting the entire cell is more common. Some aptamers have been developed for purposes other than detection, such as therapeutic or basic research applications. This technology opened the way for innovative possibilities that are not only cost-effective but also capable of overcoming the limitations posed by conventional antibodies or various probes and drugs.

The methyl erythritol phosphate (MEP) metabolic pathway of isoprenoid biosynthesis is essential for many pathogenic bacteria, both gram-positive and gram-negative, such as *B. anthracis*, *Clostridium* spp., *L. monocytogenes*, *S. enterica*, *Vibrio cholerae*, *Shigella* spp., *E. coli* and *Pseudomonas aeruginosa*. Recently a DNA aptamer, D10, was developed against 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), the second enzyme of MEP pathway [188]. This aptamer modified with a fluorescent label allowed a strong specific staining of MEP+ bacterial cells (*E. coli* and *P. aeruginosa*) while no staining was observed with bacterial lacking the DXR enzyme (e.g., *E. faecalis*). It presents, thus, a potential to replace the specific antibody for future therapeutic and diagnostic applications.

More recently, an antagonist aptamer for SEB neutralization was selected through the SELEX [189]. SEB is a critical virulence factor in staphylococcal toxic shock syndrome (TSS), triggering a robust release of proinflammatory cytokines by activating T lymphocytes. The aptamer demonstrated effectiveness in inhibiting SEB-induced proliferation and cytokine secretion in human peripheral blood mononuclear cells. Their results highlight the potential therapeutic value of the novel aptamer antagonist in mitigating SEB-mediated TSS, and presenting a promising avenue for future therapeutic strategies.

Another expanding aptamer application was described by Stoltenburg and his co-workers [105], who explored an alternative approach develop aptamers that bind a bacterial surface protein. In

contrast to the Cell-SELEX which selects specific aptamers that bind unknown target at the bacterial cell surface, the procedure was based on selecting aptamers that recognize a purified surface protein of *S. aureus*, called protein A. This protein A is a major component of the cell wall of *S. aureus*. Obtained selected protein-A binding G-quadruplex aptamer was integrated into Enzyme-linked OligoNucleotide Assay (ELONA). They demonstrated that the aptamer and its truncated versions bound to the Protein A expressed by intact cells of *S. aureus*. Some structural information of the aptamer used was also gathered because the aptamer functionality was shown to be tightly linked to their structure. Such insights of the structural basis of the aptamer functionality enhance our understanding of the molecular bases of interactions and pave the way for the development of novel more robust aptamer-based diagnostic tools.

Many bacterial virulence factors are exported by external vesicles. All Gram-negative bacteria secrete outer membrane vesicles (OMVs), proteoliposomes derived from their outer membrane with sizes between 20 and 250 nm. OMVs play crucial roles during host-microbe interactions because they transport a broad range of molecularly diverse cargoes, including adhesins, toxins and membrane embedded and associated proteins, small molecules, lipids, peptidoglycan and nucleic acids. Shin et al, have selected aptamers that bind OMVs of multiple Gram-negative bacteria [147]. The aptamers selected through the Cell-SELEX showing the highest affinity to bind Gram-negative bacterial cells were conjugated with horseradish peroxidase (HRP) and used in an enzyme-linked aptamer assay (ELAA) to detect OMVs. A LoD of 25 ng/mL was obtained. The recognition of OMVs was expected because secreted vesicles contain similar proteolipids as their cell of origin. They suggested that detection of bacterial OMVs was more effective than detection of bacterial cells in clinical samples.

5. Conclusions

In the realm of pathogenic bacteria, biosensors for their rapid and accurate identification and monitoring are important added-value in preventing spreading, reducing treatment expenses and ensuring food safety. Biosensors offer numerous advantages compared to traditional microbiological methods for their enhanced sensitivity and rapidity. One of the most significant challenges in bacterial pathogen control is the time required for the detection and identification of particular agents. Currently, identification of a microorganism typically takes between one and several days, when plating or PCR based protocols are employed. In contrast, biosensors may provide a complete microbiological analysis within several hours or even minutes [190,191]. Biosensors also hold the potential to be portable, multiplex and accessible to a wide range of users [192,193]. Biosensors are thus the most promising tool for efficient microbial detection and their point-of-need testing [194].

Aptamers have been used extensively in recent years as an alternative to conventional antibodies in various biosensing strategies including electrochemical, optical and microfluidic chip-based platforms. Compared to antibodies, aptamers have a shorter production time, lower cost, higher stability in complex matrices, and possess ability to bind a variety of specific targets which enable their tailored applications. As outlined here, various aptamers are available for different bacterial targets, including whole cells, toxins, intracellular and secreted biomarkers. Regarding the latest research, trends in aptasensors for bacterial pathogens detection include development of portable biosensors, such as electrochemical or paper-strip based devices coupled to easy or no sample preparation and high cost-affordability biosensing strategies. The specificity of detection and accuracy of overall analysis may be improved by using dual aptamer probes, where the first aptamer is capturing probe and second is detection probe. This configuration allows the use of aptamers with different chemical modifications i.e., the capturing probe may carry a thiol for its covalent immobilization onto the gold surface of the biosensor, while the detection probe may be conjugated with a fluorescent or redox probe to enable signal read-out. Moreover, a mix of various aptamers, each targeting different epitopes of the same bacterium can be employed to achieve higher accuracy of detection.

Signal amplification strategies employ aptasensors coupled with different nanomaterials, catalysis amplification, hybridization chain reaction, and rolling circle amplification [30]. Alternatively, aptamers adopting specific structural 3D conformations, such as G-quadruplex, are

shown to provide high signal intensities. The utilization of aptamers of specific 3D structures as recognition elements seems to be a smart and cost-effective approach to develop sensitive biosensors [195]. In the same time, aptamers structural changes upon target binding may induce a signal transduction and/or result in target triggered amplification, which highly simplify the device development [196]. Interestingly, although primary sequence of many validated and published aptamers suggests that they adopt a specific 3D structure (G-rich or C-rich sequences), their structure was usually not investigated. It is especially case for aptamers adopting I-motive (C-rich sequence) [61].

6. Future directions

Aptasensors still have to cross a long way to become a standard technique for microbiological applications. Indeed, there is a significant number of papers presenting proof-of-concept, but still, there are only a few commercially available aptasensors that can be utilized at the level of the food industry or hospitals. First, aptamer technology has some shortcomings. Most SELEX procedures are performed using the same oligonucleotide pool, and not all selected aptamers are very selective. Second, the selection and structural characterization of aptamers are usually performed under defined conditions, but their configuration and binding properties can be modified upon temperature, ionic strength or pH variations, resulting in a significant reduction of binding affinities. Consequently, although some aptasensors provide efficient bacterial detection in the buffer, they are inefficient when applied in biological or food samples. Still, aptamer active conformation can be stabilized through chemical modifications. However, there are only few reports where such optimization was performed. Third, aptamers as oligonucleotides can be degraded in biological matrices containing DNase or RNase enzymes. Again, chemical modification of aptamer molecules can be performed to prevent such degradations, but are rarely done. The future trend in aptamer selection, characterization and structural optimization will require closer collaboration between molecular biophysicists, microbiologist and biosensor developers.

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