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CRISPR/Cas12a-based biosensors for environmental monitoring and diagnostics

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ABSTRACT

Contaminants, such as nucleic acids or toxic small molecules, threaten both human health and the environment. Precise and highly sensitive identification of such contaminants holds paramount importance across diverse domains, including safeguarding food safety, facilitating diagnostics, and monitoring environmental conditions. Traditional methodologies, encompassing spectroscopy, chromatography, sequencing, and metagenomics, have served pivotal roles in detection processes. Nevertheless, these methods have encountered recurring challenges related to sensitivity, specificity, and portability. This review focuses on the groundbreaking CRISPR/Cas-based biosensors. These biosensors leverage the incredible precision and programmability of the CRISPR/Cas system to recognize specific targets. Here, we comprehensively assess the fundamental mechanisms that enable specific and accurate detection, covering topics from guide RNA design to optimization of collateral cleavage activity. The versatility of CRISPR/Cas12a biosensors becomes evident through their diverse applications. These applications encompass medical diagnostics, food safety, and environmental monitoring. The transition from conventional detection methods to biosensors and ultimately to CRISPR/Cas-biosensors represents a significant milestone in diverse contaminant detection. By incorporating molecular biology, nanotechnology, and bioinformatics, these biosensors have the potential to reshape the landscape of water safety, diagnostics, and environmental monitoring. CRIPSR-Cas diagnostics is a transformative technology that paves the way for a safer and healthier future for the environment and human life.

1. Introduction

The generation of waste from agricultural practices, industrial effluents, and human and animal activities is increasingly blurring the distinction between clean water sources and wastewater, leading to a decline in the availability of freshwater resources for human use (Kabir et al., 2023; Martinez-Burgos et al., 2021; Tarpeh and Chen, 2021). Water ecology plays a vital role in providing essential services, including food production (Ding et al., 2023), flood regulation (Zhao et al., 2023), habitat creation (Warner, 2023), nutrient

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cycling (Lothmann and Sewilam, 2023), soil formation (Lin et al., 2023) and water purification (Huang et al., 2023). Nucleic acid and non-nucleic acid contaminants in tap and drinking water have been linked to the ecological imbalance (Bojago et al., 2023; Jena et al., 2023; Nirmala et al., 2023; Sabzchi-Dehkharghani et al., 2023). Consequently, the development and implementation of swift and very sensitive detection methodologies are of utmost importance to safeguard safe and uncontaminated water supply. The impact of an unsafe water supply on human health is evident through the transmission of contagious diseases, such as hepatitis (Takuissu et al., 2023), influenza (Ahrens et al., 2023), SARS (Parida et al., 2023), pneumonia (Aydin et al., 2023), gastric ulcers (Duan et al., 2023), and pulmonary disorders (Wang and Xia, 2023). In urban settings, a plethora of human activities contributes to the prevalence of numerous non-nucleic acid contaminants in the water supply, with examples including agrochemicals (Molpeceres et al., 2023), heavy metals (Yan Li et al., 2023d), plastics (Tasseron et al., 2023), and antibiotics (Linghu et al., 2023). These non-nucleic acid pollutants constitute commonly detected substances, posing significant challenges to urban areas in maintaining water quality. Water quality assessment technologies are extensively employed to identify pollutants in diverse water ecosystems, encompassing applications in water treatment plants and distribution systems (Li et al., 2023f; Liu et al., 2021; Mosquera-Romero et al., 2023). Considerable research efforts have been undertaken in previous years to enhance the reliability and efficiency of pollutant detection methodologies while minimizing operational expenses and energy consumption (Shakeel et al., 2022; Zhang et al., 2021). Notably, advancements in biosensor technology and spectroscopy have led to notable enhancements in the sensitivity of detection, both qualitatively and quantitatively. Moreover, the availability of multiple detection analyses and on-site measurement tools have significantly broadened the scope of water monitoring applications, facilitating the integration of advanced techniques into handheld sensing devices. This integration has substantially improved the portability of real-time contaminant detection, encompassing various hazardous substances such as heavy metals, microbes, chemical pesticides, and inorganic as well as organic constituents (Atkinson et al., 2022; Kadam and Hong, 2022a; Reddicherla et al., 2022).

The advent of CRISPR/Cas technology has pushed the boundary of molecular biology, particularly in the realm of gene editing and genetic engineering (Hinge et al., 2021; Shelake et al., 2022; Atif Khurshid Wani et al., 2022c). However, the capabilities of CRISPR/Cas technology extend far beyond genome editing, extending into the realm of biosensing and environmental monitoring (Liu et al., 2022c; Mao et al., 2022; Phelps, 2019). Of particular interest is the CRISPR/Cas12a system, which has arisen as a versatile method for the detection of nucleic acids, including genes, pathogens, and other genetic markers (Leung et al., 2022; Qiu et al., 2022; Zhao et al., 2023). Leveraging its inherent ability to target specific DNA sequences, the Cas12a enzyme can be repurposed to initiate a molecular response upon binding to a target sequence, thereby enabling nucleic acid detection with unparalleled sensitivity and precision. CRISPR/Cas12-based methods present a suite of advantages in the realm of biosensor detection. Their high precision and specificity in recognizing target sequences, facilitated by the design of guide RNAs, ensure accurate and reliable results (Peng et al., 2020). The programmability of CRISPR/Cas systems allows for versatility, enabling the detection of various targets through tailored guide RNA sequences. Moreover, these biosensors exhibit notable sensitivity and are capable of detecting low concentrations of targets, a crucial feature in applications like early disease diagnosis and environmental monitoring. The ability to perform multiplexing, or simultaneous detection of multiple targets, enhances their utility in complex scenarios (Li et al., 2022; Zhou et al., 2018). Additionally, the relatively quick detection turnaround time makes CRISPR/Cas-based methods suitable for applications requiring rapid results, such as point-of-care diagnostics. The CRISPR/Cas12 system has been harnessed in parallel and equally groundbreaking development for non-nucleic acid contaminants detection (Feng et al., 2021a; Su et al., 2023; Wu et al., 2021). This innovative application leverages the Cas12 enzyme's collateral cleavage activity, which can be triggered in response to the existence of particular non-nucleic acid analytes (Feng et al., 2023a). Such analytes encompass various molecules, including proteins, small molecules, and other environmental indicators (Cheng et al., 2022). This expansion of the CRISPR/Cas12 technology's functionality to non-nucleic acid detection presents an unprecedented opportunity to address the comprehensive array of contaminants that threaten our ecosystems and human health (Atçeken et al., 2022; GyuáPark, 2022). This paper delves into the innovative application of CRISPR/Cas12 systems as revolutionary sensors, specifically focusing on their role in nucleic and non-nucleic acid contaminant detection within water sources and beyond. The convergence of biotechnology and molecular biology with this cutting-edge technology has paved the way for a profound enhancement in detection methods' sensitivity, specificity, and versatility. As we delve into the intricacies of CRISPR/Cas12-based sensing, we uncover its potential to address critical contamination monitoring and analysis challenges, ushering in a new era of precision environmental assessment. This review focuses exclusively on the application of CRISPR/Cas12a technology in the realms of environmental monitoring and diagnostics. Providing a thorough exploration of Cas12a's unique attributes, the review offers a detailed analysis of its role in biosensing applications designed specifically for environmental contexts. Its distinct advantages include a targeted emphasis on CRISPR/Cas12a-based biosensors, offering an up-to-date and comprehensive overview of their contributions to environmental sciences. Through this exploration, we aim to highlight the transformative capabilities of CRISPR/Cas12 sensors and their pivotal contribution to advancing analytical methodologies for a cleaner and safer world.

2. Conventional detection systems for non-nucleic acid contaminants

Although significant endeavors have been directed towards regulating the application of agrochemicals, the worldwide agricultural consumption of pesticides has shown a persistent increase, reaching an approximate value of 4.2 million metric tons. Among these, fungicides account for 17.5%, insecticides for 29.5%, herbicides for 47.5%, and the remaining 5.5% comprises other types of pesticides (Sharma et al., 2019). In the realm of water monitoring, the landscape of analytical technologies has diversified significantly. Numerous methodologies have emerged, each serving distinct purposes. These encompass traditional instrumental analysis (Carstea et al., 2016), sensor deployment strategies (Delgado et al., 2021; Zhang et al., 2020), model-based event detection (Wu et al., 2022), microfluidic devices (Rai et al., 2022), spectroscopic methodologies (Shi et al., 2022), and biosensor applications (Hui et al., 2022;

Wani et al., 2023a). The appropriateness of a particular detection approach is contingent upon the specific objectives of the analysis, necessitating careful consideration of whether a quantitative, qualitative, or hybrid measurement is required. The identification of water contaminants has predominantly occurred through manual procedures within water laboratory settings. Skilled personnel have traditionally performed analyses employing advanced and state-of-the-art technologies. Commonly utilized instruments at the laboratory level include the multiple fermentation tube technique (Eckner, 1998), filtration methods (Zhai et al., 2022), field-flow fractionation (Tan et al., 2023), chromatography (Yanghui Xu et al., 2022), and mass spectrometry (MS) (Manne et al., 2022; Shen et al., 2021). These methodologies have been extensively employed to detect several unwanted constituents present in water samples. These tools play a vital role in identifying and quantifying agrochemical residues present in agricultural samples such as crops, soil, and water. Each method operates based on distinct scientific principles and possesses different levels of sensitivity known as the Limit of Detection (LOD), which varies from nanogram to milligram per liter (Table 1). Several factors significantly impact the accuracy of these analytical tools. Sample complexity, the presence of interferences from other compounds, proper calibration of instruments, the expertise of the operator, and the quality of the instrumentation itself all influence the reliability of the results obtained.

2.1. Agrochemical detection

A pioneering approach, integrating Fourier-transform infrared spectroscopy (FTIR) analysis with fingerprint analysis, has been innovated and verified to enable thorough assessment and swift verification of pesticides. The proposed strategy demonstrated high efficacy, achieving a 90% accuracy rate and a minimal error rate of 3.33% for 30 samples, as evaluated against liquid chromatography (LC) data (Xia et al., 2022). Depth-profiling FTIR photoacoustic spectroscopy was effectively employed as an on-site, non-destructive,

Table 1
Detection of non-nucleic acid contaminants through conventional detection methods.

Sample site and/or sample type	Non-nucleic acid contaminant	Detection method	Detection value	References
Agrochemicals				
Milk	Aminopyralid, picloram, fluroxypyr, and clopyralid	Molecularly imprinted polymer (MIP) and LC-MS/MS	0.124 $\mu g \cdot L^{-1}$	(Tan et al., 2019)
Agro-products	Sulfonylurea herbicide	MIP and LC-MS/MS	0.005–0.07 μgL^{-1}	(Feng et al., 2021b)
Agricultural sample	Malathion	Colorimetry	4.03 ng mL^{-1}	(Sahu et al., 2023)
Surface water of Fyris River, Sweden	Organohalogens	LC-HRMS	0.005 μgL^{-1}	(Menger et al., 2021)
Water of Japaratuba River, Brazil	Diuron	Aqueous two-phase system	75–1900 $ng \cdot L^{-1}$	(Buarque et al., 2019)
Wastewater in Spain and Portugal	Metformin and acetaminophen	LC-MS/MS	0.1 $pgmL^{-1}$	(Montes et al., 2023)
Drinking water	Metsulfuron-methyl	Differential Pulse Cathodic Stripping Voltammetry	0.04 mgL^{-1}	(JIANG et al., 2020)
Water of Kurose and Ashida Rivers, Japan	Diroun, and butamifos in Kurose. Diuron, and futolanil	SPE, GCMS and LCMS	970 ngL^{-1} and 4.7 ngL^{-1}· 860 ngL^{-1}, and 5.5 ngL^{-1}	(Derbalah et al., 2020)
Llobregat River basin, Spain	Carbendazim	Stir bar sorptive extraction and GCMS	1.9 μgL^{-1}	(Postigo et al., 2021)
Drinking water, Netherlands	Acetamiprid and thiamethoxam	LC-MS/MS	1.1 μgL^{-1}, and 0.4 μgL^{-1}	(Sjerps et al., 2019)
Streamwater of southwestern Ontario, Canada	2,4-D; bentazon, and Thiamethoxam	GCMS and LCMS	0.014 μgL^{-1}, and 0.010 μgL^{-1}	(Raby et al., 2022)
Surface water of Lake Ontario (St. Lawrence)	Glyphosate, and atrazine	SPE and UHPLC-MS/MS, and LCMS/MS	27 ng L^{-1}, and 11 ng L^{-1}	(Montiel-León et al., 2019)
Other industrial contaminants				
Melegnano, Lambro River Italy	Polystyrene	μFT-iR	1.3 ± 0.7 plastics/m^2	(Magni et al., 2021)
Po River Delta (Italy)	Plastic debris	Unmanned aerial vehicle imaging	–	(Taddia et al., 2021)
Alcedo atthis, Ticino River, Italy	Polyethylene	μ-FTIR and SEM-EDS	3.09/m^2	(Winkler et al., 2020)
Pearl river Delta	Organophosphate, phthalate esters	LC–MS/MS	610–49,400 μg of additives/g of plastic	(Xiaotu Liu et al., 2021)
Wastewater	Chlorophenoxy acid	SPE, and LC-ultraviolet detector	2.92–21.41 μgL^{-1}	(Chen et al., 2021)
Sediments of Black Sea region of Turkey	Iron, manganese, and Zinc	ICP-MS	8135.27, 272.56, and 25.68 mg kg^{-1}	(Ustaoğlu et al., 2020)
Groundwater of Southern Saudi Arabi	Iron, lithium, and Arsenic	ICP-MS	0.025, 0.424, and 0.945 mgL^{-1}	(Alfaifi et al., 2021)
Surface water of Bohai Sea	Mercury, and Chromium	ICP-MS	0.63 ± 0.25, and 0.047 ± 0.012 μg L−1	(Liu et al., 2019)
Surface sediments from the Yangtze River	Copper, Cobalt, and Zinc	ICP-MS	20.94, 15.28, and 81.53 μgg^{-1}	(Zhuang and Zhou, 2021)
Surficial sediments of Gorgan Bay	Cadmium, and Lead	AAS	1.82, and 15.51 $mgkg^{-1}$	(Abadi et al., 2019)

and expeditious technique for the detection of tricyclazole residues on aluminum, copper, and iron substrates. The findings revealed that a moving mirror velocity of 0.95 cm s^{-1} yielded optimal results for depth profiling. The acquired spectra exhibited a prominent absorption band approximately at 1200 cm-1, corresponding to the tricyclazole C-N bond. This specific band has the potential for detecting tricyclazole residues on plant surfaces as well (Lv et al., 2018). The primary analytes identified in the water systems of the Samambaia River sub-basin, located in the Federal District and eastern Goiás, were metribuzin, atrazine, clomazone, and haloxyfop-methyl. Detection was achieved through the utilization of LC with a tandem MS system, incorporating high-efficiency liquid chromatography (HPLC) (Correia et al., 2020). In Southeast Brazil, the environmental mobility of glyphosate was evaluated using a flow injection spectrophotometer, which enabled the monitoring of surface runoff with detectable concentrations between 0.5 and 8.7 mg.L^{-1}. A study focused on assessing Atrazine pollution and its spatial-seasonal fluctuation in surface water bodies within an agricultural river basin. The analysis was performed using HPLC-MS/MS of Tandem Triple Quadrupole. The quantification frequencies observed varied between 67% and 100% during spring and ranged from 33% to 67% during autumn (Bachetti et al., 2021). A novel approach was formulated to quantitate pesticides in water by employing carbon nanotubes, followed by HPLC coupled with Diode Array Detection. The LOD for these pesticides ranged from 0.1 to 1.0 ng mL^{-1}, indicating the sensitivity and precision of the developed technique (Zhao et al., 2011). Rösch et al. (2019) established a highly sensitive analytical technique to detect insecticides in surface waters using a liquid-liquid extraction process along with n-hexane followed by applying GCMS with atmospheric pressure chemical ionization technique. The method's exceptional sensitivity is evident from its lower limits of quantification, which range between 12.5 and 125 pg L^{-1}(Rösch et al., 2019). Using the solvent-loaded metal-organic framework, a comprehensive analytical method based on UHPLC-MS/MS was employed to detect and measure five chlorophenoxy acid herbicides with a detection limit of 2.66–19.7 ng·L^{-1} (Tan et al., 2021). Conventional agrochemical detection tools exhibit certain limitations, encompassing sensitivity, specificity, interference from sample matrices, time consumption, cost, and the need for skilled operators. Although these methods hold value, they may encounter challenges in detecting trace levels of agrochemicals and differentiating between structurally similar compounds. As a result, ongoing research endeavors strive to ameliorate these constraints by either enhancing existing methodologies or innovating novel approaches to bolster agrochemical analysis.

2.2. Industrial contaminant detection

Industrial pollutants can also contaminate water resources, posing environmental and health risks. Common contaminants include heavy metals, plastics, organic compounds, and industrial byproducts (Feng et al., 2023b; Wu et al., 2020). The prevailing techniques employed for the detection of chromium in water samples primarily comprise lab-based methodologies, notably atomic absorption spectroscopy (AAs) and MS (Ahmed et al., 2022; Guo et al., 2023). While these methods exhibit remarkable selectivity and sensitivity, they necessitate costly maintenance and the expertise of well-trained personnel. In a research investigation, an optimized colorimetric method utilizing 1,5-diphenylcarbazide dye was developed for integration into a microfluidic detection system. The analytical performance of the method was assessed, and it demonstrated compliance with Beer's law within the concentration range of 0.03–3 mg·L^{-1}. The calculated quantitation limit and detection limit for chromium were determined to be 0.076 and 0.023 mg·L^{-1}, respectively (Lace et al., 2019). Colorimetric detection methods have been used to detect various heavy metals, including lead, mercury, cadmium, copper, zinc, chromium, iron, and nickel. These methods involve the use of specific colorimetric reagents that form colored complexes with the target metals. The simplicity and cost-effectiveness of these methods make them valuable for environmental monitoring and water quality assessment. Researchers have developed a fast and non-destructive technique for assessing the presence of heavy metals in crayfish exoskeletons using ATR-FTIR analysis (Volpe et al., 2020). This technique offers a beneficial, rapid, and economical approach to sensing heavy metal contamination in water. However, it is imperative to note that ATR-FTIR analysis is non-specific and cannot distinguish between different types of heavy metals. While it can indicate the presence of heavy metals, further specific testing is needed to identify and quantify individual heavy metal species in the water samples. Inductively coupled plasma (ICP) optical emission spectroscopy and AAS are employed to analyze heavy metals in water, sediment, and human blood samples. These techniques are utilized to measure heavy metal concentrations, offering valuable insights into their presence and potential impacts on the environment and human health (Gupta et al., 2023). ICP-MS (inductively coupled plasma mass spectrometry) is a preeminent and widely employed heavy metal analysis technique. Its operational principles bear resemblance to those of ICP-atomic emission spectrometry (ICP-AES), primarily concerning the atomization plasma. The distinguishing feature of MS detection lies in the necessity to extract ions emanating from the plasma, typically accomplished through a quadrupole system using a sequence of cones, directing them into a mass spectrometer. This instrument quantifies the mass-to-charge ratio of the ions, resulting in signal intensities that are directly proportional to the quantity of ions generated. A research investigation evaluated the concentration levels of As, Cd, Cr, Cu, Hg, Ni, Pb, and Zn in Olt River sediments by employing ICP-MS. The findings revealed elevated contents of As, Cd, Cu, Hg, Ni, Pb, and Zn, exceeding the national quality standards for sediments by factors of 8.28, 1.53, 4.40, 3.71, 2.46, 1.10, and 1.15, respectively (Iordache et al., 2022).

Mercury, owing to its elevated vapor pressure, can be quantified through direct gaseous atom trapping in a gold trap, serving as a pre-concentration process, and subsequent liberation via heating, followed by transportation towards the measurement cell in atomic fluorescence spectrometry (Atasoy et al., 2023). Other methods, such as plasma-induced (Liu et al., 2023c), electrochemical (Sakthi Priya et al., 2022), and photochemical vapor generation (Borowska and Jankowski, 2022), have also been utilized, with recent advancements involving nanomaterials, albeit on a relatively smaller scale. Raman scattering signals arise from species adsorbed on roughened nanoparticles and metal surfaces, particularly on silver and gold nanostructures serving as substrates (Sun et al., 2023). This improvement originates from the highly localized intensification of electromagnetic fields in the vicinity of nanostructures, principally near edges or between adjacent nanoparticles. Owing to its exceptional sensitivity, the distinctive molecular fingerprints

inherent in Raman spectroscopy, and its non-destructive data acquisition capability, Surface-enhanced Raman spectroscopy has evolved into a broadly utilized spectroscopic method for detecting and identifying various chemicals (Guo et al., 2023; Terry et al., 2022; Yao et al., 2023). This technique has become instrumental in nano-optical-based detections for the chemical speciation of heavy metals in water samples, even at trace concentrations. Substrates featuring nanostructures and tailored anchors promote precise binding with heavy metal ions, keeping them close to the surface that enhances signals, ultimately allowing for their highly amplified detection. Traditional methods for analyzing heavy metal content encounter several constraints related to their sensitivity, selectivity, detection limits, sample preparation procedures, and time requirements. These techniques can be destructive, costly, and lack portability. Furthermore, they often fail to furnish comprehensive speciation data and are vulnerable to interference from complex environmental matrices.

Detection of plastic and heavy metals differs significantly because of their distinct chemical and physical properties. Plastic is non-metallic, flexible, and lightweight, while heavy metals are dense metallic elements with specific properties (Qian et al., 2018). Detection techniques involve visual inspection, spectroscopy, and monitoring for plastic, while heavy metal detection relies on methods like AAS, ICP-MS, and X-ray fluorescence spectroscopy. Plastic pollution is a primary environmental concern due to its non-biodegradable nature (Campanale et al., 2020; Wani et al., 2023b). Sample collection and preparation vary for each, and their detection occurs at different scales. Both detections are crucial in addressing environmental pollution and its impact on ecosystems and human health. The commonly employed visual and spectroscopic techniques, such as optical microscopy (Kankanige and Babel, 2020), FTIR (Caldwell et al., 2022), and Raman spectroscopy (Lin et al., 2022b), exhibit evident limitations in accurately detecting microplastics of small sizes and offering quantitative data. A new approach, which eliminates the need for sample pretreatment, was devised to achieve precise quantification of polyethylene using a modified Pyr-GC-MS/MS method. The developed strategy demonstrated satisfactory recovery rates of 97–110%, exceptional sensitivity with a LOD of 1 pg, and reliable precision with relative standard deviations between 3% and 13% (Luo et al., 2023). The drinking water samples of an urban area in Norway were examined to identify microplastics with a size of $\geq$ 1 μm. A specialized modular filtering sampling device was made to enable sequential on-site sample preparation before conducting pyr-GCMS analysis of the samples. The analysis revealed a total polymer concentration between 6.1 and 93.1 μg/m3 at different sites. Elevated levels of polyethylene, polyamide, and polyester were found in the raw water. However, effective reduction rates between 43% and 100% were observed after the water treatment processes, subject to the type of polymer used (Gomiero et al., 2021). Sub-samples were subjected to analysis using two highly sophisticated and reliable analytical techniques: Py-GCMS and μFTIR imaging. Both methods effectively identified low concentrations of microplastics in the drinking water samples. Nevertheless, it was observed that Py-GCMS and μFTIR revealed different polymer types in samples with overall low microplastic content (Kirstein et al., 2021). While conventional analytical methods exhibit favorable sensitivity and reproducibility for detecting trace agrochemicals, they face challenges such as portability, high costs, slow processes, and significant consumption of organic solvents. Consequently, achieving in-situ and real-time monitoring using these methods becomes challenging. In response, researchers have been exploring novel and alternative approaches for agrochemical detection, including enzyme-linked immunosorbent assay, electrochemical methods, and optical sensor techniques. These new methods offer the potential for improved portability, cost-effectiveness, and rapid analysis, making them promising candidates for real-time and in-situ monitoring of agrochemicals.

3. Nucleic acid-based contaminant detection by conventional methods

Molecular diagnostic tools like real-time PCR and Reverse Transcriptase PCR have been conventionally used to detect various microbes. The molecular diagnostic techniques are accurate and sensitive but require professional instruments, costly reagents, and trained personnel (Li et al., 2023e). Furthermore, molecular diagnostic techniques are inappropriate for point-of-care testing (Li et al., 2022). Similarly, culture-based methods using differential, chromogenic, or selective media are also conventionally used for microbial detection (S. Wang et al., 2023). Biochemical assays such as substrate utilization tests or enzyme tests like oxidase or catalase tests have been used conventionally for the detection of various microbes (Bascomb and Manafi, 1998). Culture-based and biochemical methods are easy and inexpensive but can be laborious, time-consuming, and lack specificity and sensitivity (Vasavada et al., 2020). Another method that is used conventionally for the detection of microbes is microscopy, which can be used to detect viable but non-culturable microbes and other microbes that are hard to culture (Vasavada et al., 2020). Immunoassays like enzyme-linked immunosorbent assay, gold-labeled immunosorbent assay, and reversed passive latex agglutination assay can also be used to detect various microbes (Priyanka et al., 2016). Other tools that can be used for microbial detection are biosensors. Various biosensors, such as optical, piezoelectric, or electrochemical, have been employed to detect microbes from different samples such as water, food, clinical, and other environmental samples (Castillo-Henríquez et al., 2020; Kotsiri et al., 2022). Whole genome sequencing, 16 s RNA sequencing, and metagenomics can be used to identify and detect unculturable microbes. Metagenomics, an unculturable approach, is used to detect nucleic acid contaminants in water and other environments. It involves sequencing genetic material directly from samples without the requirement of culturing organisms. The process includes sample collection, DNA extraction, sequencing, and bioinformatics analysis. Researchers can identify potential contaminants and assess their prevalence by comparing the obtained sequences to databases. Metagenomics offers a comprehensive view of microbial communities and their functions, aiding in contaminant detection and environmental monitoring. Despite its complexities, metagenomics is valuable for its unbiased and thorough assessment of nucleic acid contaminants (Azli et al., 2022; Wani et al., 2022d, 2022a, 2022b; Wani et al., 2023c). Conventional culture methods for detecting nucleic acid contaminants suffer from biases, slow processing, selectivity, and viability limitations (Gazdik et al., 2016). Sequencing technology, while powerful, has challenges, including short reads, errors, cost, and computational demands (Chiara et al., 2021). Metagenomics, although innovative, presents complexities in data analysis, detection limits, reliance on reference databases, and functional inference (Lombard et al., 2011; Wani et al., 2022).

4. Novel biosensors for contaminant detection

Electrochemical biosensors have gained significant attention as a substitute for traditionally used sensing tools for environmental contaminant detection. The main focus when synthesizing these biosensors is to improve surface conductivity, enabling efficient electron transfer amid the analytes and surface of the biosensor (da Silva et al., 2017; Rhouati et al., 2022). Thus, it is crucial to incorporate conductive elements during electrochemical biosensor fabrication. To address this limitation, a range of modifiers, such as carbon nanostructures, metallic nanoparticles, and redox pairs, have been employed to enhance the conductivity of the biosensors. Moreover, to achieve greater selectivity and sensitivity in such sensors, biomolecules such as DNA, RNA, antibodies, aptamers, and enzymes have been employed (Baig et al., 2019; Gumpu et al., 2015; Islam et al., 2021; Lawal, 2016; Zhou and Guo, 2015). These biomolecules interact specifically with analytes, enhancing the biosensor's selectivity. Various electrochemical biosensors have been developed to detect biological and non-biological environmental contaminants (Fig. 1). A novel electrochemical sensor has been developed, employing a composite of Fe3O4 functionalized graphene oxide and gold nanoparticles (AuNPs/Fe3O4-APTES-GO) for the detection of catechol (CC) and hydroquinone (HQ), commonly co-occurring phenolic compounds in environmental samples. The sensor demonstrated linear current responses within the concentration ranges of 2–145 μM for CC and 3–137 μM for HQ. Moreover, the detection limits were determined to be 0.8 μM for CC and 1.1 μM for HQ (Erogul et al., 2015). Mariana et al. (2016) developed a lab-on-a-chip platform for highly sensitive electrochemical detection and degradation of the atrazine. They used boron-doped diamond (BDD) electrodes in combination with a competitive magneto-enzyme immunoassay (EIA). By modifying a BDD electrode with platinum nanoparticles (PtNPs), they achieved a remarkable limit of detection (LOD) of 3.5 pM for atrazine, one of the lowest values reported (Medina-Sánchez et al., 2016). Tain et al. (2018) synthesized CuO-TiO2 hybrid nanocomposites decorated on a glass carbon electrode to develop a very selective and sensitive electrochemical biosensor to detect methyl parathion. Cyclic voltammetry confirmed the sensor's electrochemical capabilities for detecting methyl parathion in water samples. By employing differential pulse voltammetry with carefully tuned parameters, the sensor demonstrated high sensitivity in detecting methyl parathion across a broad concentration range spanning from 0 ppb to 2000 ppb, boasting an impressive low detection threshold of just 1.21 ppb (Tian et al., 2018). In another research, a new electrochemical sensor was developed for detecting paraoxon ethyl (PE), a type of nitroaromatic organophosphate. It used a graphene-based NiFe bimetallic phosphosulfide nanocomposite as a unique electrocatalytic modifier. This combination enhanced the sensor's capacity for detecting PE by promoting strong interactions between PE and the sensor's surface. The sensor attained a highly linear detection range (12.3–10,000 nmol L^{-1}) and a low detection limit (3.7 nmol L^{-1}) (Aghaie et al., 2019). Another platform for detecting bisphenol A (BPA) using carbon-doped TiO_2 nanotube arrays decorated with gold nanoparticles (TiO_2/Au NTAs) has been developed. The addition of gold nanoparticles enhanced the photoelectrochemical properties and the electrocatalytic activities of the resulting hybrid nanotube arrays. Under UV irradiation, the electrochemical detection of BPA on the TiO_2/Au NTAs electrode was notably enhanced, as it provided a continuously fresh reaction surface, resulting in increased photocurrent due to improved separation efficiency of photogenerated electron-hole pairs, achieved through the consumption of holes by BPA. This research revealed that the TiO2/Au NTAs electrode holds promise for long-term BPA monitoring, with a low detection limit of 6.2 nM (Hu et al., 2016). A very sensitive and selective electrochemical biosensor for detecting $Hg2^{+}$ and $Pb2^{+}$ ions was developed

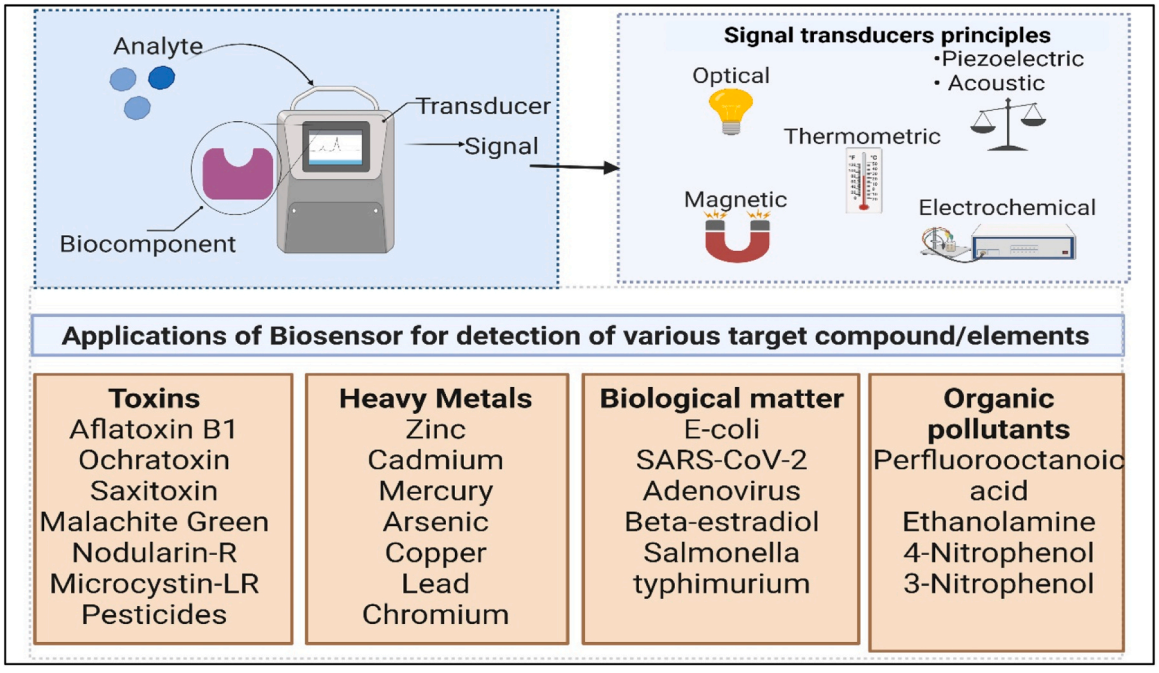

Fig. 1. Working principle of biosensors and their application for the detection of various target molecules.

in a different study. This study labeled DNA-based aptamers with ferrocene (or methylene blue) and thiol groups at their ends. These aptamers were attached to gold electrodes, and changes in their shape upon binding to Hg^{2+} and Pb^{2+} ions increased electron transfer, enabling electrochemical detection. The biosensor could detect these ions at low concentrations (0.1 ng/mL or 0.1 ppb) and showed high specificity, with affinity constants of about 9.10−7 mol for the aptamers used (Abu-Ali et al., 2019). In another study, a practical and reusable electrochemical aptasensor for detecting mercury ions (Hg^{2+}) was developed. It utilized thymine-Hg^{2+}-thymine (T-Hg^{2+}-T) coordination chemistry and nanoporous gold (NPG) for signal amplification. By assembling a thiol-modified T-rich hairpin capture probe onto the NPG-modified electrode, it could hybridize with a ferrocene-labeled T-rich probe in the presence of Hg2+, leading to a quantitative detection of Hg^{2+} in a wide range (0.01–5000 nM) with an extremely low detection limit (0.0036 nM), meeting strict water quality standards (Zeng et al., 2017). An electrochemical sensor for detecting Hg^{2+}, Pb^{2+}, and Tl^{+} ions using a composite electrode made of graphene, OPFP, and a new synthetic phosphorus ylide has been developed. The sensor can simultaneously detect these ions with high selectivity and stability without the need for prior separation from complex mixtures. The sensor shows low detection limits (3.86×10^{-10} mol L^{-1} for Hg^{2+}, 4.50×10^{-10} mol L^{-1} for Pb^{2+}, and 3.57×10^{-10} mol L^{-1} for Tl^{+}) and has been successfully applied to detect these trace ions in water and soil samples (Bagheri et al., 2015).

Detecting microbial contaminants in food and water has always been challenging. Superparamagnetic iron oxide nanoparticles (SPIONs) conjugated with *Escherichia coli*-specific aptamer-I for detecting of *E. coli*, both qualitatively and quantitatively, have been developed. The study utilized magnetic separation to isolate the *E. coli*-SPION complex. The presence of *E. coli* was confirmed by standard methods and Confocal Laser Scanning Microscopy (CLSM) using Aptamer II labeled CdTe-MPA quantum dots (QDs). The study also introduced a biosensor prototype based on Aptamer II labeled CdTe-MPA QDs and an ATmega328P microcontroller. This biosensor prototype demonstrated the capability to detect *E. coli* quantitatively and qualitatively in water samples at low bacterial counts (down to 1 × 102 cfu) (Pandit et al., 2022).

Table 2
Biosensors for detection of various toxic molecules in water and other environments.

Biosensor	Target	Sample matrix	Type of sensor	LOD	References
Schiff Base Ligand modified screen-printed carbon electrode (SPCE)	Zn	Water	Electrochemical	1 μM	(Bressi et al., 2022)
Smartphone-based aptamer and (AuNPs)	Cd	Water	Electrochemical	0.02 pm	(Xu et al., 2019)
ATR-SEIRAS technique and immobilized aptamer	Hg	Drinking water	Surface-enhanced infrared absorption spectroscopy	0.01 μM	(Liu et al., 2018)
Single walled carbon nanotube (SWCNT) biosensor	Oxytetracycline	Environmental and industrial samples	Electrochemical	2.5 nM	(Yildirim-Tirgil et al., 2019)
FRET-based turn-on fluorescent aptasensor	17β-estradiol (E2)	Milk, urine, and water	Fluorescence resonance energy transfer (FRET)	0.35 nM	(Zhang et al., 2018)
DNAzyme sensor	Pb	Water	Electrochemical	1.26 fM	(Meng et al., 2021)
Screen-printed electrochemical aptamer-based sensor	Chlorpyrifos	Fruits	FTIR, Electrochemical, Voltametry	0.097 ng/mL	(Inam et al., 2022)
Aptasensor based on an Exo III-assisted catalytic hairpin assembly (EACHA)	Chlorpyrifos	Water, fruit and vegetable	Self-fluorescence	0.93 ng/mL	(Yuancong Xu et al., 2022)
Single-stranded DNA-based fluorescence polarization aptamer	Organophosphorus	Food	Fluorescence	13.4 nM to 23.4 nM	(Zhang et al., 2014)
AuNP-based multi-aptasensor	edifenphos and Iprobenfos	Food	Colorimetric	10 nM for edifenphos and 5 nM for Iprobenfos	(Kwon et al., 2015)
Single-labeled multifunctional aptamer	Cd	Water	Fluorescence	2.15 nM	(Zhu et al., 2017)
Aptamer-AuNPs-based colorimetric assay with a smartphone-coupled optical unit	As	Soil	Colorimetric	14.44 ppb for aqueous samples and 1.97 ppm for soil samples	(Siddiqui et al., 2020)
Magnetic Halloysite Nanotube-Based SERS Biosensor	Bisphenol	Water	SERS	0.75 pg/mL	(Sen Li et al., 2022)
DNA aptamer-nanopore sensors	*SARS-CoV-2* and *adenovirus*	Water, saliva, and serum	Conductometric	1 Pfu/mL and 1 × 10^4 copies/mL, respectively	(Peinetti et al., 2021)
Photoelectrochemical aptasesor	*E. coli* O15J_HJ	Water	Photoelectrochemical	200 CFU/mL	(Zheng et al., 2021)
Aptamer-coated Fe3O4 magnetic particles (Apt-MNPs) and QD-labeled ssDNA2	*Salmonella typhimurium*	Water and food	Fluorescence	1 CFU/mL	(Ren et al., 2019)
Aptamer-based lateral flow test strip	*S. typhimurium*, *E. coli* and *Staphylococcus aureus*	Food	Colorimetric	≤10^4 CFU/mL	(Lu et al., 2020)

Another study developed a real-time and continuous method for detecting single bacterial cells using aptamer-conjugated fluorescent nanoparticles (A-FNPs) and an optofluidic particle-sensor platform. A-FNPs specifically labeled target bacteria like *E. coli*, allowing them to be counted in the optofluidic sensor. The system achieved rapid and continuous detection of individual bacterial complexes with high accuracy (~85%). It demonstrated a strong affinity for *E. coli* but did not bind to other strains lacking aptamer affinity. This approach could be extended to detect other microorganisms like viruses when conjugated with A-FNPs, highlighting the versatility of optofluidic systems for microbial cell detection (Kadam et al., 2022). Ghalkhani et al. (2022) presented a new aptasensor utilizing a nanocomposite (Ag–Cs-Gr QDs/NTiO2) for the quick and highly sensitive detection of Staphylococcus aureus. The sensor, constructed on modified screen-printed carbon electrodes, exhibits a detection limit of 3.3 CFU/mL and outperforms conventional methods when tested with human serum samples. It offers a straightforward and biocompatible approach, ensuring precise S. aureus detection without biomolecule interference in real samples (Ghalkhani et al., 2022). Zhan and colleagues introduced an innovative colorimetric platform for quickly detecting bacteria. This method employed silver nanoplates as a chromogenic substrate and capitalized on aptamers' exceptional specificity and affinity. By integrating catalase into a sandwich structure established through a dual-aptamer recognition strategy, they translated bacterial detection signals into observable shifts in localized surface plasmon resonance (LSPR) and changes in color. This approach enabled the naked-eye detection of *S. aureus* at concentrations as low as 60 CFU/mL (Zhan et al., 2022). An array of studies have been conducted in the realm of environmental toxin and contaminant detection aimed at addressing this crucial concern. Several comprehensive reviews of the literature have been published that delve deeply into the extensive body of research dedicated to detecting various forms of organic and inorganic pollutants in our environment (Goswami, 2020; Kadam and Hong, 2022b; Khanmohammadi et al., 2020; Van Dorst et al., 2010). Furthermore, beyond the broad spectrum of studies in this field, Table 2 has been included to spotlight pivotal and noteworthy research endeavors specifically.

5. CRISPR/Cas12a as a sensing tool

The Cas12a nuclease, a prominent member of the CRISPR system, has emerged as a pivotal molecular tool in the field of genome editing and nucleic acid detection. Characterized by its endonucleolytic activity, Cas12a, also known as Cpf1, exhibits unique features that distinguish it from the extensively studied Cas9 counterpart. The Class 2 Type, V-A Cas12a system, is structured with a sequential arrangement of cas12a-cas4-cas1-cas2-CRISPR array components (Mukama et al., 2020). Resembling Cas9 in both dimensions and configuration, the Cas12a protein showcases a pair of RuvC nuclease domains that display the potential for direct alignment (Yan et al., 2019). Within its structural framework, the Cas12a protein encompasses a unique nuclease domain, which is introduced into a position akin to the RuvC-like domain, albeit not precisely identical, diverging from the conventional HNH domain configuration (Sasnauskas et al., 2023). Computational predictions have indicated that the FnCas12a crRNA sourced from *Francisella novicida* encompasses 19 nucleotide DR fragments, a spacer sequence spanning 23–25 nt, and a solitary stem-loop configuration accompanied by a pseudoknot structure. In vitro investigations have demonstrated that Cas12a autonomously accomplishes crRNA maturation. Notably, Cas12a can cleave both RNA and DNA substrates (Swarts and Jinek, 2019). Preceding DNA cleavage events, RNA cleavage occurs, facilitated by the presence of crRNA stemming from the initial reaction. The mechanism underpinning PAM recognition in the Cas12a system has been elucidated as a composite of base and conformational recognition (Nguyen et al., 2022). Single RuvC domain activity, as opposed to the concerted action of both RuvC and the nuclease domain, is necessary to cleave both the target and non-target DNA strands (W. X. Yan et al., 2019). Cleavage by FnCas12a occurs after the 18th base on the non-target strand and at or after the 23rd base on the target strand, situated distal to the PAM. CRISPR/Cas12 biosensors have emerged as powerful tools for fast and sensitive detection of specific sequences of DNA and RNA (Xiexie Liu et al., 2021).

Guide RNA design is a critical step in CRISPR/Cas12a biosensor development. It involves selecting a guide RNA that is complementary to the target DNA or RNA sequence of interest. The choice of guide RNA affects the specificity and efficiency of the biosensor, and various strategies are employed to optimize its design to minimize off-target effects and enhance target recognition. Upon binding to the target DNA or RNA through the guide RNA, Cas12 undergoes conformational changes that activate its endonuclease activity (Y. Wu et al., 2023). The utility of DNAzyme in Pb^{2+} detection is pivotal; however, its efficacy in recognizing the target is consistently influenced by interfering substances within intricate matrices. To enhance DNAzyme performance, an intelligent integration of DNA nanocage is implemented to formulate a novel probe, denoted as DNA nanocage confined DNAzyme (ncCDzy) (Lai et al., 2022). The spatial confinement effect significantly enhances the thermodynamic stability of ncCDzy, resulting in a 30% increase in catalytic activity compared to free DNAzyme. When coupled with the CRISPR-Cas12a system, this synergistic approach gives rise to a novel, specific, and sensitive detection method for Pb^{2+}, exhibiting a linear range of 10–800 nM and a LOD of 1.025 nM. Furthermore, the developed method proves effective in detecting Pb^{2+} spiked in milk, showcasing its applicability for the analysis of heavy metal ions in complex samples (Yu et al., 2024). Understanding the activation process is crucial for improving biosensor efficiency and specificity and minimizing false positives or negatives. One remarkable feature of activated Cas12 is its ability to cleave neighboring DNA or RNA molecules nonspecifically. This collateral cleavage activity can lead to signal amplification, but it may also introduce unwanted background noise (Kim et al., 2021). Understanding the underlying mechanisms of collateral cleavage is essential for optimizing the biosensor's design and minimizing off-target effects. Reporter molecules are linked to the target DNA or RNA, indicating the biosensor's activity. When Cas12 cleaves the target, it releases the reporter molecules, generating a detectable signal. The choice of reporter molecule and its linkage to the target sequence impact the sensitivity and specificity of the biosensor (Tian et al., 2020). Signal amplification, a critical aspect of CRISPR/Cas12 biosensors, enhances the detectability of the target sequence. Multiple reporter molecules can be released from a single Cas12 complex, leading to signal amplification and improved sensitivity of the biosensor. The released reporter molecules produce a signal indicating the detection of the target DNA or RNA. Various methods, such as colorimetric, fluorescence, and electrochemical detection, are employed to measure this signal (Wang et al., 2019). The choice of detection method

depends on the application and the desired sensitivity of the biosensor. Type V CRISPR-Cas12-based diagnostic methods utilize the single-stranded DNA (ssDNA) trans-cleavage capabilities of Cas proteins in combination with dual-labeled ssDNA reporters containing both a fluorescence signal and a quencher (FQ-reporters) (Xie et al., 2021). In the presence of target nucleic acids, Cas12 proteins cleave the FQ-reporters, generating detectable fluorescent signals. Alternatively, the ssDNA reporters can be tagged with different molecules, like biotin and fluorescein isothiocyanate, and signal detection can be achieved through lateral flow assays (Leung et al., 2022; Li et al., 2019). In addition to its application in target nucleic acid detection, the trans-cleavage activity of Cas12 has been harnessed as a sensitive reporter for detecting various small molecules (Fig. 2). The CaT-SMelor method combines Cas12a with allosteric transcription factors (aTFs) to detect diverse small molecules like uric acid and p-hydroxybenzoic acid (Liang et al., 2019). The aTF competes with Cas12a for binding to the same target DNA, and in the absence of small molecules, the aTF occupies the DNA. When the small molecules are present, they bind to the aTF, inducing a conformational change leading to the release of the aTF from the target DNA. This allows the Cas12a/crRNA complex to bind to the target DNA and activate the trans-cleavage activity. Moreover, Cas12a can be integrated with aptamers to detect various other targets, such as ATP, exosomes, metal ions, small organic molecules, and extracellular vesicles (Xiong et al., 2020; Zhao et al., 2020b, 2020a). The presence of these non-nucleic acid targets causes a conformational change in the aptamers, making them suitable targets for Cas12a. Consequently, this activation leads to the Cas12a trans-cleavage reaction.

CRISPR-Dx has been employed for noninvasive in vivo detection of disease-associated proteases. Chemically stabilized ssDNA barcodes are joined to nanobodies using specific linker peptides that can be precisely cleaved by in-vivo proteases. This cleavage results in the release of barcodes, enabling Cas12a-based sensitive detection for disease-associated proteases in vivo (Hao et al., 2023). An innovative approach for the identification of interactions between proteins and small molecules has been established, employing the collateral cleavage activity of CRISPR/Cas12a. This methodology centers on the utilization of a singular-stranded activator DNA molecule that has been modified with a distinct small molecule. After attaching to the complementary crRNA sequence within the CRISPR/Cas12a system, this modified activator DNA triggers the CRISPR/Cas12a cleavage activity. However, in the presence of the target protein binding to the small molecule on the activator DNA, the attached protein obstructs the physical access of the activator DNA to the crRNA sequence. Consequently, this obstruction leads to a reduction in the CRISPR/Cas12a cleavage activity. By adhering to this distinct design principle, the efficacy of this process has been reported in the successful determination of two model protein-small molecule interactions: streptavidin-biotin and anti-digoxigenin-digoxigenin pairs. These interactions were accurately quantified at concentrations as low as 0.03 nM and 0.09 nM, respectively (Kim et al., 2021). Unlike Cas9, which generates blunt ends

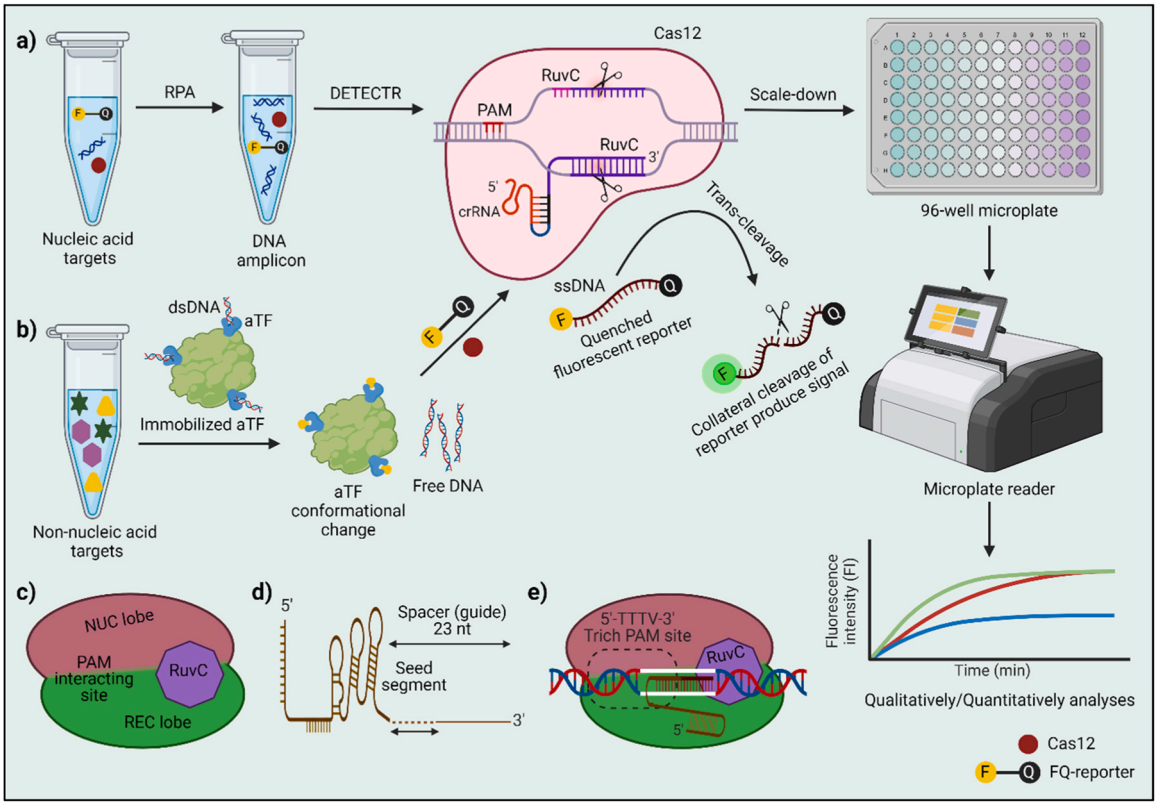

Fig. 2. Schematic representation of CRISPR/Cas12 as a sensing tool for the detection of (a) nucleic acids, involving direct amplification of DNA/RNA; (b) non-nucleic acid contaminants, achieved through signal conversion into nucleic acid signals, followed by probe recognition and molecule detection; (c) Site of interaction between REC lobe and NUC lobe; (d) Spacer guide of signals; and (e) Signal generation at RuvC site.

Table 3
Detection of industrial and biological contaminants using CRISPR-Cas12 and other effectors including Cas9, Cas13, and Cas14.

Effector	Contaminant	Methodology	LOD	Reference
Cas12a	Polychlorinated biphenyls	CRISPR-Cas12a with catalytic hairpin assembly	4.5 pM	(Deng et al., 2023a)
	Acetamiprid, and atrazine	Molecular logic gate operation based on CRISPR-Cas12a and signal amplification	2.5 and 0.2 pM	(Yan et al., 2022)
	Kanamycin	CRISPR-Cas12a with a glucometer	1 pM	(Chen et al., 2023)
	Kanamycin	Metal-Tagged CRISPR/Cas12a	4.06 pM	(Hu et al., 2021a)
	Deoxynivalenol	CRISPR-Cas12a based luminescence resonance energy transfer aptasensing platform utilizing gold nanoparticle	0.64 ng/mL	(Lin et al., 2022c)
	Sulfadimethoxine	CRISPR-Cas12a fluorescent aptasensor based on magnetic MoS_2 microspheres	2.86 pg mL^{-1}	(Lv et al., 2023)
	Deoxynivalenol	Lollipop particle counting immunoassay based on antigen-powered CRISPR-Cas12a dual signal amplification	0.061 ng/mL	(Li et al., 2023a)
	Protamine	A fluorometric biosensor based on CRISPR/Cas12a collateral cleavage	0.03 μg/mL	(Ji et al., 2023)
	Endotoxin	Mxene coupled with crispr-cas12a	11 pg/mL	(Sheng et al., 2021)
	Listeria monocytogenes	CRISPR/Cas12a-based fluorescence detection in combination with recombinase-aided amplification	1.35 $\times 10^2$ CFU/mL	(Li et al., 2021)
	Escherichia coli O157:H7	Recombinase Aided Amplification Assisted CRISPR/Cas12a System	4.4 CFU/g	(Xiao et al., 2023)
	Escherichia coli O157:H7	Filtration-based loop-mediated isothermal amplification -CRISPR/Cas12a system	1.22 × 100 CFU/mL	(Lee and Oh, 2022)
	Escherichia coli O157:H7	Thermophilic helicase-dependent amplification-based CRISPR/Cas12a system	103 CFU/g	(Kim et al., 2023)
	S. aureus	Rolling circle amplification through one pot method	50 CFU/g	(Wu et al., 2023)
	SARS-CoV-2	CRISPR/Cas12a based aptamer with trans-cleavage activity	1.5 pg mL^{-1}	(Xing et al., 2023)
	SARS-CoV-2	CRISPR-Cas12a with the integration of PAM having trans-cleavage activity	40 aM (RNA detection)	(Shi et al., 2023)
	SARS-CoV-2	CRISPR/Cas12a with isothermal recombinase-aided amplification	104 copies/reaction	(Lin et al., 2022a)
	SARS-CoV-2	Fluorescent signal readout of CRISPR-Cas12a	100 fM	(Liu et al., 2022b)
	SARS-CoV-2	CRISPR-Cas12a coupled with universal gold nanoparticle strand-displacement probe	2.7 × 10^2 copies/mL	(Liu et al., 2023a)
	SARS-CoV-2	MnO_2 Nanozyme-Mediated CRISPR-Cas12a	10 copies/μl	(L et al., 2022)
	COVID-19 nucleocapsid protein	CRISPR/Cas12a aptasensor with polyA-methylene blue electrochemical reporter	16.5 pg mL^{-1}	(Han et al., 2022)
	Monkeypox virus	CRISPR-Cas12 with recombinase polymerase amplification	22.4 aM (13.5 copies/μl)	(Q et al., 2023)
	Trichomonas vaginalis	CRISPR-Cas12a coupled with RPA-based field detection	1 copy/μl	(Shan Li et al., 2022)
	Mycoplasma hominis	recombinase polymerase amplification-Cas12a detection system	3 copies/μl	(Chen et al., 2022a)
	Salmonella	CRISPR/Cas12a with graphene oxide	5 × 10^1 copies	(Wang et al., 2023a)
	Pseudomonas aeruginosa	CRISPR/Cas12b with One Fluid-Handling Step	10 copies	(Qiu et al., 2023)
Cas9	Cadmium hyperaccumulator *Sedum plumbizincicola*	Transcriptional regulation of hyperaccumulation	-	(Zhang et al., 2023a, 2023b)
	Cabazitaxel, docetaxel and enzalutamide, resistance	Genome-wide CRISPR/Cas9 knockout	-	(Haldrup et al., 2023)
	Elution-free DNA detection	CRISPR/Cas9 based light-up aptamer transcription	Cell lysate (0.92 aM), urine (7.7 aM), and plasma (94.6 aM)	(Song et al., 2023)
	S. typhimurium and *Escherichia coli*	Lateral flow strip combined with Cas9 nickase	100 CFU/mL	(Wang et al., 2020)
Cas13	OXA-48 and GES Carbapenemases	LAMP-CRISPR-Cas13a-based assay	-	(Ortiz-Cartagena et al., 2023)
	HIV-1	Self-digitization Through Automated Membrane-based Partitioning Based Digital CRISPR-Cas13a for Amplification-Free Quantification	2000 copies/mL	(Nouri et al., 2023)
	SARS-CoV-2	Chemical additive-Enhanced Single-Step Accurate CRISPR/Cas13 Testing system	1 copy/μL	(Wang et al., 2023b)
	SARS-CoV-2	Cas13 Assisted Saliva-based & Smartphone Integrated Testing	~200 copies	(Azmi et al., 2021)
Cas14	Creatine kinase MB	DNA hydrogel based on EXPAR and CRISPR/Cas14a	-	(Chen et al., 2022b)
	Pathogenic bacteria	Collateral cleavage activity of CRISPR-Cas14a and tag-specific primer extension	1 cfu/mL	(Song et al., 2021)
	Burkholderia pseudomallei	CRISPR/Cas14a-based electrochemical biosensor with PtPd@PCN-224 nanoenzymes	12.8 aM	(Li et al., 2023b)

upon cleavage, the creation of staggered ends by Cas12a offers potential benefits for specific applications, mainly when aiming to integrate DNA sequences with meticulous orientation. Furthermore, Cas12a exhibits the capacity to produce staggered ends by cleaving crRNA arrays, thereby generating its own functional crRNAs. This intrinsic capability for crRNA processing enables the utilization of a singular tailored crRNA array, streamlining the process of multiplexed genome editing by incorporating multiple crRNAs simultaneously.

5.1. Detection of non-nucleic acid molecules using CRISPR/Cas 12a

Nucleic acid aptamers represent a novel category of recognition entities comprised of either DNA or RNA molecules that adopt intricate three-dimensional conformations, enabling precise and high-affinity binding to designated targets. Extensive endeavors have concentrated on utilizing and manipulating aptamers as foundational components for the construction of sensors (Liu et al., 2022a). An exemplar of an aptamer-driven CRISPR-Cas12a biosensor has been devised for detecting small molecules, notably demonstrated through the discerning identification of ATP with a high degree of sensitivity and selectivity. This biosensor harnesses the inherent capabilities of a widely utilized DNA aptamer, known for its adeptness in binding small molecules, to serve as the recognition component (Peng et al., 2020). Moreover, it capitalizes on the recently ascertained trans-cleavage mechanism inherent in CRISPR-Cas12a for the purpose of signal transduction and amplification. In this context, the biosensor effectively translates ATP-induced signals into nucleic acid-based signals (Fig. 2). The resulting fluorescence readout from this prototype biosensor is characterized by its expeditiousness, simplicity, and sensitivity. The portability of this biosensor's fluorescence-based output allows for direct readings through a handheld fluorescent sensor, thus enhancing its practicality for real-time, on-site detection scenarios (Ma et al., 2017). Due to the capacity of aptamers to bind a diverse spectrum of targets in vitro, ranging from minute organic molecules and metal ions to complex proteins, viruses, and even cells, the versatility of the random molecular aptamer-dependent CRISPR-assist reporter approach has been solidified (Niu et al., 2021). This method proves effective for detecting small molecules and broadening the scope of target identification. Any analyte harboring the corresponding aptamer can be accommodated within this framework. Moreover, through the substitution of the F-Q probe with a reporter molecule like MB-ssDNA modified on the electrode, the aptamer-based strategy can be extrapolated to electrochemical biosensors, thereby expanding its potential applications and achieving enhanced sensitivity. By utilizing the CRISPR-Cas12a system, coupled with the principles of rolling circle amplification and the catalytic function of gold nanoparticles, an aptasensor employing colorimetric indicators has been developed. This innovative sensor exhibits exceptional sensitivity in detecting aflatoxin. When aflatoxin is present, the activity of CRISPR-Cas12a is quelled, prompting the formation of substantial ssDNA structures on the surface of AuNPs through the application of T4 DNA ligase and phi29 DNA polymerase. The outcomes demonstrated a pronounced specificity of the biosensor for aflatoxin, showcasing its potential for highly accurate detection with an impressive LOD of 0.05 ng/L (Abnous et al., 2021). A novel strategy has been reported, wherein the integration of "aptamer-locker" DNA with CRISPR/Cas12a-based biosensing enables the swift and highly sensitive detection of melamine. The method demonstrates an impressive LOD of 38 nM, which notably falls beneath the regulatory threshold of 1.0 mg/kg established for permissible melamine content in infant milk products (Qiao et al., 2021). The integration of CRISPR/Cas12a technology with strand displacement amplification has been documented to achieve remarkably sensitive aptamer-based sensing of cadmium (II) ions. This innovative methodology has been proven effective in discerning the presence of Cadmium (II) ions at levels as low as 60 pM, showcasing its exceptional selectivity. The functionality of this aptasensor has been successfully verified through assessments conducted on authentic water and rice samples, affirming its practical utility (X. Ma et al., 2023). A CRISPR-Cas12a system has been developed for colorimetric Pb^{2+} detection, aided by DNAzyme and nanozyme components, yielding a detection sensitivity as low as 0.54 nM (Xu et al., 2023). An electrochemical biosensor employing the CRISPR/Cas12a system has been innovatively constructed, incorporating the distinctive capabilities of the GR-5 DNAzyme with a specific recognition for Pb^{2+}. In this devised approach, the GR-5 DNAzyme functions as an intermediary for signal transduction, facilitating the conversion of Pb^{2+} ions into nucleic acid signals. Consequently, these signals manifest as ssDNA, instigating a subsequent strand displacement amplification reaction. The method thus formulated demonstrates a notably low LOD, reaching an impressive threshold as diminutive as 0.02 pM (Yue et al., 2023; Fu et al., 2022) has introduced a fluorescence-based assay designed to detect organophosphorus pesticides, utilizing a strategic implementation of the CRISPR-Cas12a system. In this approach, acetylcholinesterase catalytically hydrolyzes acetylthiocholine, yielding thiocholine as a subsequent product. This thiocholine-mediated process triggers the degradation of MnO_2 nanosheets, causing the liberation of ample Mn^{2+} ions essential for the activation of an Mn^{2+}-dependent DNAzyme. This activated DNAzyme, acting as the catalytic product, further instigates the initiation of the CRISPR-Cas12a system, effectively inducing the cleavage of a DNA reporter probe marked with a fluorophore-quencher label. This cleavage event results in a noticeable escalation of fluorescence intensity within the solution. Following meticulous optimization, the limits of detection have been established for distinct organophosphorus pesticides: paraoxon, dichlorvos, and demeton, with respective values of 270, 406, and 218 pg/mL. The MnO_2 nanosheets and the implementation of three successive rounds of enzymatic signal amplification, this proposed fluorescence-based assay holds significant promise for the precise detection of organophosphorus pesticides within agricultural products. Inspite of the various application of CRISPR/Cas12 as a biosensing tool for detecting agrochemicals and environmental contaminants, it presents challenges related to specificity, assay optimization, sample complexity, signal amplification, quantitative analysis, multiplexing, field usability, regulatory approval, stability, and ethical considerations (Mir et al., 2022). Table 3 gives a deeper insight into the applicability of Cas12 effector in detecting various contaminants in water and other environments.

5.2. Detection of nucleic acid-based contaminants using CRISPR/Cas12a

The expeditious identification of nucleic acids holds pivotal significance in the clinical assessment of an extensive array of both infectious and non-infectious maladies. A dual-mode biosensor utilizing CRISPR-Cas12a was engineered to achieve highly sensitive and cross-validated detection of pathogenic bacteria. In a straightforward process, the amplicons derived from the invA sequence specific to *Salmonella* initiated a CRISPR-Cas12a-mediated degradation of single-stranded DNAs. These DNAs were originally intended to connect two pairs of gold nanoparticle probes, altering aggregation to dispersion. The resultant observable color changes were further enhanced following centrifugation (Ma et al., 2021). Although CRISPR-based diagnostic systems have been firmly established as proficient ways for the prompt and precise detection of nucleic acids, they confront a challenge in terms of limited detection sensitivity due to the lack of a preliminary target pre-amplification process. Broughton et al. have designed a CRISPR-Cas12-based lateral flow assay that can detect SARS-CoV-2 within 40 minutes from RNA isolated from the respiratory swabs. The CRISPR-Cas12-based SARS-CoV-2 detection has been reported to be faster and have comparable accuracy to the real-time RT-PCR assay (Broughton et al., 2020). CRISPR-Cas12a-based techniques have also been successful in detecting the various variants of concern of SARS-CoV-2 (Fasching et al., 2022; Liang et al., 2021). The CRISPR-Cas system's amplification-free detection is a novel cutting-edge method that eliminates the need for traditional DNA amplification techniques. This approach harnesses the precision of the CRISPR technology, using engineered Cas enzymes to detect and cleave target DNA or RNA sequences directly. The process involves specific target recognition, binding, and cleavage, leading to the generation of detectable signals (Qian et al., 2022; Shinoda et al., 2021). Scientists developed a SERS-signaled bioassay, incorporating CRISPR/Cas technology, designed to detect amplification-free and interference-resistant detection of SARS-CoV-2 in food and environmental samples. This innovative approach utilizes a single tube-in-tube vessel. Experimental validation demonstrated the capability of our proposed nanobioassay to detect SARS-CoV-2 at concentrations as low as 200 copies/mL within a rapid timeframe of 45 minutes without the need for pre-amplification processes (Ma et al., 2023c).

Similarly, another Cas12-based lateral flow assay and fluorescent readout techniques detected the Hepatitis B virus with 100% sensitivity and specificity within 10–20 minutes with a LoD of 1 copy/μL (Ding et al., 2021). Another CRISPR-Cas12a-based diagnostic platform that uses a real-time fluorescence instrument or lateral flow biosensor has been able to detect nucleic acid from *Mycobacterium tuberculosis* complex (MTC) within an hour with 100% specificity and LOD of 10 copies of MTC genomic template per test (Wang et al., 2021). Recently, FELICX, comprising flap endonuclease, Taq ligase, and CRISPR-Cas12, detected SARS-CoV-2 and Epstein-Barr virus. "FELICX represents a significant advancement over current CRISPR-based diagnostic techniques due to its remarkable capability of swiftly detecting nucleic acids precisely, without being dependent on PAM sequences (Aggarwal et al., 2023). Li et al. integrated the Cas12 system with recombinase polymerase amplification (RPA) in one tube to detect methicillin-resistant *Staphylococcus aureus* within 20 minutes, where the results can be visualized by lateral flow assay or fluorescent readout techniques (Li et al., 2022). Another study also reported the use of RPA integrated with the Cas12 system to detect *Leptospira spp* with 100% specificity, 92.7% accuracy, and 85.2% sensitivity in one tube (Jirawannaporn et al., 2022). The one-tube RPA integrated with the Cas12 system also detected *Helicobacter pylori* gene fragments as low as 2 copies/μl within 30 minutes (Dai et al., 2022). A PCR-based CRISPR/Cas12 nucleotide detection method detected *Klebsiella pneumonia* within 2 hours (S. Wang et al., 2023). G-quadruplex-probing CRISPR-Cas12 technique detected the food-borne pathogen *Salmonella enterica* as low as 20 colony-forming units (CFU) (Xia et al., 2021). The proximal DNA probe-based CRISPR-Cas12 technique also detected *S. enterica* with an LoD of 619 CFU without any DNA amplification process (T. Zhang et al., 2021). The creation of a DNA biosensor utilizing the CRISPR-Cas12a system, devoid of the requirement for nucleic acid amplification, was achieved through the integration of Au nanoparticle-facilitated metal-enhanced fluorescence and colorimetric analysis. This swift and exceedingly discerning sensor possesses the capability to be extended for the quantification of diverse nucleic acid biomarkers, including viral DNA. This adaptable sensor system can be beneficial for deployment in both field-based and point-of-care testing setups (Choi et al., 2021). Researchers have successfully crafted a CRISPR-Cas12a-based aptasensor tailored for the sensitive and selective detection of ATP. Within this aptasensor framework, an ATP-binding aptamer was meticulously designed to serve as the specific ssDNA target for Cas12a. Consequently, in the presence of ATP, the ATP-binding aptamer undergoes occupation by ATP, resulting in reduced processing of the target ssDNA by Cas12a. This event leads to an ATP concentration-dependent alteration in fluorescence, stemming from the trans-cleavage of a doubly labeled ssDNA reporter. The dynamic detection range spans from 1 μM to 200 μM, with a limit of detection quantified at 400 nM (Peng et al., 2020). In a separate investigation, researchers devised SMART-Cas12a (Small-Molecule Aptamer Regulated Test using CRISPR/Cas12a), a versatile biosensing platform for detecting non-nucleic acid small molecules. The approach involves triggering a hybridization chain reaction cascade signal amplification initiated by a functional nucleic acid (aptamer) upon target binding. Subsequently, the CRISPR/Cas system is integrated to identify the amplified products, leading to activation of trans-cleavage. ATP was chosen as the model target, and optimal conditions yielded excellent analytical performance with a linear range spanning 0.1–750 μM and a detection limit of 1.0 nM (Ma et al., 2023a).

The use of the CRISPR-Cas12 system in the detection of foodborne pathogens represents a powerful and innovative approach. This system, known for its precision in nucleic acid recognition, has been harnessed to specifically identify and detect pathogens associated with foodborne illnesses. By leveraging the programmable nature of the Cas12 enzyme, researchers have developed biosensors that can rapidly and accurately detect the presence of foodborne pathogens in various samples (Mao et al., 2022). A microfluidic paper-based analytical device was engineered to integrate recombinase polymerase amplification for the SERS detection of pathogenic bacteria. This innovative device demonstrated superior sensitivity, specificity, and quantification capabilities, achieving rapid detection of *S. typhimurium* in under 45 minutes (Zhuang et al., 2022). Scientists have innovated a versatile biosensing platform, denoted as SCENT-Cas, designed for the highly sensitive detection of pathogenic bacteria. This platform combines ratiometric fluorescence derived from silver nanoclusters as outputs along with the CRISPR-Cas12a system. SCENT-Cas exhibits the capability to detect

S. typhimurium at concentrations as low as 1 CFU/mL with a dynamic range spanning from 1 to 10^8 CFU/mL (L. Ma et al., 2023b).

6. Other Cas effectors (Cas9, Cas13, and Cas14) for detection and diagnostics

6.1. Cas9

The Cas9 nuclease, a cornerstone of the revolutionary CRISPR system, has revolutionized the landscape of genome editing and genetic engineering. Derived from the bacterial immune system, Cas9 is renowned for its unparalleled precision in recognizing and cleaving specific DNA sequences within a target organism's genome. Initially discovered in *Streptococcus pyogenes*, Cas9's utility stems from its programmable nature, guided by short RNA sequences known as gRNAs (Jiang and Doudna, 2017). The fundamental tools for detecting DNA and RNA rely predominantly on fluorescent systems, which augment the equipment costs essential for signal detection (Xiao et al., 2022). It is plausible to extensively employ the principles underpinning DNA metabolism to broaden the potential of DNA sensors reliant on electrochemical signal reactions for molecular applications. Notably, the CRISPR-Cas9 method, acknowledged with the 2020 Nobel Prize in Chemistry, holds promise as a versatile analytical technique in this context (Strzyz, 2020). Moreover, attaching DNA-modifying enzymes to a substrate through covalent bonds or crosslinking offers viable solutions to issues concerning enzyme reusability, consumption, and ecological considerations. Cas9, also referred to as "CRISPR-associated protein 9," is an enzymatic molecule that utilizes CRISPR sequences as molecular guides to specifically recognize and cleave DNA strands that possess complementarity to these CRISPR sequences (Lujan et al., 2020). Through the collaborative action of Cas9 enzymes and CRISPR sequences, an influential technology called CRISPR-Cas9 emerges, offering the capability to modify genetic material within organisms. This process of genetic alteration holds diverse implications spanning fundamental biological research, the advancement of biotechnological commodities, and therapeutic intervention in medical conditions.

The pioneering development of the CRISPR-Cas9 genome editing technique garnered the esteemed Nobel Prize in Chemistry in 2020, an accolade attributed to the contributions of Emmanuelle Charpentier and Jennifer Doudna (Doudna and Charpentier, 2014, p. 9). Utilizing the Cas9 effector to detect biological and industrial contaminants involves harnessing the CRISPR/Cas9 system's specificity to identify target molecules of interest. By customizing the guide RNA sequence, Cas9 can be directed to bind to specific contaminant sequences. This binding triggers the Cas9 enzyme to induce a double-strand break at the target site. Detection methods can then exploit this enzymatic activity to signal the presence of the contaminant. The Bio-SCAN methodology (biotin-coupled specific CRISPR-based assay for nucleic acid detection) exhibits the capability to rapidly identify the SARS-CoV-2 genome within a time frame of less than one hour, starting from the initial sample collection and culminating in the test outcome. In contrast to alternative CRISPR-Cas-based assays for detecting pathogens, the implementation of Bio-SCAN in conjunction with the Cas9 effector obviates the necessity for supplementary reporters, probes, enhancers, reagents, or intricate apparatuses for the interpretation of results. Notably sensitive, Bio-SCAN effectively identified synthetic SARS-CoV-2 RNA genomes at a clinically pertinent concentration of 4 copies/μL, underlining its diagnostic precision and efficiency (Ali et al., 2022; Marsic et al., 2021). A CRISPR/Cas9 abrogation mechanism was engineered and subsequently integrated into both RT-PCR and PCR platforms. This innovative integration demonstrated remarkable precision in the identification of African swine fever virus and SARS-CoV-2 through a user-friendly lateral flow assay (Lin et al., 2021). Numerous methodologies for nucleic acid detection necessitate an extensive array of reagents and intricate, costly instrumentation. In a recent study, researchers unveiled a graphene-based field-effect transistor that leverages CRISPR technology to enable the binary detection of a designated genetic sequence within intact genomic material. Upon encountering genomic DNA harboring the specific target gene, the CRISPR–Chip platform elicits a discernible signal amplification, achieving this outcome within a brief 15-minute timeframe and attaining a heightened sensitivity level of 1.7 fM, all without necessitating the conventional amplification steps. This advancement extends the purview of CRISPR–Cas9 technology toward the domain of on-chip electrical identification of nucleic acids (Hajian et al., 2019). The diagnostic potential of the CRISPR-Cas9 system was broadened through the creation of Bio-SCAN V2, an innovative CRISPR-Cas platform responsive to ligands. This advanced platform facilitated the detection of non-nucleic acid small molecule targets like theophylline. Utilizing the Bio-SCAN V2, researchers successfully identified theophylline with an impressive LOD extending to 2 μM in 15 minutes (Jiang et al., 2023). Hu and colleagues have introduced an innovative diagnostic approach termed the Dual-Cas Tandem Diagnostic Platform that ingeniously combines the utilization of Cas9 nickase and Cas12a enzymes. The Dual-Cas Tandem Diagnostic Platform operates through the orchestration of the Cas9n-sgRNA complex, which introduces a nick in the target strand's double-helix structure. Subsequently, DNA polymerase displaces the ssDNA, setting off a sequence of DNA replication cycles encompassing nicking, displacement, and extension events. Detection ensues as the ssDNA comes under scrutiny by the Cas12a-crRNA complex. This engagement triggers trans-cleavage, eventually causing the emission of a fluorescent signal by the attached fluorescent reporter molecule. Notably, the Dual-Cas Tandem Diagnostic Platform displays remarkable attributes including heightened sensitivity (1 CFU/mL), exquisite specificity, and impeccable accuracy (100%) (Hu et al., 2023). Cas9 cleaves DNA via sgRNA and requires a PAM sequence, while Cas12 cleaves DNA via crRNA and recognizes T-rich PAM. Cas9-based biosensing requires signal amplification for sensitivity, while Cas12's collateral cleavage provides intrinsic signal amplification. Cas12 offers advantages like multiplexing and sensitivity due to collateral cleavage, making the choice dependent on application specifics. The efficacy of a CRISPR experiment, encompassing factors like targeting precision and cleavage efficiency, predominantly hinges on the judicious selection of the most suitable CRISPR RNA molecule. This RNA molecule should be adeptly tailored to match the intended target site with the least possible occurrence of off-target effects. In recent times, a plethora of computational tools have been formulated to facilitate the strategic design of optimal CRISPR RNAs. These tools are instrumental in ensuring the Cas protein's accurate engagement with a predetermined genomic region, across diverse species. Notwithstanding the escalating array of available resources, it remains conspicuous that a significant majority of contemporary crRNA design tools are primarily tailored to accommodate the Cas9 nuclease type (Nidhi et al.,

2021). In contrast, a limited selection of tools has emerged to facilitate the formulation of crRNAs targeting diverse CRISPR-Cas systems, including but not confined to, Cas12 or Cas13. Notable examples of these tools encompass CHOPCHOPv3, CRISPOR, and Cas13design (Concordet and Haeussler, 2018; Labun et al., 2019; Wessels et al., 2020).

The dCas9 serves as an alternative effector of Cas9. Unlike the active Cas9 enzyme, dCas9 is a catalytically dead variant that lacks endonuclease activity. Instead, dCas9 retains its ability to specifically bind to target DNA or RNA sequences due to its preserved gRNA recognition capability. Researchers leverage dCas9 in various applications, utilizing its binding specificity without inducing DNA cleavage. This alternative effector is particularly valuable for applications such as transcriptional regulation, epigenome editing, and as a molecular scaffold for the development of biosensors. The dCas9 is strategically coupled with signal transduction modules, such as fluorescent reporters or other detection mechanisms, to enable the precise identification of target DNA or RNA sequences (Safdar et al., 2022; Uygun and Atay, 2021). This biosensing strategy leverages the programmable and highly specific binding capability of dCas9, allowing for the detection and monitoring of genetic elements or gene expression levels. The integration of a dCas9-polymer dot--copper sensor with isothermal amplification demonstrates specificity in detecting nucleic acids associated with multidrug-resistant bacteria, including antibiotic-resistance genes such as *kpc-2* and *mecA*. The sensor, denoted as dCas9-PD-Cu, exhibits notable sensitivity, enabling the detection of approximately 54 femtograms of target nucleic acids. This sensitivity is attributed to the precise measurement of changes in the resistivity of silicon electrodes resulting from the capture of target nucleic acids by dCas9 (Im et al., 2024). A biosensing strategy has been devised using dCas9, designed for on-site, visual, and bimodal detection of foodborne pathogenic bacteria. This method, enabled by dCas9, demonstrates remarkable sensitivity, detecting bacterial concentrations as low as 1 CFU/mL and features a dynamic detection range spanning from 1 to 10^9 CFU/mL (Li et al., 2023c).

6.2. Cas13

In 2016, the enzyme Cas13a, previously called C2c2, originating from the microorganism *Leptotrichia shahii*, underwent comprehensive characterization. The Cas13 nuclease, an integral component of the CRISPR system, stands out for its unique and transformative capabilities in RNA manipulation. Cas13 distinguishes itself by its RNA-targeting specificity, in contrast to the DNA-targeting functionalities of Cas9 and Cas12. The ability of Cas13 to specifically cleave RNA sequences has positioned it as a powerful tool for RNA interference and nucleic acid detection (Deng et al., 2023b). Cas13a constitutes an RNA-guided endonuclease specific for RNA molecules and does not exhibit activity towards DNA molecules (Abudayyeh et al., 2016). Specifically, Cas13a interacts with ssRNA substrates. The process involves the guidance of Cas13a by its cognate crRNA to a target ssRNA molecule, facilitating binding and subsequent cleavage of the target (Han et al., 2020). Notably, akin to Cas12a, Cas13a remains bound to the initial target and consequently carries out indiscriminate cleavage of other ssRNA molecules in proximity. This attribute of collateral cleavage has been strategically harnessed for the advancement of diverse diagnostic methodologies. Aside from its primary function of cleaving target RNA molecules, Cas13 possesses the ability to nonspecifically cleave adjacent RNA molecules subsequent to binding with the target RNA. This phenomenon, called collateral cleavage, underscores an exceedingly efficient mechanism for converting and amplifying signaling processes, which is particularly advantageous for advancing biosensor technologies (Shihong Gao et al., 2021). Consequently, Cas13a has been broadly leveraged to identify RNA-based pathogens, including viruses like ZIKA and COVID (Ackerman et al., 2020; Freije et al., 2019; Rauch et al., 2021; Yin et al., 2022). The comprehensive genomic assessment known as Whole-genome Assay utilizing Tiled Surveillance of Nucleic acids exhibits the capability to identify cell-free DNA originating from the pathogenic agent Mycobacterium tuberculosis using CRISPR/Cas13. This identification takes place within the plasma of individuals afflicted with active pulmonary tuberculosis (Thakku et al., 2023, p. 13). A complex SUMO fusion tag, constructed from the integration of His, Twinstrep, and Smt3 tags, effectively mitigates the target-triggered activation of Cas13a through the obstruction of crRNA binding. This inhibitory effect is alleviated upon the catalysis of proteolytic cleavage facilitated by proteases. The modular architecture of this composite tag can be adjusted to achieve tailored responses to distinct proteases. The engineered biosensor, SUMO-Cas13a, demonstrates the capacity to discern a wide spectrum of protease Ulp1 concentrations, displaying a 48.8 pg/μL LOD within an aqueous buffer medium (Liu et al., 2023b). Zhang et al. (2023a, 2023b) have devised a notably specific and high-sensitivity identification system, amalgamating PCR, the CRISPR-Cas13a system, and lateral flow dipstick technology. This innovative system facilitates the rapid and precise detection of *Locusta migratoria manilensis* DNA (Zhang et al., 2023a, 2023b). The CRISPR-Cas13a-based detection strategy is harnessed for the visual discrimination of *L. migratoria manilensis* across distinct developmental stages, as well as with the utilization of incomplete samples. This diagnostic approach yields discernible test outcomes within a concise timeframe of 2 hours. Remarkably, the PCR-Cas13a-LFD detection system exhibits a heightened sensitivity, achieving a detection threshold of 0.1 ng/μL, which is tenfold greater than conventional PCR coupled with electrophoresis methods. Aralis and colleagues have delineated a CRISPR-Cas13-derived technique denoted as CREST (Cas13-based, robust, impartial, scalable testing), designed to identify SARS-CoV-2. This method possesses distinct qualities, including specificity, sensitivity, and extensive accessibility (Aralis et al., 2022). Scientists also showcased the efficacy of a CRISPR-Cas13-based approach against SARS-CoV-2 by proficiently degrading viral RNA. They commenced with a cellular infection experimentation, conducted a comprehensive screening of CRISPR-associated RNAs (crRNAs) designed to target conserved viral regions, and subsequently validated the functionality of these crRNAs using an in vitro model. The tailored Cas13d effectors, reprogrammed to target NSP13, NSP14, and nucleocapsid transcripts, achieved an impressively high silencing efficacy exceeding 99% in human cells afflicted with coronavirus 2 infection. Notably, this efficacy was sustained across various viral variants that have emerged over the past two years, including B.1, B.1.1.7 (Alpha), D614G B.1.351 (Beta), and B.1.617 (Delta) (Patchsung et al., 2023). Cas13 enzymes have limited use in detecting non-nucleic acid contaminants because their primary function is to target and cleave RNA molecules. These enzymes have evolved to be highly specific for RNA, and their design doesn't facilitate interactions with non-nucleic acid substances. Additionally, the risk of generating false positive signals due to potential off-target interactions with non-nucleic acid

entities hinders their application in this context. Although ongoing research explores engineering possibilities, Cas13's natural RNA-targeting nature presents challenges for detecting non-nucleic acid contaminants effectively.

6.3. Cas14

Cas12f, also called Cas14, constitutes an atypically compact exemplar of the type V-F CRISPR-Cas nucleases, demonstrating a size approximately fifty percent smaller than comparable nucleases of its classification (Harrington et al., 2018). This specific enzyme forms an asymmetrical dimeric configuration with Cas12f molecules, binding to a singular sgRNA to recognize and subsequently cleave dsDNA substrates harboring a PAM sequence enriched in thymidine (Khan et al., 2019). Despite its dimeric nature, Cas12f adopts a conserved activation mechanism reminiscent of other type V nucleases. This mechanism necessitates coordinated structural alterations instigated by creating a heteroduplex between the crRNA and the target DNA, which encompasses a transition from a closed to an open conformation within the lid motif of the RuvC domain (Tang et al., 2021). In the context of the Cas12f dimer, solely one of the RuvC domains undergoes activation upon substrate recognition, leading to the confinement of the substrate within the structurally discernible activated RuvC domain. Intriguingly, structural insights have indicated that a truncated sgRNA, with a length of less than half of the original sgRNA, retains functional capability for cleaving target DNA, bolstered by guidance from the elucidated structural framework (Xiao et al., 2021). Jennifer Doudna's research team made a significant breakthrough by uncovering the existence of Cas14, which is predominantly found within a specific group of extremophilic archaea known as a superphylum (Wang and Doudna, 2023). This recently identified Cas14 variant exhibits a unique capability for targeting ssDNA despite its size being only half that of the well-known Cas9 protein. This attribute suggests its potential role in defense against viruses that possess ssDNA genomes. Moreover, the team combined the inherent ssDNase cleavage function of Cas14 with an isothermal amplification technique (referred to as DETECTR-Cas14). This innovative fusion presents a promising avenue for accurate genotyping of DNA single-nucleotide polymorphisms, known for their role in genetic variations. Additionally, this method could be a promising approach for detecting ssDNA viruses that hold unquestionable significance in clinical, ecological, and economic contexts, as these viruses infect hosts across all three major domains of life (Aquino-Jarquin, 2019; Hu et al., 2021b). Currently, CRISPR-based biosensors primarily utilize Cas12 and Cas13 proteins, with the biosensing capabilities of the recently discovered Cas14 not yet extensively explored. In this regard, a novel fluorometric biosensor called HARRY (highly sensitive aptamer-regulated Cas14 R-loop for bioanalysis) has been created. HARRY involves a designed diblock ssDNA containing both the activating sequence of Cas14 and the aptamer sequence particular to target molecules. When no target molecules are present, the ssDNA triggers the activation of Cas14a, which subsequently cleaves a fluorescent reporter, causing the enhancement of fluorescence intensity. Conversely, in the presence of target molecules, the aptamer interacts with the ssDNA, developing an ssDNA-target complex that prevents Cas14a activation. Through this mechanism, HARRY can effectively detect molecules like aflatoxin B1, ATP, thrombin, Cd^{2+}, and histamine, achieving detection limits in the low nanomolar range. Notably, HARRY demonstrates enhanced sensitivity and versatility compared to aptasensors based on Cas12a. This improvement can be attributed to the unique ssDNA specificity of Cas14a. The study further establishes a direct correlation between the detection limit of HARRY and the binding affinities of the aptamers used in the sensor design. As a result, this research unveils the untapped potential of Cas14a for multifaceted aptamer-based sensing applications. These findings offer a basis for the potential development of CRISPR-based biosensors utilizing the Cas14a variant (B. Zhou et al., 2023). The precise and highly sensitive identification of microcystin-LR holds significant significance due to its considerable toxicity and widespread prevalence. In a study, a groundbreaking and adaptable fluorescence-based sensing mechanism (termed the Cas14-pMOFs fluorescence sensor) has been innovated through the integration of the CRISPR/Cas14a system with two-dimensional porphyrin metal-organic framework nanosheets (2D-pMOFs) for the purpose of microcystin-LR detection. By leveraging the exceptional capabilities of the Cas14-pMOFs fluorescence sensor in quantifying cDNA, an extraordinary LOD of 0.12 nM was achieved. Consequently, this sensor displayed remarkable sensitivity in detecting MC-LR within a concentration range spanning from 50 picograms per milliliter (pg/mL) to 1 microgram per milliliter (μg/mL), with a LOD of 19 pg/mL. Notably, this investigation not only introduced a fresh perspective for the development of fluorescence sensors that incorporate 2D-pMOFs in conjunction with CRISPR/Cas14a but also showcased the sensor's adaptability in discerning targets encompassing both nucleic acid and non-nucleic acid compositions (P. Wu et al., 2022). Hu and colleagues introduced an innovative detection framework that utilizes element probes within the context of CRISPR/Cas14 technology to identify non-nucleic-acid targets. By merging metal isotope detection with CRISPR/Cas14 biosensing, the platform exhibits the capability to achieve sensitive detection of targets that do not involve nucleic acids. The researchers meticulously devised and refined the element probe, revealing that Cas14 can cleave longer segments within the element probe structure. By implementing this approach, they accomplished the quantitative detection of minute concentrations of aqueous ampicillin within 45 minutes at ambient temperature (25 °C). The method achieves an exceptional detection sensitivity, showcasing a remarkably low detection limit of 2.06 nM. The technology also demonstrated outstanding performance in tests targeting interference and complex matrices, further attesting to its robustness and reliability (Hu et al., 2021b). The inherent molecular mechanisms of Cas14 offer valuable potential for biotechnological utilization while also prompting significant inquiries for subsequent investigation. The unveiling of Cas14 provides tangible empirical support for the conjecture surrounding the emergence of Class 2 effectors from a precursor akin to RuvC. Furthermore, as substantiated by the recent identification of additional Type V effectors, an evolutionary perspective centered around RuvC could prove to be a promising avenue for unearthing further variant discoveries (Hillary and Ceasar, 2023). The evolutionary revelations rendered by Cas14 and its akin proteins, coupled with adept molecular biological methodologies, will empower biochemists to harness the intrinsic modularity of Cas proteins, thereby fine-tuning and optimizing their potential for diverse biotechnological applications.

7. Future perspectives

In recent years, CRISPR/Cas12a-based systems have undergone substantial advancements, evolving into a widely accessible and influential method, particularly prominent as molecular scissors in biosensing applications. These systems, leveraging the cis- and trans-cleavage activity of Cas12a, have resulted in the development of various CRISPR/Cas biosensors. Notably, while the cis-cleavage of Cas12a is limited to a single turnover, the trans-cleavage can achieve several turnovers per second. Consequently, the trans-cleavage of Cas12a is frequently employed to cleave the reporter probe, amplifying the fluorescence signal. Efforts to enhance detection performance involve modifications to PAM, target sequences, crRNA, Cas nuclease, reporter probes, and reaction conditions within the CRISPR/Cas12a detection system. Despite these advancements, future work remains essential to improve detection sensitivity further, reduce background signals, broaden the scope of detectable targets, enable intracellular imaging, and develop point-of-care testing platforms (Kasputis et al., 2024; Lei et al., 2024). The potential for off-target effects, where unintended sequences may be cleaved, poses a risk of false-positive results and reduces overall specificity. Delivery of CRISPR/Cas components to target cells presents another hurdle, demanding efficient and effective methods. CRISPR/Cas-based methods' implementation cost can be higher than traditional biosensor techniques (Jiménez and Crosetto, 2023; Wessels et al., 2023). The integration of complementary systems involving CRISPR-Cas and aptamers represents a potent scheme for enhancing the efficacy of non-nucleic acid detection methodologies. Over the past decade, the amalgamation of these two systems has progressively supplanted conventional detection approaches.

Nevertheless, the amalgamation of diverse Cas proteins with varying aptamer methodologies, including allosteric probe-triggered catalysis and proximity-enhanced readouts, has predominantly been confined to nucleic acid detection paradigms. Consequently, forthcoming research endeavors should be undertaken to propel the advancement of contaminant detection predicated on non-nucleic acid targets within environmental contexts. Incorporating CRISPR/Cas systems into electrochemical biosensors also exhibits considerable promise, presenting augmented adaptability, heightened precision and selectivity, abbreviated recovery intervals, and the capacity to capture and discriminate analytes at minimal concentrations (J. Ma et al., 2023). Further, Integrating CRISPR/Cas assays with microfluidic platforms represents an advanced avenue for precise detection, with potential extensions to wearable technologies and smartphone-based assay readouts. In contrast to solution-based detection techniques, surface-bound electrode sensing methodologies offer distinct advantages in terms of minimizing high native concentrations of target biomarkers on the sensor area. Moreover, incorporating multiplexed chip designs, integrating smartphones for digital detection, amalgamation with machine learning algorithms, and data logging within wearable devices collectively hold significant promise for the comprehensive detection of nucleic acid and non-nucleic acid contaminants (Avaro and Santiago, 2023; Lu et al., 2023; H. Zhou et al., 2023). The requirement for a PAM sequence can limit the target selection range, as a specific PAM is essential for Cas12 recognition and cleavage. The reaction kinetics and turnover rates of Cas12a, particularly during trans-cleavage, can impact the overall efficiency and speed of the detection process (Deng et al., 2024).

8. Conclusions

The journey from conventional methods to the revolutionary CRISPR/Cas12 biosensors represents a remarkable advancement in nucleic and non-nucleic acid contaminant detection in water and beyond. The emergence of CRISPR/Cas12 biosensors has brought a paradigm shift, offering unparalleled specificity, sensitivity, and rapidity in identifying contaminants, thereby addressing many of the shortcomings of traditional methods. The comprehensive exploration of various applications ranging from environmental monitoring to medical diagnostics underscores the versatility and transformative potential of CRISPR/Cas12 biosensors. Furthermore, the integration of these biosensors with cutting-edge technologies like microfluidics, nanoparticles, and smartphone-based readouts enhances their feasibility for widespread deployment, particularly in resource-limited settings. Challenges related to robustness, multiplexing, off-target effects, and regulatory approval must be systematically addressed to ensure their reliability and acceptance. Additionally, ethical considerations surrounding genetic manipulation and biosafety necessitate ongoing deliberation as these biosensors become more prevalent. By synergizing the power of molecular biology, nanotechnology, and bioinformatics, these biosensors can revolutionize how we monitor and ensure the safety of our water sources and how we approach diagnostics and surveillance across diverse fields. This review paper serves as a comprehensive roadmap that navigates the evolution, promise, challenges, and ethical dimensions of the transition from conventional methods to CRISPR/Cas12 biosensors.

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Declaration of Competing Interest

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

Data Availability

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

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