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Review

Nanozyme-Assisted CRISPR/Cas Biosensing for Environmental Pollutant Monitoring

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Abstract: Environmental pollution increasingly threatens human health in concealed forms, such as the spread of pathogenic microorganisms and antibiotic resistance genes (ARGs), as well as the widespread presence of low-concentration small-molecule pollutants in water environments. These evolving challenges demand detection technologies with higher selectivity and sensitivity, making the development of advanced sensing platforms a critical research priority. The CRISPR/Cas system can be rationally engineered to recognize specific nucleic acid sequences, offering significant potential for detecting pathogens and ARGs. Its utility has been further extended to small-molecule sensing through the incorporation of aptamers. Nanozymes, which mimic the catalytic functions of natural enzymes while exhibiting unique physicochemical properties, can substantially enhance the performance of CRISPR/Cas-based biosensors. This review systematically examines the fundamental mechanisms and construction strategies of nanozyme-assisted CRISPR/Cas sensing platforms, details their applications in environmental pollutant monitoring, discusses current challenges and potential solutions for real-world implementation, and outlines future prospects. The aim is to provide valuable insights for further research and practical deployment of this innovative technology.

Keywords: nanozyme; CRISPR/Cas biosensing; environmental monitoring; pollutants

1. Introduction

With the deep advancement of global industrialization and urbanization, and the continuous expansion of human production and living scales, the emission forms, occurrence characteristics, and hazard pathways of environmental pollutants have become increasingly complex and diverse [1], posing a core bottleneck constraining global ecological security, sustainable development, and human health protection. Currently, humanity faces more diverse environmental pollution problems. Pathogenic microorganisms and viruses can cause outbreaks and the spread of infectious diseases, endangering human safety. Antibiotic abuse leads to the widespread dissemination of antibiotic resistance genes (ARGs) in environmental media, enhancing the drug resistance of pathogens and posing great difficulties for clinical treatment [2]. Heavy metal ions such as lead, mercury, and cadmium, after accumulating in the environment, can enter the human body through the food chain, causing nervous system damage, kidney diseases, and other problems [3]. Refractory organic pollutants such as pesticides [4], polycyclic aromatic hydrocarbons (PAHs) [5], and persistent organic pollutants (POPs) [6] persist in the environment at low but widely distributed levels, causing long-term harm to ecosystems and human health. Moreover, the current forms of environmental pollutants are more stealthy. Therefore, the current environmental pollution issues impose higher demands on pollutant detection technologies.

Developing highly selective, sensitive, and convenient monitoring technologies is crucial for ensuring environmental safety and human health.

Environmental monitoring technology, as the "eyes and ears" and "cornerstone" of the environmental management system, serves as the core support for pollution source tracing, process supervision, effectiveness evaluation, and risk warning. Its technical level directly determines the scientificity and effectiveness of environmental governance. Traditional environmental monitoring technologies such as chromatography-mass spectrometry (MS) [7], polymerase chain reaction (PCR) [8], Raman spectroscopy [9], enzyme-linked immunosorbent assay (ELISA) [10], and genomic sequencing [11], although possessing high detection accuracy, rely on advanced laboratory equipment, cumbersome sample pretreatment, and professional technical personnel, forming a high-cost and time-consuming detection process [12]. They cannot meet current needs for trace, rapid, in situ, and high-throughput monitoring. In this context, biosensing technology, with its advantages of strong specificity, simple operation, rapid response, and low cost, has gradually become a research hotspot in the field of environmental pollutant monitoring, providing a new path to address the bottlenecks of traditional technologies. Over the past few decades, sensors capable of converting specific physical and chemical changes into usable signal outputs have become viable alternatives, among which biosensors and microfluidic sensors have been used to detect pathogens [13], heavy metals [14], organic pollutants [15], etc., in the environment. Furthermore, single-component biosensing technologies still confront multiple technical challenges: natural enzymes are susceptible to inactivation and exhibit inadequate stability, thereby restricting the service life and environmental adaptability of sensing systems; conventional signal amplification strategies suffer from insufficient efficiency, rendering accurate detection of ultra-trace pollutants challenging; interference from complex environmental matrices frequently impairs the specificity and accuracy of detection. To address these bottlenecks, the integration of advanced functional materials with molecular biology technologies has emerged as an inevitable development trend.

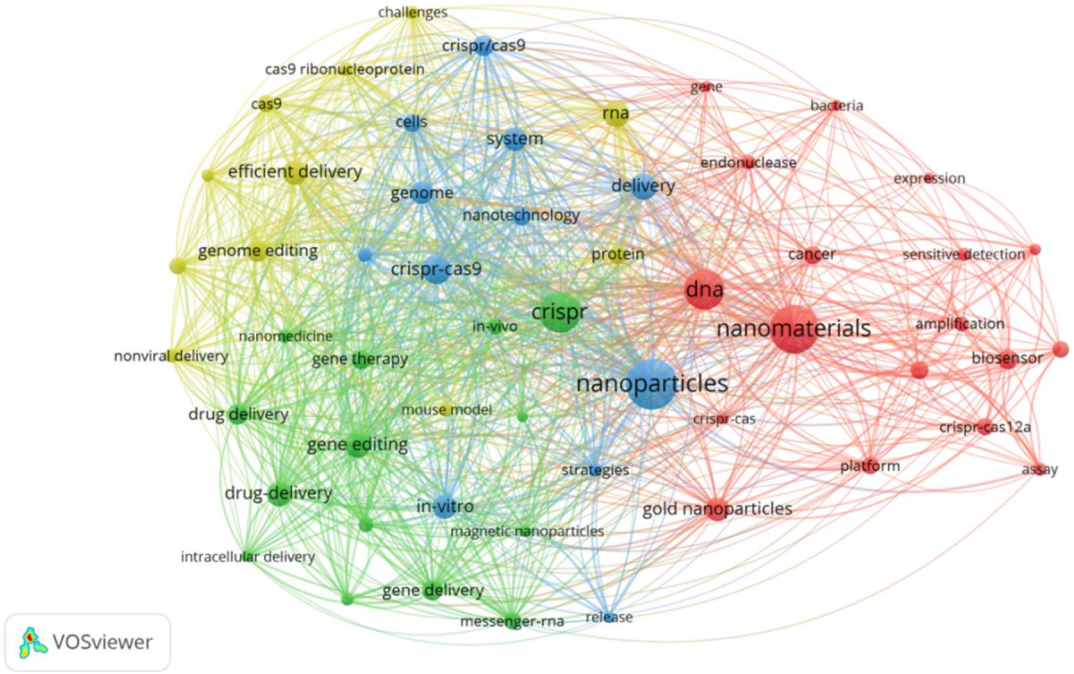

Figure 1. Figure label: Statistical analysis of literature related to nanozyme-assisted CRISPR/Cas biosensors. Keyword co-occurrence network analysis (VOSviewer) of 304 Web of Science articles (2012–2025) containing the subject terms "CRISPR" and "nanozyme".

Nanozymes, as a category of nanomaterials endowed with enzyme-mimetic catalytic properties, integrate the catalytic efficiency of natural enzymes with the inherent merits of nanomaterials—including superior stability, cost-effectiveness, and facile modifiability [16]. They can maintain high catalytic activity under complex environmental conditions, providing new technical support for signal amplification in biosensing systems [16]. Since 2007, when Fe_3O_4 nanoparticles were discovered to possess peroxidase activity, nanozyme research has developed rapidly, showing broad application prospects in fields such as food safety detection [17], environmental monitoring [18], and biosensing, with advantages including simple synthesis, low cost, good stability, and tunable catalytic activity. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology, with its characteristics of high specificity, high sensitivity, and programmability, has gradually expanded into the field of biosensing after achieving revolutionary breakthroughs in gene editing [19]. The Cas proteins (such as Cas12, Cas13) in the CRISPR/Cas system can recognize target nucleic acids under the specific guidance of guide RNA (gRNA), which activates non-specific nuclease activity, thereby achieving efficient amplification of the target signal and providing a molecular recognition basis for the high-specificity detection of pollutants. The CRISPR/Cas system has become a powerful tool in the field of biosensing. By coupling with this system, biosensors can improve their detection sensitivity, specificity, and response speed. Besides directly targeting nucleic acids, it can also utilize functional nucleic acids, allosteric transcription factors, antibodies, etc., to achieve the conversion of nucleic acid and non-nucleic acid targets, and thus be used for detecting small molecules, proteins, cells, etc. [12]. The

distinctive integration of nucleic acid detection mechanisms and signal transduction principles positions CRISPR/Cas-based biosensors at the cutting edge of diagnostic innovation [18]. CRISPR/Cas biosensors integrated with colorimetric [20], fluorescent [21], and electrochemical [22] detection methods have achieved high sensitivity and specificity. These characteristics make them widely applicable in fields such as biosensing, clinical diagnosis, and environmental monitoring [23]. Currently, most detection platforms based on the CRISPR/Cas system, such as SHERLOCK [24], DETECTR [25], and CDetection [26], primarily relies on isothermal amplification processes. Combining the efficient catalytic activity of nanozymes with the high-specificity recognition ability of CRISPR/Cas technology to construct nanozyme-assisted CRISPR/Cas biosensing systems can significantly improve the sensitivity, specificity, and stability of the sensing system through the synergistic effect of "specific recognition—signal amplification—catalytic enhancement", effectively solving the detection challenges of trace pollutants in complex matrices. Nanozyme-CRISPR/Cas sensing platforms can achieve pollutant detection through various signal output modes such as colorimetric [20], fluorescent [21], and electrochemical [22]. Besides directly targeting nucleic acids, they can also utilize functional nucleic acids, allosteric transcription factors, antibodies, etc., to achieve the conversion of nucleic acid and non-nucleic acid targets, enabling the detection of small molecules, proteins, cells, etc. (**Figure 1**), and providing new technical directions for environmental pollutant monitoring. Currently, nanozyme-assisted CRISPR/Cas biosensing technology has shown great application potential in the detection of environmental pollutants such as heavy metal ions [3], antibiotics [27], persistent organic pollutants [28], and viruses [29], becoming a frontier direction in the field of environmental monitoring technology. This technology can not only achieve trace, rapid, and in situ detection of pollutants but also meet the needs of high-throughput and portable monitoring, providing key technical support for the early warning, precise control, and emergency response of environmental pollutants. It holds substantial theoretical and practical significance for promoting the intellectualization and miniaturization of environmental monitoring technologies, optimizing the ecological environment monitoring network system, and elevating the scientific level of environmental governance.

While previous reviews have summarized the advances of nanozymes and CRISPR/Cas systems in the field of food safety, a systematic review focusing on the integration of nanozyme-assisted CRISPR/Cas systems for monitoring environmental pollutants is still lacking. This article aims to fill this gap by summarizing recent research on nanozyme-assisted CRISPR/Cas biosensors designed for environmental pollutant detection. We focus on several pressing classes of environmental contaminants, including pathogenic microorganisms and viruses, antibiotic resistance genes, heavy metal ions, persistent organic pollutants, and toxin residues. The application of nanozyme-enhanced CRISPR/Cas platforms for detecting these targets is reviewed, and the current research landscape, along with future development prospects are discussed.

2. Core Mechanisms and Platform Construction of Nanozyme-Assisted CRISPR/Cas Sensing

2.1. Fundamentals of the CRISPR/Cas System

The CRISPR/Cas system is an adaptive immune mechanism that originated in bacteria and archaea to defend against invasive nucleic acids [30], such as those from bacteriophages. Comprising clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nucleases (Cas proteins) [31], the system directs precise recognition and cleavage of specific nucleic acid sequences through a guide RNA (gRNA) [32]. Owing to its high specificity and programmability, CRISPR/Cas has evolved rapidly from a third-generation genome-editing tool into a pivotal platform for precise nucleic acid detection [33].

CRISPR/Cas systems are broadly classified into two categories based on their effector composition [34]: Class 1 (types I, III, and IV) utilizes multi-subunit protein complexes for cleavage, whereas Class 2 (types II, V, and VI) employs a single effector protein. The structural simplicity and ease of engineering of Class 2 systems have made them the preferred choice for molecular detection. Key representative effectors include Cas9 [35], Cas12 [25], and Cas13 [24]. Cas9 is directed by a single-guide RNA (sgRNA) to cleave double-stranded DNA in a PAM-dependent manner [36]. Cas12, upon target DNA recognition, performs both specific cis-cleavage and nonspecific trans-cleavage of single-stranded DNA [37]. Cas13 targets RNA and likewise exhibits promiscuous RNase (tran-cleavage) activity upon activation, enabling highly sensitive RNA detection [38].

The programmability and trans-cleavage activity of these Cas proteins provide the foundation for highly sensitive and specific nucleic acid detection. The trans-cleavage behavior, first clearly demonstrated for Cas12a in 2018, is structurally linked to target-induced conformational changes [25]. This activity converts a specific binding event into an amplifiable signal, facilitating rapid, amplification-free detection strategies. By effectively coupling target recognition with signal output, this mechanism paves the way for integrating CRISPR systems with nanomaterials such as nanozymes, thereby advancing the performance of environmental biosensing platforms.

2.2. Nanozyme-Enhanced CRISPR/Cas Biosensing

Nanozymes exhibit distinct advantages over natural enzymes, including high stability, lower cost, and being easy to prepare and purify [39]. Beyond their intrinsic enzyme-like catalytic activity, their nanoscale properties confer unique physicochemical characteristics, making them particularly suitable for biosensing applications [16]. In such contexts, nanozymes can significantly enhance signals generated from biorecognition events [40]. By catalyzing reactions triggered by target analytes, they convert biochemical interactions into quantifiable outputs—such as colorimetric, fluorescent, electrochemical, and Raman signals—thereby enabling faster, simpler, and more sensitive detection [41–43]. This signal-amplification capability is central to improving both sensitivity and accuracy in sensing platforms. With ongoing advances in nanotechnology, nanozyme-based systems hold strong

potential for developing integrated and multifunctional detection platforms [18].

The Integration of nanozymes with CRISPR/Cas systems substantially enhances biosensor performance and facilitates practical deployment. In such combined platforms, nanozymes primarily serve two complementary roles: (1) as signal labels, where their inherent optical or electrochemical properties enable direct visual or instrument-based readouts, and (2) as signal amplifiers, where their catalytic activity dramatically boosts the output from CRISPR-mediated recognition events [44,45]. This synergy supports the development of visual, user-friendly, and highly sensitive detection methods, broadening the application scope of CRISPR/Cas technology.

A typical nanozyme-assisted CRISPR/Cas sensing process involves four consecutive steps: (1) nucleic acid extraction and isothermal amplification (e.g., via recombinase polymerase amplification (RPA) or reverse transcription-recombinase polymerase amplification (RT-RPA) to enrich target sequences; (2) CRISPR/Cas-mediated recognition, where the Cas complex specifically binds and cleaves target DNA or RNA while exhibiting collateral trans-cleavage activity; (3) nanozyme-mediated signal enhancement, in which nanozyme catalysis amplifies the output signal; and (4) analysis and readout through colorimetric, fluorescent, electrochemical, or other measurable signals [33,46]. These platforms thus support multimodal detection, with each output format offering distinct advantages: colorimetric for simplicity and visual interpretation, fluorescent for high sensitivity, and electrochemical for portability and rapid response [34,47].

To further improve the specificity, stability, and field applicability of nanozyme-CRISPR/Cas platforms, strategic modifications can be introduced. These include functionalizing nanozymes with recognition elements (e.g., aptamers or antibodies) for selective target binding, optimizing gRNA designs to enhance CRISPR specificity, and integrating the sensing components into microfluidic or paper-based devices. Such developments pave the way for portable, automated systems capable of on-site, real-time environmental monitoring.

3. Applications in Environmental Pollutant Monitoring

3.1. Pathogenic Microorganism and Virus Monitoring

Pathogenic microorganisms and viruses are widely present in environmental media such as water, soil, and air, posing multiple threats to human health, ecosystems, and environmental engineering management. Pathogenic microorganisms (bacteria, fungi, parasites, etc.) can spread through contaminated water and food, causing acute infections such as cholera and hemorrhagic enteritis. Fungi and parasites can also cause chronic tissue damage. These microorganisms can also infect plants and animals, disrupting ecological balance, competing with native microorganisms for resources, reducing environmental self-purification capacity, and increasing the costs and secondary pollution risks of wastewater treatment and soil remediation. Due to their small size and strong resistance, viruses spread more efficiently than bacteria. Viruses such as SARS-CoV-2 and norovirus can cause large-scale acute infectious diseases, and some viruses can lead to chronic infections or even cancer [48]. Plant and animal viruses can cause huge losses in agriculture and aquaculture. Conventional water treatment processes often fail to effectively remove viruses. Their existence via

adsorption onto particles further increases the risk of cross-media transmission, posing severe technical challenges for environmental engineering management [48].

The core challenges in the detection of pathogenic microorganisms and viruses in environmental monitoring are as follows: the difficulty in detecting low-abundance targets, the susceptibility of specificity to interference from complex matrices, the high requirements for operational simplicity and response speed in on-site monitoring, and the need to simultaneously satisfy both quantitative accuracy and intuitive screening demands. The nanozyme-CRISPR/Cas sensing platform offers an innovative technical approach to address these challenges through the synergistic mechanism of "gRNA-targeted recognition - Cas activation - nanozyme signal amplification". However, the adaptability and limitations of different strategies vary significantly, necessitating precise evaluation in conjunction with specific application scenarios.

The dual-mode (fluorescence + colorimetric) detection strategy proposed by Arshad et al. [49,50] specifically addresses the core demand of "simultaneous quantitative accuracy and rapid screening" in environmental monitoring. Pre-enrichment based on recombinase polymerase amplification (RPA) effectively overcomes the bottleneck of low-abundance target detection. Two distinct nanozyme-based configurations were employed in these two studies, respectively: nano-hybrid composites (silica nanobeads combined with magnetic nanoparticles) and CeO_2 nanozymes, each integrated into the CRISPR/Cas12a system to enable dual-signal output—fluorescence signals generated via probe cleavage post-Cas activation, and colorimetric signals produced through the peroxidase-mimicking activity of nanozymes. These two configurations achieved highly specific detection of Campylobacter (Fluorescence LOD = 0.98 pg/μL, Colorimetric LOD = 0.96 pg/μL) and Salmonella (Fluorescence LOD = 0.88 pg/μL, Colorimetric LOD = 1.28 pg/μL), respectively (**Figure 2a**). Nevertheless, their limitations are equally notable: the preparation processes of these two nanozyme-based materials are complex and costly; the coordinated regulation of signal thresholds in the dual-signal system is challenging, which may compromise the stability of on-site detection; and the interference of heteroproteins in complex environmental matrices (e.g., sewage, soil leachate) on the catalytic activity of nanozymes remains unresolved.

The work by Wu et al. [29] focuses on optimizing the simplicity of on-site visual detection. Targeting the core limitation of traditional peroxidase-mimicking systems that rely on H_2O_2, this study employs the oxidase-like activity of MnO_2 nanorods to construct an H_2O_2-free colorimetric system, and leverages Cas12a trans-cleavage to regulate the binding efficiency between nanozymes and magnetic beads, thereby enabling visual detection of SARS-CoV-2 (**Figure 2b**). It simplifies the catalytic system, and forms a complement to Arshad et al.'s complex nanomaterial-enhanced signal strategy. However, the reliance on reverse transcription-RPA (RT-RPA) pre-amplification impairs the core advantage of "point-of-care testing (POCT)": the nucleic acid extraction and amplification processes not only extend the detection time but also increase instrument dependence, restricting its application in emergency monitoring under non-laboratory conditions.

In the high-demand scenario of clinical virus detection, the study by Chen et al. [51], by integrating GO@Pt two-dimensional nanozymes, CRISPR/Cas13a, and microfluidic technology, a "one-step RPA-Cas13a trans-cleavage-based sandwich

immune complex" system was established. Nanozymes catalyze the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) for color development, achieving a detection limit of 1 copy/μL for monkeypox virus within 60 minutes. Furthermore, this system exhibits 100% consistency with real-time quantitative PCR (qPCR) results in 40 clinical samples (**Figure 2c**). The core value of this study lies in verifying the anti-interference capability of the nanozyme-CRISPR/Cas strategy in complex clinical matrices. However, the complex microfabrication process and high cost of large-scale production of microfluidic chips pose major obstacles to its popularization in primary clinical monitoring, failing to address the industry challenge of translating high-end technologies to grassroots applications.

The aggregation-induced emission (AIE) MOFzyme dual-functional probe strategy developed by Qiu et al. [52] achieves an upgrade in the signal regulation mechanism from "cleavage regulation" to "phase transition release". This dual-functional probe (integrating aggregation-induced emission (AIE) and peroxidase-mimicking activity) is embedded in a DNA hydrogel to construct a Cas12a-responsive platform; upon CRISPR/Cas12a activation, the hydrogel undergoes phase transition to release the probe, thus achieving fluorescence-colorimetric dual-signal amplification for Salmonella enterica detection with higher sensitivity than traditional single-signal strategies (**Figure 2d**). Nevertheless, the core limitation of this system is its inadequate environmental adaptability: the phase transition response of the hydrogel is susceptible to environmental factors such as temperature and ionic strength; additionally, the stability of AIE MOFzyme in real environmental samples (e.g., high-salinity sewage) has not been validated. The synergistic optimization of "signal amplification - environmental anti-interference" remains a key unresolved issue.

Research in the field of electrochemical detection extends the nanozyme-CRISPR/Cas strategy to meet the demand for "ultra-sensitive quantification and high-throughput detection". Both the Cr-MOF/PPy@Au electrode substrate strategy proposed by Hui et al. [53] and the PtPd@PCN-224 nanozyme strategy developed by Li C. et al. [54] realize pathogen detection based on changes in electrochemical signals through Cas-mediated regulation of nanozyme immobilization. Specifically, the latter achieves precise immobilization of nanozymes via Zr-O-P coordination interactions, resulting in a detection limit of 12.8 aM for Burkholderia pseudomallei DNA—an achievement that highlights the ultra-sensitive advantage of the synergistic effect between bimetallic MOF nanozymes and Cas14a. However, such strategies generally suffer from a "disconnection between technical advancement and on-site applicability": electrochemical detection relies on specialized instruments; moreover, the synthesis of bimetallic MOF nanozymes is costly and exhibits poor reproducibility, making it difficult to meet the core requirements of "low cost and easy operation" in environmental monitoring. This exposes a methodological gap in the field regarding the "synergy between ultra-sensitive detection and on-site convenience".

In summary, existing nanozyme-CRISPR/Cas sensing strategies have addressed specific challenges in environmental monitoring (e.g., low-abundance detection, visual screening, and clinical compatibility) through signal mode innovation (dual-mode, electrochemical), material design optimization (dual-functional probes, two-dimensional nanozymes), and technical integration (microfluidics, pre-amplification). Nevertheless, three key methodological gaps persist: first, insufficient research on

anti-interference mechanisms in complex environmental matrices; second, a lack of synergistic optimization between high sensitivity and low cost/easy operation; third, unmet requirements in the design of simultaneous multi-pathogen detection systems. Future research should focus on the synergistic optimization of "environmental adaptability - cost control - multi-target detection" to facilitate the translation of this technology from the laboratory to practical environmental monitoring applications.

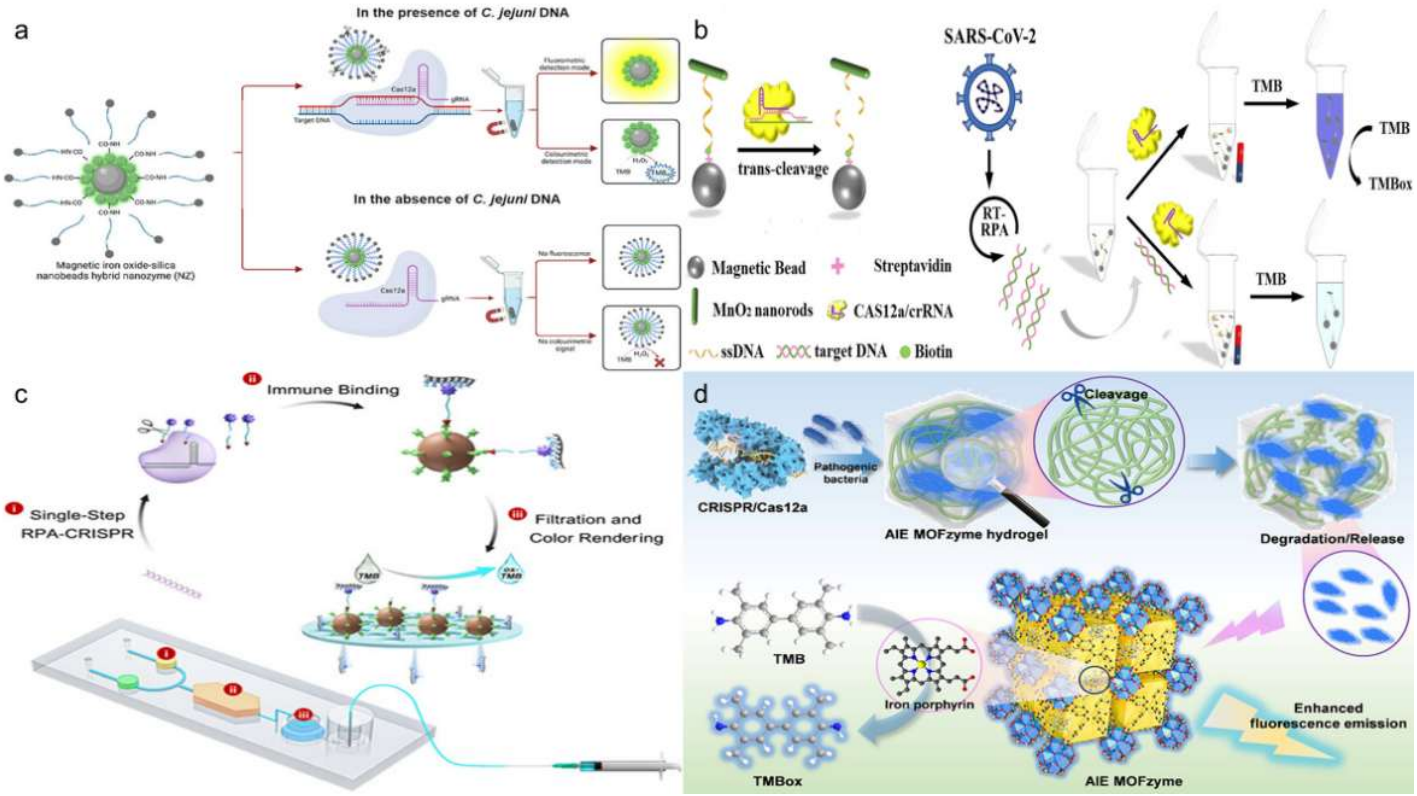

Figure 2. Applications of nanozyme-assisted CRISPR/Cas biosensors in pathogenic microorganism and virus monitoring. **(a)** Nanozyme-assisted CRISPR/Cas biosensor for detecting Campylobacter. Reprinted (adapted) with permission from [49], American Chemical Society, 2025; **(b)** Nanozyme-assisted CRISPR/Cas biosensor for detecting SARS-CoV-2. Reprinted (adapted) with permission from [29], American Chemical Society, 2022; **(c)** Nanozyme-assisted CRISPR/Cas biosensor for detecting monkeypox virus. Reprinted (adapted) with permission from [51], American Chemical Society, 2025; **(d)** Nanozyme-assisted CRISPR/Cas biosensor for detecting foodborne pathogens. Reprinted (adapted) with permission from [52], American Chemical Society, 2025.

3.2. Screening of Antibiotic Resistance Genes in Environmental Media

Antibiotic abuse has led to the widespread dissemination of antibiotic resistance genes (ARGs) in environmental media [55], such as β-lactamase genes (e.g., blaTEM, blaCTX-M), tetracycline resistance genes (e.g., tetM, tetO), and sulfonamide resistance genes (e.g., sul1, sul2). These resistance genes can confer drug resistance to pathogenic bacteria through horizontal gene transfer, posing a serious threat to public health security. For human health, ARGs can enter the human body through drinking water, food, or aerosols, and confer drug resistance to pathogenic bacteria via horizontal gene transfer [55]. For example, tetracycline ARGs remaining in soil may be transferred to intestinal pathogens, leading to clinical antibiotic failure, increasing treatment difficulty for infections, and even posing a risk of "superbug" infections. At

the ecosystem scale, antibiotic resistance genes (ARGs) disrupt the structure of microbial communities: Enrichment of ARGs in aquatic environments can suppress the metabolic activity of nitrifying and denitrifying bacteria, thereby impeding the nitrogen cycling process; ARGs present in soil matrices can reduce the abundance of beneficial microbial taxa such as nitrogen-fixing bacteria, resulting in impaired soil fertility, and can also undergo trophic level transfer via the food chain, thereby posing adverse impacts on the health of plants and animals.

The detection of antibiotics and antibiotic resistance genes (ARGs) in environmental monitoring poses more intricate challenges: the coexistence of trace antibiotics and low-abundance ARGs necessitates synergistic dual-target recognition; the high complexity of environmental matrices (e.g., sewage, soil, and livestock wastewater) readily interferes with detection specificity and signal stability; and there is an urgent demand for integrating "on-site rapid screening", "ultra-sensitive quantification" and even "pollution remediation" to form a full-chain governance solution. Endowed with flexible target recognition and multi-modal signal amplification capabilities, the nanozyme-CRISPR/Cas sensing platform provides a viable technical pathway to address these challenges. To guide practical applications, the adaptability of different strategies to specific scenarios and their inherent limitations requires critical evaluation.

To address these challenges, Zhang et al. [2] proposed the "separation-recognition-multi-signal output" technical framework for synergistic monitoring of antibiotic-ARGs dual targets, which specifically targets the core challenge of simultaneous screening of antibiotics and their corresponding resistance genes in on-site environmental monitoring. By employing Fe_3O_4@PDA (FP) as the magnetic separation unit to mitigate matrix interference and Prussian blue@UiO-66@MnO_2 (PUM) as the signal amplification unit, fluorescence, colorimetric, and photothermal tri-modal detection was achieved via aptamer-mediated ampicillin (AMP) recognition and Cas12a-driven blaTEM gene trans-cleavage (AMP LOD = 1.03 pM, blaTEM LOD = 1.03 pM). Integration of smartphone-assisted visual detection further enhanced the on-site applicability of the system (**Figure 3a**). Nevertheless, this strategy exhibits notable limitations: the tri-modal signal system involves complex signal threshold coordination, which may compromise detection stability in complex matrices; additionally, the catalytic activity of PUM nanozymes is prone to inhibition by heavy metal ions and organic matter in natural environments.

Zhang et al. [27] achieved targeted technological upgrading for high-sensitivity dual-target detection in complex environmental matrices. Aiming at the monitoring requirements of sulfamethazine (SMZ) and the sul1 resistance gene, they introduced the highly active MOF-818@PtPd (MPP) nanozyme, which significantly improved signal amplification efficiency. Combined with magnetic separation and the high specificity of Cas12a, the detection limits for SMZ and sul1 were optimized to 0.67 pM and 7.6 fM, respectively (**Figure 3b**). This study achieved optimized performance for dual-target detection in complex environmental matrices, forming a complementary technical system for dual-target detection of different classes of antibiotics (β-lactams, sulfonamides) and their corresponding ARGs. However, the high synthesis cost of MPP nanozymes (attributed to the incorporation of precious metals Pt and Pd) and the complex preparation process restrict their large-scale

practical application, failing to strike a balance between "high performance" and "cost-effectiveness"—a critical prerequisite for environmental monitoring technologies.

Jiang et al. [56] addressed the specialized challenge of accurate detection of veterinary antibiotic residues. Through directed evolution technology, they successfully screened a truncated aptamer (N-20) with high affinity for netilmicin (NET) from the parental aptamer. Integrating the truncated aptamer with enzyme-assisted strand displacement amplification (EASDA) further enhanced signal intensity, activating Cas12a for trans-cleavage to release NMOF-Pt nanoenzyme using amplified products, and the released NMOF-Pt nanozymes realized colorimetric quantitative detection (linear range: 0.003–30 ng/mL, LOD = 1.15 pg/mL). The core advantage of this strategy lies in its high adaptability to the veterinary antibiotic residue detection scenario; however, it suffers from poor universality: the truncated aptamer is specific to NET, and the developed optimization strategy cannot be directly extrapolated to other veterinary antibiotics. Furthermore, the EASDA pre-amplification step prolongs the detection time and increases instrument dependence, thereby compromising the on-site rapid detection capability.

Chen et al. [57] overcame the limitation of "single-molecule detection" and targeted the core demand for on-site rapid diagnosis of drug-resistant bacteria in clinical and environmental emergency monitoring. They used a nanozyme-coupled CRISPR/Cas12a strategy for direct detection of drug-resistant bacteria without bacterial culture, leveraging the peroxidase-mimetic activity of Au-Fe_3O_4 nanozymes for visual colorimetric detection. This strategy achieved identification of various drug-resistant bacterial strains (e.g., kanamycin-resistant, ampicillin-resistant strains) within 1 hour, with a resistance gene LOD < 0.1 CFU μL^{-1} and a resistant bacteria LOD ≈ 10^2 CFU mL^{-1}. It also supports high-throughput detection in 96-well plates and smartphone-based portable measurement, filling the technical gap in on-site rapid diagnosis of drug-resistant bacteria. Nevertheless, the lack of strain-specific recognition may lead to false-positive results in the presence of multiple coexisting bacterial strains.

Liu et al. [58] achieved a functional upgrade. They constructed an Fe-C-N@Au multifunctional nanozyme platform that simultaneously enables aptamer-mediated azithromycin (AZM) recognition and Cas12a-mediated ermB resistance gene identification, coupled with colorimetric-photothermal dual-modal sensing (AZM colorimetric LOD = 2.34 pM, ermB photothermal LOD = 8.46 pM) and portable device-based synchronous quantification. More importantly, the nanozyme exhibits AZM degradation functionality (>90% degradation rate within 60 minutes), extending the technological value from detection to pollution control. However, this integrated system faces significant practical obstacles: the degradation efficiency of the nanozyme may be affected by environmental factors (e.g., pH, temperature, and coexisting pollutants).

The intrinsic connections and evolutionary logic of the aforementioned studies are distinctly reflected in three aspects: first, complementary target coverage, which gradually expands from single antibiotics/ARGs to antibiotic-ARGs dual targets and drug-resistant bacteria, achieving full-chain coverage from "molecular targets" (antibiotics, ARGs) to "living organism targets" (drug-resistant bacteria) for diverse

antibiotic classes (β-lactams, sulfonamides, macrolides); second, continuous optimization of nanozyme performance, with nanozyme materials evolving from traditional composite nanomaterials to highly active MOF-based and single-atom-modified nanozymes, significantly enhancing signal amplification efficiency; third, progressive upgrading of functional positioning, advancing from simple "detection" to "detection + on-site visualization" and further to "detection + pollution remediation". However, inter-study differences also highlight the fragmented nature of current research: most strategies are tailored to specific antibiotics or targets, lacking a universal technical framework.

In terms of methodological gaps, three key unresolved issues persist: first, the absence of a universal dual-target/triple-target detection framework for diverse antibiotics and ARGs limits large-scale screening of antibiotic pollution in environmental settings; second, the adaptability of nanozyme-CRISPR/Cas systems in ultra-complex matrices (e.g., composite pollution scenarios involving multiple antibiotics, heavy metals, and organic matter) needs to be further improved; third, the "detection-remediation" integrated system is still in the laboratory stage, and its practical application potential in natural environments requires in-depth verification. Future research should focus on the development of universal target recognition modules, optimization of nanozyme-environment interaction mechanisms, and systematic evaluation of integrated system performance to facilitate the translation of this technology from laboratory research to practical application in antibiotic pollution control.

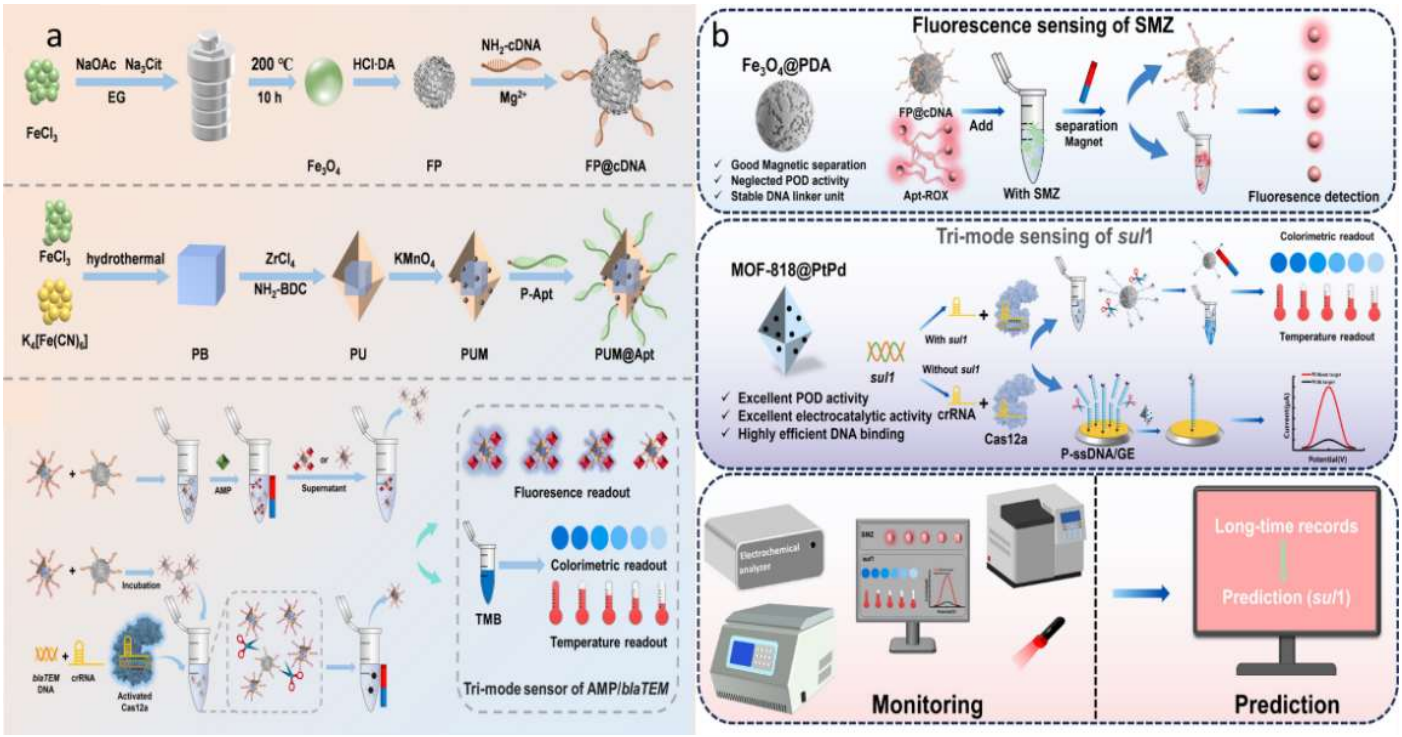

Figure 3. Applications of nanozyme-assisted CRISPR/Cas biosensors in the detection of antibiotic resistance genes in environmental media. **(a)** Nanozyme-assisted CRISPR/Cas biosensor for detecting ampicillin and resistance genes, Reprinted (adapted) with permission from [2], American Chemical Society, 2025; **(b)** Nanozyme-assisted CRISPR/Cas biosensor for detecting sulfamethazine and sulfonamide resistance-related genes, Reprinted (adapted) with permission

from [27], American Chemical Society, 2025.

3.3. Highly Sensitive Detection of Heavy Metal Ions

Heavy metal ions (e.g., mercury, lead, cadmium, chromium, arsenic) are typical persistent environmental pollutants, widely present in water bodies, soil, and sediments. They possess bioaccumulative and non-degradable properties, with hazards spanning the entire process of human health, ecosystems, and environmental management. For human health, heavy metal ions enter the human body through drinking water and food, accumulating long-term in organs such as the liver, kidneys, and bones: mercury ions (Hg^{2+}) damage the central nervous system, causing cognitive impairment and limb tremors; lead ions (Pb^{2+}) affect children's intellectual development and bone growth, and long-term exposure in adults can easily lead to anemia and kidney damage [59]; cadmium ions (Cd^{2+}) can disrupt bone metabolism, induce "Itai-itai disease", and various heavy metals (e.g., hexavalent chromium Cr^{6+}, arsenic As^{3+}) have clear carcinogenicity, increasing the risk of lung cancer and skin cancer. At the ecosystem level, heavy metal ions disrupt the balance of the food chain: heavy metals in water can damage fish gills, reduce reproductive capacity, and even cause mass mortality; simultaneously, they transfer across trophic levels through the food chain. For example, birds ingesting lead-contaminated fish may experience thinner eggshells and reduced chick survival rates, damaging biodiversity. Traditional detection methods, such as atomic absorption spectroscopy (AAS) and inductively coupled plasma mass spectrometry (ICP-MS), although highly sensitive, have drawbacks such as expensive equipment and complex operation.

Heavy metal ions detection in environmental monitoring faces five core challenges limiting practical application: first, ultra-trace heavy metal ions levels in natural water and soil leachates demand extreme detection sensitivity; second, complex matrix components (e.g., humic acids, nucleases, coexisting metal ions) easily cause non-specific interference or signal attenuation; third, on-site emergency monitoring requires rapid response, simple operation, and low reliance on large-scale instruments; fourth, CRISPR/Cas system background interference (e.g., "DNA breathing"-induced false activation) impairs detection reliability; fifth, large-scale pollution surveys need high-throughput screening capability. The three CRISPR/Cas-based strategies target different subsets of these challenges, but their inherent trade-offs and methodological gaps require in-depth critical analysis.

Li et al. [3] addressed core challenges of "nuclease interference in complex matrices", "ultra-trace detection", and "on-site applicability", proposing a strategy integrating spherical nucleic acid (SNA) fluorescent probes, CRISPR/Cas12a signal amplification, and bionic photonic crystal chips. SNA probes (Au nanoparticle-DNAzyme probes) with excellent nuclease resistance mitigate nuclease interference in complex samples, ensuring Pb^{2+} recognition specificity. Cas12a-mediated trans-cleavage of SNA signal probes enables efficient signal amplification, lowering the limit of detection (LOD) to 86 fM. Additionally, combining bionic periodic photonic crystal chips with smartphones and portable UV lamps achieves on-site detection (on-site LOD = 24 pM), breaking reliance on large laboratory instruments (**Figure 4**). Inherent limitations exist: complex and costly preparation of SNA probes and photonic

crystal chips limits large-scale deployment in routine environmental monitoring; Cas12a-dependent signal amplification may prolong detection cycles.

Ren et al. [60] focused on the critical flaw of "unavoidable background signals and poor reliability" in conventional DNAzyme-Cas12a Pb^{2+} detection systems, proposing a strategy based on a split activator-driven Cas12a switch. The split activator with a flap region effectively blocks Cas12a activation without Pb^{2+}, suppressing "DNA breathing"-induced background noise. Pb^{2+}-dependent activation of GR-5 DNAzyme cleaves the flap, enabling Cas12a activator reconstitution and triggering trans-cleavage for signal amplification. This strategy achieves a Pb^{2+} LOD of 615 pM with high specificity against interfering metal ions, with core merits including: (1) resolving traditional system background interference to improve detection reliability; (2) eliminating DNA amplification and nanoparticle modification requirements, enabling rapid ambient-temperature detection and simplifying operations. However, it has obvious trade-offs: its LOD (615 pM) is three orders of magnitude higher than Li et al.'s (86 fM), failing to meet ultra-trace Pb^{2+} detection requirements in high-standard scenarios (e.g., drinking water source monitoring).

Wen et al. [61] targeted the core demand for "ultra-sensitive and high-throughput Pb^{2+} detection" in large-scale environmental pollution surveys, constructing a CRISPR-Cas12a sensor based on a dual-functional deoxyribozyme (df-DNAzyme) probe. The df-DNAzyme duals as a Pb^{2+} recognition element and Cas12a activator; combining with gold nanoparticles (AuNPs) forms a DNAzyme/AuNP nanoprobe, enabling one-to-multiple Cas12a activation. Pb^{2+} exposure cleaves df-DNAzyme, causing the nanoprobe to dissociate from magnetic beads. Released nanoprobes with multiple double-stranded DNA activators efficiently trigger Cas12a activity, initiating fluorescence-quenched reporter DNA cleavage for signal amplification. Quantification via qPCR enables simultaneous high-throughput detection of 96/384 samples, achieving an ultra-low LOD of 1 pg/L (linear range: 1 pg/L–10 μg/L), exceeding WHO and US EPA standards. Practical application is severely limited by instrument dependence: qPCR reliance precludes on-site detection; additionally, df-DNAzyme/AuNP nanoprobe preparation and magnetic separation increase sample pretreatment complexity.

The logic of the three strategies reflects the field's progression from "single performance breakthrough" to "multi-demand adaptation": Li et al. use "sensitivity + on-site applicability + complex matrix adaptation"; Ren et al. focus on "background control + operational simplification"; Wen et al. pursue "ultra-sensitivity + high-throughput". However, fragmented research highlights three key methodological gaps: First, a lack of a balanced framework between "high performance" and "practical applicability": high-sensitivity strategies (Li et al., 86 fM; Wen et al., 1 pg/L) either have complex preparation and high costs (Li et al.) or strong instrument dependence (Wen et al.), while the simplified strategy (Ren et al.) sacrifices sensitivity. Second, insufficient ultra-complex matrix adaptability verification: although Li et al. note complex sample suitability via nuclease resistance and Ren et al. validate tap/drinking water accuracy, no strategy has systematically evaluated performance in composite pollution scenarios (coexisting Pb^{2+}, other heavy metals, antibiotics, and humic acids). Third, lack of multi-target universality: all strategies are Pb^{2+}-specific, and recognition element design logic (SNA-DNAzyme, GR-5 DNAzyme, df-DNAzyme) cannot be

directly extended to other heavy metals. These gaps indicate future research should focus on developing low-cost, simplified ultra-sensitive recognition modules, systematically verifying matrix anti-interference mechanisms, and constructing universal multi-target frameworks to advance CRISPR/Cas technology translation from laboratory to practical environmental monitoring.

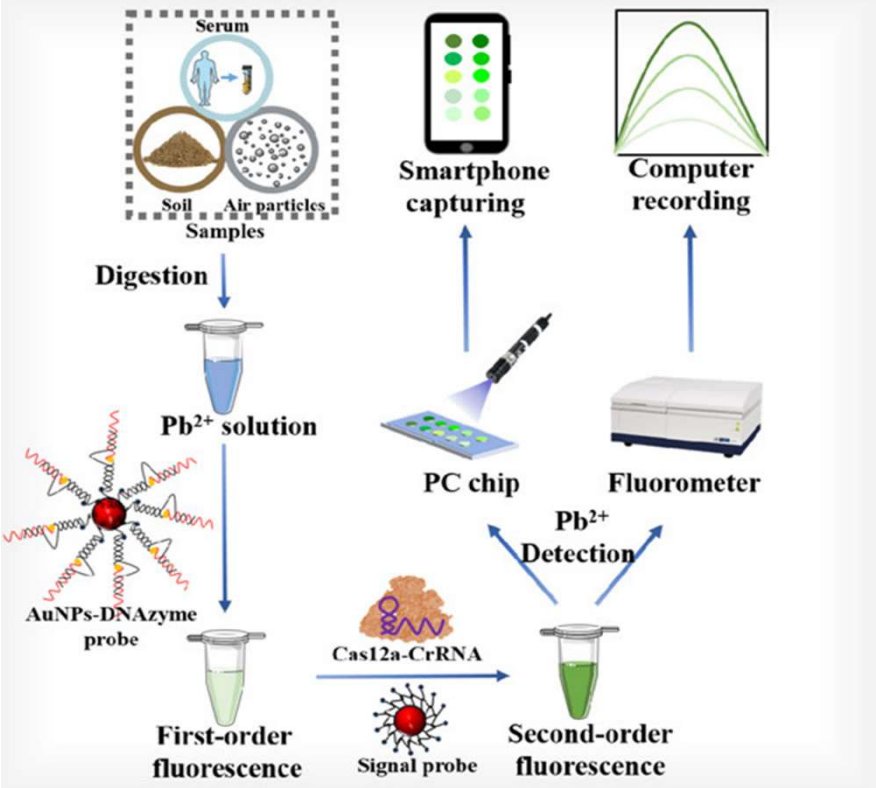

Figure 4. Applications of nanozyme-assisted CRISPR/Cas biosensors in heavy metal ion monitoring. Nanozyme-assisted CRISPR/Cas biosensor for detecting Pb^{2+} based on the fluorescence method. Reprinted (adapted) with permission from [3], American Chemical Society, 2022.

3.4. Detection of Refractory Organic Pollutants and Biotoxins

Refractory organic pollutants (polycyclic aromatic hydrocarbons (PAHs), persistent organic pollutants (POPs), pesticide residues) and biotoxins (phycotoxins, mycotoxins, bacterial toxins), due to their strong stability and high toxicity, seriously threaten human health, ecosystems, and environmental management. Refractory organic pollutants are lipophilic and accumulative, entering the human body through food and drinking water: PAHs (e.g., benzo[a]pyrene) are strongly carcinogenic, increasing lung cancer risk; POPs disrupt the endocrine system, causing abnormal development in children; pesticide residues cause neurotoxicity. At the ecological level, they inhibit algal photosynthesis, kill beneficial soil bacteria, and also bioaccumulate through the food chain. In management, conventional processes struggle to degrade them, requiring advanced oxidation technologies, which are costly and may produce toxic intermediates. Biotoxins are highly toxic and act quickly: cyanobacterial toxins damage the liver, aflatoxins (contaminating grains) are

carcinogenic, and botulinum toxin causes muscle paralysis. Ecologically, cyanobacterial blooms in water bodies release toxins that kill aquatic life; mycotoxins in soil inhibit crop growth and transfer through the "soil—crop—animal—human" chain.

The detection of refractory organic pollutants and biotoxins in environmental monitoring is constrained by three core challenges that hinder practical application: first, the ultra-trace abundance of target analytes (often at ng/L-pg/mL levels) imposes extreme requirements on detection sensitivity; second, the complex and variable environmental matrices (e.g., soil leachate, surface water with humic acids) easily cause non-specific interference, undermining detection specificity; third, on-site emergency monitoring scenarios demand a balance between "highly sensitive quantification" and "simple operation, low cost, and rapid response". The nanozyme-CRISPR/Cas sensing platform achieves target detection by designing gRNAs targeting specific metabolites or related genes of pollutants and combining them with the catalytic signal output function of nanozymes [62]. However, the adaptability of different technical routes to the above challenges varies significantly, and their inherent trade-offs and methodological gaps require in-depth critical scrutiny.

Esmaelpourfarkhani et al. [62] targeted the core challenge of "simplified on-site signal transduction" and proposed a colorimetric detection strategy based on flower-like MnO_2 nanozymes. By utilizing the oxidase-mimetic activity of MnO_2 nanozymes and regulating their catalytic efficiency through Cas12a-mediated cleavage or non-cleavage of ssDNA, the strategy achieved quantitative detection of the mycotoxin AFM1 (LOD = 2.1 ng/L). This strategy's core advantage lies in establishing a concise "nanozyme switch-type signal transduction" model, which simplifies the signal output process and reduces dependence on complex instruments, aligning with the demand for on-site rapid detection. Nevertheless, this strategy has inherent limitations reported in the original study: the oxidase-mimetic activity of MnO_2 nanozymes is easily inhibited by reductive substances in environmental matrices (e.g., sulfides in industrial wastewater); in addition, the single metal oxide nanozyme has limited catalytic activity, which restricts the further improvement of detection sensitivity.

Liu et al. [63] aimed at addressing the limitations of "low catalytic activity and high background signal" of traditional metal oxide nanozymes, and achieved a key upgrade of the nanozyme system. They combined Fe–N–C single-atom enzymes (SAzymes) with CRISPR/Cas12a, and paired them with Fe–Co magnetic nanoparticles to reduce background signal and detection cost. Leveraging the high peroxidase activity of SAzymes, the strategy realized highly sensitive colorimetric detection of aflatoxin B1 (AFB1). This study promotes the iteration of nanozymes from traditional metal oxides to high-activity, low-background single-atom systems, which effectively improves detection sensitivity and signal stability. However, the strategy still has unresolved defects reported in the original study: the synthesis process of Fe–N–C SAzymes involves complex heteroatom doping and high-temperature calcination steps, resulting in high synthesis costs; moreover, the magnetic separation process adds an additional operation step, which prolongs the detection cycle.

Esmaelpourfarkhani et al. [64] further optimized nanozyme performance and

detection convenience, proposing a label-free colorimetric sensor based on ZrFe bimetallic MOF nanozymes for sulfadimethoxine (SDM) detection. By leveraging the reversible peroxidase-mimetic activity of ZrFe bimetallic MOFs, the strategy regulates the binding efficiency between ssDNA and MOFs based on the activation state of Cas12a, thereby affecting the oxidation effect of o-phenylenediamine (OPD) and achieving a low detection limit of 1.5 pM. This strategy's core merits are reflected in two aspects reported in the original study: the reversible regulation of bimetallic MOF nanozymes enhances the controllability of signal output, and the label-free design simplifies the experimental process and reduces detection costs. However, this strategy still faces limitations: the binding between ssDNA and MOFs is easily affected by non-target organic molecules in complex environmental matrices; in addition, the peroxidase-mimetic activity of MOFs is sensitive to environmental pH.

Wu et al. [65] focused on breaking the limitation of "single colorimetric signal mode" and meeting the demand for high-sensitivity on-site detection of biotoxins. They used Pd@PCN-222 nanozymes as reporter agents—the Pd@PCN-222 nanozymes' excellent H_2O_2 reduction catalytic activity significantly amplifies the electrochemical signal—achieving highly sensitive detection of ochratoxin A (LOD = 1.21 pg/mL) without pre-amplification. The development of a microfluidic electrochemical chip further promotes the practical application of on-site detection, filling the gap of scenario-based electrochemical CRISPR/Cas sensors. This strategy effectively improves detection sensitivity and on-site applicability, but its limitations are also prominent as reported in the original study: the preparation of Pd@PCN-222 nanozymes involves precious metal Pd, resulting in high synthesis costs; the microfluidic chip requires precise processing technology; in addition, the electrochemical signal is easily interfered by electroactive substances in the matrix (e.g., phenols in industrial wastewater).

Han et al. [28] targeted the core challenge of "high specificity detection of herbicides" and proposed an electrochemical biosensor based on Ni-SPNC single-atom nanozymes. By fabricating highly active Ni-SPNC single-atom nanozymes through a heteroatom doping strategy and integrating the dual recognition and signal transduction functions of nucleic acid aptamer-deoxyribozyme motors, the strategy achieved detection of atrazine with a detection limit as low as 2.3 fg/mL. This strategy's core advantages are breaking the sensitivity bottleneck of herbicide detection and improving detection specificity through dual recognition. However, this strategy has obvious practical limitations: the dual recognition system (aptamer + deoxyribozyme) has complex design and synthesis processes; the electrochemical detection process requires professional operation of the instrument.

The evolutionary logic of the aforementioned studies reflects the field's exploration process from "basic technology construction" to "performance optimization and scenario adaptation": in terms of core components, nanozymes have evolved from traditional metal oxides (MnO_2) to high-activity single-atom enzymes (Fe–N–C, Ni–SPNC) and bimetallic MOFs (ZrFe), realizing the upgrading of catalytic activity, controllability and signal-to-noise ratio; in terms of signal modes, the field's research has expanded from single colorimetry to electrochemical signals, meeting diverse detection needs from rapid screening to high-sensitivity quantification; in terms of application scenarios, the field's research has progressed from generalized

biotoxin detection to precise adaptation to specific targets (herbicides). However, the fragmented nature of the research is also prominent, highlighting three key methodological gaps based on the existing studies:

First, the lack of a universal technical framework: all strategies are tailored to specific targets, and the gRNA design, nanozyme selection and signal regulation mechanisms cannot be directly extrapolated to other types of refractory organic pollutants. Second, the adaptability of the system in ultra-complex matrices is insufficient: most studies focus on performance verification in standard solutions or simple simulated matrices, and the anti-interference mechanism of nanozyme-CRISPR/Cas systems in composite pollution scenarios (coexistence of multiple biotoxins, heavy metals and organic matter) has not been systematically explored. Third, the balance between "high performance" and "practical applicability" has not been achieved in current technical routes: strategies with high sensitivity (e.g., Han et al.'s [28] 2.3 fg/mL LOD) tend to have complex processes and high costs, while simplified strategies (e.g., Esmaelpourfarkhani et al.'s [62] MnO_2-based method) may sacrifice sensitivity. These gaps indicate that future research should focus on the development of universal gRNA design modules and multi-specific recognition systems, the optimization of nanozyme-matrix anti-interference mechanisms, and the integration of low-cost, miniaturized detection devices to promote the translation of this technology from laboratory research to practical application in environmental monitoring.

4. Challenges and Solutions for Practical Applications

4.1. Challenges in Environmental Application

The nanozyme-assisted CRISPR/Cas biosensing platform still confronts multiple core challenges in practical environmental applications, which originate not only from the inherent limitations of the technology itself but also from the mismatch between the complexity of environmental matrices and the diverse requirements of application scenarios. Firstly, the specificity advantage of the CRISPR/Cas system is restricted by potential off-target interference, a problem that is particularly pronounced in the detection of low-concentration environmental pollutants. Sequence mismatches between gRNA and non-target nucleic acids may trigger non-specific recognition and cleavage, directly leading to false-positive results that could misguide environmental risk assessment [12]. More notably, most existing CRISPR/Cas-based detection systems depend on auxiliary amplification technologies; non-specific products and cross-contamination generated during the amplification process often amplify the adverse impacts of off-target effects, forming a "synergistic superposition of off-target effects and amplification biases" that severely compromises the detection reliability of the platform.

The synthesis and fabrication of nanozymes are plagued by substantial technical bottlenecks and practical drawbacks. Currently, the synthesis of enzyme-mimetic nanozymes primarily relies on trial-and-error approaches and empirical accumulation, which is not only inefficient and resource-intensive but also hampers precise modulation of catalytic performance [46]. Although automated and rational design have emerged as mainstream trends in nanozyme research and development, existing

technologies still struggle to accurately predict the structure-activity relationship of nanozymes, impeding the discovery of novel high-efficiency nanozymes. Additionally, batch-to-batch variations in nanozyme synthesis, combined with the disruption of environmental factors (temperature, pH, ionic strength) on the stability of nanozymes and the activity of CRISPR/Cas components, collectively result in insufficient stability and reproducibility of the sensing platform's detection results. For single-atom nanozymes, the precise regulation of single-atom loading and long-term dispersion stability remains unsolved core technical challenges, directly affecting their catalytic efficiency and service life [66]. Therefore, there is an urgent need to improve performance through the optimization of preparation processes and the modification of storage conditions.

The complexity of real environmental samples and the limitations of application scenarios further aggravate the practical predicament of the platform. Environmental samples (e.g., soil, sewage, biological tissues) have complex compositions, and their intrinsic interfering substances - such as proteins, polysaccharides, and salts - tend to interact with nanozymes or CRISPR/Cas components, leading to reduced catalytic activity or impaired recognition specificity. Maintaining the platform's anti-interference capability in complex matrices has thus become a key challenge in current research [12]. From the perspective of application scenarios, most existing detection methods rely on standardized PCR procedures and thus struggle to achieve direct, high-precision on-site quantitative detection. They also lack multiplex diagnostic capabilities and are highly dependent on professional operators [34]. This "laboratory-centered" detection model is severely disconnected from the core demands of environmental monitoring (e.g,. on-site, rapid, high-throughput), greatly restricting the practical promotion and application scope of the platform.

4.2. Countermeasures and Solutions

To address the inherent limitations of the CRISPR/Cas system, a coordinated optimization strategy focusing on source design, reaction control, and amplification compatibility is essential. First, specificity can be enhanced at the source by employing bioinformatics-assisted gRNA design to minimize off-target homology and by selecting high-fidelity Cas protein variants. Second, at the operational level, introducing a dual-gRNA targeting strategy provides a "double-verification" mechanism for cleavage activity, while fine-tuning Cas protein concentration and reaction duration helps prevent non-specific cleavage caused by enzyme over-accumulation [12,46]. Furthermore, adopting isothermal amplification techniques such as RPA and loop-mediated isothermal amplification (LAMP) enables efficient amplification of trace nucleic acids under constant temperature, eliminates the need for thermal cyclers, and reduces non-specific amplification products. This makes them more suitable for on-site rapid detection, although strict protocols are required to prevent cross-contamination [67].

The consistency in synthesis and performance of nanozymes is a critical bottleneck for their practical application. Breakthroughs are needed across multiple dimensions, including rational design, process optimization, and standardization of conditions. On one hand, moving beyond traditional trial-and-error methods toward

an integrated approach combining computational simulation and high-throughput screening can establish an automated, design-oriented platform for the efficient and controllable synthesis of nanozymes. On the other hand, optimizing synthesis protocols and surface modification strategies, such as tuning surface charge and hydrophobicity, can improve intrinsic stability and catalytic activity [68]. Concurrently, standardizing the preparation and purification processes for nanozymes, along with systematic optimization of reaction parameters (e.g., temperature, pH, ionic strength) and implementation of improved storage solutions (e.g., sealed low-temperature storage with stabilizers), can significantly reduce batch-to-batch variation and enhance detection reproducibility. For more structurally precise single-atom nanozymes, it is crucial to develop precise atomic loading techniques and effective dispersion stabilization strategies, maintaining long-term performance through carrier modification and active site protection [66].

To overcome the complexity of real environmental samples and meet the demands of diverse field monitoring scenarios, an integrated workflow solution from sample pretreatment to final device integration must be developed. For interference resistance, a three-tiered strategy of "sample pretreatment - specific recognition - component tolerance enhancement" can be implemented: preliminary removal of matrix interferents via physical methods such as centrifugation and filtration; use of high-affinity recognition elements like aptamers or antibody-nucleic acid conjugates to specifically convert target pollutants into detectable nucleic acid signals; and engineering of both CRISPR/Cas components and nanozymes to improve their tolerance to inhibitors in complex matrices [12]. For field adaptability, developing ready-to-use kits (e.g., lyophilized reagents) coupled with user-friendly readout devices (e.g., smartphones or portable detectors) can greatly reduce the need for specialized expertise. Multiplex detection of pollutants can be achieved by exploiting orthogonal Cas enzymes and designing multi-channel probes. Furthermore, integrating the entire assay into microfluidic or paper-based platforms can create "sample-in-answer-out" portable and automated systems, ultimately fulfilling the requirements for rapid on-site monitoring in field and grassroots settings.

5. Conclusion

Nanozyme-assisted CRISPR/Cas biosensing technology integrates the efficient catalytic properties of nanozymes with the precise recognition capability of the CRISPR/Cas system, providing a novel, efficient, sensitive, and convenient detection strategy for environmental pollutant monitoring. This paper systematically elaborates on the core mechanism and platform construction strategy of this technology, comprehensively summarizes its applications in fields such as pathogenic microorganism and virus monitoring, screening of antibiotic resistance genes in environmental media, highly sensitive detection of heavy metal ions, and detection of refractory organic pollutants and biotoxins, deeply analyzes the challenges and countermeasures in its practical environmental application, and prospects its future development trends. In general, this technology holds significant application potential in environmental monitoring but still faces issues such as technical bottlenecks and

insufficient scenario adaptability, which urgently need to be addressed through in-depth research and technological innovation.

From the perspective of core technology iteration, the future development of the nanozyme-assisted CRISPR/Cas biosensing platform will focus on the functional upgrading of the CRISPR/Cas system and the innovative exploration of Cas proteins. On one hand, develop integrated on-site detection platforms to simplify sample preparation processes, simplify or eliminate pre-amplification steps for high-concentration target detection, improve detection efficiency, and reduce pollution risks, while retaining optimized isothermal amplification for trace target analysis; develop temperature-tolerant CRISPR/Cas reaction systems to broaden their application range under varying environmental temperatures; and achieve simultaneous multi-target monitoring through Cas enzyme modification or specific probe design. More importantly, the in-depth integration of the CRISPR/Cas system with complementary technologies (e.g., microfluidics, electrochemical detection) is expected to yield portable and commercial biosensors, promoting their transformation from laboratory research to practical application. On the other hand, the in-depth exploration and functional modification of novel Cas proteins will provide core support for platform performance breakthroughs—screening Cas proteins with higher specificity, sensitivity, and multi-recognition capabilities can break the platform's performance bottlenecks at the core component level.

At the level of functional integration and scenario adaptation, multifunctionalization and integration will become core development directions. Integrating multiple target recognition elements (e.g., multi-gRNA panels, orthogonal Cas proteins) with complementary detection modes (e.g., colorimetry, fluorescence, electrochemistry) can enable the simultaneous detection of multiple pollutants, overcoming the current platform's limitation of insufficient multiplex diagnostic capability; integrating the platform with microfluidic chips (for automated sample handling and reaction control), paper-based chips (for low-cost sample separation), and smartphones (for automated signal acquisition and data analysis) to construct portable, automated detection systems will break the limitations of professional operations and large instruments, meeting the monitoring needs of diverse environmental scenarios. Simultaneously, attention should be paid to the research and development of green preparation technologies for nanozymes and low-cost large-scale production processes for CRISPR components (gRNA, Cas proteins). Selecting environmentally friendly nanozyme materials and simplifying preparation processes can reduce the platform's production costs and environmental risks, laying the foundation for its large-scale promotion.

In the direction of intelligent upgrading and application expansion, integration with artificial intelligence (AI) and big data technologies will endow the platform with higher application value. Combining these cutting-edge technologies with the sensing platform enables in-depth analysis and mining of detection data, accurately completing the rapid identification, quantitative analysis, and risk assessment of target pollutants. More importantly, constructing pollutant databases and risk prediction models can upgrade the platform from a "single detection tool" to an "environmental governance decision support system" with dynamic early warning and precise regulation functions. This will provide a scientific basis for environmental management and policy

formulation, promoting the transformation of environmental monitoring toward "precision, intelligence, and prospectiveness".

In summary, although nanozyme-assisted CRISPR/Cas biosensing technology still faces numerous challenges in practical application, with continuous breakthroughs in core technologies, continuous optimization of scenario adaptability, and in-depth advancement of interdisciplinary integration, this technology is expected to play an important role in environmental monitoring, providing strong support for ensuring environmental safety and human health. Future research should focus on the core pain points in practical applications, strengthen technological innovation and achievement transformation, and promote the transition of this technology from laboratory research to large-scale, regular application.

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