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Argonaute proteins-powered biosensors and on-site analytical devices: A promising detection tool for food safety analysis

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ABSTRACT: As a core issue of global public health, food safety is of great importance. With the globalization of food trade, there is an increased risk of contamination with hazardous substances at all stages in the food supply chain. Therefore, the development of accurate and on-site detection methods is crucial to ensure food safety. Notably, Argonaute protein (Ago)-based biosensing technology offers innovative solutions for food safety detection due to its high specificity and programmability. In recent years, Ago-based biosensing technologies have been successfully integrated with portable detection equipment, opening up new pathways to achieve efficient on-site food safety detection. This review provided a summary of recent advances in Ago-based biosensing technologies and their portable detection devices, highlighting their potential applications in food safety detection. Firstly, the working principle of the Ago system was introduced and compared with the CRISPR/Cas systems. Subsequently, the nucleic acid amplification strategies and the signal amplification techniques employed in Ago-based biosensors were considerately summarized. Most importantly, the progress on the applications of Ago-based biosensing technologies in food safety detection was comprehensively discussed. Notably, this review also provided an outlook on the future prospects and challenges of this emerging field. Ago-based biosensing technology showed great potential for food safety detection. Impressively, the combination of Ago-based sensing technology and portable devices made effective on-site detection possible. As research continues, Ago-based biosensing technologies and portable detection devices are expected to provide more reliable and convenient detection solutions for the food industry.

Keywords: Argonaute; Biosensing technologies; Portable detection devices; Food safety analysis

1. Introduction

With the rapid development of the food industry and the globalization of the food supply chain, the risk of food contamination is increasing[1-2]. Pathogens, toxins, and other contaminants that may be present in food pose a serious threat to the health of consumers[3-4]. Therefore, the development of sensitive, rapid, and accurate food safety detection technologies has become a key means to ensure food safety[5]. A variety of

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detection techniques (e.g., instrumental analysis, immunoassay, and molecular diagnostic techniques) have been used to detect food hazards. The advantages and disadvantages of current food safety detection methods were summarized in Table 1. Instrumental analysis techniques represented by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) have the advantages of high sensitivity and accuracy but require expensive equipment and professional operators, which are difficult to meet the demand for rapid on-site detection[6-8]. Enzyme-linked immunosorbent assay (ELISA) is a typical immunoassay that is favored for its high sensitivity and high throughput[9]. However, it is susceptible to interference from food matrices, leading to false-negative or false-positive results[10]. Traditional molecular diagnostic methods, such as polymerase chain reaction (PCR), are considered the 'gold standard' for nucleic acid detection. However, the reliance on expensive equipment and the ease of contamination caused by improper operation have hindered their application in on-site detection[11]. Therefore, the development of reliable and accurate on-site detection techniques and portable equipment is essential to ensure food safety.

Table 1 Intestinal microorganisms producing indole derivatives and their functional properties

Aspect	Ago-based detection technology	Conventional methods	Comparative analysis
Detection time	Minutes to 2 h	• Plate culture: 1-5 d; • ELISA: 2-4 h; • PCR/qPCR: 1-3 h	Ago-based detection technology eliminates thermal cycling and multi-step incubation steps, simplifying the process. Its key advantage is combining high sensitivity with on-site detection speed.
Cost and portability	Relatively low reagent cost; requires only constant temperature.	• Plate culture: Low cost, high time/labor, incubator • ELISA: Moderate cost (antibodies), plate washer/reader • HPLC/MS: Large benchtop systems • PCR: Moderate cost, Thermal cycler	Ago-based detection technology eliminates the need for costly antibodies (ELISA), precision thermocyclers (PCR), and complex instrumentation, enabling on-site detection.
Specificity	Programmable guide DNA allows for single-nucleotide discrimination.	• Plate culture: phenotypic • ELISA: epitope-dependent • PCR: primer-dependent	Similar to PCR, the specificity of Ago-based detection technology is determined by sequence design; offering flexibility and programmability but it can be operated isothermally; surpassing antibody cross-reactivity (ELISA) and phenotypic ambiguity (plate culture).
Sensitivity/LOD	Based on nucleic acid amplification technology, Ago-based detection technology achieves PCR-level sensitivity. Its performance significantly surpasses that of plate culture-based methods (require target proliferation) and ELISA (limited by antibody affinity), enabling the detection of trace analytes.		

In recent years, Argonaute proteins (Agos) have emerged as novel tools for biosensing systems due to their excellent biological properties, such as programmability, nucleic acid targeting capability, high sensitivity, and sequence specificity[5, 12]. Similar to the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein (CRISPR/Cas) system, Ago is a class of emerging programmable nucleic acid-guided endonucleases[13]. Agos is widely found in prokaryotes and eukaryotes and is able to protect host cells by interfering with the invasion of foreign nucleic acids[14]. Several identified Agos can specifically cleave complementary target nucleic acids between the 10th and 11th bases of the guide DNA (gDNA)[15]. Compared with the CRISPR/Cas system, Agos is not subject to the limitations of specific sequence motifs (e.g., the Protospacer Flanking Sites (PFSs) and Protospacer Adjacent Motif (PAM) sequences) and can flexibly target many different nucleic acid sequences by designing different gDNA, thus enabling the

simultaneous detection of multiple targets in complex samples[16]. In addition, most Agos use a low synthetic cost and high stability single-stranded DNA (ssDNA) as a guide for targeting, which increases the flexibility, stability, and adaptability of the detection[17]. Due to its high programmability, Ago-based biosensing can be integrated with nucleic acid amplification technologies (e.g., PCR, loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA)) or amplification-free signal amplification strategies to achieve higher sensitivity[18-20]. Currently, Ago-based biosensing technologies have been combined with a variety of signal output strategies (e.g., fluorescence, colorimetric, and electroanalytical methods), opening up more avenues for Ago-based research and practical applications[21-23]. Furthermore, Ago-based detection methods have been integrated with lateral flow analysis (LFA) techniques, microfluidic chips, and nanopores, which further improve the sensitivity and convenience of the detection, and offer a promising prospect for effective on-site monitoring[24-25]. In addition, emerging technologies such as machine learning and artificial intelligence (AI) have also been applied in Ago-based biosensing technologies to further improve the accuracy and efficiency of data analysis[26-27].

Although Ago-based biosensing technologies were still in their infancy compared to that of CRISPR/Cas technologies, they have made significant progress in the field of food safety detection in recent years. To realize on-site detection, Ago-based biosensors have been integrated with portable devices, which were expected to be the platform for the next generation of food safety detection. Although several literatures have been reviewed on emerging programmable nuclease-based biosensing technologies[12-13, 28], the promising applications of Ago-based biosensing technologies and their portable detection devices in the field of food safety detection have never been specially summarized. Therefore, this review summarized the latest advances in Ago-based biosensing technologies and portable detection devices and their applications in the field of food safety (Fig. 1). Firstly, the working principle of Agos was briefly introduced, and the advantages and limitations of Ago-based technologies compared with those of the CRISPR/Cas system were highlighted. Then, the nucleic acid amplification and signal amplification strategies used in Ago-based biosensing techniques were comprehensively discussed. Finally, the promising applications of Ago-based biosensing technologies and portable detection devices for food safety detection were summarized. On this basis, the remaining challenges and future chances of Ago-based biosensing technologies in terms of design, development, and applications were proposed.

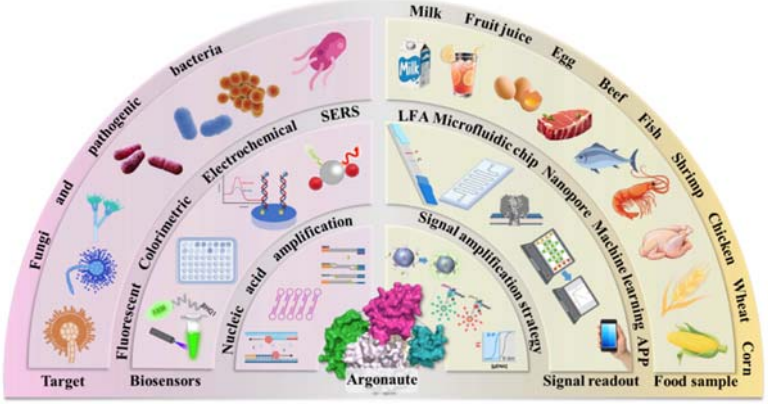

Fig. 1. Schematic illustration of Ago-based biosensing strategies and portable detection devices for food safety analysis.

2. Functional mechanisms of Argonautes

In recent years, programmable nucleases have gradually become important tools in the field of detection due to their significant biological advantages. Among them, the CRISPR/Cas system and Agos have been successfully used to construct biosensors. However, there are significant differences between these two nucleases in terms of DNA recognition, cleavage mechanisms, and practical applications. Therefore, an in-depth discussion on the differences between the CRISPR/Cas system and Agos is important for the design and optimization of biosensors. In this section, the functional mechanisms of Agos were described, and their differences from the CRISPR/Cas system were also discussed.

2.1. Functional mechanisms of Agos

Similar to the CRISPR/Cas system, Agos is a defense mechanism of microorganisms in nature to resist invasion by phages and other organisms[14]. According to their origins, Agos can be classified into eukaryotic Agos (eAgos) and prokaryotic Agos (pAgos)[29]. eAgos is a key component of the RNA-induced silencing complex (RISC) and plays an important role in the RNA interference (RNAi) mechanism. The structure of eAgos is more uniform and highly conserved, primarily consisting of four spherical domains (N-terminal; PAZ, PIWI Argonaute Zwille; MID, middle domain; and PIWI, P element-induced wimpy testis) and two linkers[30]. In contrast to eAgos, pAgos from archaea or bacteria exhibit more functional and structural diversity[14, 31]. pAgo is able to participate in the host defense processes through DNA interference and may also play roles in homologous recombination, DNA replication, and other pathways in the hosts[31, 32]. Based on structural length, pAgos can be classified into three categories: short pAgos, long pAgos, and PIWI-RE proteins (prokaryotic PIWI with only conserved R and E residues)[17, 33]. The structure of long pAgos is very similar to that of eAgos[33]. However, pAgos is more inclined to recognize DNA targets than eAgos[34].

pAgo can target and cleave specific DNA sequences under the guidance of one or more gDNAs (5'-phosphorylated modified (5'-P) ssDNA or 5'-hydroxylated modified (5'-OH) ssDNA)[35]. When cleaving double-stranded DNA (dsDNA), multiple distinct pAgo-gDNA complexes can target different strands of the same dsDNA for primary cleavage, resulting in the breakage of the dsDNA to generate a short ssDNA fragment. The resulting ssDNA can serve as a new gDNA to guide pAgo to perform a secondary cleavage and shear a molecular beacon that is dual-labeled with a quencher and the fluorescent group, thus generating a fluorescent signal for detection[36]. This “double guarantee” mechanism based on the two-step pAgo cleavage triggering detection signal ensures the specificity and accuracy of the detection[14]. Notably, by designing different gDNA and molecular beacons labeled with different fluorophores, Agos can target multiple target sequences at the same time, allowing multiplexed detection in complex samples[37].

In recent years, more and more structural features and cleavage properties of pAgos have been intensively studied and confirmed. Based on the host adaptation to the environment, pAgos can be classified into thermophilic pAgos and mesophilic pAgos[38]. Thermophilic pAgos typically exhibit cleavage activity at high temperatures. Compared to mesophilic pAgos, thermophilic Agos have higher endonuclease activity. This is

due to the fact that the structure of thermophilic Ago is more loosely packed at high temperatures, and its four structural domains are more likely to interact with substrates[39]. Among them, thermophilic Agos of *Thermus thermophilus* Ago (*Tt*Ago) and *Pyrococcus furiosus* Ago (*Pf*Ago) have been widely reported [28]. *Pf*Ago is the first pAgo protein whose crystal structure has been reported, exhibiting significant bioactivity within a high temperature range of 87 ℃-99.9 ℃, and it can effectively reduce plasmid DNA transformation to function in host defense[31, 40]. *Pf*Ago can use 15-31 nucleotides (nt) of 5'-P ssDNA as gDNA to precisely cleave the phosphodiester bond (on ssDNA or dsDNA) located between the 10th and 11th nucleotides from the 5' end of the gDNA[31, 33]. Excessively long or short gDNA sequences may affect its binding to pAgo, thereby impacting cleavage activity. Currently, *Pf*Ago-based food safety detection methods primarily focus on the detection of foodborne pathogens and mycotoxins. The high reaction temperature required by *Pf*Ago facilitates the denaturation of dsDNA, thus demonstrating significant potential in nucleic acid-based detection of foodborne pathogens[41]. *Tt*Ago is another thermophilic Argonaute protein, with an optimal reaction temperature range of 65 ℃ to 86 ℃, which is lower than that of *Pf*Ago[40]. Similar to *Pf*Ago, *Tt*Ago can specifically cleave DNA or RNA targets after binding to 13-25 nt of gDNA[17]. Notably, *Tt*Ago has the same binding affinity for gDNA and dsDNA, which makes it difficult for the *Tt*Ago-gDNA complex to bind to dsDNA, resulting in higher cleavage efficiency for ssDNA than for dsDNA[17]. In contrast, mesophilic Agos (e.g., *Clostridium butyricum* Ago (*Cb*Ago), *Clostridium perfringens* Ago (*Cp*Ago), and *Kurthia massiliensis* Ago (*Km*Ago)) exhibit cleavage activity at moderate temperatures (around 37 ℃)[17]. This feature not only avoids the limitations of high-temperature reactions, allowing the detection process to be performed without heat, but also simplifies the operational process, increases adaptability, and reduces costs[14]. Among these mesophilic pAgos, *Cb*Ago is the most widely used in detection. Guided by gDNA, it is capable of cleaving ssDNA or dsDNA at moderate temperatures, providing operational flexibility in food-safety detection. However, compared to thermophilic Agos such as *Pf*Ago and *Tt*Ago, *Cb*Ago exhibits relatively lower cleavage activity owing to its unique crystal structure, which may limit its practical application[41]. Therefore, the temperature dependence of Agos must be taken into account when designing detection methods using different types of Agos. Overall, with these excellent properties, Agos shows great potential for applications in biosensing.

2.2 Comparison between Agos and CRISPR/Cas systems

Both Agos and CRISPR/Cas systems are derived from microbial defense mechanisms that protect host organisms by recognizing and cleaving foreign nucleic acids[13, 42-43]. Similar to Cas proteins, Agos also function as nucleic acid-guided restriction endonucleases.

Although Agos and Cas proteins exhibit some similarities in target recognition and cleavage activities, their different catalytic mechanisms lead to differences in performance in biosensing applications (Table 2). To date, constructed CRISPR/Cas detection methods primarily employed Cas12a or Cas13a as effector molecules[13, 44]. While most Ago-based detection methods focus on the application of *Pf*Ago, *Tt*Ago, and *Cb*Ago[45-46]. Compared with the CRISPR/Cas system, pAgos has the following significant advantages:

Table 2 Intestinal microorganisms producing indole derivatives and their functional properties

Feature	pAgo Systems	CRISPR/Cas Systems (e.g., Cas12, Cas13)
Core component	Ago protein and guide DNA	Cas protein and crRNA
Target recognition	DNA-guided target (DNA or RNA) recognition.	RNA-guided target DNA (Cas9, Cas12) or target RNA (Cas13) recognition.
Guide DNA/RNA	Short, synthetic 5'-P or 5'-OH DNA (gDNA). Cheap, stable, easy to synthesize/modify.	Longer, crRNA (often with tracrRNA). Requires *in vitro* transcription or chemical synthesis.
PAM/PFS Requirement	PAM-independent. Dramatically simplifies target site selection and multiplexing.	PAM (Cas12) or PFS (Cas13) dependent. Restricts targetable sequences.
Operational temperature	Broad range available: • Thermostable (*Pf*Ago: 87~99.9 ℃) • Thermostable (*Tt*Ago: 75~86 ℃) • Mesophilic (*Cb*Ago: ~37 ℃)	~37 ℃
Cleavage characteristics	In contrast, Agos cleaves the substrate in a sequence-specific manner, requiring a "two-step" sequence-specific cleavage process to complete the detection. The initial cleavage by Agos detects the target, and the cleavage products can be used as a "secondary guide" to further guide a second round of cleavage of the complementary target, resulting in a detection signal.	Cas12a and Cas13a both have cis- and trans-cleavage activities. Once the target is recognized, Cas proteins can be used to achieve one-step detection with their trans-cleavage activity.
Primary use in biosensing	pAgo systems can specifically recognize and cleavage corresponding target sequences. Can be coupled with pre-amplification for ultra-sensitivity.	In the CRISPR/Cas system, crRNA can recognize the specific cleavage sites and Cas effectors can cleave the target nucleic acids. Utilizing *trans*-cleavage activity to achieve exponential signal amplification, enabling single-molecule sensitivity. Almost always requires a pre-amplification step.
Multiplexed detection	The use of short DNA (rather than longer crRNA) not only simplifies the design of guide strands but also allows for the design of various guide strands based on the principle of complementary base pairing. In addition, it has the flexibility to target multiple different nucleic acid sequences in a sequence-specific manner, thus enabling multiple detections.	The trans-cleavage of the CRISPR/Cas system is nonspecific, making it difficult to accurately distinguish between different targets, which poses challenges for multiplex detection.
Instrument dependency	No thermal cycling.	No thermal cycling.

Firstly, in the initial recognition step, the cleavage of Agos is strictly dependent on the complementarity between the guide strand and the target rather than on specific sequences (e.g., PAM or PFSs), which allows it to target a broader range of nucleic acid regions than the CRISPR/Cas system[47]. Agos typically utilize shorter 5'-P or 5'-OH DNA or RNA guide sequences for target recognition[48]. Compared to the longer crRNA guide strands used by Cas nucleases, DNA guide strands offer lower synthesis costs and higher stability[49]. Furthermore, the compact size of pAgos (about half that of Cas9 and Cas12a, at 75-85 kDa) enhances the delivery efficiency of the ribonucleoprotein complex into the desired host, providing favorable conditions for their broader application[33, 34].

Secondly, the structures of Agos and Cas proteins change at different stages of target recognition. Cas12a and Cas13a both have cis- and trans-cleavage activities[50]. Once the target is recognized, Cas proteins can be used to achieve one-step detection with their trans-cleavage activity[51-52]. In contrast, Agos cleaves the substrate in a sequence-specific manner, requiring a "two-step" sequence-specific cleavage process to complete the detection[53]. The initial cleavage by Agos detects the target, and the cleavage products can be used as a "secondary guide" to further guide a second round of cleavage of the complementary target, resulting

in a detection signal[54]. This "double-check" mechanism allows each step to be guided by a different base-pairing sequence, which significantly enhances the specificity of the detection[12].

Lastly, Ago-based approaches are more advantageous in handling the challenges of multiple nucleic acid detection. The use of short DNA (rather than longer crRNA) not only simplifies the design of guide strands but also allows for the design of various guide strands based on the principle of complementary base pairing. In addition, it has the flexibility to target multiple different nucleic acid sequences in a sequence-specific manner, thus enabling multiple detections[55-56]. In contrast, the trans-cleavage of the CRISPR/Cas system is nonspecific, making it difficult to accurately distinguish between different targets, which poses challenges for multiplex detection[57]. It is worth noting that the Ago-based approach provides the solution to this problem, effectively avoiding the signal attribution problems caused by trans-cleavage activity in the CRISPR/Cas system.

3. Nucleic acid amplification strategies in Ago-based detection system

Direct detection of trace DNA is challenging due to the fact that actual samples usually contain minute amounts of the target nucleic acid. To increase the sensitivity of the detection, it is often needed to amplify the target nucleic acid to a detectable level prior to detection[58]. As the most commonly used nucleic acid amplification technology, PCR has been successfully combined with Ago-based sensing strategies for the detection of spoilage bacteria and pathogenic bacteria in food samples[59- 60]. Although the PCR exhibited good specificity and sensitivity, the reliance on sophisticated instruments (thermal cyclers) and the complex operational procedures had limited its wide application. Consequently, several emerging isothermal nucleic acid amplification techniques were also combined with Ago-based biosensors to improve the sensitivity of detection, including loop-mediated isothermal amplification (LAMP)[22, 25, 61-63], recombinase polymerase amplification (RPA)[64-69], recombinase aided amplification(RAA)[70], and exponential isothermal amplification reaction (EXPAR)[71]. They used strand displacement polymerase to amplify the target under constant temperature conditions, which eliminated the need for a precise thermal cycler[14]. Despite the advantages of amplification efficiency and high sensitivity, the isothermal nucleic acid amplification methods were prone to false-positive results due to mispriming or template-independent amplification in practical applications[72]. Combined with nucleic acid amplification, the Ago-based two-step cleavage approach could be used as a 'double-checking' mechanism to improve the specificity and accuracy of the detection. However, this two-step cleavage method also had limitations, such as the prolonged detection time, increased complexity/cost due to the introduction of multiple gDNAs, and reduced sensitivity due to the dependence of the reaction order[31]. To address these issues, several Ago-based one-step cleavage methods have been reported[73]. For instance, Li et al. developed a *Pf*Ago-based one-step cleavage fluorescence detection platform by integrating Exonuclease I (Exo I) and Tag-specific primer extension. In this strategy, the target gene was first amplified using Tag-specific primers, and then the remaining primers were digested by Exo I. Finally, the amplification products with the Tag sequence could be used as a gDNA that would guide *Pf*Ago to cleave the fluorophore-quencher reporter, resulting in efficient fluorescence signal output[21].

4. Nucleic acid amplification-free strategies in Ago-mediated detection system

Although the nucleic acid amplification step significantly increased the sensitivity of the detection by enhancing the target concentration, the reliance on additional amplification steps not only prolonged the analysis time and increased the complexity and cost of the reaction but also introduced the risk of cross-contamination and aerosol contamination[31]. More critically, the formation of off-template products or secondary structures during the amplification process could easily lead to false-negative/false-positive results, which seriously affected the accuracy of the detection[54]. Therefore, researchers developed a one-pot detection strategy by integrating target pre-amplification and specific sensing by nucleases in a single reactor[61, 66-67]. However, this method sacrificed the performance of individual reactions to some extent. Based on this, the researchers turned to constructing a variety of signal amplification strategies without nucleic acid pre-amplification strategies to break through the dependence on amplification steps in Ago-based biosensing.

4.1 Signal amplification based on entropy-driven circuit (EDC)

EDC was an isothermal enzyme-free signal amplification technology that exhibited higher stability and lower cost compared to enzyme-based amplification techniques[39]. The EDC reaction employed a series of multifunctional linear ssDNA strands to provide an amplification circuit, triggering a toehold-mediated strand displacement reaction (TMSDR) that released single-stranded oligonucleotides[18]. Due to its advantages of simple design, high stability, low cost, and modularity, EDC has been widely used as an emerging signal amplification strategy in biosensing. Considering that the ssDNA products could be designed as an initiator for downstream reaction systems, EDC could be cascaded with Ago to enhance signal amplification efficiency. For example, Lu et al. proposed a magnetic Fe_3O_4@metal-organic framework (MOF)@Aptamer-mediated entropy-driven fluorescence biosensor combined with the *Cb*Ago for multiplex detection of pathogenic bacteria. Prepared aptamer-modified magnetic Zr-based MOF recognized pathogens and released complementary DNA (cDNA) to activate EDC reactions. This process converted low concentrations of cDNA input into high concentrations of gDNA output, triggering efficient cleavage by *Cb*Ago and effectively amplifying the fluorescent signal[39]. In another study, Lin et al. developed an EDC-*Tt*Ago powered fluorescence single-particle aptasensing platform for the detection of fumonisin B_1 (FB_1). When the target was presented, the aptamer specifically bound to the target molecule and released a trigger sequence, which mediated the EDC reaction to generate a large number of 5' P output sequences. These sequences could serve as gDNA to activate *Tt*Ago, enabling the signal probe (red fluorescence-encoded microspheres (RFEMs)-ssDNA-Biotin) to be specifically cleaved. This process led to an increase in the number of RFEMs remaining in the final reaction supernatant after magnetic separation, ultimately enabling amplification of the fluorescent signal[18].

4.2 Signal amplification based on enzyme-assisted methods

The incorporation of other enzymatic reactions into Ago-based detection has also emerged as an effective signal amplification strategy, which could significantly enhance the application prospects of Ago-based biosensing technologies. For example, Lu et al. developed a *Cb*Ago-based aptamer fluorescence platform utilizing the Exo I cyclic signal amplification to achieve multiplexed detection. When the target bound specifically to the aptamer, it triggered the release of gDNA from the surface of the millimeter-sized Polystyrene Sphere (PS_{mm}) and the generation of target-aptamer complexes. Subsequently, the resulting complexes were digested by Exo I to release the target, which triggered the next cycle. After several cycles, the number of gDNA enriched on the surface of the PS_{mm} increased, which significantly enhanced the cleavage efficiency of the FQ reporter by *Cb*Ago, thus displaying a signal amplification effect[74]. In addition, enzyme-mediated cascade amplification could considerably increase the sensitivity of the detection. For example, Chen et al. developed a double-enzyme (*Pf*Ago and CRISPR/Cas12a)-mediated cascade signal amplification detection platform by utilizing the specific cleavage activity of *Pf*Ago and the highly efficient trans-cleavage activity of CRISPR/Cas12a. This platform first activated the trans-cleavage activity of Cas12a through the release of tDNA from phage lysis, triggering the detachment of alkaline phosphatase (ALP) from the surface of magnetic nanoparticles (MNPs). By adjusting the amount of ALP available for dephosphorylation, the specific cleavage efficiency of *Pf*Ago on FQ could be controlled, thereby inducing changes in fluorescent signals. This process of reading out the signal realized the cascading amplification[19].

4.3 Signal pre-amplification based on signal probes

In the detection system based on Agos, researchers have successfully developed a series of high-fluorescence-load probes, which significantly improve detection sensitivity through a signal pre-amplification mechanism, thereby eliminating the need for nucleic acid amplification steps. For example, Wei et al. developed a *Cb*Ago-mediated fluorescent biosensor using fluorescence-enriched microspheres with encoded signals. The fluorescence-encoded microsphere designed through on-tetrahedron rolling circle amplification (RCA) effectively achieved amplified enrichment of fluorescence. By reading signals using *Cb*Ago-mediated decoding technology, ultrasensitive detection of the methicillin-resistant *Staphylococcus aureus* (MRSA) genes (*mecA* and *femA*) was achieved[75]. Similarly, Wang et al. created multi-signal probes using tetrahedron DNA (TDNA)-enhanced hybridization chain reaction (TDNA-HCR), generating ultra-bright polystyrene microspheres (PS) labeled with various fluorophores, which significantly enhanced the signal intensity. When the target triggered the *Cb*Ago cleavage reaction, the fluorescent signal on the surface of the microspheres responded to the target through a "fluorescence quenching-recovery" mechanism, achieving one-to-many signal amplification without DNA amplification[76]. In another study, Wang et al. constructed botryoidal-like fluorescent PS dots using a primer exchange reaction (PER), namely DNA-PER-PS-dots. PER could greatly increase DNA binding sites through self-assembly, thereby enhancing the DNA binding sites of the fluorescent probes and effectively amplifying the signal. In this digital detection method based on DNA-PER-PS-dots, the DNA sequences generated after precise cleavage of pathogen DNA by *Cb*Ago could serve as bridges for molecular hybridization reactions. This allowed DNA-PER-PS-dots to specifically bind to the surfaces of

DNA sequence-modified liposome-decorated magnetic beads (DNA-LP-MBs) of different sizes through hybridization reactions, thus enabling simultaneous detection of multiple pathogens[77].

4.4 Signal amplification based on fluorescent signal enrichment strategies

As an important nanomaterial, magnetic beads (MBs) have emerged as promising candidate carriers for digital detection due to their unique properties, such as superparamagnetic behavior, site-specific targeting, and controllable magnetic fields [78]. Improving the binding efficiency of fluorescent molecules to the surface of MBs was also an effective strategy for signal amplification. For example, a phage and *Cb*Ago-mediated fluorescent biosensing system had been developed. This system initially utilized phages to specifically capture and lyse viable *Salmonella typhimurium* to release genomic DNA. Subsequently, the resulting tDNA could activate *Cb*Ago to cleave biotinylated fluorescent probes, which enabled the accumulation of fluorescent probes on the surface of the fluorescent signal enrichment carrier (PS microspheres), achieving signal amplification[26]. Subsequently, the Chen's team developed a digital carrier system that utilizes homogeneous MBs as encoding beads in place of the monodisperse droplets used in standard digital nucleic acid detection. In this platform, each of the three foodborne pathogen-specific gDNA-*Cb*Ago complexes activated cleavage of the corresponding FQ reporter probes with biotin-modified fluorescent ends. Through the interaction between streptavidin (SA) and biotin, the resulting biotinylated fluorescent products were specifically encoded onto the surface of the MB-tyramine-SA conjugate, resulting in signal enrichment without amplification. This approach overcomes the limitations of conventional amplification methods that rely on high-intensity fluorescence readouts for signal detection[27].

4.5 Multiple signal amplification strategies

To further enhance the sensitivity of the amplification-free biosensors, Wang et al. proposed a DNA and adenosine triphosphate (ATP) was synergistically triggered fluorescent signals enhanced biosensor for the detection of viable *Salmonella*. On the one hand, the genomic DNA produced by phage lysis could directly serve as the gDNA of *Pf*Ago, which triggered the cleavage of the FQ fluorescent probe. On the other hand, the ATP released by phage lysis could induce the release of gDNA through the recognition of bivalent aptamers, thereby triggering the cleavage of the FQ reporter by *Pf*Ago. This double cleavage by DNA and ATP synergistically triggered in a single tube, which greatly improved the sensitivity of the detection[15]. To construct a universal non-nucleic acid target detection technology, biorecognition molecules such as aptamers and antibodies have been introduced into the Ago system for universal and efficient signal conversion. For example, Chen's team developed a programmable platform by combining a TDNA@MNPs encoding system with *Cb*Ago[32]. This platform employed two signal conversion systems (antibody–antigen immunoreaction system and aptamer recognition of targets system) to release gDNA complexes (gDNA-bovine serum albumin (BSA)-mycotoxin conjugate, for mycotoxin detection) and gDNA (for pathogen detection), respectively. The released gDNA could guide *Cb*Ago to recognize and cleave the initial DNA (iDNA) on TDNA-functionalized MNPs (iDNA-TDNA@MNPs), resulting in targeted blockade of different HCRs (containing hairpins

modified with different fluorescent groups). The HCRs on TDNA@MNPs could change the number of fluorescent hairpins in the supernatant, thus altering the fluorescence signal. The platform combined the gDNA-BSA-mycotoxin probe synthesized through the antigen-enriched gDNA strategy with the *Cb*Ago cleavage system and HCR, effectively achieving a triple signal amplification effect[32]. Furthermore, Chen's team also developed a multiplex detection platform based on micropore resistance counting of size-encoded PS microspheres for the detection of mycotoxin. When the concentration of mycotoxin was high, the binding of gDNA-BSA-mycotoxin to antibodies was reduced, resulting in more gDNA-BSA-mycotoxin remaining in the supernatant. Subsequently, the resulting gDNA-BSA-mycotoxin could activate *Cb*Ago protein to cleave the corresponding tDNA on the surface of PS-tDNA conjugates (PS microspheres modified with different tDNAs) of different particle sizes. This process weakens the interaction between MNP-SA and PS-tDNA (tDNA was modified with biotin), which altered the concentration of PS microspheres remaining in the supernatant after magnetic separation. Measurement of the concentration and size of PS microspheres by microporous resistance counting allows simultaneous detection of various targets. The biosensing platform combined gDNA-enriched antigen with the *Cb*Ago system, achieving dual-signal amplification and enhancing the sensitivity of the biosensor[79].

4.6 Signal amplification based on Ago' built-in

In recent years, Ago-based biosensing methods have also been widely explored due to their built-in signal amplification properties. For example, Huang et al. introduced ALP into the system using the *Pf*Ago's preference for 5'-P gDNA and proposed a regulatory strategy based on gDNA dephosphorylation for the detection of aflatoxin B_1. When the target was present, immunolabeled ALP catalyzed the hydrolysis of the 5'-P gDNA, thus effectively locking the cleavage activity of *Pf*Ago on the FQ reporter and inhibiting the generation of fluorescent signals[80]. In another study, Chen's research team designed a novel gDNA containing DNAzyme segments ($gDNA_{zyme}$) by investigating the key factors affecting the activity of Agos. This $gDNA_{zyme}$ significantly enhanced the cleavage efficiency of *Cb*Ago. Based on this, they further constructed a $gDNA_{zyme}$-enhanced *Cb*Ago-mediated aptasensor, which could detect three pathogenic bacteria simultaneously without DNA extraction and amplification[81].

4.7 Signal amplification-free strategy

To address the throughput limitations of traditional microdroplet analysis, Wang et al. developed a *Cb*Ago-mediated multiplex nucleic acid platform by integrating a deep-learning-powered programmable, amplification-free system (PASS), which could rapidly quantify nucleic acid at ambient temperature in one pot. In this platform, activated fluorescent signal mediated by *Cb*Ago was transferred into a polydisperse emulsion, which allowed the fluorescent signal to be concentrated in tiny droplets through the confinement effect of the microdroplets. Compared to traditional homogeneous reaction systems, PASS achieved high sensitivity and precision in both single and multiplex detections without signal amplification[82].

5. Promising applications of Agos-based detection technologies in food safety detection

In recent years, frequent food safety incidents have triggered widespread concern in the community about food contamination. Foodborne contaminants not only posed a threat to food safety but also presented significant risks to human health. In view of the excellent performance and potential of Ago in the field of biosensing, researchers have successfully developed a variety of Ago-based biosensors, which have been widely used in the detection of major contaminants such as foodborne pathogens and mycotoxins. To further promote the development of on-site rapid detection technologies, portable detection devices based on Ago technology (e.g., LFA, microfluidic chips, and nanopores) have been developed and successfully applied to on-site food safety detection. This section focused on the latest application advances and research highlights of Ago-based biosensing technologies and their portable detection devices in the field of food safety detection, especially the exploration of the integration of multiple detection technologies and machine learning algorithms.

5.1 Ago-based biosensors

5.1.1 Ago-based fluorescent biosensors

Fluorescent biosensors are mainly based on target-induced changes in fluorescence intensity, such as fluorescence enhancement/quenching (i.e., "turn-on"/"turn-off") and fluorescence resonance energy transfer (FRET)[83-84]. FRET refers to the non-radiative transfer of energy from an excited donor molecule to an acceptor molecule. In this process, the donor absorbs light and is excited, and then transfers this energy to the acceptor, which subsequently emits fluorescent light. FRET usually occurs under the condition that the emission spectrum of the donor fluorescent molecule and the absorption spectrum of the acceptor fluorescent molecule overlap sufficiently and are close enough (usually less than 10nm)[85]. Fluorescent biosensors have been extensively used in the field of analysis due to their advantages of simple operation, high sensitivity, and fast response. Currently, fluorescence sensing has been successfully introduced into Ago to develop novel Ago-based fluorescent biosensors. Ago could be used as a biomolecular recognition element to specifically recognize target analytes and convert this recognition event into a detectable fluorescent signal, enabling quantitative and qualitative analysis of the target analyte. Typical Ago-based fluorescence biosensing strategies used fluorescent dye and quencher dual-labeled ssDNA as the signal probe. When a target was present, Agos performed primary cleavage of the target nucleic acid under the guidance of gDNA. The resulting short DNA products served as a secondary guide sequence that triggered the secondary cleavage of the FQ probe by Ago, which released a detectable fluorescent signal by separating the fluorophore from the quencher. Through a combination of various nucleic acid amplification techniques, this strategy has been successfully used to detect foodborne contaminants such as *Enterocytozoon hepatopenaei*[64], *Vibrio parahaemolyticus* carrying the acute hepatopancreatic necrosis disease (AHPND) virulence gene in shrimp[65], *Alicyclobacillus acidoterrestris* in fruit juices[59], and *Salmonella*[60, 68]. The corresponding limit of detection (LOD) and linear ranges were listed in Table 3.

Table 3 Summary of representative Ago-enabled biosensing technologies for detecting food contaminants

Analytes	Ago category	Amplification	Signal readouts	Detection time	Linear range	LODs	Sample matrix	Ref.
Alicyclobacillus acidoterrestrsis	*Pf*Ago	PCR	Fluorescence	-	10^2-10^5 CFU/mL	10^1 CFU/mL	Fruit juice	[59]
Enterocytozoon hepatopenaei	*Pf*Ago	RPA	Fluorescence	1.5 h	-	-	Shrimp	[64]
The *PirA^{vp} and PirB^{vp}* gene from acute hepatopancreas necrosis disease	*Pf*Ago	RPA	Fluorescence	75 min	-	-	Shrimp	[65]
The *hly gene* from *Listeria monocytogenes*	*Pf*Ago	LAMP	Fluorescence and LFA	-	-	1.8×10^1 copies (under UV/blue light), and 1.8×10^2 copies (using lateral flow strips)	Beef; Chicken; Milk	[62]
Salmonella	*Pf*Ago	RPA	Fluorescence	40 min	1-1.05×10^8 CFU/mL	1.05×10^1 CFU/mL	Milk; Potable water; Chicken; Salad	[68]
Salmonella	*Pf*Ago	-	Fluorescence	-	1.0×10^2-1.0×10^7 CFU/mL	20 CFU/mL	Chicken; Pork; Beef; Eggs	[15]
Salmonella	*Cb*Ago	-	Fluorescence	-	50-10^7 CFU/mL	40.5 CFU/mL	Chicken; Lettuce; Pork; Fish; Milk; Orange juice; Environmental water sample	[26]
Salmonella typhimurium	*Pf*Ago	-	Fluorescence	-	50-1×10^7 CFU/mL	23 CFU/mL	Pork; Fish; Eggs; Milk; Orange juice	[19]
Salmonella typhimurium	*Pf*Ago	LAMP	Fluorescence and LFA	45 min	10^0-10^8 CFU/mL	1 CFU/mL	Milk; Eggs	[22]
Salmonella typhimurium	*Pf*Ago	LAMP	SERS	60 min	10^0-10^8 CFU/mL	1 CFU/mL	Milk; Eggs; Orange juice	[96]
Salmonella typhimurium	*Pf*Ago	LAMP	Colorimetric	-	10^1-10^8 CFU/mL	1 CFU/mL	Milk; Chicken; Eggs; drinking water	[20]
Salmonella typhimurium	*Pf*Ago	LAMP	Fluorescence	-	10^1-10^7 CFU/mL	1 CFU/mL	Milk; Meat; Eggs; drinking water	[109]
The *hilA* gene from *Salmonella*	*Pf*Ago	PCR	Fluorescence	-	1×10^2-10^3 copies	3×10^0 copies and 2.7×10^0 CFU (cells)	Chicken; Milk	[60]
Staphylococcus aureus	*Pf*Ago	RAA	Fluorescence, LFA	-	10^0-10^8 CFU/mL	1 CFU/mL	Milk; Eggs	[70]

Staphylococcus aureus	*Tt*Ago	LAMP	Fluorescence, microfluidic chip	17 min	10^0-10^7 CFU/mL	1 CFU/mL	Milk; Eggs	[25]
Aquatic pathogens	*Pf*Ago	LAMP	Fluorescence, handheld isothermal fluorescence detector	40 min	-	-	Fish; Shrimp	[103]
Vibrio parahemolyticus	*Tt*Ago	EXPAR	Fluorescence, microfluidic chip	40 min	3.5×10^0 to $\times 10^7$ CFU/mL	10^0 CFU/mL	Fish; Shrimp; Shellfish; Crab; Cured fish	[110]
FB_1	*Tt*Ago	-	Fluorescence	-	1-10^5 pg/mL	0.89 pg/mL	Corn; Plant-based meat analogues	[18]
ALP; AFB_1	*Pf*Ago	-	Fluorescence	-	10-1000 U/L (ALP); 0-500,000 pg/mL (AFB_1)	2.70 U/L (ALP); 29.89 pg/mL (AFB_1)	Wheat; Corn	[80]
The *MecA* genes and *nuc* genes from *Staphylococcus aureus*	*Pf*Ago	LAMP	Fluorescence	-	1-10^8 CFU/mL	1 CFU/mL	Milk; Eggs	[61]
The *MecA* genes and *nuc* genes from *Staphylococcus aureus*	*Pf*Ago	RPA	Fluorescence	55 min	10^1-10^9 copies/μL	10^2 copies/μL	Clinical sample; Milk; Beef	[67]
The *MecA* genes and *nuc* genes from *Staphylococcus aureus*	*Pf*Ago	LAMP	Fluorescence	<65 min	10-10^7 CFU/mL	10 CFU/mL	Egg; Milk; Meat	[63]
The *MecA* genes and *FemA* genes from *Staphylococcus aureus*	*Cb*Ago	-	Fluorescence	-	1-1000 fM	0.63 fM (*mecA* genes); 0.48 fM (*femA* genes)	Milk; Eggs; Pork	[75]
The *hly* genes and *ide* genes from *Listeria monocytogenes*	*Pf*Ago	LAMP	Fluorescence	45 min	10^0-10^8 CFU/mL	1 CFU/mL	Egg; Beef; Shrimp	[98]
Staphylococcus aureus; Salmonella	*Pf*Ago	-	Fluorescence	-	1-1×10^8 CFU/mL	1 CFU/mL	Orange juice; Eggs	[21]
Salmonella; *Listeria monocytogenes*	*Cb*Ago	EXPAR	Fluorescence	-	10-10^7 CFU/mL; (*Salmonella*) 10^2-10^7 CFU/mL;	7 CFU/mL (*Salmonella*); 22 CFU/mL	Chicken; Pork; Milk	[71]

					(*Listeria monocytogenes*)	(*Listeria monocytogenes*)		
Salmonella typhimurium; *Listeria monocytogenes*	*Cb*Ago	RPA	Fluorescence	<45 min	9.3-9.3×10^6 CFU/mL (*Salmonella typhimurium*); 8.4-8.4×10^6 CFU/mL (*Listeria monocytogenes*)	2 CFU/mL (*Salmonella typhimurium*); 1CFU/mL (*Listeria monocytogenes*)	Chicken; Pork	[66]
Escherichia coli; *Staphylococcus aureus*	*Cb*Ago	-	Fluorescence	-	10^2-10^6 CFU/mL	79 CFU/mL (*Escherichia coli*); 68 CFU/mL (*Staphylococcus aureus*)	Chicken; Pork	[39]
Staphylococcus aureus; Escherichia coli; *Salmonella*	*Cb*Ago	-	Fluorescence	-	10-10^6 FU/mL	46 CFU/mL (*Staphylococcus aureus*); 76 CFU/mL (*Escherichia coli*); 75 CFU/mL (*Salmonella*)	-	[81]
Listeria monocytogenes; *Staphylococcus aureus*; *Salmonella typhimurium*	*Cb*Ago	-	Fluorescence	1~1.5 h	10^3-10^6 CFU/mL (*Listeria monocytogenes*); 10^2-10^5 CFU/mL (*Staphylococcus aureus*); 10^2-10^6 CFU/mL (*Salmonella typhimurium*)	3 CFU/mL (*Listeria monocytogenes*); 2 CFU/mL (*Staphylococcus aureus*); 5 CFU/mL (*Salmonella typhimurium*)	Eggs	[82]
Listeria monocytogenes; *Staphylococcus aureus*; *Salmonella typhimurium*	*Cb*Ago	-	Fluorescence	1.5 h	10^2-10^6 CFU/mL (*Staphylococcus aureus* and *Listeria monocytogenes*); 10^3-10^6 CFU/mL (*Salmonella typhimurium*)	2 CFU/mL (*Listeria monocytogenes*); 4 CFU/mL (*Staphylococcus aureus*); 6 CFU/mL (*Salmonella typhimurium*)	Live shrimp; Frozen shrimp; Live fish; Clinical sample	[77]

Listeria monocytogenes; *Staphylococcus aureus*; *Salmonella*	*Cb*Ago	-	Fluorescence	-	10^1-10^7 CFU/mL	6 CFU/mL (*Listeria monocytogenes*); 6 CFU/mL (*Staphylococcus aureus*); 7 CFU/mL (*Salmonella typhimurium*)	Live shrimp; Frozen shrimp; Eggs; Frozen chicken	[27]
Listeria monocytogenes; *Staphylococcus aureus*; *Salmonella typhimurium*; *Escherichia coli*	*Cb*Ago	-	Fluorescence	-	10^3-10^8 CFU/mL (*Listeria monocytogenes*); 10^2-10^7 CFU/mL (*Staphylococcus aureus*); 10^3-10^7 CFU/mL (*Salmonella typhimurium*); 10^2-10^7 CFU/mL (*Escherichia coli*)	101 CFU/mL (*Listeria monocytogenes*); 39 CFU/mL (*Staphylococcus aureus*); 92 CFU/mL (*Salmonella typhimurium*); 46 CFU/mL (*Escherichia coli*)	Fish; Clinical sample	[76]
Mycotoxins; Pathogenic bacteria	*Cb*Ago	-	Fluorescence	-	0.05-200 ng/mL (AFB_1); 0.1-500 ng/mL (Ochratoxin A); 0.1-200 ng/mL (Zearalenone); 10^2-10^7 CFU/mL (*Staphylococcus aureus*; *Escherichia coli*; *Salmonella typhimurium*)	28.5 pg/mL (AFB_1); 50.0 pg/mL (Ochratoxin A); 56.0 pg/mL (Zearalenone); 46 CFU/mL (*Staphylococcus aureus*); 39 CFU/mL (*Escherichia coli*); 64 CFU/mL (*Salmonella typhimurium*)	Maize	[74]
Mycotoxins; Pathogenic bacteria	*Cb*Ago	-	Fluorescence	-	10-10^5 pg/mL (AFB_1 and AFG_1); 10^2-10^6 pg/mL (DON); 10^2-10^7 CFU/mL (*Escherichia coli*); 10^2-10^8 CFU/mL (*Staphylococcus aureus)*;	5.48 pg/mL (AFB_1); 4.88 pg/mL (AFG_1); 59.65 pg/mL (DON); 46 CFU/mL (*Escherichia coli*); 59 CFU/mL (*Staphylococcus aureus*);	Wheat; Corn	[32]

Mycotoxins; Biomarker	*Cb*Ago	-	Fluorescence	-	10^2-10^7 CFU/mL (*Salmonella typhimurium*) 10 pg/mL-100 ng/mL (AFB_1); 100 pg/mL-1 μg/mL (OTA); 100 pg/mL-1 μg/mL (DON); 10 pg/mL -10 ng/mL (Procalcitonin); 5 pg/mL-1 ng/mL (Interleukin-6)	70 CFU/mL (*Salmonella typhimurium*) 2.81 pg/mL (AFB_1); 30.05 pg/mL (OTA); 14.27 pg/mL (DON); 3.22 pg/mL (Procalcitonin); 2.73 pg/mL (Interleukin-6)	Corn; Blood sample	[79]
Mycotoxins	*Tt*Ago	-	Nanopore	-	1 to 1 × 10^3 nM (AFB_1 and OTA); 1×10^{-1} to 1×10^2 nM (ZEN)	67.18 fM (AFB_1); 13.32 fM (OTA); 11.69 fM (ZEN)	Maize	[102]
Mycotoxins	*Tt*Ago	-	Nanopore	-	1 to 10^4 nM (AFB_1); 10^{-1} to 10^3 nM (OTA); 1 to 10^4 nM (ZEN)	24.28 pM (AFB_1); 0.11 pM (OTA); 0.89 pM (ZEN)	Milk	[111]
Foodborne toxins	*Tt*Ago	NEMA	Colorimetric	-	100 pg/mL-50 *μ*g/mL (tetrodotoxin (TTX)) 2 fM-2 nM (miR-21)	47 pg/mL (tetrodotoxin (TTX)) 1.2 fM (miR-21)	Mice serum	[88]

In addition, by integrating Exo I and Tag-specific primer extension, Li et al. established a fluorescent biosensor based on one-step Ago cleavage (named NOTE-Ago). As shown in Fig. 2A, the target gene was first amplified using Tag-specific primers. Exo I could specifically hydrolyze the remaining primer (ssDNA) from the 3' end. When the enriched amplification products were mixed with the *Pf*Ago system, the Tag sequence on the amplification products served as a guide, enabling *Pf*Ago to recognize and cleave the FQ-reporter, thereby altering the fluorescence intensity. With this method, *Staphylococcus aureus* could be detected between 1 CFU/mL to 1×10^8 CFU/mL and an LOD of 1 CFU/mL[21]. In another study, by combining the effective trans-cleavage activity of CRISPR/Cas12a with the specific cleavage activity of *Pf*Ago, Chen et al. developed a fluorescent biosensor for the sensitive detection of *Salmonella typhimurium* (Fig. 2B). In this biosensor, the $MNP_{1\mu m}$-phage captured and lysed *Salmonella typhimurium* to release DNA. When *Salmonella typhimurium* was present, a complementary sequence bound to crRNA and activated Cas12a. The activated Cas12a non-specifically cleaved the ALP-labeled ssDNA1 on the surfaces of the MNP, releasing free ALP into the solution. After magnetic separation, the number of ALP molecules on the MNP surface was reduced, which inhibited the removal of the 5′-P group from the gDNA by ALP. This caused the specific cleavage of the ssDNA-FQ by *Pf*Ago be activated, which effectively amplified the fluorescent signal. The intensity of the generated fluorescent signal was linearly correlated with the concentration of *Salmonella typhimurium*. This approach could detect the *Salmonella typhimurium* with a linear range of 50 CFU/mL to 1×10^7 CFU/mL, and the LOD was 23 CFU/mL[19]. More interestingly, Wang et al. developed a phage-assisted DNA and ATP synergistically Ago-mediated fluorescent biosensor for detection of *Salmonella* **(**Fig. 2C). First, the MNP-phage specifically captured and lysed viable *Salmonella* in the sample to release DNA and ATP. Subsequently, by coupling a designed ATP bivalent aptamer with MNPs formed MNP-BAPT complexes could bind to ATP and release gDNA, which triggered the cleavage reaction of *Pf*Ago to produce fluorescent signals. This fluorescent signal generated by the DNA and ATP synergistically triggering the cleavage reaction of *Pf*Ago significantly enhanced the sensitivity of *Salmonella* detection. Using this biosensor, *Salmonella* was detected in the range of 1.0×10^2 CFU/mL to 1.0×10^7 CFU/mL, and the LOD was 20 CFU/mL[15]. Although Ago-based fluorescent biosensors have the advantages of simplicity of operation, high sensitivity, and fast response, their application in complex food sample matrices remains limited by the influence of visible light and spontaneous background signals, as well as the issue of non-specific adsorption of substances in real samples[86]. In addition, the reliance on sophisticated fluorescence detection equipment makes it difficult to achieve on-site detection.

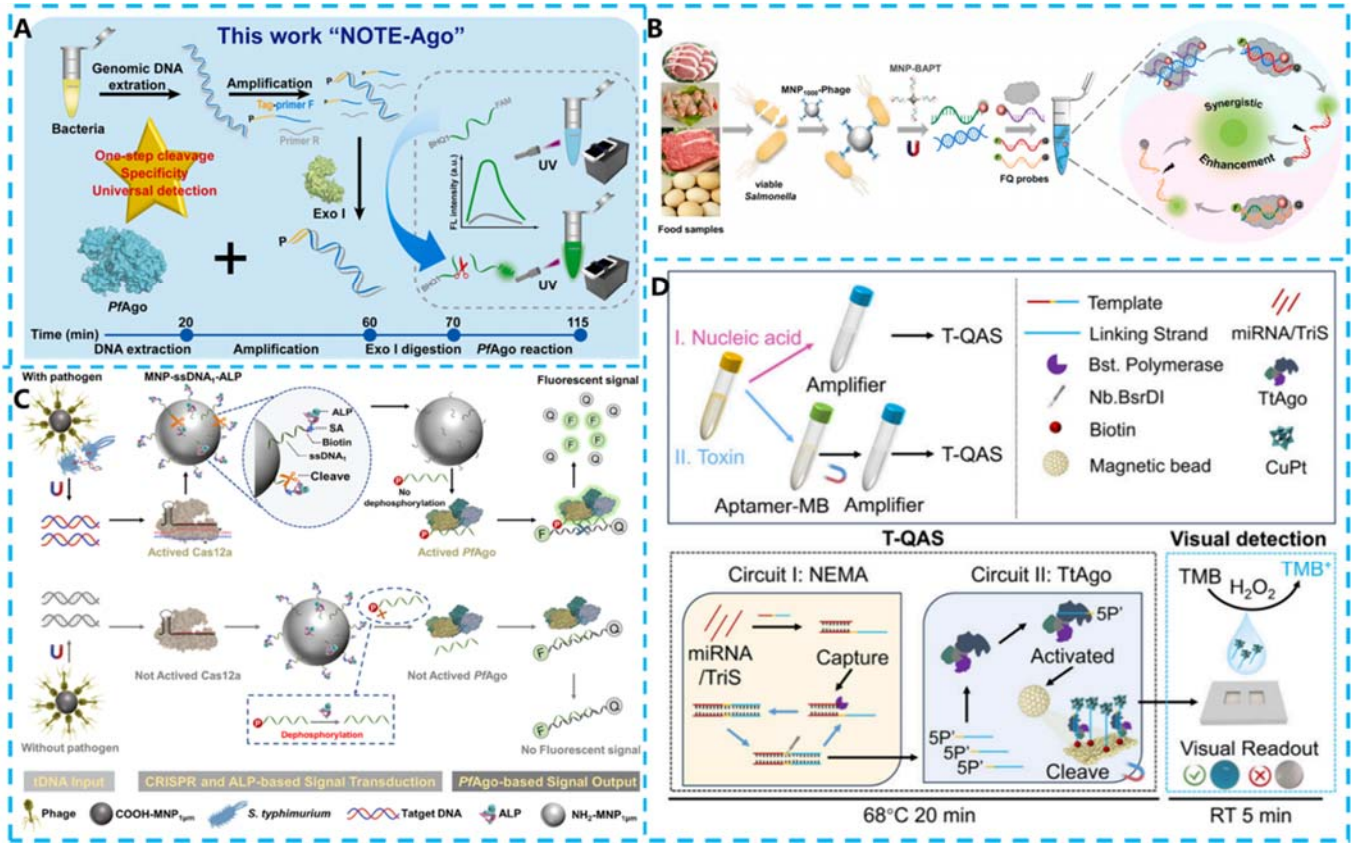

Fig. 2. (A) Schematic diagram of the proposed NOTE-Ago assay based on the PfAgo system. Reproduced with permission[21]. Copyright 2023, Elsevier. (B) Schematic diagram of the phage-assisted DNA and ATP synergistically triggered Argonaute-mediated fluorescent (PDAAF) biosensor for the detection of viable Salmonella. Reproduced with permission[15]. Copyright 2024, Elsevier. (C) Detection strategy and workflow of the double-enzyme (CRISPR/Cas12a and PfAgo)-mediated cascade signal amplification method for the detection of Salmonella typhimurium. Reproduced with permission[19]. Copyright 2023, American Chemical Society. (D) All-in-one isothermal enzymatic signal transduction amplifier (AIESTA) powered by T-QAS and CuPt nanozyme for visual profiling of nucleic acid and toxin. Reproduced with permission[88]. Copyright 2025, American Chemical Society.

5.1.2 Ago-based colorimetric biosensors

As one of the most common signal modes in biosensor development, colorimetry has gained a lot of attention owing to its convenient operation, easy readout, low cost, and rapid visual detection by portable devices (e.g., smartphones) or even the naked eye[87]. It can detect target analytes based on color changes during analyte-mediated chemical reactions. Benefiting from their advantages, colorimetric biosensing has been used to construct novel Ago-based biosensors. For example, Shen et al. constructed an AIESTA platform (all-in-one isothermal enzymatic signal transduction amplifier) by integrating a *Tt*Ago-mediated thermostable quadratic amplification system (T-QAS) with CuPt nanozymes for the detection of foodborne toxins (tetrodotoxin (TTX)) (Fig. 2D). In this platform, T-QAS was established by integrating a thermostable nicking-enzyme-mediated amplification strategy with *Tt*Ago. In the presence of TTX, the aptamer bound to TTX and released a trigger strand (TriS). Subsequently, the T-QAS amplifier was specifically triggered by the TriS to synthesize a large number of ssDNA products. These products could activate *Tt*Ago to cleave the linking strand on the MBs, thereby releasing the CuPt nanozymes anchored on the MBs. After magnetic separation, the CuPt nanozymes in the supernatant catalyze the oxidation of 3,3',5,5'-tetramethylbenzidine

(TMB) in the presence of H_2O_2, leading to a color change. This color change could be captured by a smartphone and analyzed by a relevant color recognition program. The established platform was used for the detection of TTX. The linear dynamic range was between 1×10^2 pg/mL to 5×10^7 pg/mL and the LOD of 47 pg/mL[88]. However, the sensitivity of Ago-based colorimetric biosensors is generally lower than that of fluorescence or electrochemical methods. Moreover, color stability is susceptible to environmental factors such as pH and temperature, resulting in limited quantitative capability and challenges in achieving high-precision detection of low-concentration targets. Although Ago-based colorimetric biosensors have been employed for sensitive, rapid, and intuitive detection of food hazards, their practical application in the field of food safety remains relatively limited. In the future, it is necessary to further improve the accuracy and sensitivity of Ago-based colorimetric biosensors to enhance their applicability for detecting complex food samples.

5.1.3 Ago-based electrochemical biosensors

Electrochemical biosensing is established by converting specific molecular recognition processes into quantifiable current or impedance signals, thereby enabling the qualitative or quantitative analysis of target substances[89, 90]. To date, Ago-based detection technologies have been efficiently utilized in the development of electrochemical biosensors[23, 91-92]. For example, by integrating tetrahedral DNA nanostructures with *Natronobacterium gregoryi* Argonaute (*Ng*Ago), Yang et al. constructed a wearable electrochemical biosensor that enabled long-term stable monitoring of ultratrace unamplified nucleic acids. Tetrahedral DNA nanostructures were used as scaffolds to immobilize *Ng*Ago-gDNA complexes on the surface of graphene-modified microneedles. When target nucleic acids (cell-free DNA and RNA) were present, the *Ng*Ago-gDNA complexes specifically recognized and bound to them, thereby generating electrochemical signals. The biosensor enabled the detection of cell-free DNA with an LOD of 3×10^{-16} mol/L, within a linear range of 3×10^{-16} mol/L to 3×10^{-13} mol/L[91]. In another study, Kong et al. developed Ago-mediated graphene field-effect transistor (GFET) platform (named Ago-GFET) for the detection of single-nucleotide variations (SNVs). *Tt*Ago provided a nanoscale binding channel to preorganize the DNA probe. In the presence of the target nucleic acid, the *Tt*Ago-gDNA complex could rapidly recognize SNVs with single-nucleotide resolution. When a mismatch occurred in the seed region of gDNA, it affected the binding affinity of the *Tt*Ago-gDNA complex with the target sequence, leading to a change in the conductivity of the graphene channel in the GFET. With this biosensor, miR-21, ctDNA, viral RNA, and reverse transcribed cDNA could be detected with an LOD of 3×10^{-20} mol/L, 3×10^{-20} mol/L, and 20 copies/mL (viral RNA and reverse transcribed cDNA), respectively. The linear detection ranges were 3×10^{-20} mol/L to 1×10^{-8} mol/L for miR-21, 1×10^{-21} mol/L to 1×10^{-14} mol/L for ctDNA, and 10 copies/mL to 10^6 copies/mL for viral RNA and reverse transcribed cDNA[92]. More interestingly, Mao et al. developed a dual electrochemical signal amplification DNA sensor for the detection of B19 parvovirus by combining *Cb*Ago and PCR. Briefly, when the B19 DNA was present, its PCR amplification products were specifically recognized and cleaved by *Cb*Ago, producing short ssDNA (5'P-gn). These molecules could serve as secondary gDNA, triggering *Cb*Ago to cleave the SH-

cDNA probe connected to single-walled carbon nanotubes (SWCNT). This results in the release of SWCNT from the surface of the glassy carbon electrodes (GCEs) modified with the MnO_2/CMK−3/g−C_3N_4/AgNPs nanocomposite, subsequently reducing the electron transfer rate at the GCEs surface and decreasing the peak current response. By virtue of the dual electrochemical signal amplification effect of the nanocomposite and SWCNT, the sensitive detection of B19 DNA was achieved. This approach could detect the B19 DNA with a linear range of 1×10^{-15} mol/L to 1×10^{-10} mol/L, and the LOD was 2×10^{-16} mol/L[23]. Ago-based electrochemical biosensors offer high sensitivity, rapid response, ease of miniaturization, and the potential for integration with portable devices. However, their performance remains influenced by factors such as the stability of electrode surface modification, non-specific adsorption, and electrochemical interferents. Despite the promising applications of this technology in disease diagnosis and biomarker detection, its practical application in the field of food safety detection remains relatively limited. In the future, with continuous advancements in related technologies and the deepening of in-depth research, Ago-based electrochemical biosensors are expected to further expand their applications in food safety detection.

5.1.4 Ago-based SERS biosensors

Surface-Enhanced Raman Scattering (SERS) is a highly sensitive and specific sensing technology that integrates Raman spectroscopy with nanotechnology, capable of significantly enhancing Raman signals[84, 93]. The enhancement mechanism primarily originates from the synergistic amplification effect between localized surface plasmon resonance and chemical enhancement induced via charge transfer. This synergistic effect is generated when target molecules are positioned within “hot spots” regions formed by nanomaterials (such as silver and gold nanoparticles)[94]. Currently, this technology has been combined with the Ago-based biosensing system, demonstrating outstanding detection sensitivity and rapid response capabilities[95-96]. For example, Lu et al. proposed a triple-cascade amplified detection strategy named PASS, which integrated LAMP target amplification, *Pf*Ago-mediated signal conversion and cleavage, and synergistic SERS enhancement generated by mixing nano SERS probes (AuNPs/AgNPs@PVA), enabling highly sensitive and specific detection of the *invA* gene of *Salmonella typhimurium*[96]. When the target was present, its amplification products (1st signal amplification) were recognized by *Pf*Ago, which triggered targeted and programmable nucleic acid cleavage. This cleavage leads to the degradation of a pre-designed linker sequence, preventing the conjugation of the SERS probe with the magnetic probe and converting the nucleic acid signal into a detectable Raman signal (2nd amplification). Finally, by hybridizing the AuNP-SERS nanoprobes with AgNPs@PVA to form mixed nano SERS probes with synergistic enhancement effects, the Raman signal intensity is significantly amplified (3rd amplification). This method could be completed within 60 min, achieving a detection limit of 1 CFU/mL for *Salmonella typhimurium* and a linear range from 10^0 to 10^8 CFU/mL[96]. For multiplex detection, Liu et al. developed a novel sensing platform based on the integration of *Cp*Ago with SERS technology (*Cp*Ago-SERS) to achieve high-sensitivity multiplex nucleic acid detection without nucleic acid amplification[95]. Its principle relied on the sequence-specific recognition and two consecutive rounds of cleavage of target DNA by the *Cp*Ago/gDNA complex to achieve signal amplification.

Meanwhile, SERS tags were constructed by encapsulating Raman reporter molecules with distinct characteristic peaks into polystyrene (PS) nanoparticles. In the presence of target DNA, *Cp*Ago was activated and initiated cleavage of the linker ssDNA in MB@PS, leading to the release of the SERS tags. Following magnetic separation and tetrahydrofuran treatment, a large number of Raman reporter molecules were released from the PS nanoparticles and generated significantly enhanced Raman signals on the AuNPs substrate. Utilizing multiple distinguishable Raman signals, this platform enabled simultaneous multiplex nucleic acid detection in one tube without pre-amplification, achieving a LOD of 0.497 ng/μL. It was successfully applied to identify animal-derived food authentication, demonstrating excellent specificity, sensitivity, and potential for on-site detection[95]. Ago-based SERS biosensors offer significant advantages, including extremely high detection sensitivity and the ability to provide molecular-specific "fingerprint" information. However, their performance still faces several challenges. On the one hand, the signal intensity and reproducibility are largely limited by the uniformity and enhancement efficacy of the nanostructured substrates, often resulting in unstable performance. On the other hand, the technique requires relatively stringent detection conditions and sample preparation procedures, and is prone to background interference in complex matrices.

5.2 Ago-based portable detection devices

Although Ago-based biosensors have been successfully applied to food safety detection, the fluorescence readout method on which they mainly rely has limited their application in on-site detection due to the need for precision instruments. In recent years, portable detection platforms (such as LFA, microfluidic chips, and nanopores) have been integrated with Ago-based sensing technologies by relying on their advantages of high system integration and good miniaturization, providing innovative strategies for rapid food safety on-site detection. In this section, the applications of Ago-based portable detection devices in the field of food safety detection were summarized, and their advantages and limitations were discussed.

5.2.1 LFAs

LFAs is an efficient detection technology that utilizes capture antibodies to immobilize target analytes and produces a clear visual signal on the test strip through the sandwich binding of detection antibodies with signal reporter molecules, thereby accurately detecting the target analytes[24]. The lateral flow test strip (LFS), as the core component of LFA, mainly consists of four components: sample pad, conjugate pad, absorbent pad, and nitrocellulose membrane[97]. To date, Ago-based biosensors have been successfully integrated into the LFA platform, enabling rapid and on-site detection of foodborne pathogens (e.g., *Salmonella typhimurium*, *Listeria monocytogenes*, and *Staphylococcus aureus*)[22, 62, 70]. For example, Qiao et al. developed a lateral flow biosensor to detect *Salmonella typhimurium* by integrating LAMP with *Pf*Ago (Fig. 3A). In this biosensor, DNA was first extracted from the sample, and then the *invA* gene of *Salmonella typhimurium* was amplified using LAMP. Subsequently, the specific gDNA induced *Pf*Ago to cleave the amplified DNA fragments. The fragment produced by cleavage served as a new gDNA to activate the *Pf*Ago cleavage of the double-labeled ssDNA reporter molecule, which separated the fluorescent marker from the quenched molecule

and generated fluorescence. By replacing the reporter molecule with FAM-ssDNA biotin, a colorimetric signal could be observed using LFS. For negative samples, the AuNP-anti-FAM antibody bound to the FAM-ssDNA-Biotin reporter and was intercepted by the biotin ligand on the control line, causing a red color due to AuNP aggregation. In contrast, for positive samples, *Pf*Ago cleaved the FAM-ssDNA-Biotin reporter gene, leading to the separation of FAM-ssDNA and Biotin-ssDNA modified portions. As a result, the AuNP-anti-FAM antibody bound to the free FAM-ssDNA and accumulated on the test line, while the accumulation on the control line was reduced. Using this biosensor, *Salmonella typhimurium* could be determined with an LOD of 1 CFU/mL in a linear range from 10^0 CFU/mL to 10^8 CFU/mL[22]. Similarly, Yu et al. engineered a LFA for highly sensitive detection of *Listeria monocytogenes hly* gene by integrating LAMP with *Pf*Ago and the LOD was 1.8×10^2 copies[62]. Although isothermal amplification methods have made significant progress in on-site nucleic acid detection, they still suffer from a number of limitations in practical application, such as carryover cross-contamination[70]. To address these challenges, Li et al. developed an Ago-mediated bio-barcode bioassay for on-site and one-tube detection of *Staphylococcus aureus* by integrating RAA with *Pf*Ago. Firstly, the genomic DNA of *Staphylococcus aureus* was amplified using RAA technology, and a bio-barcode with a Tag sequence was introduced during the process. In the presence of a target, the FAM and biotin-labeled ssDNA-FB were cleaved by *Pf*Ago, leading to the separation of FAM and biotin. Subsequently, the reporter molecule containing the biotin groups were captured by SA on the control line. While the reporter molecule containing the FAM group first bound to the colloidal gold-labeled anti-FAM antibody preloaded on the sample pad and continued to flow towards the test line and bound to the antibodies and thus displayed the color. A highly sensitive LFA platform was constructed for the detection of *Staphylococcus aureus* with a detection range of 10^0 CFU/mL to 10^8 CFU/mL and an LOD as low as 1 CFU/mL [70]. In addition, Tang et al. innovatively integrated *Pf*Ago with a dual-mode rLFTS with cross-validating performance, constructing a portable platform for rapid, highly sensitive, and multiplex detection of foodborne pathogens[98]. Specifically, when the target gene was present, the linker DNA (linker 1 and linker 2) was specifically cleaved by *Pf*Ago, which prevented AuNPs@DNA probes from binding to the capture probes (T1-Biotin-DNA/R6G and T2-Biotin-DNA/R6G) on the test lines. At that point, neither the T1 line (for detecting the *hly* gene of *Listeria monocytogenes*) nor the T2 line (for the *lde* gene) showed color development, while R6G remained fluorescent without quenching. Meanwhile, the AuNPs@DNA probes could still hybridize with the C-Biotin-DNA/R6G capture probes on the control line (C line), leading to aggregation of the gold nanoparticles and producing a visible red band. Conversely, in the absence of the target gene, the linker DNA remained intact and could hybridize with the capture probes, allowing AuNPs@DNA probes to bind and form a “sandwich” complex. This results in visually detectable colorimetric signals on both T1 and T2 lines, while the fluorescence of R6G was quenched. The dual-mode output of colorimetric and fluorescence signals enabled cross-verification of results, greatly enhanced the specificity and reliability of the detection. By combining a portable 3D-printed visualizer with smartphone-based analysis, this method completed detection from sample to result within 45 min, achieving simultaneous multiplex analysis of pathogenic and drug-resistance genes of *Listeria*

monocytogenes at single-cell sensitivity (1 CFU/mL) with a linear range of 1–10^8 CFU/mL[98]. The LFA technology has significant advantages, such as rapid response, easy operation, and visual detection results, which significantly enhance the flexibility and field applicability of detection technology[99]. However, they generally suffer from limited sensitivity and quantitative capability, making it difficult to detect targets at extremely low concentrations. Furthermore, in multi-target detection, the bioreceptors (aptamers or antibodies) employed often struggle to distinguish between structurally similar targets, which may lead to false-positive results [34]. Furthermore, most current LFA technologies still rely primarily on qualitative or semi-quantitative outputs and have not yet achieved high-precision quantitative analysis.

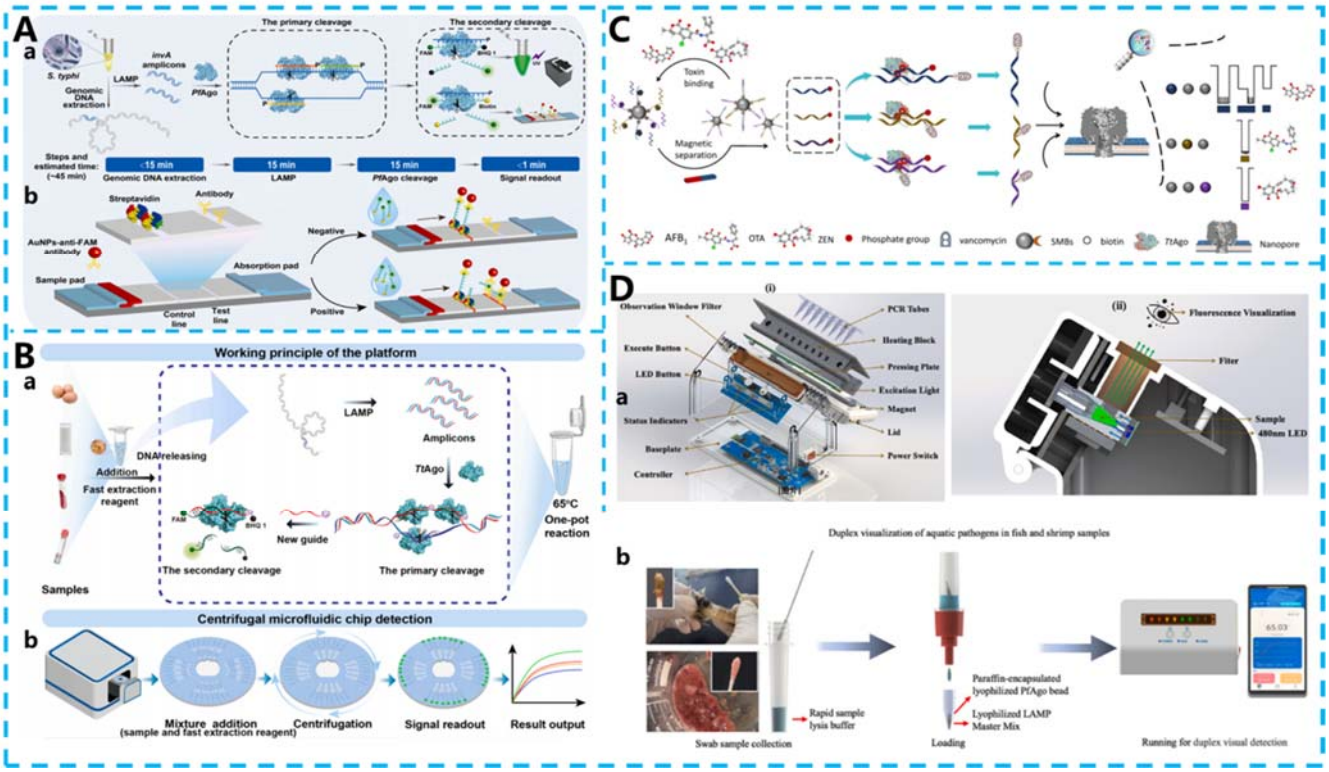

Fig. 3.(A) The schematic diagram of the platform for *Salmonella typhimurium* detection. a) The working principle of this biosensor and the estimated operational time for each step. b) The schematic of the lateral flow strip. Reproduced with permission[22]. Copyright 2024, Elsevier. (B) The illustration of the proposed Sample-in Answer-out detection of Pathogen *Staphylococcus aureus*. Reproduced with permission[25]. Copyright 2024, Elsevier. (C) Basic principle of the developed encoding strategy based on *Tt*Ago and nanopores for the multiplexed detection of three mycotoxins. Reproduced with permission[102]. Copyright 2025, American Chemical Society. (D) a) The schematic diagram of the handheld isothermal fluorescence detector, presenting its mechanical and electronic components. b) Duplex visualization of aquatic pathogens in fish and shrimp samples using LAMP-*Pf*Ago. Reproduced with permission[103]. Copyright 2024, Elsevier. .

5.2.2 Microfluidic chips

A microfluidic chip is a miniaturized device that utilizes microfabricated channel structures to achieve precise manipulation and biochemical detection of fluids at the microliter/nanoliter scale[84]. The microfluidic chip integrates multiple functional modules, including microchannels, reservoirs, and detection zones, successfully integrating basic operational units such as sample preparation, reaction, and detection onto a micrometer-sized chip platform through miniaturized system design[100]. With its outstanding high-throughput detection performance, integration, and versatility, microfluidic technology has been successfully integrated with Ago-based biosensors for on-site detection of foodborne pathogens. For example, Wang et al. developed

a *Tt*Ago-powered centrifugal microfluidic chip reaction biosensor for highly sensitive and multiplexed detection of *Staphylococcus aureus*. As shown in Fig. 3B, the sample was first mixed with a nucleic acid fast extraction reagent and then injected into the sample cell of the centrifugal microfluidic chip. Then, the mixture was centrifuged into the reaction cell preloaded with LAMP and *Tt*Ago systems. In the reaction cell, the species-specific *nuc* genes of *Staphylococcus aureus* were amplified isothermally using LAMP technology. Subsequently, *Tt*Ago recognized the amplicons and performed site-specific cleavage with the help of gDNA. This cleavage cut the doubly labeled fluorescent ssDNA reporter to release fluorescent signals for detection. The proposed biosensors could detect the *Staphylococcus aureus* with high specificity in the range of 10^0 CFU/mL to 10^7 CFU/mL and displayed an LOD of 1 CFU/mL[25]. Although Ago-based microfluidic biosensors integrate sample processing and detection functions, offering advantages such as low reagent consumption, high automation, and potential for high-throughput analysis, they also face several challenges. Their design and fabrication are often complex and costly. Furthermore, the technique demands precise optimization of reaction conditions, such as flow rate and temperature, and often encounters difficulties in fluid control and signal stability when handling complex samples. Additionally, most current technologies rely on external pumps, valves, and detection modules. The development of fully integrated, portable devices still requires further breakthroughs.

5.2.3 Nanopore-based detection devices

As an emerging single-molecule detection technology, nanopore-based detection equipment has attracted widespread attention in the food safety field owing to its advantages of high resolution, high sensitivity, portability, and preamplification-free[101]. The detection principle is that when a target molecule (e.g., nucleic acids and proteins) passes through a nanopore, it causes specific changes in the current or ion flow in the pore due to the different characteristics of the molecule, such as size, shape, and charge, so as to realize the identification and detection of the molecule. In recent years, based on the unique fingerprints of different analytes, nanopore devices have been successfully integrated with Ago systems for multiple detection of analytes[102]. For example, Chen et al. innovatively proposed a *Tt*Ago-assisted nanopore encoding strategy for the simultaneous detection of ochratoxin A (OTA), aflatoxins B_1 (AFB_1), and zearalenone (ZEN) (Fig. 3C). In this strategy, gDNA was hybridized with aptamers to form dsDNA. This dsDNA was then conjugated to SA-modified MBs (SMBs) to form dsDNA-SMB conjugates. When the target mycotoxin was present, the toxin bound specifically to the aptamer, resulting in the release of gDNA from the SMBs. The produced gDNA bound to *Tt*Ago and specifically cleaved different encoding reporters. Using nanopore technology, encoding reporters induced changes in ionic currents when passing through α-hemolysin nanopores under applied voltage. By analyzing the parameters of the characteristic events, different encoding reporters could be distinguished, thus enabling qualitative and quantitative detection of different mycotoxins. This strategy exhibited low LODs of 67.18 fM for AFB_1, 13.32 fM for OTA, and 11.69 fM for ZEN, respectively. The wide linear ranges were 1 to 1×10^3 nmol/L for AFB_1 and OTA, and 1×10^{-1} to 1×10^2 nmol/L for ZEN, respectively[102].

5.2.4 Others

In addition, Ago-based biosensors have also been integrated into other portable detection devices for on-site detection of food contaminants [103]. For example, Pang et al. developed a handheld isothermal fluorescent detector to detect aquatic pathogens by integrating an enhanced one-pot LAMP-*Pf*Ago detection (Fig. 3D). Briefly, samples were mixed with rapid sample lysis buffer and used directly in the reaction. In the reaction system, the target nucleic acids of aquatic pathogens were first amplified by LAMP technology at 65 ℃. Subsequently, the paraffin-encapsulated lyophilized *Pf*Ago protein was released and activated at 95 ℃ and cleaved the amplicons under the guidance of gDNA, thereby generating secondary gDNAs. These secondary gDNA molecules triggered the cleavage of reporters to produce fluorescent signals for detection. This enhanced one-pot *Pf*Ago-based detection method simplified the operational procedure and prevented aerosol contamination. When used in conjunction with the developed device, its real-time smartphone interaction function allowed real-time observation of fluorescence changes during the reaction, effectively enabling on-site detection [103].

5.3 Research highlights in this fields

5.3.1 Multiplex detection of nucleic acid targets

In practical detection processes, the same food matrix often contains multiple foodborne pathogens simultaneously. However, many detection methods can only detect a single target, which not only prolongs the detection time but also increases the risk of false positives/negatives due to cross-contamination of multiple pathogens[104]. Therefore, the development of novel detection methods with multiplexing capabilities is of great significance. As a new generation of nucleic acid detection tools, Agos provide a solution for the challenge of simultaneous detection of multiple targets in complex samples by virtue of its precise targeting, programmability, and freedom from PAM sequence restrictions[35]. To date, Ago-based multiplexed detection systems have been developed for the detection of foodborne pathogens[39, 69, 71, 81]. For example, Chen et al. established a duplex in-one-tube detection method with color indication for multiplex detection of white spot syndrome virus (WSSV) and AHPND by combining RPA and *Pf*Ago. In this method, double RPA amplification of the target was performed in the same reaction mixture using specific primers for WSSV and AHPND gene fragments. The purified RPA amplification products were then cleaved by *Pf*Ago under the guidance of specific and distinct gDNA. The resulted DNA fragments further guided *Pf*Ago to cleave different fluorescent probes to generate distinct fluorescent signals. Specifically, in the presence of WSSV, *Pf*Ago cleaved molecular beacon-WSSV labeled with the FAM fluorophore, resulting in a green fluorescent signal. When AHPND was present, *Pf*Ago cleaved molecular beacon-AHPND labeled with the Texas Red fluorophore, which produced a red fluorescent signal. When both WSSV and AHPND were present, *Pf*Ago cleaved both molecular beacon-WSSV and molecular beacon-AHPND, producing a yellow fluorescent signal[69]. By utilizing the remarkable programmability of *Cb*Ago-assisted EXPAR, He et al. developed a fluorescent biosensor for the simultaneous detection of *Salmonella* and *Listeria monocytogenes*. As shown in

Fig. 4A, the genomic DNA of the pathogen was first cleaved by *Cb*Ago under the guidance of gDNA to generate ssDNA. The generated ssDNA hybridized with the template, triggering polymerization in the presence of Vent (exo-) DNA polymerase to produce dsDNA. This dsDNA contained three functional regions: the recognition sequence of the endonuclease Nt.BstNBI, a DNA fragment identical to the ssDNA produced by *Cb*Ago cleavage, and DNAzyme fragments. Specifically, the Nt.BstNBI recognition sequence could locate the cleavage site, the fragment identical to ssDNA was used to re-initiate EXPAR, and the DNAzyme fragments were used to recognize and cleave the reporter molecules. Then, Nt.BstNBI cleaved the dsDNA at four bases from the 3' end of its recognition site to produce ssDNA and DNAzyme. The generated DNAzyme specifically recognized and cleaved the reporter molecules with the help of metal ions, thereby generating a fluorescent signal. Additionally, the continuous hybridization of ssDNA with the DNA fragment identical to that produced by *Cb*Ago cleavage initiated new amplification cycles, continuously generating a large number of DNAzyme fragments. This process led to the extensive cleavage of reporter molecules, producing a strong fluorescent signal. By rationally designing two templates for *Salmonella* and *Listeria monocytogenes*, 8-17 DNAzyme and 10-23 DNAzyme could be successfully exported by the EXPAR conversion. The two DNAzymes with different cleavage properties could act on reporter molecules, which were labeled with different fluorophores (FAM for *Salmonella* and ROX for *Listeria monocytogenes*), thus achieving specific detection of the two pathogens. This biosensor showed a good selectivity for detecting *Salmonella* with a wide linear range from 10 to 10^7 CFU/mL, exhibiting a low LOD of 7 CFU/mL. It could also detect *Listeria monocytogenes* could be detected with an LOD of 22 CFU/mL within a linear range of 10^2 to 10^7 CFU/mL[71].

Moreover, aptamers have also been integrated into Ago-based systems for the multiplex detection of foodborne pathogens due to their strong specificity, high stability, and high designability[39, 71, 81]. For example, Chen's group invented a novel gDNA containing DNAzyme segments (named $gDNA_{zyme}$), which could significantly enhance the cleavage efficiency of *Cb*Ago. On this basis, the ultrasensitive detection of foodborne pathogens (i.e., *Salmonella*, *Escherichia coli*, and *Staphylococcus aureus*) was achieved simultaneously by designing three gDNAs with completely different sequences (Fig. 4B). Briefly, when the target was present, each pathogen first bound to its respective aptamer rather than to the cDNA. Subsequently, the remaining cDNA induced conformational transition in the double strand formed by the hairpin structures (HPs) and cHP. The resulted double strand was recognized by Nt.BstNBI at a specific cleavage site, which led to the output of three distinct gDNA sequences and DNAzyme segments. Subsequently, *Cb*Ago was added to form a binary complex, which activated its cleavage of tDNA labeled with different fluorophores, generating fluorescent signals for multiplex pathogen detection. In the absence of pathogens, the aptamers hybridized with cDNA, which inhibited the output of gDNA, thus preventing the cleavage of tDNA to generate fluorescent signals. The gDNAzyme-activated *Cb*Ago-mediated aptasensor had a linear range of 10 to 10^6 CFU/mL for *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella*, with LODs of 46, 76, and 75 CFU/mL, respectively[81]. In another study, Lu et al. designed a magnetic Fe_3O_4@MOF@Aptamer-based fluorescent biosensor for the simultaneous detection of *Staphylococcus aureus* and *Escherichia coli* by combining it with

*Cb*Ago. As shown in Fig. 4C, the phosphorylated aptamer was stably conjugated to the surface of magnetic Fe_3O_4/SiO_2/UiO66 via Zr-O-P bonds to form the Fe_3O_4/SiO_2/UiO66/Apt complex. The aptamer in this complex could specifically recognize target pathogenic bacteria and had a higher affinity for pathogenic bacteria than cDNA. When pathogenic bacteria were present, the aptamer bound to the bacteria, causing the dissociation of cDNA from the complex into the solution. If no pathogenic bacteria were present, the aptamer hybridized with cDNA. After the reaction, magnetic separation was performed, and the content of cDNA in the solution was positively correlated with the number of pathogenic bacteria. Subsequently, the three-strand DNA duplex complex (Q/P/gDNA) containing the Q strand, P strand, and gDNA were mixed with cDNA. The cDNA bound to the single-stranded toehold of the Q strand in Q/P/gDNA, thereby releasing the P strand and forming the Q/gDNA/cDNA complex. Then, the F strand bound to the toehold of the Q/gDNA/cDNA complex, releasing gDNA and cDNA. The regenerated cDNA could trigger a new cycle, achieving the cyclic amplification of cDNA through the EDC reaction and generating a large amount of target-related gDNA. The 5' end of the F strand was labeled with biotin. After the reaction, the Q/F complex was removed by SA-modified MNP_{150}-SA to avoid interference with subsequent *Cb*Ago cleavage reactions. The generated gDNA bound to *Cb*Ago and guided it to cleave the reporter DNAs, which were modified with different fluorophores and quenchers. In the presence of different pathogens, the aforementioned reaction could generate corresponding gDNA, which could guide *Cb*Ago to cleave the corresponding reporter DNAs, thus releasing fluorophores and generating different fluorescent signals. This proposed biosensor showed an excellent linear relationship for *Escherichia coli* and *Staphylococcus aureus* with a concentration range of 10^2 to 10^6 CFU/mL, and the LOD could reach 79 CFU/mL and 68 CFU/mL, respectively[39].

More interestingly, Ago-based biosensors have also been used for the dual-gene detection of foodborne pathogens[61, 63, 67, 75]. For instance, Li et al. developed a detection platform for highly sensitive and simultaneous dual-gene (*mecA* and *nuc*) detection of MRSA by combining LAMP with *Pf*Ago (Fig. 4D). First, the genomic DNA of *mecA* and *nuc* of MRSA was extracted and amplified using the LAMP. Subsequently, the LAMP amplification products were added to the *Pf*Ago reaction system. Then, the three gDNAs guided *Pf*Ago to cleave the nucleic acid target, generating a 16-nt ssDNA. The generated ssDNA could serve as a new guide strand, triggering the cleavage of biotin-DNA-DNAzyme-1 and biotin-DNA-DNAzyme-2 by *Pf*Ago. After adding SA-MNPs magnetic separation, the DNAzyme-1 and DNAzyme-2 were released into the supernatant. Upon addition of Mg^{2+} to the supernatant, DNAzyme-1 and DNAzyme-2 were activated to cleave the double-labeled (FAM-BHQ1 and ROX-BHQ2) fluorescent reporters, respectively, thereby generating a fluorescent signal. The established method could effectively detect the *mecA* and *nuc* genes of MRSA with an LOD of 1 CFU/mL in a linear range from 1 CFU/mL to 10^8 CFU/mL[61]. Similarly, by integrating LAMP with *Pf*Ago, Kou et al. developed a portable biosensor for detecting the *mecA* and *nuc* genes of MRSA (Fig. 4E). This platform achieved a satisfactory LOD of 10 CFU/mL in a linear range from 10 to 10^7 CFU/mL[63]. In another study, Chen et al. established a fluorescent detection platform by combining RPA with *Pf*Ago. The established method could effectively detect the *nuc* and *mecA* genes of MRSA with an

LOD of 10^2 copies/μL in a linear range from 10^1 to 10^9 copies/μL[67]. In addition, Wei et al. constructed a fluorescent biosensor for ultrasensitive detection of the *mecA* and *femA* genes of MRSA by combining fluorescent-encoded microspheres with *Cb*Ago (Fig. 4F). First, the sequences of MRSA target genes (i.e., *mecA* and *femA*) were simultaneously hybridized with two proximity probes to form a three-way junction. Subsequently, the sticky ends in the generated three-way junction were filled by DNA polymerization to generate a dsDNA barcode with a cleavage site, which was then released specific gDNA by a nicking cleavage reaction. This process continues in a cycle, producing a large amount of gDNA from a single target and amplifying the detection signal. For detection of the *femA* and *mecA* genes, 6-FAM and Texas Red encoded microspheres were used as signal probes, respectively. Under the guidance of target-specific gDNA, the activated *Cb*Ago cleaved the phosphodiester bonds of the PS-TDNA-RMB probe (PS-TDNA-supported, RCA-assisted MB probes) in a multi-turnover mode. This triggered a detectable fluorescent signal and enabled the decoding of the fluorescent signal. Using this biosensor, the *mecA* and *femA* genes could be detected in a linear range from 1 fM to 1000 fM, with LODs of 0.63 fM and 0.48 fM, respectively[75].

5.3.2 Multiplex detection of non-nucleic acid targets

In addition to nucleic acid targets (e.g., pathogenic bacteria), non-nucleic acid contaminants (e.g., mycotoxins, antibiotics, and metal ions) also pose a serious threat to human health and food safety[105, 106]. However, relatively few applications of Ago-based sensors in the field of non-nucleic acid target detection have been reported, mainly because there are still many challenges in effectively converting non-nucleic acid signals into nucleic acid signals recognizable by Ago[107]. Therefore, constructing an efficient and universal signal conversion mechanism and developing a versatile platform applicable to multiple non-nucleic acid detections are important to ensure food safety and human health. In recent years, researchers have successfully developed Ago-based multiplex detection methods for the detection of mycotoxins in food products[32, 74, 79, 80]. For instance, Lu et al. constructed a multiplex detection platform for the detection of mycotoxins and pathogenic bacteria by combining *Cb*Ago, Exo I, and aptamers (Fig. 5A). First, the gDNA was modified with phosphate at 5' termini and biotin 3' termini. The modified gDNA was then assembled onto the surface of the PS_{mm}-SA conjugate through biotin-SA interactions. Subsequently, the aptamer was hybridized with the gDNA to form dsDNA. Owing to higher affinity of the aptamer for the target than for the gDNA, the aptamer dissociated from the dsDNA upon target addition, forming a target-aptamer complex in the reaction solution. This complex was then cleaved by Exo I from the 3' end, releasing free mononucleotides and the target. The released target could participate in the next cycle. After multiple cycles, the amount of gDNA remaining on the surface of the PS_{mm} increased, which allowed *Cb*Ago to assembled with it. The assembled *Cb*Ago then specifically cleaved the fluorophores and quenchers-modified reporter DNA, separating the fluorophores and quenchers to generate a fluorescent signal. Using this platform, ZEN, OTA, and aflatoxin A were detected with LODs of 56 pg/mL, 50 pg/mL, and 28.5 pg/mL, respectively. The linear detection ranges were from 0.1 pg/mL to 200 pg/mL for ZEN, from 0.1 pg/mL to 500 pg/mL for OTA, and from 0.05 pg/mL to 200 pg/mL for aflatoxin A. In the same way, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium* could

be detected with LODs of 46 CFU/mL, 39 CFU/mL, and 64 CFU/mL, respectively, within a linear range of 10^2 to 10^7 CFU/mL[74]. In another study, by combining a dephosphorylation-regulated *Pf*Ago biosensor with immunolabeled ALP, Huang et al. developed a *Pf*Ago-based immunosensor for the detection of ALP and AFB_1 (Fig. 5B). First, AFB_1-BSA was coated on a microplate. In the absence of AFB_1, the mouse-anti-AFB_1 antibody (AFB_1-Ab) and ALP-labeled goat anti-mouse IgG (ALP-Ab_2) were sequentially immobilized on the microplate. Subsequently, ALP-Ab_2 hydrolyzed the phosphate groups on the 5'-P gDNA, preventing *Pf*Ago from cleaving the ssDNA-FQ reporter molecule, resulting in a weak fluorescent signal. When AFB_1 was present, it bound to AFB_1-Ab instead of pre-coated AFB_1-BSA, which led to a decrease in immobilized ALP-Ab_2. This process allows *Pf*Ago to be guided by more 5'-P gDNA to cleave the ssDNA-FQ reporter molecule, resulting in a strong fluorescent signal. The established biosensor effectively detected ALP with an LOD of 2.70 U/L in a linear range of 10-1000 U/L. Similarly, the LOD for AFB_1 was 29.89 pg/mL in a linear range from 0 to 500,000 pg/mL[80]. In addition, Li et al. constructed a platform for multiplex detection of mycotoxin and three foodborne pathogens by combining *Cb*Ago with DNA tetrahedra-functionalized MNPs (iDNA-TDNA@MNP) (Fig. 5C). For pathogen detection, when the target bacteria were present, they bound to aptamerson polystyrene lollipops, resulting in the release of gDNA. For mycotoxin detection, mycotoxins competed for binding with antibodies immobilized on polystyrene lollipops and altered the gDNA-BSA-mycotoxin conjugate, thereby releasing the unbound gDNA complexes. The gDNA in the aforementioned supernatant further activated *Cb*Ago, which targeted and cleaved iDNA onto the iDNA-TDNA@MNP system, thus achieving targeted blockade of different HCRs (containing different fluorescent-group-modified hairpins). By designing different gDNA, aptamers, iDNA-TDNA-MNP encoding systems, and different hairpin DNAs, multiplex detection of target analytes could be achieved. This platform was capable of simultaneously detecting three mycotoxins with a linear range of 10 pg/mL to 100 ng/mL for AFB_1 and aflatoxin G_1 (AFG_1) and 100 pg/mL to 1 μg/mL for deoxynivalenol (DON). The LOD of DON, AFG_1, and AFB_1 were 59.65 pg/mL, 4.88 pg/mL, and 5.48 pg/mL, respectively. In addition, this platform exhibited good detection capability for three pathogens with linear ranges of 10^2–10^7 CFU/mL. The LODs of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium* were 46 CFU/mL, 59 CFU/mL, and 70 CFU/mL, respectively[32]. More interestingly, Li et al. developed a biosensing platform for the multiple detection of three mycotoxins and biomarkers by combining *Cb*Ago with PS (Fig. 5D). First, PS with different tDNAs (PS-tDNA) modified on the surfaces were synthesized. Meanwhile, the tDNAs were labeled with biotin, enabling them to specifically bind to SA-labeled MNPs (MNP-SA). When a target (e.g., mycotoxin) was present, an immuno-competitive reaction was triggered on the "lollipop" carrier. The mycotoxin and gDNA-BSA-mycotoxin competitively bound to the antibody. When the concentration of mycotoxin was higher, it occupied more antibody binding sites. This caused more gDNA-BSA-mycotoxin to remain in the supernatant, which activated the cleavage activity of *Cb*Ago on the complementary tDNA on PS-tDNA. Similarly, when detecting biomarkers, subsequent reactions were triggered by the specific binding between the biomarker and the gDNA-modified detection antibody (Ab_2-gDNA). When the tDNA was cleaved, the binding between PS-

tDNA-biotin and MNP-SA weakened. Consequently, after magnetic separation, more PS microspheres remained in the supernatant The target concentration was quantified by measuring the concentration of PS microspheres in the supernatant. Moreover, by using PS microspheres of distinct sizes, each associated with a specific target, multiplexed detection of various targets was achieved. This Ago-based fluorescence platform simultaneously detected AFB_1, OTA, and DON with linear ranges of 0.1−10 ng/mL, 0.5−50 ng/mL, and 0.5−50 ng/mL, respectively. The LOD for AFB_1, OTA, and DON were 71.22 pg/mL, 190.94 pg/mL, and 335.63 pg/mL, respectively[79].

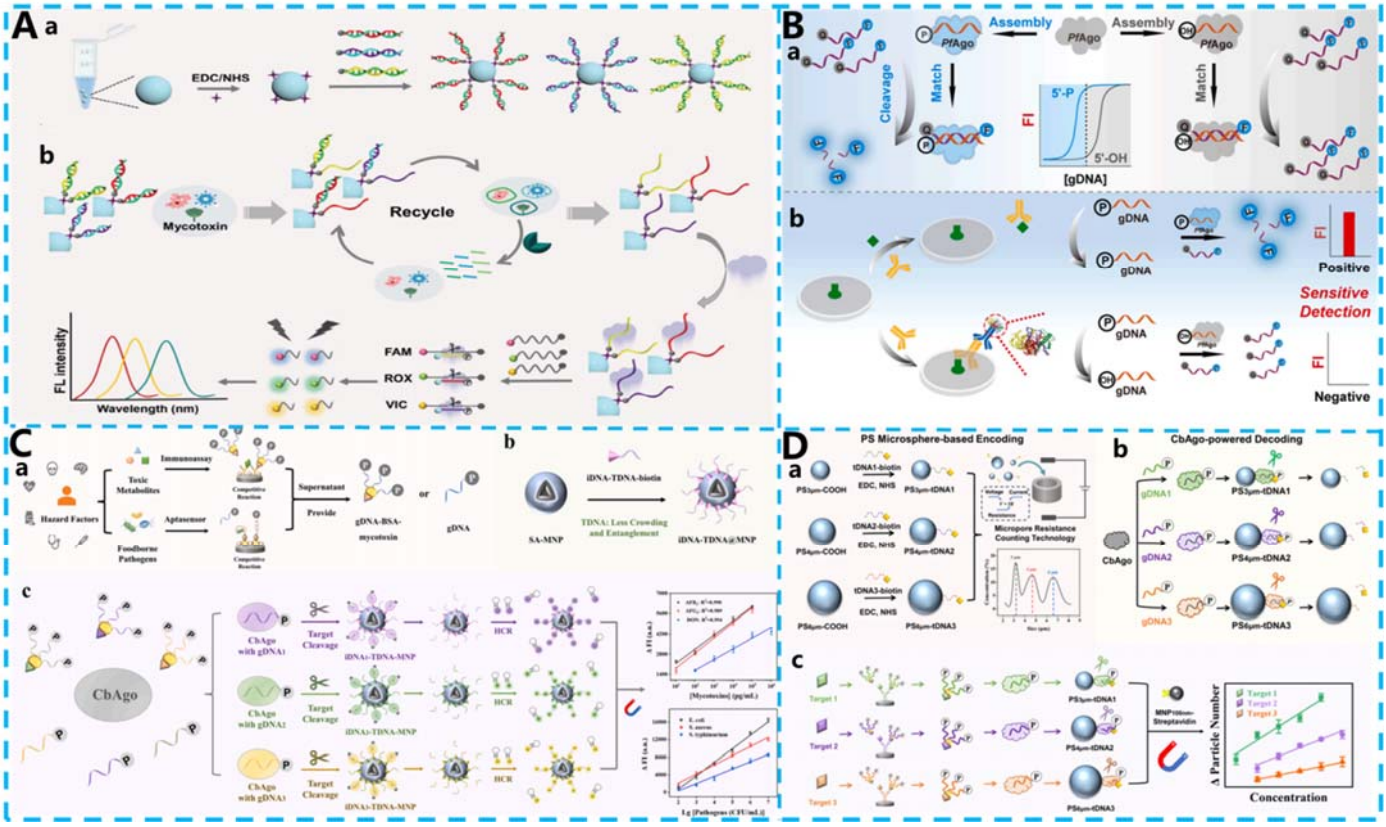

Fig. 5.(A) Illustration of the MARPS platform for simultaneous detection of three non-nucleic acid targets (AFB_1, OTA, and ZEN): a) Preparation of PS_{mm}-probe complexes: Aptamers of AFB1, OTA, and ZEN are hybridized with corresponding gDNA to form dsDNA, which is then assembled onto the PS_{mm} surface via biotin–SA interactions. b) Detection process: Simultaneous detection of AFB_1, OTA, and ZEN is performed using the MARPS platform. Reproduced with permission[74]. Copyright 2024, Wiley-VCH. (B) Illustration of the *Pf*Ago-based immunosensor for AFB_1 detection. a) Probing the phosphorylation preference of *Pf*Ago for the 5-terminal end of gDNA. b) *Pf*Ago-based immunosensor detection mechanism for AFB_1. Reproduced with permission[80]. Copyright 2024, Elsevier. (C) Schematic illustration of the APP platform for sensitive and multiplexed detection. a) Signal conversion system: Different non-nucleic acid hazard factors are detected by converting target signals into gDNA signals via immunoassay and aptasensor. b) Synthesis of iDNA-TDNA@MNP: TDNA enhances cleavage and HCR efficiency. c) Multi-target detection principle: *Cb*Ago cleaves iDNA on TDNA@MNPs, and signal changes are amplified by HCRs and converted into fluorescent signals. Different gDNAs drive *Cb*Ago activity on encoded TDNA@MNPs, enabling multiplexed detection through distinct HCRs. Reproduced with permission[32]. Copyright 2024, Elsevier. (D) Workflow of the micropore resistance counting platform based on PS microsphere size encoding and *Cb*Ago decoding. a) PS tDNA encoding system. The construction of the encoding system was achieved by loading different tDNA on PS microspheres of distinct sizes. Micropore resistance counting resolved peaks corresponding to PS microspheres with different diameters; b) *Cb*Ago decoding system. Interaction of gDNA with *Cb*Ago enables specific cleavage of the corresponding tDNA, separating the tDNA from the PS microspheres; and c) schematic illustration of a multiplexed micropore resistance counting detection platform based on encoding-decoding recognition. Replacing the tDNA with a gDNA input, combined with the PS microsphere encoding system and the *Cb*Ago decoding system, allows simultaneous quantitative multi-mycotoxin identification. Reproduced with permission[79]. Copyright 2025, American Chemical Society.

5.3.3 Machine learning-assisted intelligent detection

Recently, the rapid development of machine learning, AI, and deep learning algorithms has greatly enhanced the analysis and imaging capabilities of biosensing technologies[17]. These technologies have not only improved the signal-to-noise ratio of fluorescent images but also enhanced the recognition capabilities, especially for low-resolution fluorescent images[108]. Therefore, machine learning has been successfully integrated with Ago-based food safety detection platforms to further enhance detection efficiency and sensitivity[18, 26, 27, 76-77]. For example, Zhao et al. constructed a phage and *Cb*Ago-mediated fluorescent biosensor for the detection of *Salmonella typhimurium* by integrating machine learning with a single microsphere (Fig. 6A). First, *Salmonella typhimurium* was captured and lysed using MNP-phage conjugates and lysis buffer, respectively. Subsequently, released bacterial DNA was cleaved by *Cb*Ago under the guidance of gDNA to obtain a 16-base tDNA, which could function as a new gDNA to direct the secondary specific cleavage of biotinylated fluorescent probe (6-FAM(Bio)-ssDNA-BHQ1). Afterwards, the generated fluorescent signal was accumulated on the SA-modified single microsphere. Finally, the entire detection process was analyzed using machine vision and learning algorithms, resulting in a highly sensitive detection of *Salmonella typhimurium*. The LOD of *Salmonella typhimurium* was 40.5 CFU/mL, and the linear range was from 50 CFU/mL to 10^7 CFU/mL[26]. Similarly, Lin et al. established a *Tt*Ago-powered fluorescent platform for ultrasensitive detection of FB_1 by combining deep learning and EDC (Fig. 6B). In this platform, biotin-modified ssDNA and RFEMs were used as signal probes, and SA-conjugated MBs were used as separation carriers. In the presence of a FB_1, it bound to the aptamer in the aptamer-trigger (Apt-T) duplex, thereby releasing the trigger sequence to initiate the EDC cycle. This progress generated a large number of 5'-P output sequences. These sequences could be further used as gDNA to activate *Tt*Ago, and thus activated the cleavage signal probe (RFEM-ssDNA-Biotin). After adding SA-MBs and performing magnetic separation, the number of fluorescent microspheres in the final reaction supernatant increased. After obtaining the fluorescent images of RFEM in the supernatant by laser confocal microscopy, the number of red bright particles in the confocal fluorescence images was counted using the YOLOv9 deep learning model to achieve sensitive and specific detection of FB_1. The established method was able to effectively detect FB_1 in a linear range from 1 pg/mL to 10^5 pg/mL with an LOD of 0.89 pg/mL[18]. In addition, AI techniques have been integrated into Ago-based multiplex detection systems to enhance detection accuracy and speed[27, 76-77]. For instance, Wang et al. developed a digital detection method based on botryoidal-like fluorescent PS-dots for multiplex detection of pathogenic bacteria. As shown in Fig. 6C, the target ssDNA sequences of pathogens were precisely cleaved by Ago. The cleaved ssDNA could be used as bridges for conjugating DNA-PER-modified botryoidal-like PS dots (DNA-PER-PS-dots) and DNA sequence-modified liposome-decorated MBs (DNA-LP-MBs) with different sizes, forming LPMB-PER strand-modified PS dot complexes (LP-MBs@PER-PS-dots). Finally, the generated LP-MBs@PER-PS-dots were identified and counted using an AI fluorescent microshere counting algorithm. By distinguishing the dots based on their distinct colors and particle sizes, thus enabling the simultaneous detection of multiple pathogens. This biosensor was able to

detect two pathogenic bacteria (e.g., *Listeria monocytogenes* and *Staphylococcus aureus*) between a range of 10^2 CFU/mL to 10^6 CFU/mL with an LOD of 2 CFU/mL and 4 CFU/mL, respectively. Similarly, *Salmonella typhimurium* could be detected in a linear range from 10^3 CFU/mL to 10^6 CFU/mL with an LOD of 6 CFU/mL[77]. By combining *Cb*Ago with fluorescence-encoded MBs and AI, Wang et al. constructed a digital carrier system for multiplexed and ultrasensitive detection of food-borne pathogens. In the presence of target pathogenic bacteria, *Cb*Ago could precisely target and cleave FQ reporters (fluorescent end modified with biotin) corresponding to different bacteria through a two-step process. Subsequently, different single biotinylation fluorescent probes were encoded to the surface of the MB-tyramine-SA (MB-TA-SA) conjugates through the interaction between SA and biotin. After magnetic separation, fluorescence images of encoded MBs were observed and captured using confocal laser scanning microscopy, and these images were decoded by AI to achieve quantitative signal analysis. The established method could enable the simultaneous detection of three food-borne pathogens with an LOD of 6 CFU/mL for *Staphylococcus aureus* and *Listeria monocytogenes*, and 7 CFU/mL for *Salmonella typhimurium*. The linear range of detection was from 10^1 CFU/mL to 10^7 CFU/mL[27]. In another study, Wang et al. established an advanced microscopy imaging platform for multiplex detection of pathogenic bacteria by combining PS microspheres and an Ago-based decoding system (Fig. 6D). First, multi-signal probes were constructed using the TDNA-enhanced HCR. Specifically, TDNA with two sticky ends could bind to the designed hairpin probes by sticky end hybridization. Both sides of hairpin 1 were labeled with fluorophores and quenchers, respectively. The iDNA with biotin was linked to the surface of PS microspheres through the biotin-SA interaction, triggering the HCR reaction of the two hairpin structures on TDNA. When the iDNA was present, the hairpins opened, which separated the fluorophore from the quencher and generated a fluorescent signal. These PS-TDNA HCR probes were encoded by spectrally distinct fluorophores and differing particle sizes. When pathogenic bacteria were present, they were specifically recognized by their respective aptamers. This recognition event triggered the release of specific phosphorylated gDNA. Subsequently, *Cb*Ago bound to this gDNA and specifically cleaved the iDNA bridge on the surface of the PS microsphere under the guidance of the gDNA. This resulted in the dissociation of the TDNA-HCR complex from the PS microspheres, reducing the number of fluorophores on the PS-TDNA-HCR probe and thus amplifying the signal. Finally, the fluorescent PS microspheres of different colors and sizes were counted by a computer vision-based algorithm to quantify pathogenic bacteria. The microscopy imaging platform successfully detected four pathogenic bacteria, with a linear range of 10^3 CFU/mL-10^8 CFU/mL for *Listeria monocytogenes*, 10^2 CFU/mL-10^7 CFU/mL for *Escherichia coli*, 10^3 CFU/mL-10^7 CFU/mL for *Salmonella typhimurium*, and 10^2 CFU/mL-10^7 CFU/mL for *Staphylococcus aureus*, respectively. The LODs were 101 CFU/mL for *Listeria monocytogenes*, 46 CFU/mL for *Escherichia coli*, 92 CFU/mL for *Salmonella typhimurium*, and 39 CFU/mL for *Staphylococcus aureus*, respectively[76].

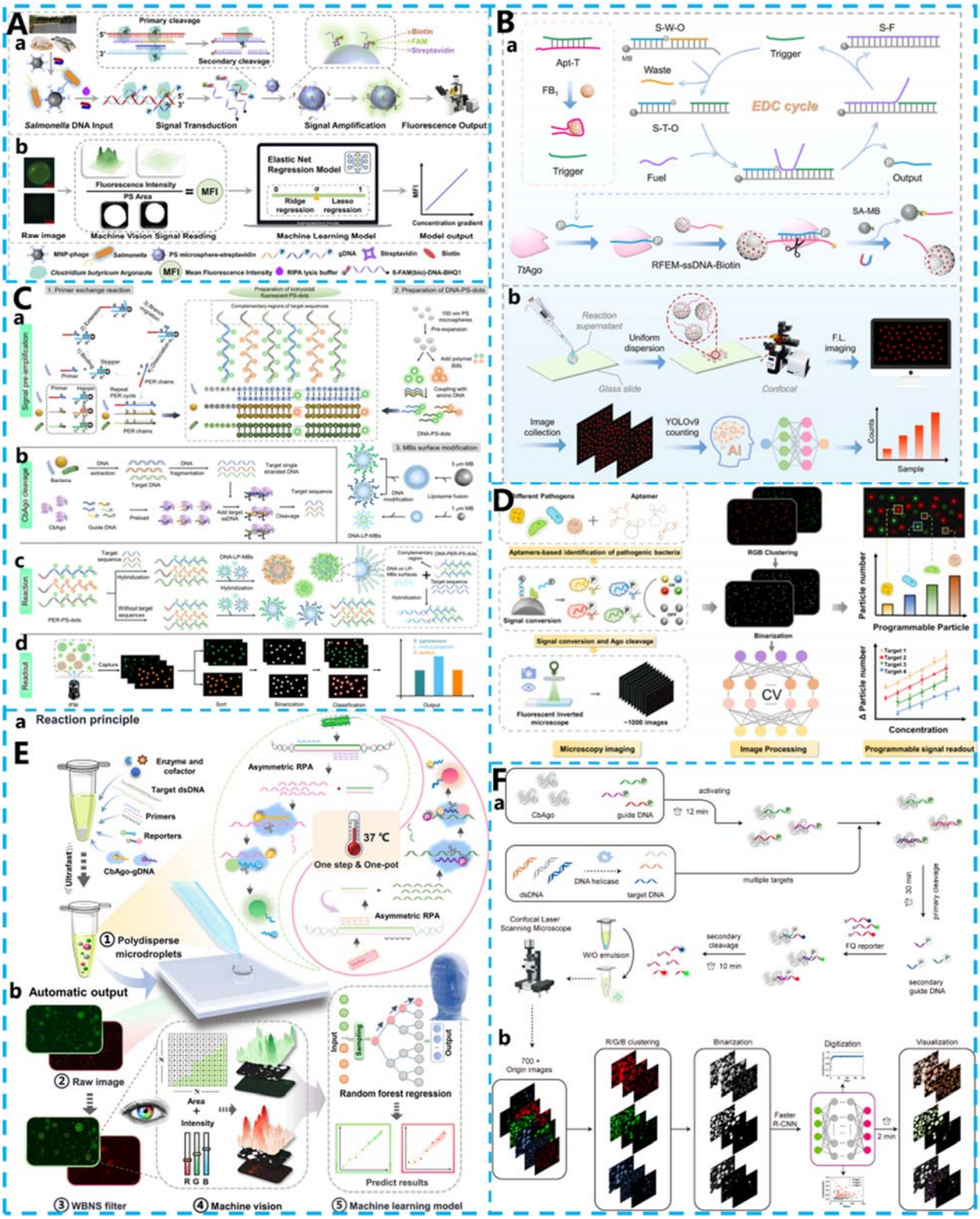

Fig. 6.(A)Schematic diagram of the AFPAM biosensor for detecting viable *Salmonella typhimurium* without convoluted DNA extraction and amplification procedures. a) Schematic diagram of AFPAM biosensor for detecting *Salmonella typhimurium*. b) The machine vision and machine learning-mediated image processing and data analysis. The scale is 0.2 mm. Reproduced with permission[26]. Copyright 2024, Elsevier. (B) Detection principle of deep learning-assisted fluorescence single-particle detection for FB_1. Reproduced with permission[18]. Copyright 2025, American Chemical Society. (C) Overview of the digital assay for multiplexed detection of pathogens. a) Preparation of the fluorescent botryoidal-like PS-dots for signal preamplification (1. The primer exchange reaction for DNA binding site amplification, e.g., *Salmonella typhimurium*. 2. The preparation of PS dots. 3. The preparation of liposome fusion-modified magnetic beads). b) Primary cleavage reaction mediated by *Cb*Ago for production of the target DNA sequence (PDB: 6QZK). c) Hybridization reaction between DNA-PER-PS-dots, target sequences from pathogens, and DNA-LP-MBs to fabricate the digital probe. d) Workflow of fluorescent images captured and processed by an AI-based fluorescent microsphere counting algorithm for a digital readout. Reproduced with permission[77]. Copyright 2024, American Chemical Society. (D)

Schematic diagram of the programmable microscopy imaging platform for multiple pathogenic bacteria multiple pathogenic bacteria detection: Aptamers are used to convert the information on pathogenic bacteria into gDNAs recognizable by the *Cb*Ago system. The encoded PS microsphere probes are then decoded using the activated *Cb*Ago-based specific system. Finally, multiplex detection is achieved by establishing quantitative associations between different encoded PS microspheres and concentrations of pathogenic bacteria. Reproduced with permission[76]. Copyright 2025, American Chemical Society. (E) The schematic workflow of DRAGON for the o one-pot simultaneous detection of different foodborne pathogenic bacteria. a) The reaction principle of polydisperse microdroplet reactors-assist one-step asymmetric RPA driven mesophilic *Cb*Ago report digital platform (DRAGON) for one-pot dual target nucleic acid detection. b) Dual parameters (microdroplet area and fluorescence intensity)-assist automatic machine learning-based image processing and result outputs. Reproduced with permission[66]. Copyright 2024, Elsevier. (F) Schematic illustration of the *Cb*Ago-mediated multiplexed nucleic acid assay based on a deep learning processed multivessel microdroplet signal readout system. a) The multiple cleavage reaction mechanisms of *Cb*Ago occur in the W/O emulsions. b) The workflow of the deep learning-triggered multivessel microdroplet signal readout. Reproduced with permission[82]. Copyright 2024, American Chemical Society.

In addition, Ago-based systems have been successfully integrated with emerging nucleic acid detection reactions in microdroplets and have enabled multiplexed quantitative analysis of low-concentration foodborne pathogens with the aid of machine learning[66, 82]. For instance, Wen et al. developed a digital detection platform for simultaneous detection of *Salmonella typhimurium* and *Listeria monocytogenes* by integrating *Cb*Ago, microdroplet reactors, and machine learning technologies (Fig. 6E). This platform utilizes the asymmetric RPA technology to generate large amounts of ssDNA at 37 ℃. For different target pathogens, specific gDNAs and fluorescence-modified reporter probes were designed, respectively. When the target was present, the gDNA guided *Cb*Ago cleave the ssDNA to generate new gDNA, which further cleaved the reporter probes and released different fluorescent signals to distinguish the targets. The platform used the polydisperse microdroplet reactor system to separate the reaction system into tiny reaction units, reducing background signal interference and enhancing the detection sensitivity. In addition, a random forest regression (RFR) machine learning model was constructed based on the dual parameters of microdroplet area and fluorescence intensity. This model could accurately analyze the signals and achieve intelligent quantitative detection of multiple pathogens in a one-step and one-pot manner. The established RFR model could detect *Salmonella typhimurium* with an LOD of 2 CFU/mL in a linear range of 9.3-9.3×10^6 CFU/mL. In addition, *Listeria monocytogenes* could be effectively detected with an LOD of 1 CFU/mL in a linear range of 8.4-8.4×10^6 CFU/mL[66]. Similarly, Wang et al. constructed a PASS platform based on mesophilic *Cb*Ago and deep learning for multiplex detection of nucleic acids from foodborne pathogens (Fig. 6F). On the one hand, *Cb*Ago could bind to gDNA to cleave the un-prelinearized tDNA, and the resulted DNA fragment could be used as a guide sequence for secondary cleavage to cleave FQ probes. On the other hand, portions of the *Cb*Ago opened double-stranded plasmids were linearized with the assistance of *Escherichia coli* RecQ helicase. This linearized plasmid and the DNA fragments generated from the primary cleavage acted as new guides to activate the secondary cleavage reaction of the FQ probe by *Cb*Ago. The entire reaction system was transferred in a water-in-oil (W/O) emulsion, with each microdroplet serving as an independent reaction unit. Meanwhile, deep learning technology was introduced. Through processes such as grayscale conversion, binarization, and region detection, the fluorescence signals were accurately analyzed to improve the detection accuracy. The constructed platform successfully detected three pathogenic bacteria, with a linear range of 10^2 CFU/mL-10^6 CFU/mL for *Salmonella typhimurium*, 10^3 CFU/mL-10^6 CFU/mL for *Listeria monocytogenes*,

and 10^2 CFU/mL-10^5 CFU/mL for *Staphylococcus aureus*, respectively. The LOD was 5 CFU/mL for *Salmonella typhimurium*, 3 CFU/mL for *Listeria monocytogenes*, and 2 CFU/mL for *Staphylococcus aureus*, respectively[82].

6. Challenges and perspectives

In recent years, Agos have provided a new insight in the field of biosensing due to their flexible programmability, excellent cleavage efficiency, and high specificity. To date, numerous of Ago-based biosensors and portable detection devices have been successfully developed, demonstrating significant potential for food safety detection. However, despite these advances, the technology still faces some challenges. Future studies could explore the following directions:

(1) Exploration of novel Agos. Currently, Ago-based detection methods primarily rely on thermophilic pAgos (such as *Pf*Ago and *Tt*Ago). While they exhibit high cleavage activity, their requirement for high temperatures limits widespread application, especially for on-site rapid screening. To address this, it is crucial to explore mesophilic pAgos (e.g., *Cb*Ago, *Km*Ago, *Mycobacterium bovis* Ago (*Mbp*Ago), and *Limnothrix rosea* Ago (*Lr*Ago)) that can operate at ambient temperature. It is noteworthy that currently reported mesophilic pAgos (such as *Cb*Ago) exhibit relatively low cleavage activity due to their structural constraints. Therefore, by integrating synthetic biology with AI technologies to conduct directed evolution and rational design of Ago proteins, it is promising to develop novel Ago proteins that combine high-efficiency cleavage capability, strong stability, and excellent adaptability. This will further advance the application of this technology in fields such as food safety.

(2) Development of non-nucleic acid target detection methods. Currently, Ago-based strategies for food safety detection primarily focus on nucleic acid detection. However, non-nucleic acid targets (e.g., biotoxins, antibiotics, pesticide residues, heavy metals, and other small molecules) that are widely present in food also pose serious threats to public health. Although progress has been made in detecting these targets, challenges remain, including cumbersome operational steps, complex separation processes, and insufficient sensitivity at ultra-low concentrations. Notably, the continuous advancement of molecular recognition and conversion elements, such as antibodies and functional nucleic acids (e.g., aptamers and DNAzymes), has provided effective tools for signal transduction. In the future, by integrating Ago (particularly mesophilic pAgo suitable for ambient temperature reactions) with novel biological recognition molecules and signal transduction elements, it is expected to enable highly sensitive and efficient detection of various non-nucleic acid targets in food samples, thereby further expanding the application prospects of Ago technology in this field.

(3) Exploration of multiplex detection. In complex food samples, relying solely on single target detection often makes it difficult to accurately determine the presence of hazardous substances. Simultaneous detection of multiple analytes can improve detection efficiency, reduce costs, and enhance the reliability of results, making the development of multiplex detection technologies essential. In Ago-based methods, the cleavage of reporter genes exhibits strict sequence dependence. This characteristic allows the signals generated from cleavage to be clearly distinguished, providing a unique advantage for constructing biosensors capable of

detecting multiple targets simultaneously. Although a few Ago-based multiplex detection methods have been reported, related research remains relatively scarce. Therefore, it is particularly important to expand the multiplex detection capabilities based on Ago and explore novel detection strategies.

(4) Development of amplification-free technologies. Currently, Ago-based detection methods typically rely on nucleic acid pre-amplification steps to enhance sensitivity. However, this approach increases operational complexity, detection costs, and analysis time. More importantly, aerosol contamination may occur during pre-amplification, leading to false-positive or false-negative outcomes and significantly affecting detection accuracy. To reduce contamination risks, some studies have integrated pre-amplification and sensing steps into a "one-pot" detection method. Nevertheless, this approach still requires the solution of compatibility issues between extraction components and subsequent amplification or sensing steps. Moreover, the complexity of the reaction process may reduce detection specificity and sensitivity. To date, although Ago-based amplification-free detection methods have been successfully developed, their variety and performance remain limited. By incorporating amplification strategies such as protein-protein cascades, protein-nucleic acid cascades, and microsphere aggregation signals, it is promising to achieve higher sensitivity in nucleic acid detection without preamplification, thereby expanding the application scope of this technology.

(5) Construction of novel Ago-based biosensing platforms. Although Ago-based biosensing technologies have been applied to the detection of food contaminants, the application of Ago in the field of biosensing is still in its early stages. Existing methods primarily rely on fluorescence signal output, which limits their broader application potential. Therefore, there is a need to further develop novel signal transduction and readout modes to expand their applicability. At the same time, challenges such as low nucleic acid content in real samples and the difficulty of detecting trace targets impose higher requirements for detection sensitivity and specificity. To address these issues, it is crucial to develop efficient target enrichment techniques, signal amplification strategies, and optimized biosensors to further enhance detection performance. For example, signal response and stability can be significantly enhanced by incorporating functional nanomaterials (e.g., gold nanoparticles, nanozymes, improved fluorescent probes) and adopting advanced readout modes (e.g., SERS, electrochemiluminescence, electrochemical). Additionally, the synergistic integration of Ago with the CRISPR/Cas system offers a new technical pathway for constructing multifunctional and highly sensitive biosensing platforms.

(6) Development of on-site detection devices. Currently, most Ago-based biosensors rely on laboratory precision instruments for signal readout, making it difficult to meet the needs of rapid on-site food safety detection. Therefore, the development of novel, miniaturized, and portable on-site detection devices is particularly important. Integrating portable platforms (such as lateral flow strips, nanopore devices, and microfluidic chips) with intelligent readout equipment (such as smartphones and electronic thermometers) can fully leverage their advantages of compact size, portability, and simple operation. Such integration strategies not only can enhance the analytical performance of Ago-based biosensors but also strongly promote their practical application in on-site detection.

(7) Integration of emerging technologies. In recent years, rapid advances in technologies such as AI, cloud computing, big data, and machine learning have provided opportunities for deep integration with Ago-based biosensing technology. Through machine learning-assisted design, it is possible to engineer pAgos with enhanced reaction kinetics, stronger substrate affinity, or higher specificity, thereby significantly improving detection accuracy and sensitivity. Moreover, the application of technologies such as cloud computing, big data, and AI can improve the efficiency of data processing and analysis for Ago-based biosensors, effectively reducing human bias, upgrade signal readout modes, and achieve comprehensive optimization of performance.

(8) Enhanced detection stability and simplified operation for complex food matrices. Food samples have complex matrices, and substances such as stabilizers, preservatives, and thickeners may interfere with the Ago-based sensing process, affecting the reliability and reproducibility the results. To reduce matrix interference, steps such as sample pretreatment, separation, enrichment, and purification of the target are usually required. These procedures are not only cumbersome but also rely on specialized personnel, thereby increasing the complexity and cost of detection. To enhance the practicality of this technology in food safety detection, future efforts should focus on two aspects: on the one hand, improving the robustness and compatibility of the Ago detection system by optimizing technical workflows and experimental conditions; on the other hand, striving to develop integrated and automated detection platforms. For example, integrating pre-processing, signal transduction, and Ago cleavage into a "one-pot" reaction, and leveraging miniaturized integration technologies (such as microfluidics), can ultimately achieve sample-in-result-out detection operational mode. This would significantly streamline operational process while ensuring the accuracy and stability of the detection.

7. Conclusions

Food safety is a core issue in the global public health field and has always received high attention from all sectors. As a key part of the food safety management system, food safety detection places higher demands on detection technologies in terms of on-site, precision, and high-throughput characteristics. Benefiting from its high catalytic activity, sequence specificity, and multi-target simultaneous detection capability, Agos has been used to construct biosensors that significantly improved the specificity and sensitivity of detection. Further integration with portable detection platforms has positioned Ago-based biosensing as a breakthrough tool for the development of rapid and accurate on-site detection. In this review, the recent research progress of Ago system-based biosensing technology and portable detection devices in the field of food safety detection was systematically summarized. Firstly, the working principle of Agos was briefly introduced, and its differences with the CRISPR/Cas system were compared. Secondly, the innovative strategies of Ago-based biosensing technology in signal amplification were comprehensively discussed from the two major technical levels of pre-amplification and amplification-free. Most importantly, the application of Ago-based biosensing technology and portable detection equipment in food safety detection was emphasized. Finally, the current challenges and prospective directions of Ago-based biosensing technology were highlighted. With the continued wide application of Agos in the field of biosensing and its continuous integration with a variety of

novel technologies, Ago-based biosensing technologies and portable detection devices were expected to provide effective solutions for food safety monitoring and rapid response to unexpected food contamination events.

Conflicts of Interest

The authors declared that they have no conflict of interest to this work.

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