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Emerging Argonaute-based nucleic acid biosensors

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Recent advances in Argonaute (Ago)-mediated biotechnology have provided new insights into the development of programmable and highly sensitive nucleic acid detection platforms. This study provides an overview of recent research on Ago-based nucleic acid detection. The potential applications of these emerging nucleic acid biosensors and the challenges associated with their use have been discussed.

Nucleic acid detection

Nucleic acid detection plays a significant role in various areas such as clinical diagnosis, food safety, and environmental pollutant testing. The development of highly sensitive and specific novel nucleic acid detection techniques has become an attractive but challenging goal, especially during a global pandemic. In addition to common nucleic acid-specific quantitative amplification techniques, programmable site-specific CRISPR-Cas endonucleases have been widely used for nucleic acid detection based on their nucleic acid binding, target cleavage, or target-activated collateral cleavage activities [1]. Although CRISPR-based detection methods have revolutionized the field of diagnosis, they face challenges, including the restriction of protospacer adjacent motifs (PAMs) or protospacer flanking sites (PFSs), and difficulties in multiplexed biosensing [1,2]. Ago protein, like CRISPR-Cas, has been proposed as a promising next-generation nucleic acid detection platform due to its ability to recognize and cleave target sequences. Ago represents an attractive way to solve the challenges encountered by CRISPR-Cas.

Principles and methods

Both eukaryotic Agos (eAgos) and prokaryotic Agos (pAgos) are nucleases that are guided by small RNA or DNA by guide-target base pairing (Figure 1A). The eAgos are involved in RNAi processes that rely on a common RNA-induced silencing complex. The pAgos are more diverse and divided into short and full-length pAgos. Recent biochemical and structural studies of several identified Agos showed that they have catalytic performance both *in vivo* and *in vitro*, with facile recognition and high cleavage efficiency. This indicates the high potential of Agos to act as programmable technology towards nucleic acid sensing platforms [3].

The cleavage activity of Agos used for nucleic acid detection involves: pre-amplification of targets (DNA or RNA) through PCR or isothermal amplification (Figure 1B-1); target amplicon cleavage of Agos guided by 5′-phosphorylated designed guide strands and the production of 5′-phosphorylated end DNA fragments as new guides for the cleavage of the fluorophore–quencher-labeled probes (Figure 1B-2, top); and the detection of signals by fluorescence spectrophotometry, quantitative real-time PCR (qPCR), droplet digital PCR (ddPCR), or Sanger sequencing (Figure 1B-3). The above strategy enables multiplex target monitoring by designing multiple guides to cut many targets and the corresponding probes (Figure 1B-2, middle). The cleavage activity of Agos could also achieve enrichment of mutant alleles by directly degrading wild-type DNA or RNA targets for diagnosis (Figure 1B-2, bottom). Furthermore, the binding activity of Agos used for nucleic acid localization *in vitro* and *in vivo* involves: the assembly of catalytically inactive fluorescently labeled Agos with designed guides (Figure 1B-4); capturing the entire region of targets onto the surface of organismic materials *in vitro* or premixing the guide with the live organism and binding of the Ago compounds with the target strand by base pairing (Figure 1B-5); and nucleic acid localization using fluorescence imaging technology (Figure 1B-6).

Applications

SARS-CoV-2 detection

The development of a rapid, accurate, and multiplex site detection platform for SARS-CoV-2 and its mutants would significantly reduce the virus incidence and provide a quick response to pandemic outbreaks. Wang and his colleagues established a rapid and high-sensitivity biosensor for SARS-CoV-2 and its mutant SARS-CoV-2 D614G by using *Pyrococcus furiosus* Ago (*Pf*Ago) integrated with reverse transcription (RT)-PCR [4]. In addition to the development of rapid, one-step approaches for point-of-care testing, Xun and coworkers reported a Scalable and Portable Testing (SPOT) system that combines RT-loop-mediated isothermal amplification (LAMP) with *Pf*Ago for on-site monitoring of multiplex SARS-CoV-2 sites [5]. These strategies achieved one-copy detection of SARS-CoV-2 and its mutants within 30 min, meeting the demands of field testing and emergency situations. Ago-mediated biosensors can be applied for the detection of any virus in the near future.

Early diagnostics for cancer

Cancer is caused by somatic mutations in normal cells, with single-nucleotide variation being one of the most common and important sources. Due to its high sensitivity towards the single-nucleotide mismatch in guide-target base pairing, Ago shows high application potential in rare mutation-associated tumor sample analysis. A recent study presented a novel approach for the early diagnosis of pancreatic cancer through highly specific enrichment of nucleic acid fractions using *Thermus thermophilus* Ago (*Tt*Ago). This approach improves the sensitivity of the downstream detection technologies

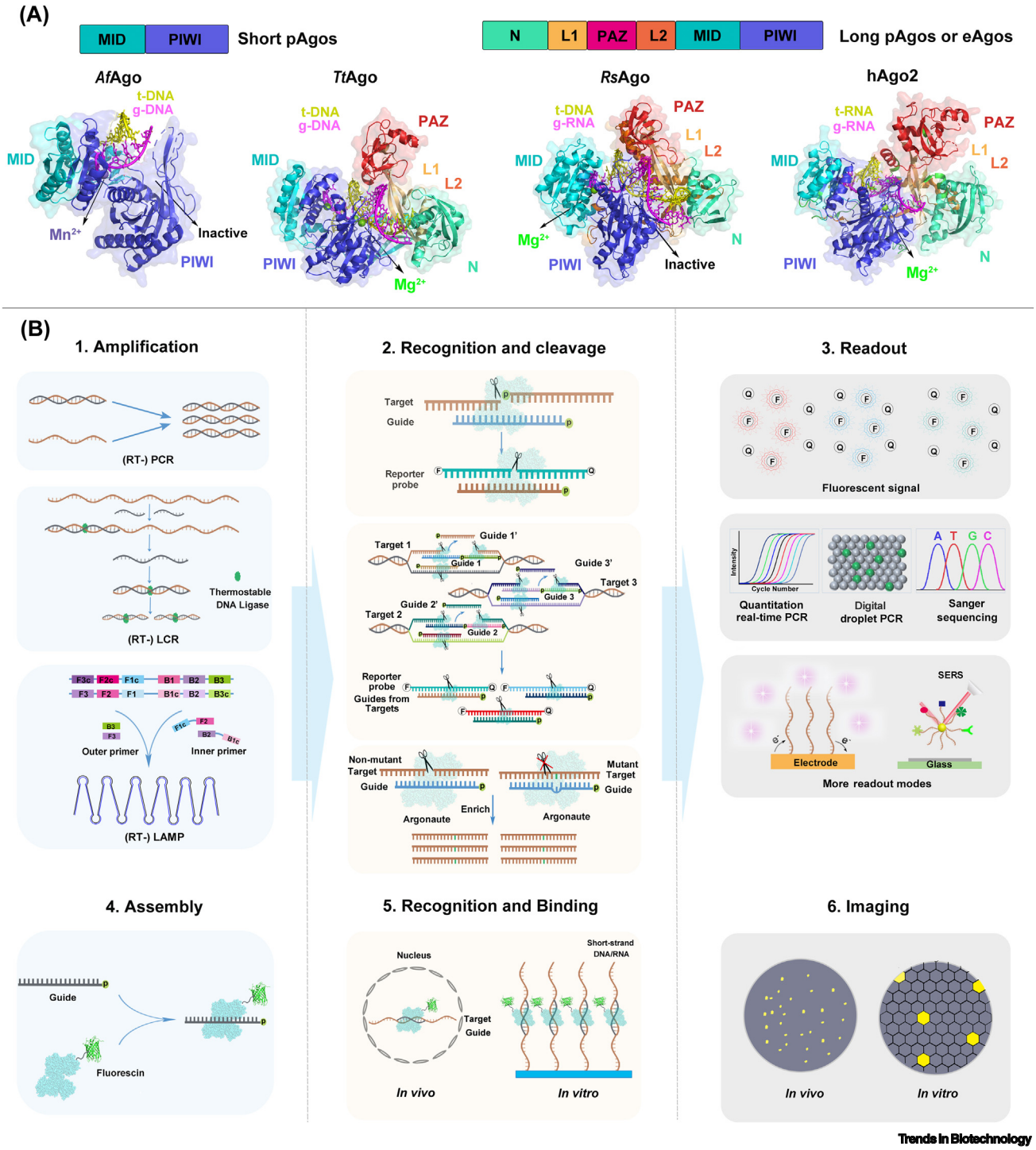

Figure 1. (A) Structural basis of Argonaute (Ago) proteins. Short prokaryotic Agos (pAgos) comprise middle (MID) and P-element-induced wimpy testis (PIWI) domains. All studied long pAgos contain all structural segments present in eukaryotic Agos (eAgos): the N-terminal, linker 1 (L1), PIWI-Argonaute-Zwille (PAZ), linker 2 (L2), MID, and PIWI domains, which form a two-lobe scaffold comprising MID-PIZI and N-PAZ lobes with the linkers L1 and L2. The catalytic site of Ago is located in the PIWI domain that mediates nucleic acid cleavage. Short pAgos and some long pAgos [e.g., *Rhodobacter sphaeroides* (*Rs*Ago)] have inactive PIWI domains that lack endonucleolytic activity. The structures of four

(Figure legend continued at the bottom of the next page.)

by utilizing the *Tt*Ago cleavage site of guide-complementary nucleic acids with single-base accuracy [6]. A similar diagnostic platform was adopted to monitor the rare mutations in tumors by *Pf*Ago, followed by a qPCR process. Because of the high activity temperature of *Pf*Ago, which is compatible with DNA polymerase, the processes of cleavage and amplification were combined into one step using this technique, achieving great efficiency for potential applications in point-of-care testing [7].

Short nucleic acid analysis

miRNA detection technology faces the challenges of complicated steps and the identification of single-base mismatches. Hohng and colleagues developed a *Tt*Ago-based multicolor. single-molecule sensing technique named Ago-FISH, which is capable of detecting purified endogenous let-7a miRNAs with high sensitivity, specificity, and reliability [8]. Zheng and coworkers developed miRNA: Ago2 cleavage molecular beacons, which achieved the target recycling and signal amplification of mir-21 microRNA [9]. Similarly, a previous study reported a *Clostridium butyricum* Ago (*Cb*Ago)-assisted DNA-PAINT used for ssDNA super-resolution imaging, which significantly accelerates target binding [10]. These strategies reduce the false-positive rates and improve target sensitivity.

In vivo imaging

The Ago system is also used for nucleic acid *in situ* labeling due to its high sequence-specific target recognition. Sun and his coworkers used the fluorescence-labeled nuclease-deficient *Tt*Ago (d*Tt*Ago) combined with 5′-phosphorylated guide DNA to label specific sequences in human and mouse cells [11]. Barriere and his colleagues used the exogenous RNAi pathway and fluorescently tagged Ago NRDE-3 to locate multiple genes at the transcription site in the cytoplasm [12].

Multiplex target monitoring

Ago-based biosensing can be performed for multiplex target monitoring by using several short guides DNA or RNA, which is valuable in point-of-care testing and clinical analysis. Wang and his colleagues demonstrated a *Pf*Ago-mediated multiplexed detection diagnostic method that includes a secondary round of site-directed cleavage of *Pf*Ago guided by the guide DNA from previous cutting reactions that had three designed initial guide DNAs. The molecular beacons with various fluorophores may be used at the following stage to indicate diverse Ago effector complexes carrying different neonatal guide DNAs. The proposed approach was successfully applied to the simultaneous detection of HPV subtypes in clinical samples [13]. A similar SPOT strategy detected multiplex SARS-CoV-2, *N* gene, and *E* gene sites [5]. Moreover, Xun and his coworkers directly obtained DNA fragments with 5′-phosphorylated ends from the stepwise cleavage of *Pf*Ago directed by a single guide DNA, which acted as a new guide and achieved a clinic diagnosis for HPV genotyping [14].

Technological advantages and outlook

Compared with traditional detection techniques, an Ago-based nucleic acid biosensor provides a number of advantages, making it a more accessible and versatile nucleic acid biosensing platform. Agos are PAM independent, which means that they recognize the targets only by base pairing with guides. Agos greatly simplifies the design of guides using short DNA or RNA. For example, DNA-guided pAgos, with high stability and simplicity, can be produced at a lower cost, making laboratory operation and point-of-care testing easier. *Pf*Ago and *Tt*Ago have been successfully applied to the highly specific diagnostics of rare single-base mutations because of their high sensitivity towards the matching between guides and target [6,7]. Finally, the Ago-based biosensor is more accessible to solving the challenge of multiplexed nucleic acid detection, even when only one

representative Agos are shown in the following text: the short *Archaeoglobus fulgidus* Ago (*AfAgo*) (PDB: 2W42); the long *Thermus thermophilus* Ago (*TtAgo*) (PDB: 4NCB); the inactive long *Rs*Ago (PDB: 5AWH); and the eAgo human Argonaute2 (hAgo2) (PDB: 4W5O). They are displayed with guide (g-) and target (t-) nucleic acid strands. The N-terminal domain is shown in cyan green, the L1 domain is light orange, PAZ is red, L2 is orange, MID is cyan, and PIWI is blue. Adapted from [3]. (B) Schematics for Ago-based nucleic acid biosensing system (5′-phosphorylated end DNA fragments as guides). The cleavage activity of Agos used for nucleic acid detection mainly includes amplification (1), recognition and cleavage (2), and readout (3). Amplification strategies include reverse transcription (RT)-PCR, (RT-)ligase chain reaction (LCR), and (RT-)loop-mediated isothermal amplification (LAMP). Ago-mediated guide recognition and target cleavage are classified as general single target detection (top), multiplex target monitoring (middle), and single-nucleotide mutation analysis (bottom). Top: Designing a short ssDNA guide, showing base pairing with the target; a new guide generated from the target under Ago catalysis; Ago used for another cleavage with the guidance of the new guide towards the fluorophore–quencher-labeled probe. Middle: Similar to (Top); several new guides were obtained from different targets by the cleavage of Ago; these new guides were used for the recognition and cleavage of corresponding probes under Ago catalysis and generated different fluorescent signals. Bottom: Ago can recognize and cleave the non-mutant target with the designed guide and enriched the mutant target due to Ago's single-nucleotide-precision base pairing between guide and target. The readout modes include fluorescent signal acquisition by fluorescence spectrophotometry and sequence information by quantitative real-time PCR (qPCR), digital droplet PCR, and Sanger sequencing. Advanced signal readout approaches like electrochemiluminescence and surface-enhanced Raman scattering (SERS) will be reported in the near future. Second, the binding activity of Agos used for nucleic acid localization *in vitro* and *in vivo* comprises the assembly of guides onto fluorescently tagged Ago (4), recognition and binding *in vitro* or *in vivo* (5), and readout by imaging technology (6). The assembly step involved pre-incubation of guides and Agos, which showed binding activity and cleavage inactivity only with the following strategies: construction of a *Tt*Ago mutant at the Asp546 residue of its PIWI domain; incubation of the *Tt*Ago system at 30°C; or adjustment of the base-pairing number below 10 nt between the guide and target bound by *Cb*Ago. The recognition and binding procedure (4) can occur *in vivo* or *in vitro*. The purified ssDNA or ssRNA was immobilized onto the sensing chip by the addition of adenosine and biotinylated poly (T) DNA to 3′-end targets and the sensing chip, respectively, and then the target was captured by the hybridization reaction. The Ago compound was bound by base pairing between the guide and target *in vitro*. By the exogenous RNAi pathway and co-incubation of the Ago compound and cells, nucleic acid target detection was achieved *in vivo* and *in situ*, respectively.

Ago is used for the simultaneous analysis of DNA and RNA targets [5,13–15]. Ago-based biosensors also have several challenges. In contrast to the self-signal amplification by the collateral cleavage activity of CRISPR-Cas, Ago-based biosensors require amplification or enrichment to improve the detection sensitivity [4,8]. The development of Ago-based biosensors needs to note the tolerated mismatches of single nucleotides in certain sites. Furthermore, the molecular and functional mechanisms of Agos, including the discovery of new Agos, new nuclease activities, and new physiological functions, need further exploration. These insights will help us develop new biosensors such as dsDNA detection at room temperature.

Concluding remarks

The application of Agos in nucleic acid biosensing remains in its infancy (Table 1). Current research focuses primarily on the application of hyperthermophilic Agos. The mesophilic Agos, such as *Cb*Ago and *Synechococcus elongatus* Ago, and the engineered Agos with thermostability and catalytic activity at room temperature are worth developing as versatile point-of-care nucleic acid testing tools. Based on their high target-binding affinity and specificity, dAgos can be used for clinical diagnosis, genome typing, and sequencing after labeling them with reporters (e.g., fluorescent proteins, peroxidases, nanozymes). Agos can be used for multiplex nucleic acid detection *in vivo* by coupling with an organism's endogenous RNA or DNA interference pathways. Moreover, with the development of smart nucleic acids (i.e., aptamer, DNAzyme), the Ago system has demonstrated the capacity to detect a wide range of non-nucleic acid analytes (e.g., proteins, bacteria, metal ions) in various fields, including clinical diagnosis, food safety, and environmental protection. The Ago system can significantly improve the sensitivity and achieve autonomous monitoring of target content by combining with nanotechnology and advanced signal readout modes such as electrochemical, electrochemiluminescence, and surface-enhanced Raman scattering.

In summary, Ago-mediated biosensors provide rapid, programmable, and highly specific detection for pathogens, genotyping,

Table 1. List of Ago-mediated nucleic acid detection

Ago name	Target	Signal amplification	Ago-based biosensing[a]	Conditions[b]	Sensitivity	Specificity	Multiplex	Readout	Refs
*Pf*Ago	SARS-CoV-2 (D614G)	RT-PCR	Single target detection	95°C, 20–30 min	1 copy per reaction	1 nt	No	Fluorescence	[4]
*Pf*Ago	COVID-19	RT-LAMP	Multiplex target monitoring	95°C, 30 min	0.44 (*N* gene) and 1.09 copies μl^{-1}(*E* gene)	1 nt	Yes	Fluorescence	[5]
*Tt*Ago	*KRAS* G12D (pancreatic cancer)	PCR	Single nucleotide mutation	83°C, 60 min	1 copy in low frequency blood samples (<0.01%)	1 nt	Yes	ddPCR etc.[c]	[6]
*Pf*Ago	Oncogenic mutations	PCR	Single nucleotide mutation	94°C[d]	1 copy in low frequency blood samples (0.01%)	1 nt	Yes	qPCR or Sanger sequencing	[7]
*Tt*Ago	Let-7a	No	*In vitro*	55°C, 30 min; 30°C	fM	1 nt	No	Fluorescence	[8]
hAgo 2	mir-122	No	*In vitro*	37°C, 1 h	13-fold	1 nt	No	Fluorescence	[9]
*Cb*Ago	ssDNA	No	*In vitro*	37°C, 20 min; RT	10-fold	No	No	Fluorescence	[10]
d*Tt*Ago	Genomic loci	No	*In situ*	47°C, 16–20 h	Simple-copy repeats	No	Yes	Fluorescence	[11]
NRDE-3	Transcription site localization	No	*In vivo*	–	–	Yes	No	Fluorescence	[12]
*Pf*Ago	HPV serotypes	PCR or tHDA	Multiplex target monitoring	95°C, 20 min	1.60 aM	1 nt	Yes	Fluorescence	[13]
*Pf*Ago	HPV serotypes	PCR	Single target detection	95°C, 30 min	fM	–	Yes	Fluorescence	[14]
*Pf*Ago	SARS-CoV-2 (D614G) or HPV	(RT-)Ligase chain reaction	Other[e]	95°C, 20 min	10 aM	1 nt	Yes	Fluorescence	[15]

[a]Ago-based biosensing strategy (Figure 1B).
[b]Reaction condition for Ago.
[c]ddPCR, peptide nucleic acid-PCR, xenonucleic acid-PCR, or Sanger sequencing.
[d]One-step with cleavage of *pf*Ago and amplification process.
[e]The strategy includes target amplification, and then the fluorophore–quencher-labeled probes were cleaved under *Pf*Ago catalysis.

rare mutations, and multiple targets. We believe Agos could become the next-generation nucleic acid biosensor, with further applications in complex conditions and distinct target monitoring.

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Declaration of interests

The authors declare no interests.

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