Review

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Duplex-specific nuclease-mediated bioanalysis

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Duplex-specific nucleases (DSNs) are promising tools for bioanalysis because of their unique ability to cleave DNA within duplexes while keeping a single strand intact. There is prevalent use of DSNs in both biomedical and biological science applications, such as cDNA library construction, circulating miRNA detection, telomeric overhang detection, and SNP recognition. We present an overview of the current knowledge of DSNs, with special emphasis on DSN-mediated isothermal signal amplification strategies for trace miRNA detection. Continued innovation to address key challenges, such as amplification-free approaches, will open up new avenues in the field of miRNA profiling, offering opportunities for improved personalized medicine, preventive medicine, and translational medicine.

Structure and characteristics of DSNs

DSNs are DNases that specifically degrade double-stranded (ds) nucleic acids. DSNs have been isolated from the digestive gland or hepatopancreas of some *Decapoda*, such as shrimp and crabs [1]. Although several DSN homologs have been isolated and purified from *Pandalus borealis* (a red shrimp) and *Penaeus monodon* (black tiger shrimp), the Kamchatka crab-derived DSN is the only industrially applicable heat-resistant DSN [2,3].

The full-length cDNA of Kamchatka crab-derived DSN is 1348 nt in length, excluding the poly(A) tail [4]. The DSN protein is a monomer with a molecular mass of 41.5 kDa and an isoelectric point of 4.2. As a member of the nonspecific DNA/RNA endonuclease family that can specifically target ds DNA-containing substrates, the structure of Kamchatka crab DSN is highly similar to that of kuruma shrimp nuclease, with 67% amino acid identity and 82% overall homology (Figure 1). However, DSN exhibits unique substrate specificity and hydrolyzes only dsDNA or DNA in DNA/RNA hybrids, regardless of nucleotide sequence, and does not cleave single-stranded (ss) DNA or RNA at all. The inability of DSN to cleave RNA differentiates it from Argonaute (Ago) proteins, which catalyze cleavage of miRNA-guided mRNA [5]. Moreover, DSN can discriminate between perfectly and imperfectly matched (up to one mismatch) short duplexes. These unique enzymatic features have made DSNs attractive in the fields of biological and biomedical sciences since their discovery in 2002 (Box 1). Reported applications have preliminarily focused on the construction of cDNA libraries, including cDNA normalization, cDNA depletion, and cDNA subtraction. Recent studies have reported the use of DSN as an effector to mediate isothermal amplification for rapid miRNA quantification [6]. Applications such as SNP detection, quantitative telomeric overhang determination, the construction of repeat-free fluorescent *in situ* hybridization (FISH) probes, and RNA sequencing have also been reported [7].

DSN-mediated bioanalysis strategies exhibit outstanding operating characteristics compared to conventional methods (Table 1). A DSN-based signal amplification assay enabled the determination of miRNA at a lower detection limit of 0.1 fM. This strategy also allows the recognition of single-base mismatched miRNAs, which could be utilized to develop assays for SNP detection. We review and assess the potential applications of DSNs to bioanalysis in biology and medicine, and as a new approach for the ultrasensitive detection of miRNAs.

DSN-based miRNA detection

miRNAs (approximately 19–23 nucleotides) are highly tissue-specific biomarkers with potential clinical applicability to profiling cancer metastases [8–10]. Circulating miRNA (cmiRNA), miRNAs present in various biological fluids, are resistant to digestion by ribonucleases (RNases), and are stable to repeated freeze–thaw cycles and prolonged storage, suggesting their potential as a novel biomarkers for disease diagnosis and prognosis [11–13]. Rapid and highly sensitive methods for trace miRNAs determination are urgently needed to permit different expression models to be studied and the regulatory mechanisms of miRNAs to be elucidated [14].

Conventional methods for miRNA quantification include northern blotting [15], *in situ* hybridization [16], microarrays [17], qRT-PCR [18], and next-generation sequencing [19]. Despite their remarkable advantages, each method is limited by either throughput or cost (Box 2) [20]. A DSN-mediated signal amplification strategy was recently developed for miRNAs in which the original detection signal could be amplified linearly without changing the quantity of the target miRNA (Box 3). Thus, no PCR amplification was involved, substantially reducing the risk of nonspecific amplification. By combining amplification with different assay platforms, miRNAs can be quantified rapidly using colorimetric, fluorescent, or electrochemical assays.

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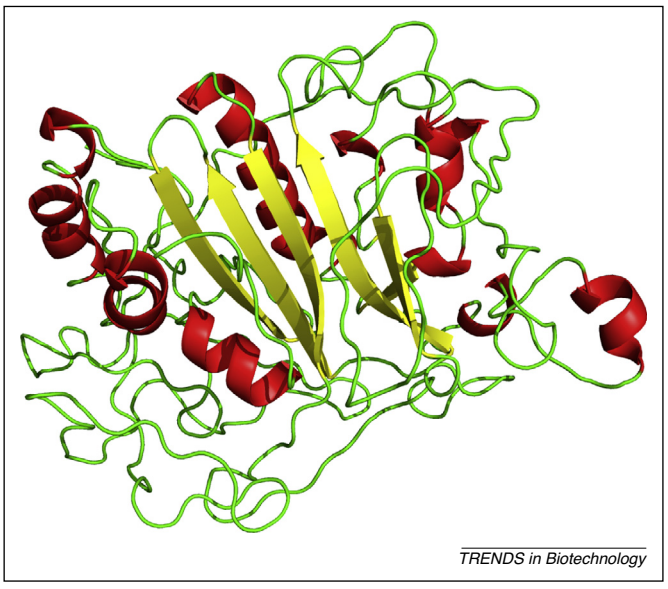

Figure 1. Simulated duplex-specific nuclease (DSN) structure based on the DSN sequence in GenBank (GenBank: AF520591.1) created using PyMol.

DSN-mediated colorimetric assays

Colorimetric analysis is a simple process because the results can be monitored with the naked eye [21]. Considerable attention has focused on the development of rapid and automated biosensors for cmiRNA detection. The first success in developing a DSN-mediated colorimetric assay for miRNA detection was reported by Tian Tian *et al.* [22]. They designed a DNA probe comprising a ss G-rich sequence with interference sequences at the two ends of the probe. The G-rich sequence bound hemin to catalyze the oxidation of $ABTS^{2-}$ [2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid] by H_2O_2 to produce $ABTS^{-}$. Upon hybridization and cleavage, the G-rich sequence was released and combined with hemin to induce peroxidase activity. The color change due to $ABTS^{-}$ formation can be observed by the naked eye or accurately measured using a colorimeter (Figure 2A). The use of DSN allowed the target miRNA to be recycled so that it can act on the remaining probes until all probes were cleaved. This strategy permitted the label-free determination of miR-141 in a linear dynamic range between 20 pM and 10 nM. The specificity of this assay was validated using miR-429, which has a sequence similar to that of miR-141.

Although colorimetric assays are amenable to point-of-care (POC) testing, their lack of sensitivity makes the primary detection of miRNA at sub-fM concentrations extremely difficult. To further lower the detection limit to fM levels, magnetic beads or gold nanoparticles (AuNPs) were introduced to enhance the analytical signals. Huimin Deng *et al.* designed magnetic bead-labeled capture probes (CPs) composed of miRNA-capturing segments and DNase sequences at their termini for sensitive detection of miRNA [23]. The DNase segments of the CPs folded into a G-quadruplex that bound hemin to form a peroxidase-like DNase, which was cleaved from the magnetic beads to catalyze the oxidation of 3-aminophthalimidohydrazine (luminol) and produce chemiluminescence (Figure 2B). Thus, miRNA could be determined at concentrations as low as 20 fM. In addition, the analytical sensitivity could be improved by integrating magnetic beads with DNase-capture probes (DZ-CP) [24]. In that study, the released DNase catalyzed the oxidation of tetramethylbenzidine (TMB) using H_2O_2. An optimized analysis on let-7b miRNA (22 nt) revealed a linear range between 0.20 fM and 50 fM, with a detection limit of 0.1 fM at a signal/noise ratio of 3.0.

An AuNP network probe has also been developed to facilitate the detection of miRNA [25]. Wei Shen *et al.* designed AuNP networks crosslinked by DNA CPs and gold nanoparticles that served as a bifunctional probe (capture probe and signal generator). After cleavage of the CPs in the miRNA/CP duplex by DSN, the released miRNA strands re-hybridized with the AuNP networks; thus, one target miRNA could cleave millions of AuNPs from the AuNP networks after incubation, and an absorption peak shift from 590 nm to 530 nm could be observed (Figure 2C). In the presence of 0.25 U DSN and 5.0 nM AuNP networks, let-7b could be detected accurately in a wide dynamic range of 0.20 fM to 10 pM with a detection limit of 0.1 fM, comparable to that of the most-sensitive qPCR assays.

DSN-mediated fluorescent assays

Fluorescence-based techniques have long been prevalent because of their remarkable advantages, including simplicity, low detection limits, and easy handling. Fluorescent analysis strategies are typically based on the measurement of the fluorescence intensity induced upon specific binding between fluorescence-labeled probes and target biomolecules. Compared with colorimetric assays, fluorescence assays enable extraordinary sensitivity and multiplex detection capability as well as automation. Because the target miRNAs are typically in low abundance, conventional fluorescence assays still fail to quantify

Box 1. Enzymatic characteristics of DSNs

Substrate specificity: strong cleavage preference for dsDNA substrates. DSNs hydrolyze only dsDNA or DNA in DNA/RNA hybrids, regardless of nucleotide sequence. Only minor activity against ssDNA has been reported, and this activity was completely inhibited in the presence of competitive dsDNA.

Substrate length requirements: a minimum of 10 bp DNA or 15 bp DNA/RNA perfect duplex is required for effective cleavage.

Optimal ionic strength: the activity of purified DSN depends on the presence of divalent cations (Mn^{2+}, Co^{2+}, or Mg^{2+}). Calcium does not activate DSN directly but significantly enhances the activating effects of Mn^{2+}, Co^{2+}, and Mg^{2+}. DSN enzymatic activity requires a minimal Mg^{2+} concentration of 5 mM (7 mM for optimal activity), and DSN enzymatic activity decreases with increasing ionic strength.

pH and temperature stability: DSN has a very broad pH optimum, with a maximum at pH ~6.6. DSN is completely inactive at pH values <3.0 or >9.0. DSN is deactivated only at 70°C or higher, and the optimal temperature for DSN activity is approximately 60°C.

Chemical tolerance: DSN is tolerant to proteinase K treatment and most aggressive chemicals. Approximately 90% of initial activity was maintained after incubation of DSN with 1% SDS, 10 mM β-mercaptoethanol, and 0.3% hydrogen peroxide at 37°C for 30 min However, SDS completely inhibits DSN activity at 60°C, and EDTA inactivates DSN completely. DSN is sensitive to polyamines and chaotropic agents.

Table 1. Comparison of different DSN-mediated detection methods

DSN application	Detection method	Target molecule	Linear range	LOD	Year	Refs.
miRNA detection	Colorimetric	miR-141	20 pM–10 nM	20 pM	2013	[22]
miRNA detection	Colorimetric	let-7b	20 fM–5.0 pM	10 fM	2013	[23]
miRNA detection	Colorimetric	let-7b	0.2 fM–50 fM	0.1 fM	2014	[24]
miRNA detection	Colorimetric	let-7b	0.2 fM–10 pM	0.1 fM	2013	[25]
miRNA detection	Fluorescent	miR-141	10 pM–100 nM	10 pM	2013	[22]
miRNA detection	Fluorescent	miR-141, miR-21, let-7d	100 pM–100 nM	100 fM	2012	[31]
miRNA detection	Fluorescent	let-7a	0.5 pM–500 pM	0.4 pM	2013	[26]
miRNA detection	Fluorescent	miR-21	0.1 pM–10 nM–	300 fM	2014	[27]
miRNA detection	Fluorescent	let-7a, miR-143	100 pM–5 nM	60 pM	2014	[28]
miRNA detection	Fluorescent	let-7b	500 fM–1 nM	160 fM	2014	[29]
miRNA detection	Fluorescent	miR-21, miR-203	5 pM–200 pM	0.2–0.3 fM	2014	[30]
miRNA detection	Electrochemical	let-7b	2.0 fM–2.0 pM	1.0 fM	2013	[6]
SNP detection	Duplex-specific nuclease preference (DSNP)	MTHFR, prothrombin, COX1, p53	NA	NA	2002	[1]
SNP detection	DSNP	kRAS nRAS, hRAS, F5, MTHFR, F2, ApoE, p53, BRCA1, prothrombin	NA	NA	2005	[63]
SNP detection	GSMapper, ssahaSNP, bwa/SAMtools	187 653 SNPs	NA	NA	2012	[64]
SNP detection	AuNP-based colorimetric assay	HBV genomic DNA	NA	NA	2011	[65]
Telomeric overhang detection	DSN-based overhang assay	BJ foreskin fibroblasts, HeLa cervical carcinoma cells	NA	12 nt	2008	[69]
RNA-Seq	DSN-based rRNA removal	Whole transcriptome in A2780 cells	NA	NA	2013	[53]
RNA-Seq	DSN-based rRNA removal	Transcriptomes of *Escherichia coli*	NA	NA	2011	[55]
RNA-Seq	DSN-based normalization	Transcriptomes of *Schmidtea mediterranea*	NA	NA	2011	[56]

low-concentration miRNAs, particularly in clinical settings. The DSN enzyme has also been adopted in fluorescence assays for improved miRNA detection.

A fluorescence biosensor prototype for trace miRNA quantification using a DSN cleavage strategy was developed using a molecular beacon (MB) probe whose opposite arms were labeled with a fluorophore and a quencher [26]. In the presence of the target miRNA, the MB probe binds to the target and produces a fluorescence signal, and the resulting MB/miRNA duplex is hydrolyzed. To avoid potential false-positive cleavage of the dsDNA, the MB was modified with 2-OMe-RNA on its stem. This method achieved a linear response between 0.5 pM to 500 pM, with a lower detection limit of 0.4 pM. Moreover, Tian Tian *et al.* designed a type of MB probe with the functional sequence from the 8-17 DNAzyme and its interfering sequence at opposing ends [22]. The presence of the target miRNA leads to the formation of a stable heteroduplex that could be cleaved by DSN. Then, the functionally released 8-17 DNAzyme hydrolyzes its molecular substrate (another hairpin probe), reversing the fluorescence quenching and enabling the target miRNAs to be quantified by measuring the increased fluorescence intensity (Figure 2D). Using the proposed assay, the detection limit of miR-141 was 10 pM, a slight increase in sensitivity compared with the previously established peroxidase assay.

In addition to using DNA probes as catalysts, various nanomaterials have been adopted as fluorescence media for developing DSN-mediated biosensors. Nanomaterials such as tungsten disulfide (WS_2) nanosheets, graphene oxide (GO), and gold nanoparticles can serve as substrates to quench the fluorescence of labeled probes. Thus, the fluorescence intensities are negligible in the absence of the target miRNAs, and are 'switched on' or increased in the presence of the target miRNAs. Qiang Xi *et al.* developed a WS_2 nanosheet-based strategy for miRNA detection by combining fluorescence quenching with DSN signal amplification (DSNSA) [27]. This strategy was based on the different affinities of the WS_2 nanosheet toward short oligonucleotide fragments (<10 nt) versus ssDNA probes (22 nt). WS_2 acts as a fluorescence quencher by adsorbing the fluorescence of a FAM-labeled DNA probe complementary to the sequence of miRNA-21. In the presence of the target miRNA-21, DSN cleaves the induced DNA/miRNA hybrids, and the resulting DNA fragments detach from the WS_2 substrate, enabling fluorescence detection. This strategy permits the simple, sensitive detection of miRNA-21 in the range of 0.001 nM to 10 nM. Several homologous miRNAs (miR-143, miR-141) were distinguished using this approach. GO-based nanobiosensors have also been developed for miRNA detection [28,29], based on the principle that DSN-cleaved short DNA fragments are weakly

Box 2. Comparison of typical miRNA detection strategies

Northern blotting: widely used to visualize the expression of miRNAs of all sizes, ranging from long primary miRNAs to mature forms, because of its reliable accuracy [70]. Its disadvantages include time consumption and lack of throughput, making Northern blotting impractical for the large-scale detection of hundreds of miRNAs [71].

Microarrays: widely utilized in miRNA profiling and can simultaneously determine the expression levels of large numbers of miRNAs in a single experiment [72]. However, the short length of miRNAs, differences in their melting temperatures (Tm), and the high sequence similarity of miRNA family members make probe design extremely difficult [73,74].

qRT-PCR: the 'gold standard' for miRNA detection that is used to validate the results obtained via other methods [18]. qRT-PCR cannot be used to quantify miRNA directly because miRNAs are only 21–23 nucleotides in length. To overcome this problem, longer precursor molecules of miRNA have been detected using a qRT-PCR approach [11]. However, this method is inherently unreliable because the amount of pre-miRNA and mature miRNA is inconsistent.

RACE RT-PCR or stem-loop RT-PCR: direct profiling of mature miRNAs by modifying the classical qRT-PCR using either RACE (rapid amplification of cDNA ends) primers or stem-loop reverse primers [75,76].

qPCR-array: combining the high sensitivity of stem-loop RT-PCR with the ability to profile large numbers of miRNAs in a single experiment has provided superior sensitivity and specificity to classic microarray methods. However, strict primer design is required because two distinct sets of primers (for reverse transcription and PCR amplification, respectively) are typically needed, and these two distinct sets of primers are liable to produce primer dimers [72]. In addition, increased variations are frequently observed during the pre-amplification of miRNAs of low abundance, which may contribute to miRNAs with a low expression level and high cycle-threshold values.

High-throughput sequencing: capable of identifying novel miRNAs, but the detection procedure is laborious, costly, and often requires the identification and removal of adaptor sequences before the miRNA sequence itself can be determined [77].

Box 3. Mechanism of the DSN-mediated signal amplification strategy

Step 1. The target miRNA hybridizes with the DNA probe to form a DNA/miRNA heteroduplex.

Step 2. DSN is introduced to selectively cleave the DNA within the hybrid duplexes. After cleavage, the target miRNA and DSN are released.

Step 3. The miRNA that is released can hybridize with the remaining DNA probes to form a second DNA/miRNA heteroduplex.

Step 4. Once the heteroduplex is formed, the released DSN can cleave the DNA in the heteroduplex. By repeating the procedures from steps 1 through to 4, a hybridization/cleavage cycle is developed to produce an isothermal signal amplification.

Step 5. Because DSN is rarely consumed during the procedure, the hybridization/cleavage cycle can persist until all DNA probes are completely cleaved. In turn, the original miRNA can be determined quantitatively by detecting the decrease in the quantity of the DNA probe.

adsorbed by GO (Figure 2E). AuNPs have been employed for miRNA biosensing in a similar manner by triggering the detachment of fluorophores from the gold surface [30]. Under optimized conditions, a linear response was observed in the range of 5–200 pM miR-203 after a 2 h incubation.

Multiplex detection is crucial in clinical sample analysis for the simultaneous analysis of several analytes. The assays described above are not capable of multiplex detection because DSN is duplex-specific rather than sequence-specific. Bincheng Yin *et al.* developed a multi-color DSN assay that can simultaneously detect several miRNAs by labeling Taqman probes with different fluorophores [31]. By integrating three Taqman probes (P-141, P-21, and P-7d) that have perfect complementarity to miR-141, miR-21, and let-7d, respectively, the miRNAs in a mixture were quantified based on the increased fluorescence intensity of FAM, TAMRA, and Cy5, respectively (Figure 2F). A fM detection limit was achieved, which is nearly five orders of magnitude lower than that of conventional MB strategies. Although only three distinct fluorescence signals were measured in the pilot experiment, this study provides a solid foundation for further development of DSN-mediated multiplex detection in mixed clinical samples. Despite its high sensitivity, the Taqman probe-based approach is limited to differentiating miRNA sequences that differ by at least four bases, making this method unsuitable for miRNA family profiling because many miRNA members possess high homology, with single-base differences.

Electrochemical assays

Electrochemical biosensor-based miRNA detection is popular owing to its low cost, ease of use, portability, and the simplicity of construction of the electrochemical biosensors [32]. The reaction is monitored electrochemically, based on the generation of a measurable current (amperometry), potential (potentiometry), or changes in the conductive properties of the medium between the electrodes (conductometry) [33]. To develop a DSN-mediated electrochemical biosensor for miRNA detection [6], thiolated ss CPs were immobilized on a gold electrode. After introducing the target miRNA and DSN into the solution, the immobilized CPs were gradually cleaved, and a distinct difference in electrochemical impedance between the working electrodes and the control was observed, enabling the label-free detection of miRNA let-7b in a linear range from 2.0 fM to 2.0 pM (Figure 2G). By integrating the advantages of isothermal amplification with label-free detection of miRNA, the proposed technique provides the possibility of directly profiling miRNA expression in clinical samples with minimal or no sample pretreatment.

Although electrochemical biosensing is convenient and rapid for miRNA detection, the limited shelf-life and stability of the biorecognition component, and the lack of specific binding, are significant weaknesses of electrochemical biosensors. The detection parameters, particularly the electrode-processing technologies and the probe-conjugation strategies, are extremely important in ensuring reliable results. Although several hand-held or portable devices are commercially available for field measurements, most electrochemical biosensors are in development and testing phases. Recent developments in nanotechnology and material science, as well as the ability to custom-engineer biorecognition components, will further advance the development of feasible and reliable biosensor devices for miRNA detection.

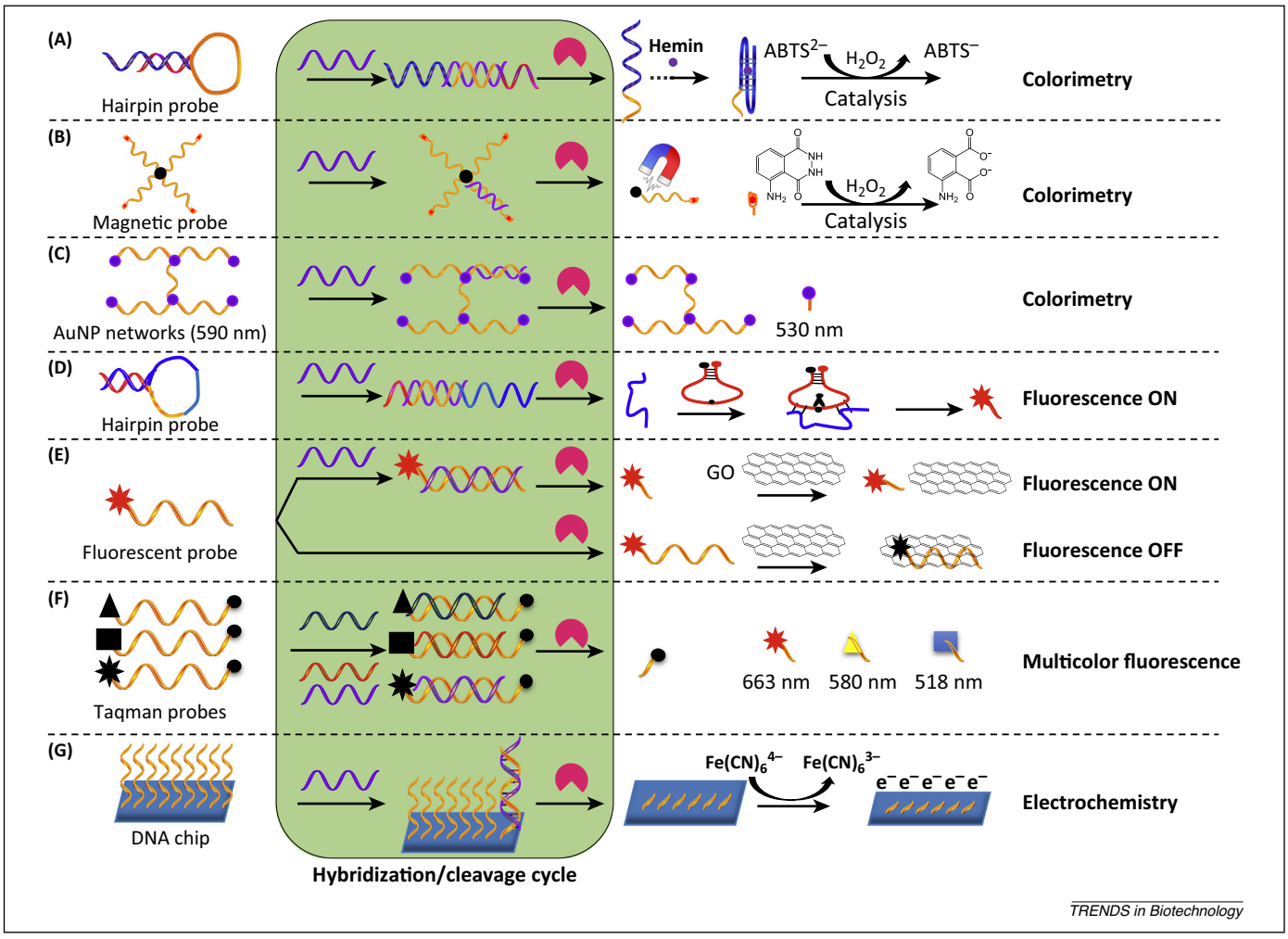

Figure 2. Mechanisms of DSN-mediated miRNA detection assays. **(A–C)** The principles of three colorimetric assays. **(D-F)** The principles of three fluorescence-based detection methods. **(G)** The principle of the electrochemical assay for miRNA quantification.

Construction of a cDNA library

cDNA normalization

Although many strategies have been developed for cDNA library preparation, the efficiency of high-throughput analysis is limited by the significant differences in the abundances of various transcripts among cell types [34]. To address this problem, DSNs were first explored for generating a full-length normalized cDNA library from the nervous tissues of *Aplysia californica* (a typical model organism in neuroscience) and human skeletal muscle [35]. DSN selectively cleaves dsDNA and DNA in DNA/RNA hybrids, thus reducing the need for physical separation of ssDNA from duplex DNA. Compared to conventional techniques, DSN-mediated normalization is easier to perform and requires fewer additional materials. Therefore, this technique is useful for the discovery and annotation of new transcripts, the analysis of transcription of bacteria lacking mRNA poly(A) tails, and the analysis of paraffin-embedded RNA fixed by formalin [7]. In addition, the integration of SMART (switching mechanism at 5′ end of RNA transcript) technology [36,37] and DSN-mediated normalization have significantly improved the reliability of cDNA libraries derived from various sources, including plants [38–41], animals [42,43], and humans [44].

cDNA subtraction

Representation differential analysis (RDA) and suppression subtractive hybridization (SSH) are two conventional methods of gene subtraction. However, both techniques are limited by the complexity and length of the cDNA samples. To address this problem, DSN-mediated transcriptome subtraction (DTS) was developed to identify the unique full-length cDNAs in complex tissue or cell types. Despite enabling the identification of an additional 1812 bp GUS gene from a complex *Arabidopsis* cDNA library [45], this approach may result in low representation of rare target cDNAs. An improved method, called DSN-mediated normalization and subtractive hybridization (DNSH), was subsequently established to investigate differential gene expression at the oocyte stage of *Artemia* [46]. The DNSH method enriched differentially expressed transcripts that comprised only approximately 0.0001% of total transcripts.

cDNA depletion

The redundant identification of transcripts during the process of expression cloning is extremely time-consuming and expensive. To minimize labor and time costs, a DSN depletion approach has been developed to efficiently

eliminate up to several hundred known sequences from cDNA before library cloning [47]. Transcriptome profiling by deep-sequencing technologies would also benefit from depletion of abundant transcripts, but a DSN approach is challenging in this case. DSN-mediated C_0T-hybridization involving heat-denaturation and re-annealing can be used to deplete high-abundance sequences from dsDNA libraries. However, the difficulty in fine-tuning the extended hybridization reaction, and the potential targeting of the ubiquitous linker/adapter sequences, make this approach unfeasible for deep sequencing. Accordingly, a probe-directed degradation (PDD) approach was recently developed by employing hybridization to DNA oligonucleotides at the ss-cDNA library stage, followed by digestion with DSN [48]. Because PDD acts at the cDNA stage, handling of fragile RNA samples can be minimized, thus providing a low-cost option for large-scale projects. This method can also be customized for different depletion targets, sample types, and organisms.

As sequencing costs decrease, DSN treatment will become less worthwhile for species with genomes less than 1 Gb, and significantly beneficial only for larger-sized genomes. The application of DSN normalization to species with very large genomes, for example, wheat (16 Gb) and lily (90 Gb), may enable efficient access to the gene space of one or more genotypes of these species [49]. The inclusion of tetramethylammonium chloride (TMAC) might also increase the applicability of the DSN method for metagenomic experiments involving multiple species with varying GC contents.

Other applications

RNA-Seq

RNA-Seq is a reliable methodology for comprehensively characterizing the transcripts present in a biological sample [50]. However, establishing standard RNA-Seq protocols for samples with either low-quantity or low-quality input RNA remains challenging [51]. DSN has been employed to generate expressed sequence tag (EST) libraries to establish normalized RNA-Seq libraries [52]. A method has also been developed for 'stranded' whole-transcriptome RNA-Seq by using DSN to remove rRNA reads from low-quantity samples [53]. The resulting data were of high quality, with an accuracy exceeding that of qRT-PCR. This method permitted the detection of splicing variants as well as SNPs because sample integrity is preserved.

Compared with several popular approaches (RNase H, Ribo-Zero, Ovation RNA-Seq, and SMART) employed in RNA-Seq from low-quality libraries, the DSN method is advantageous for the treatment of prokaryotic samples because it employs a non-poly(A) approach [54]. In contrast to eukaryotes, rRNA removal in bacteria is a key step in sequencing-based transcriptomics because bacteria do not have clear mechanisms for differentiating mRNAs from rRNAs. DSN treatment removes rRNA more efficiently than conventional subtractive hybridization methods in total *Escherichia coli* RNA populations [55]. In addition, the DSN approach works well for the assembly of a complex transcriptome without using a genome sequence, such as a full-length cDNA library from freshwater planaria [56]. Although DSN has great potential for studying bacterial transcription using next-generation sequencing, several limitations may apply. Extended hybridization time is required for DSN normalization by using a PCR instrument to pre-heat the DSN reaction buffer prior to the hybridization reaction. Second, the hybridization buffer must be optimized to obtain the proper pH without additional salts. Third, this method requires an additional day to perform and is less reliable for high-GC libraries.

SNP detection

SNPs are crucial for personalized medicine because variations in the DNA sequences of humans can affect how they develop diseases and respond to pathogens, chemicals, drugs, and other agents [57,58]. Recently, increased attention has been paid to the significance of SNPs in comparing regions of the genome between cohorts (such as matched cohorts with and without a disease) in genome-wide association studies [59]. Numerous methods for SNP detection have been reported, including restriction fragment length polymorphism (RFLP), ss conformational polymorphism (SSCP) [60], allele-specific amplification (ASA) [61], and DNA sequencing [62]. In addition to individual strengths, each assay has its own weaknesses, such as lengthy times, complicated processes, or expensive reagents.

A duplex-specific nuclease preference (DSNP) assay has been developed for SNP detection that exploits the outstanding capability of DSNs to recognize single-base mismatches. The DSNP strategy has been employed to detect SNPs in the prothrombin, cytochrome *c* oxidase subunit I (COX1), and *p53* genes in homozygous and heterozygous genomic DNA. Several other research groups have adopted the DSNP assay to study SNPs in human genes, including *kRAS*, *nRAS*, and *hRAS*; the genes for blood coagulation factor V; a deletion in the *BRCA1* gene; and the genome of the Douglas fir (*Pseudotsuga menziesii*) [63,64]. Moreover, nucleoside monophosphates (dNMPs) have been introduced to improve the specificity of SNP detection based on the principle that dNMPs can better stabilize unmodified AuNPs than conventional DNA with the same base composition and concentration [65].

The proposed DSNP assay has several advantages. The DSNP assay works well with dsDNA templates, such as unpurified PCR products, reducing the need for additional separation, purification, or centrifugation steps. Additionally, PCR failures can be easily identified using built-in internal controls that allow both alleles to be analyzed in the same tube. PCR primers and probes can be designed to enable multiple distinct assays to be performed under one set of standardized reaction conditions because DSN has no preference for template length or the position of the fluorescent nucleotide.

Repeat-free FISH probes

FISH is a powerful technique for gene mapping, the diagnosis of chromosomal abnormalities, and studying cellular structure and function. In most cases, FISH probes originating from genomic DNA contain a variable amount of repetitive DNA that must be removed or blocked before FISH analysis. A simple and reliable method has been

developed to remove repetitive DNA from FISH probes. DSN is used to hydrolyze all ds elements in the libraries developed by whole-genome amplification (WGA). This method eliminates the need to add blocking DNA to the FISH assay, which simplifies the procedure, reduces time and cost, and minimizes background fluorescence while maintaining the specificity and signal-to-noise ratio of the FISH probes [66].

Telomeric overhang detection

Telomeres terminate in 3′ overhangs, which function in end protection and in the formation of t-loops. Determining the steps and factors involved in overhang processing is compromised by the inability to easily and accurately determine overhang size in the presence of many kb of ds telomeric DNA. Although several methods of determining overhang length have been developed, obtaining accurate measurements remains challenging. The nondenaturing hybridization assay only determines the relative strength of overhang signals with respect to total DNA [67]. The telomeric-oligonucleotide ligation assay provides an imperfect representation of sizes and primarily estimates their maximum length [68]. Electron microscopy requires large amounts of purified telomeres and is unable to measure lengths shorter than <75 nt. The gp32 overhang protection assay cannot determine overhang lengths shorter than 45 nt.

A DSN-mediated strategy was developed to accurately and directly measure overhang length based on the premise that DSN digestion would preserve the telomeric overhangs while removing all dsDNA, allowing overhang detection by Southern blot [69]. This method can detect overhangs as short as 12 nt within 1 day, with no additional, unusual reagents, and thus represents a pivotal advance in accelerating the discovery of the processing events that occur at the ends of human chromosomes.

Concluding remarks and future perspectives

DSNs have emerged as versatile components in developing bioanalytical strategies for the detection of various biomolecules, such as miRNA, SNPs, and telomeric overhangs. Although colorimetric assays are convenient and intuitive, their precision for the detection of miRNA is low. AuNP networks can significantly improve this sensitivity to fM levels. High portability and affordability make electrochemical biosensors feasible in a decentralized setting for applications such as POC diagnosis. However, the above-mentioned assays can only detect a single miRNA each time, thereby restricting their clinical applications. Fluorescence-based biosensing techniques are reliable and permit multiplex detection. Their high stability and feasibility enable miRNA detection at the fM level, suggesting the potential for miRNA profiling in samples of various origins.

Although effective in miRNA detection and cDNA library construction, minor drawbacks of DSN in clinical applications have been recognized. DSN requires a minimal 10 bp hybrid substrate for effective cleavage. The divalent cation requirement of DSN makes it incompatible with some applications. Non-specific cleavage of dsDNA hybrids also makes multiplex detection very difficult. Importantly, DSN can only mediate linear signal amplification. Thus, additional strategies are needed to improve analytical sensitivity for the detection of low-abundance miRNAs. As chemical or material science progresses, further development of the applications of DSN will make it necessary to address and expand upon several important questions (Box 4).

Box 4. Outstanding questions

- How can multiplex detection of miRNAs be performed?
- How can probe coating efficiency be enhanced?
- How can miniaturization and portable devices be designed?

Because DSNs are duplex-specific rather than sequence-specific, additional specific probes are necessary to fulfill the multiplex detection of a panel of miRNAs. New chemical modification and nanoparticle-based fluorescence-labeling approaches will facilitate high-throughput miRNA detection. The sensitivity of DSN-mediated signal amplification depends largely on the quantity and density of the immobilized probes, in contrast to conventional biosensors which require optimized coating probe concentrations. Overcrowded probes decrease the detection signal because of steric hindrance among the probes. However, DSN biosensors are seldom influenced by steric hindrance because the trace target miRNA preferentially binds the peripheral probes until all of the probes are cleaved. In this case, exploring novel coating strategies that can conjugate more probes on the chips would significantly enhance the detection signals. Nanomaterials such as gold nanoparticles, graphene, carbon nanotubes, and quantum dots could be used to increase the quantity of the probes or the intensity of the detection signal.

DSN-mediated biosensors will enable rapid miRNA detection in an isothermal amplification style, decreasing the need for large PCR instruments. Portable biosensors that can be used at bedside or at home at the POC will be developed in the future. Preliminary results have demonstrated that DSN-mediated biosensors are rapid, robust, and convenient. More sensitive, specific, and high-throughput DSN biosensors or devices for detecting disease biomarkers and for personalized health care will be developed in the future.

In conclusion, the capability of highly-sensitive and specific detection of miRNAs and SNPs makes DSN a promising biomedical tool for facilitating disease diagnosis and prognosis and for elucidating the physiological mechanisms that regulate disease-related cmiRNA formation.

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