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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 non-specific 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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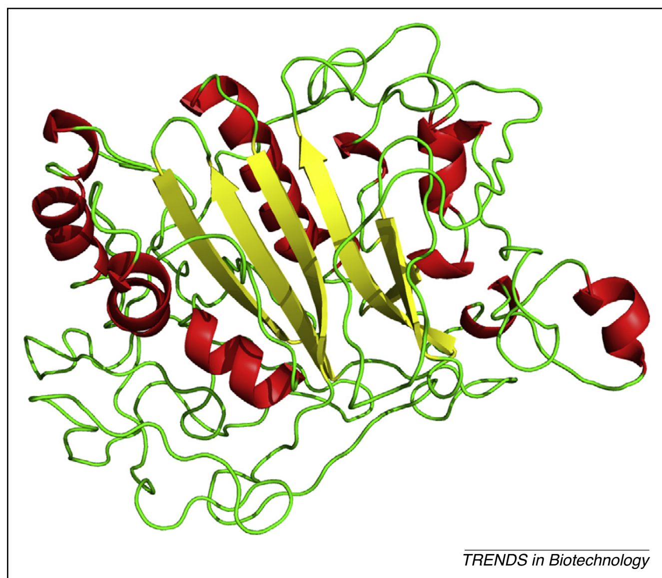

Figure 1. Simulated duplex-specific nuclease (DSN) structure based on the DSN sequence in GenBank (GenBank: AF520591.1) created using PyMol.

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.

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.

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

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