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Review

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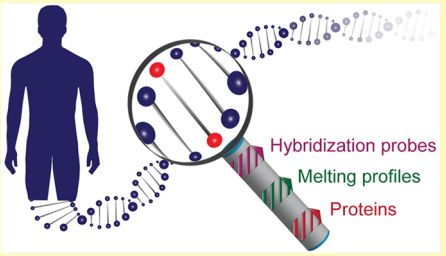
Targeted Detection of Single-Nucleotide Variations: Progress and Promise

Alireza Abi* and Afsaneh Safavi*

Department of Chemistry, Faculty of Sciences, Shiraz University, Shiraz 7194684795, Iran

ABSTRACT: Advances in nucleic acid sequencing and genotyping technologies have facilitated the discovery of an increasing number of single-nucleotide variations (SNVs) associated with disease onset, progression, and response to therapy. The reliable detection of such disease-specific SNVs can ensure timely and effective therapeutic action, enabling precision medicine. This has driven extensive efforts in recent years to develop novel methods for the fast and cost-effective analysis of targeted SNVs. In this Review, we highlight the most recent and significant advances made toward the development of such methodologies.



KEYWORDS: *single-nucleotide variation, single-nucleotide polymorphism, point mutation, genotyping, specificity, biosensor, assay development, point-of-care testing, precision medicine*

A single-nucleotide variation (SNV) occurs when one nucleotide in the human genome is substituted for another through an endogenous (e.g., errors in DNA replication) or exogenous (e.g., chemical mutagens) source of DNA damage.[1] It is the most common type of sequence change in the human genome and plays a significant role in disease susceptibility and individual response to treatment.[2] Over the past decade, numerous SNVs associated with diverse human illnesses and disorders have been identified through genome-wide association studies.[3] These disease-specific discoveries are hoped to contribute to the development of precision medicine, wherein optimal care is provided to individuals based on their unique genetic and disease profile.[4]

In spite of the substantial biological and biomedical implications of many SNVs, the intrinsically minimal impact of a single-nucleotide change on the overall physicochemical properties of a nucleic acid sequence has made SNV detection a challenging task. Next-generation sequencing methods have been widely used for the discovery of new SNVs in the human genome, as they require little or no prior knowledge of the position or identity of the variants in the DNA sequence.[1] However, in these techniques, the vast majority of the sequencing data map to non-informative wild-type sequences,[5] which increases the time and cost of the analysis. Further, the large amount of data produced by next-generation sequencing and the complexity of their analysis are not compatible with routine clinical use.[6] The widespread implementation of precision medicine requires simpler, faster, and more cost-effective technologies capable of targeted detection of SNVs at the point-of-care (POC). Ideally, these analytical tools would need no specialized infrastructure or skilled personnel to perform the analysis, and, as such, they would be easily translatable to the low-resource settings of the developing world.

Recent years have witnessed tremendous advances in non-sequencing-based detection technologies to deal with the increasing demand for routine SNV analysis. The present Review highlights these developments and the challenges that have been addressed, with emphasis on studies in the past 4 years. We discuss selected technological approaches grouped into three general categories—SNV detection through melting profile analysis, SNV detection using enhanced hybridization probes, and protein-assisted SNV detection—and discuss their working principles, merits, and limitations.

SNV DETECTION THROUGH MELTING PROFILE ANALYSIS

A double-stranded DNA (dsDNA) denatures or melts to two single-stranded DNAs (ssDNAs) when exposed to elevated temperatures, chemical denaturants, or low ionic strength media. The temperature at which the transition from the double-helical structure to the randomly coiled single-stranded state occurs within 50% of the dsDNA is termed the melting temperature (T_m) and is sequence dependent. Because of this, dsDNAs differing by only one base-pair reveal different T_m, and they can be differentiated on the basis of their melting profiles.[8] Traditional methods in this context are melting gel techniques (e.g., temperature gradient gel electrophoresis and denaturant gradient gel electrophoresis), which rely on electrophoresis of polymerase chain reaction (PCR) amplified DNA fragments while exposing them to elevated temperature and/or chemical denaturants (e.g., urea and formamide).[9] In these techniques, a DNA fragment will proceed through the denaturing area in the gel or matrix until its T_m has been reached and, as a result of its denaturation, the retardation of its electrophoretic mobility has occurred. The mobility difference between sequence variants, corresponding to the

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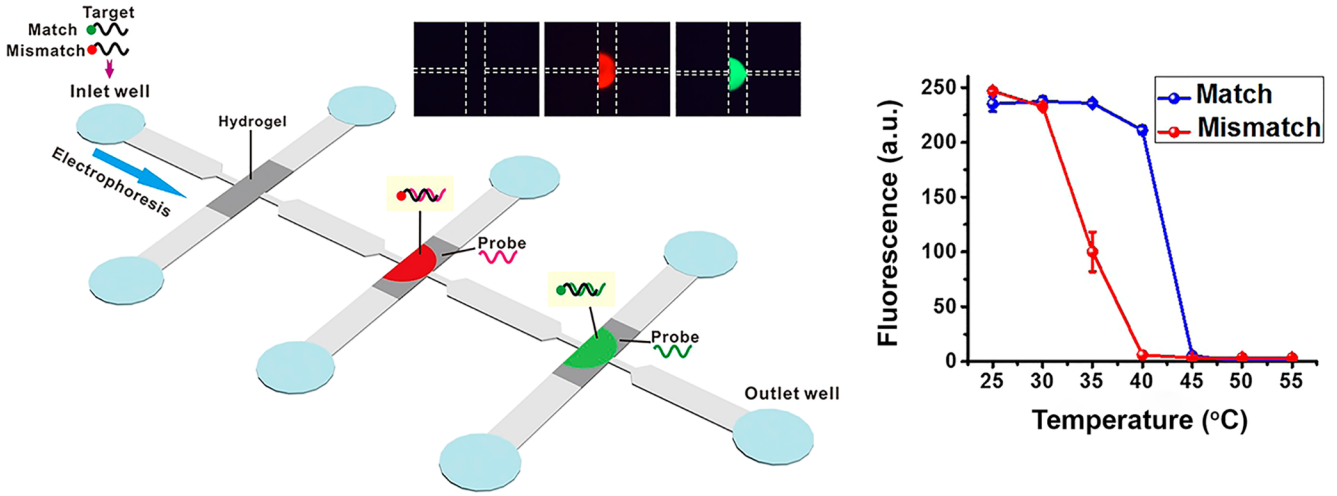

Figure 1. SNV detection based on a microfluidic hydrogel array. Fluorophore-labeled targets are transported to the probe-embedded hydrogels by electrophoresis, and SNV discrimination is achieved through a temperature gradient electrophoretic wash and melting profile analysis. Adapted with permission from ref 7. Copyright 2015 American Chemical Society.

difference in their T_m, allows the detection of SNVs in target molecules.[10,11] Nevertheless, conventional gel-based methods are difficult to automate and require long analysis times (several hours), which limit their applicability in POC test settings.

High-resolution melting (HRM) is another technique based on liquid-phase melt profiling for SNV discrimination.[12] This relatively new method involves precise monitoring of the change in the fluorescence of a DNA binding dye as dsDNAs are melted at elevated temperatures. Combined with quantitative PCR (qPCR), HRM can detect sequence variants differing by as little as a single base pair on the basis of the difference in their melting profiles.[13] In this straightforward approach, a saturating dye is added before or after the PCR, and rapid melting analysis of the PCR products is carried out. In contrast to melting gel techniques, HRM is fast and permits melting profile analysis of as high as 96 or more PCR products in minutes.[14] Taking advantage of the possibility for precise and rapid temperature control in microfluidics and the short temperature stabilization time of small sample volumes, HRM coupled with microfluidic platforms has enabled melting analysis in even shorter times, down to a few seconds or even less than a second.[15,16] Besides reducing the assay time, such high-speed melting analysis has been reported to decrease errors in genotyping of small amplicons.[16] Given that short analysis time and high accuracy are two important factors in POC testing, microfluidic chips that enable rapid PCR and HRM analysis hold great promise for such applications.[17,18] It should be noted, however, that HRM has limited capability in discriminating between sequence variants that show a subtle difference in their melting profiles, particularly base-pair neutral variants (e.g., G-C to C-G changes).[19]

Other than the liquid-phase melting approaches described above, DNA melting can be applied to surface-tethered nucleic acids in order to combine the high SNV discriminating power of melt profiling with the excellent multiplexing capability of planner microarrays. Planner DNA microarrays are platforms that employ two-dimensional surfaces with multiple DNA probes linked to different areas of them for the multiplexed detection of nucleic acid targets.[20] Melting analysis of probe–target hybrids can be used in DNA microarrays to achieve high single-base specificity based on the principle that T_m of a perfectly matched duplex is higher than that of a similar one with a mismatch. Taking advantage of this, Rizzi et al. simultaneously analyzed five mutations and four methylation sites in human melanoma cell lines by melting temperature curve analysis of the duplexes formed on a magneto-resistive sensing array.[8]

One major drawback of conventional planar microarrays is their slow on-surface hybridization kinetics, which can result in long assay times (up to 48 h).[20] Microfluidic hydrogel DNA microarrays have been recently demonstrated as a promising solution to tackle this problem.[7,21] Jung and co-workers designed a microfluidic containing probe-embedded hydrogel matrices to which fluorescently labeled ssDNA targets were transferred electrophoretically (Figure 1).[7] The dynamics of microfluidic electrophoresis enabled short hybridization times (≤20 min), and the stringent wash with temperature gradient electrophoresis allowed for the specific analysis of SNVs in the ssDNA targets. This platform was more recently extended to permit multiplexed analysis of dsDNAs in clinical samples.[21] The drawback of these analytical tools is the need for fluorescently labeled target oligonucleotides, which may increase the overall cost of the analysis.

SNV DETECTION USING ENHANCED HYBRIDIZATION PROBES

DNA hybridization probes provide a simple means of detecting complementary nucleic acid targets. However, the target binding activity of traditional hybridization probes may be nonspecific, except near the T_m, where binding of a perfect complement is energetically more favorable than binding of a mismatched strand.[22] Although it is possible to perform the hybridization or the subsequent wash steps near the T_m to obtain single-base specificity,[23] the practical implementation of this approach is associated with two major challenges. First, substantial investment in assay optimization is required, as the optimal temperature needs to be defined for the detection of every new target of interest. Second, such methods have limited multiplexing capability and may not be effective when several targets, each with its own T_m, have to be analyzed simultaneously under the same conditions. To overcome these limitations, there have been numerous attempts toward

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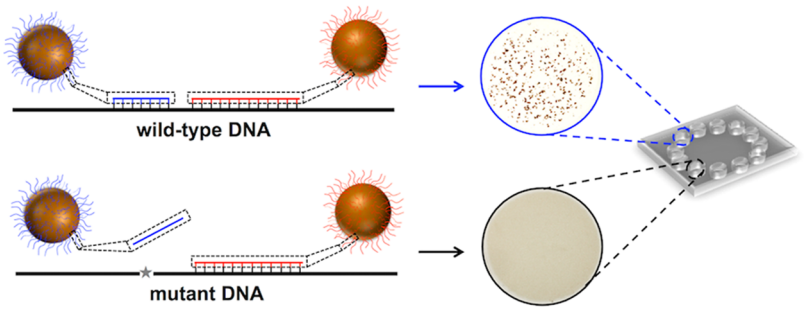

Figure 2. An SNV discrimination assay based on dual hybridization probes labeled with magnetic beads. The discriminating probe (blue) is designed such that its hybridization to the mutant DNA is thermodynamically less favorable than to the wild-type DNA. Binding of both probes to the wild-type sequence induces bead aggregation that can be visually detected. Reprinted with permission from ref 27. Copyright 2015 American Chemical Society.

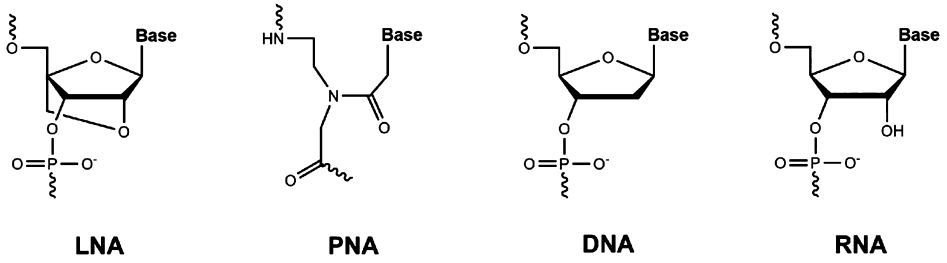

Figure 3. Molecular structures of LNA and PNA. Structures of DNA and RNA are shown for comparison.

designing hybridization probes with improved binding properties and higher specificity for single-base mismatches over a wider temperature range. These enhanced hybridization probes were developed through shortening the probes' sequence lengths, modifying the probes' chemical structures, or applying constraints to the probes.

Short Hybridization Probes. The selectivity of SNV discrimination methods relying on differential hybridization between perfectly matched and mismatched strands can be significantly improved by reducing the length of the hybridization probes. This is because the destabilization effect of a single-base mismatch is more substantial in duplexes with fewer base pairs, which makes the binding of probes to mismatched sequences thermodynamically much less favorable.[24] Nevertheless, cross-reactivity with non-target sequences that contain a short stretch of the complementary nucleotides may occur if too short hybridization probes are employed. The use of dual hybridization probes, of which at least one is short enough to possess high selectivity against single-base mismatches, can be a solution to the above problem.[25−27] In SNV assays that use dual hybridization probes, the hybridization signal is produced only when both probe segments are hybridized to the intended target.[25,26] Figure 2 represents an example of such assays in which a pair of magnetic bead-bound hybridization probes specifically bind to a complementary target sequence and enable direct visual discrimination of sequences differing by one nucleotide.[27]

An attractive class of dual hybridization probes for SNV sensing includes probe segments that can, in the presence of target, assemble into functional nucleic acid structures.[28−31] In assays that use these kinds of hybridization probes, the sequence of the functional DNA is typically split into two halves, to each of which a target binding region is added. Hybridization of the probes to the target yields the assembly of the functional structure with the ability to sensitively report the target binding event. One example of such probes is split aptameric probes that can hybridize to a target sequence and form a binding site for a low fluorescence dye.[29] Binding of the dye to the aptameric site can dramatically increase the dye fluorescence, enabling specific nucleic acid sensing down to the upper picomolar levels.[30] More sensitive detection can be achieved by employing dual hybridization probes designed to form deoxyribozymes upon target binding. Deoxyribozymes (also known as DNA enzymes or DNAzymes) are catalytic DNA molecules with specific sequences that are obtained from random-sequence DNA libraries by *in vitro* selection methods.[32] Owing to their important advantages over protein enzymes, namely high thermal stability and ease of synthesis, deoxyribozymes have attracted great attention in the field of molecular sensing as signal amplifiers.[33] By combining the signal amplification capability of deoxyribozymes (e.g., RNA-cleaving deoxyribozymes or peroxidase-like deoxyribozymes) with the high selectivity of dual hybridization probes, scientists have been able to develop specific SNV assays with sensitivity down to the lower picomolar concentrations.[31] Notably, both split aptameric and deoxyribozyme-based hybridization probes require a pair of unlabeled DNA oligonucleotides, which are relatively inexpensive and make the SNV analysis cost-effective.

Shortening of hybridization probes to a few nucleotides can, on one hand, improve the binding specificity but, on the other hand, may reduce the analysis sensitivity by decreasing the thermal stability of the probe−target duplexes. This issue may be effectively addressed by the use of a probe oligonucleotide that contains two target-specific regions of limited lengths, similar to the two-tailed primer recently introduced by Androvic and co-workers.[34] Their two-tailed primer was composed of two hemiprobes at the end of a hairpin DNA structure that were complementary to separate regions of a target microRNA (miRNA). While the hemiprobe stretches were designed relatively short (4−10 nucleotides) to provide high discriminatory power against SNVs, the hybridization cooperativity ensured overall high thermal stability of the

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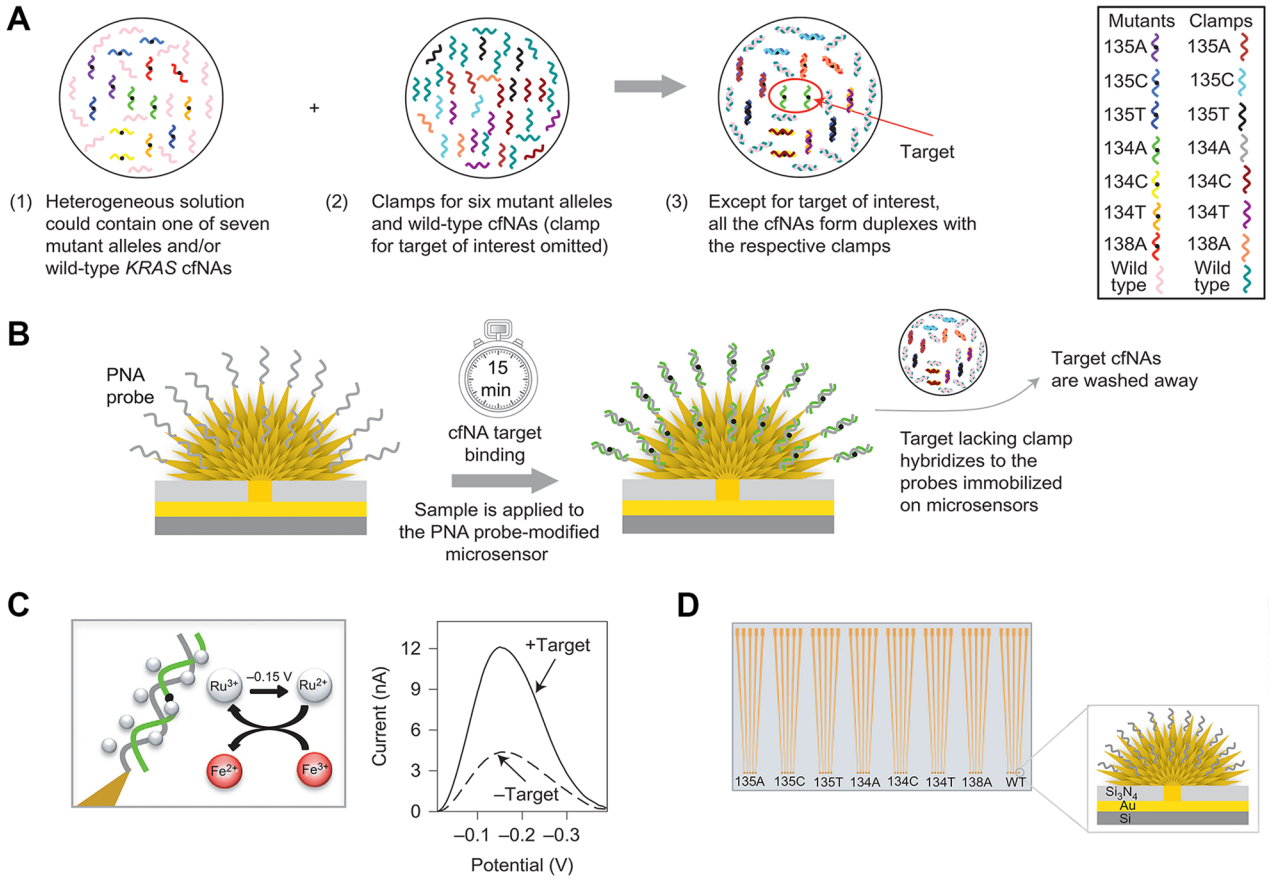

Figure 4. Schematic representation of the chip-based PNA clamp assay for the electrochemical analysis of mutant *KRAS* 134A: (A) clamp strategy, (B) on-chip hybridization, (C) electrochemical readout, and (D) microfabricated chip layout. Adapted with permission from ref 49. Copyright 2015 Springer Nature.

forming primer−target complex. By employing a two-step reverse transcription qPCR, the assay not only showed excellent specificity to discriminate between highly homologous miRNAs but also exhibited high sensitivity to detect as few as 10 copies of target miRNA molecules.[34]

Hybridization Probes with Modified Chemical Structures. Structurally modified or artificial nucleic acids are useful analogues of natural nucleic acids and have found many applications in the field of biosensing.[35] They are typically more expensive than natural oligonucleotides but possess improved binding properties. The molecular structures of two popular types of artificial nucleic acids—locked nucleic acid (LNA) and peptide nucleic acid (PNA)—are presented in Figure 3, and their usefulness as hybridization probes is discussed in the following subsections.

LNA-Based Hybridization Probes. LNA is a class of synthetic oligonucleotides containing conformationally restricted nucleotide building blocks.[36] The chemical structure of LNA monomers differs from that of natural nucleotides by a bridging group across their ribose ring, locking it into an N-type or 3′-endo conformation (Figure 3). Transforming flexible sugar moieties into rigid bicycles has important consequences for the recognition potential of LNA molecules. In terms of binding strength, replacing several or all nucleotides of a DNA oligomer with locked nucleotides can increase T_m by 3−5 and 4−8 °C per locked nucleotide when the modified oligomers hybridize to a complementary DNA and RNA sequence, respectively.[37] Importantly, locked nucleotides within oligomers were also found to stabilize perfectly matched double helices more than mismatched duplexes, thereby enhancing the SNV discrimination power of the hybridization probes.[38]

Hybridization assays have benefited from the remarkable binding properties of LNA by employing probes consisting of both DNA and LNA nucleotides, particularly in cases where unmodified DNA probes failed to offer desirable performance.[39] In this context, probes with a triplet of LNA residues centered at the mismatch site were demonstrated to provide the largest difference in the thermal stability of perfectly matched and mismatched duplexes and used for highly specific SNV detection.[38,40] When it comes to multiplexed analysis, rational incorporation of LNA nucleotides into hybridization probes is an effective approach to equalize the T_m of all probe−target duplexes. This would enable the simultaneous and specific analysis of multiple target sequences at a single elevated temperature.[41] There are, however, challenges associated with the improved binding properties of LNA probes. LNA oligonucleotides can form self-structures (e.g., hairpins, homodimers, and other undesirable secondary structures), which are especially stable if they contain LNA−LNA base pairs.[42] Cross-hybridization to other LNA oligonucleotides is also possible in multiplexed assays.[43] Therefore, special care must be taken to design LNA probes with optimized length and LNA contents so that the desirable binding properties can be achieved with minimal cross-reactivity or formation of stable self-structures.

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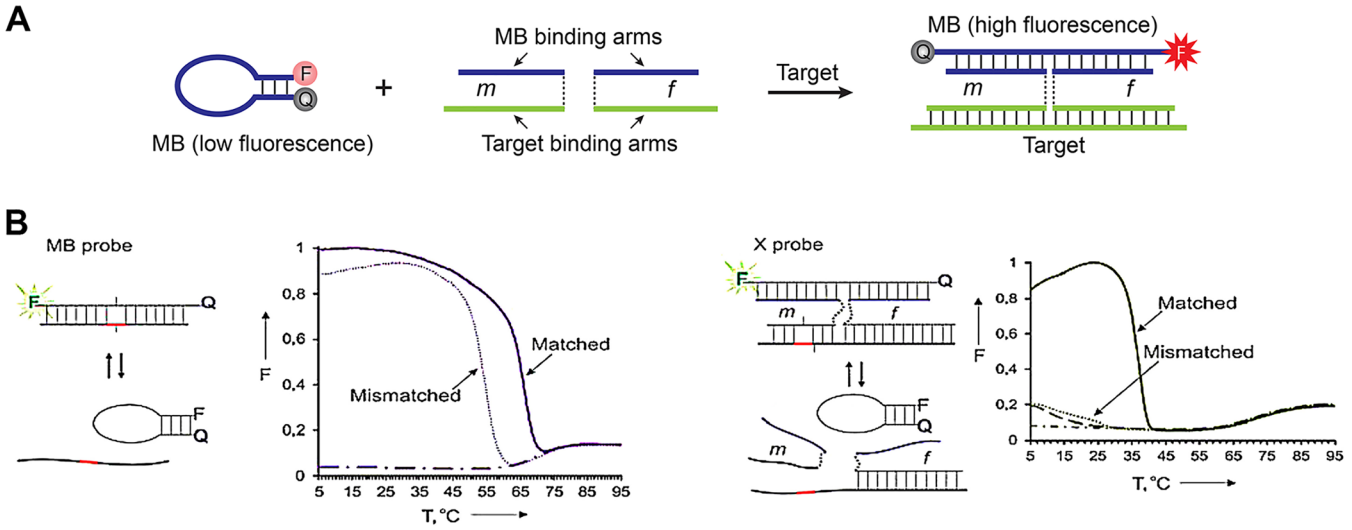

Figure 5. (A) Principle of the indirect binding of MB probe to the target sequence mediated by two unlabeled strands, *m* and *f*. (B) Design and fluorescent melting profile of a conventional MB (left) and the multicomponent X-probe (right) in the presence of matched (solid line) or mismatched (dotted line) sequences. "F" denotes fluorophore, and "Q" denotes quencher. Adapted with permission from ref 55. Copyright 2016 American Chemical Society.

PNA-Based Hybridization Probes. PNA is another synthetic analogue of natural nucleic acids, which was put forward by Nielsen et al. in 1991.[44] Instead of the negatively charged sugar−phosphate backbone of native nucleic acids, PNA uses a chain of peptide-like *N*-(2-aminoethyl)glycine units onto which usual nucleobases are attached (Figure 3). The uncharged PNA backbone causes PNA−DNA duplexes to be more thermally stable than the corresponding DNA−DNA double helices and not to be drastically dependent on the solution ionic strength. Importantly, PNA sequences possess higher single-base specificity when binding to their complementary oligonucleotides. A single-base mismatch near the middle of a 15-mer PNA−DNA duplex was found to lower the T_m by 8−20 °C (depending on the type and exact position of the mismatch), whereas the mismatch-induced decrease in the T_m of the corresponding DNA−DNA duplexes was 4−17 °C.[45] Consequently, PNA and its derivatives have been extensively used as hybridization probes to magnify the destabilization effect of SNVs within targeted molecules.[46−48] Notably, PNA sequences have also exhibited specific binding activity to form triplex structures with double-stranded targets; thereby they have provided a means for analysis of double-stranded oligonucleotides at the single-base-pair resolution.[50]

Besides serving as duplex- or triplex-forming probes, PNA can act as sequence-specific clamps to prevent the interfering effect of sequences that differ from the target by one (or more) nucleotide. In PNA clamp PCR assays, PNA sequences are used as non-extendable oligonucleotides to suppress the amplification of wild-type DNA, while the mutated sequence is selectively amplified and detected during PCR.[51] A shortcoming of these assays is that replication errors in the PNA-binding site can be amplified during PCR, and this may produce false results when using a small amount of sample or when the frequency of the mutant template in the sample is very low (e.g., <0.1%).[52] The problem can be alleviated by employing high-fidelity DNA polymerases to minimize PCR-generated errors.[53]

PNA clamping has also been employed in PCR-free approaches.[49,54] Recently, Das et al. coupled PNA clamping strategy to a chip-based hybridization sensor for the rapid (~15 min) and direct detection of tumor-specific circulating RNAs in patient serum without the need for error-prone PCR amplification.[49] The assay used a collection of PNA clamps to hybridize to and sequester wild-type and non-target mutant sequences, leaving only the targeted mutant unhybridized (Figure 4). The unhybridized mutant target was then selectively detected on a PNA probe-functionalized electrochemical chip using an electrocatalytic reporter system. Importantly, such interference-free detection of point mutations was not attainable without PNA clamping, even when the hybridization to the surface-immobilized probes was performed at an elevated temperature to enhance the destabilizing effect of single-base mismatches.[49] The assay was later extended to detect circulating tumor DNA by incorporating DNA clutch probes—pairs of ssDNA molecules that prevented re-association of the denatured target DNA strands by hybridizing to one of the two sequences from each target duplex.[54] Mutated circulating tumor DNA at a level of 0.01% relative to wild-type sequences could be detected by this method in 30 min.

Constrained Hybridization Probes. Constrained hybridization probes associate with their targets with much more specificity than unconstrained probes do. We here discuss the two most common forms of constrained hybridization probes: hairpin and strand displacement probes.

Hairpin Probes. Hairpin DNAs are single-stranded self-hybridized oligonucleotides with a stem-loop structure, which are widely used as hybridization probes or masking agents in the field of nucleic acid biosensing.[56−61] Compared to traditional linear oligonucleotides, hairpin DNAs exhibit a wider range of temperatures within which they form duplexes with perfect complements but not with single-base-mismatched sequences.[62] This feature of hairpin probes is particularly important in multiplexed assays, as sequences containing different SNVs can selectively bind to their corresponding hairpin probes at a single temperature.[63]

In fluorescence-based methods, hairpin DNAs are commonly labeled with a fluorophore at one end and a quencher at the other end.[64] These probes, which are known as molecular beacons (MBs), switch from folded stem-loop conformation

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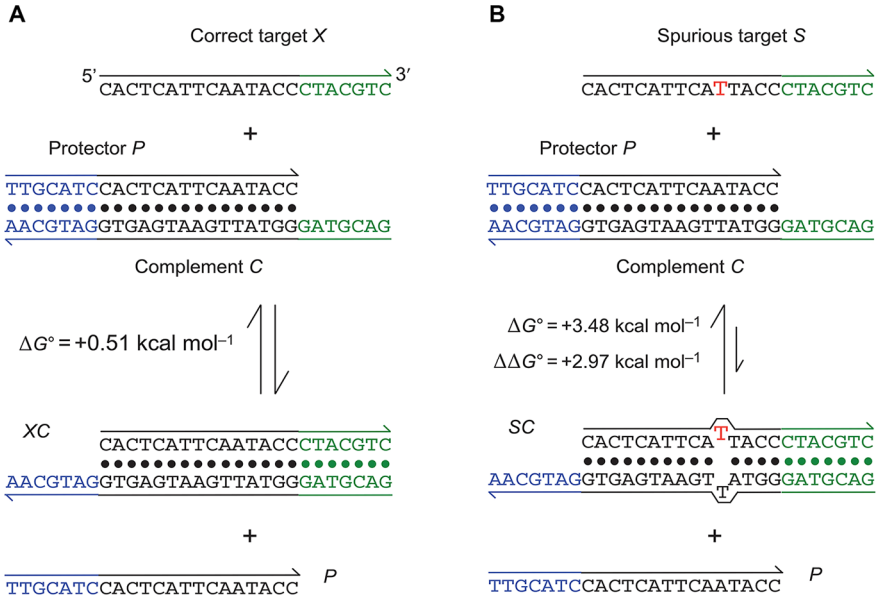

Figure 6. A toehold exchange probe capable of discriminating among single-nucleotide variants. (A) The probe was designed so that ΔG° of the forward reaction with the correct target was close to zero (ΔG° = +0.51 kcal mol^{-1}). Seven new base pairs (green base pairs) are formed, and seven existing base pairs (blue base pairs) are broken. (B) Hybridization of the probe with a spurious target containing one base mismatch was less thermodynamically favorable by +2.97 kcal mol^{-1} (ΔG° = +3.48 kcal mol^{-1}). Adapted with permission from ref 74. Copyright 2012 Springer Nature.

with quenched fluorescence to extended highly fluorescent conformation upon binding to a complementary sequence. It is, therefore, possible to specifically detect nucleic acid targets by measuring the increase in MBs' fluorescence intensity. Since their introduction in 1996 by Tyagi and Kramer,[65] MBs have been extensively applied to the field of biotechnology, particularly for SNV sensing.[66,67] A disadvantage of MBs is the requirement for dual labeling of the oligonucleotides with a fluorophore and a quencher and the subsequent purification procedures, which add to the cost of MB probes. This can limit the practical applicability of MBs, as a unique MB is required for the detection of each SNV. The problem can be alleviated by the combined use of a target-independent MB and two unlabeled DNA strands, each of which contains a short sequence region (8−10 nucleotide) complementary to the target and a binding arm complementary to the MB.[55,68] In this assay format, the two unlabeled DNA strands mediate the association of the MB probe with the target strand to form an X-shaped tetrameric complex, which is fluorescently reported (Figure 5A). As the MB does not bind the target directly, the same MB can be used to recognize any analyte simply by varying the analyte-binding arms of the unlabeled strands, which, in contrast with MBs, are inexpensive synthetic oligonucleotides.[68] Such a multicomponent X-probe has recently been demonstrated to allow for the differentiation of point mutations in the range of 5−40 °C, which is much wider than the temperature range offered by conventional MBs (Figure 5B).[55] This broad temperature range is the result of the non-equilibrium hybridization conditions and the mismatch-induced increase of the time required for the equilibration of the probe−target complex. Consequently, the complex of the X-probe with the target equilibrates faster than that with a mismatched sequence, enabling specific detection of nucleic acid targets within a broad range of ambient temperatures.[55]

Strand Displacement Probes. Toehold-mediated strand displacement (TSD) occurs when a double helix exchanges one of its strands (the protector strand) for an incoming complementary strand to form a longer, more stable duplex. This reaction, which is the core reaction of DNA-based responsive materials systems,[69] is initiated by binding of the incoming strand at a single-stranded overhanging region (known as a toehold) in the double helix, proceeds through a branch migration process, and is completed via the release of the single-stranded protector. Due to the competitive nature of the TSD reactions, single-base mismatches within the incoming sequences can slow down the displacement of the protector strand.[70] This kinetic difference between the binding of perfectly matched and single-base-mismatched sequences has offered the possibility of developing various SNV detection schemes.[71−73] However, TSD probes generally fail to effectively discriminate among sequence variants if there is small or no difference in the reaction rates of the perfectly matched and single-base-mismatched input sequences. This is the case when the SNV is located far away from the toehold region that cannot act as a kinetic barrier to the strand displacement reaction.[75]

A solution to the above limitation involves TSD probes operating at equilibrium and having high enough thermodynamic preference for the perfectly matched target over single-base-mismatched strands. Such an equilibrium-based probe was proposed by Zhang et al. and called "toehold exchange" (Figure 6).[74] The probe was designed so that its hybridization to the correct target had virtually no net thermodynamic effect: the number of single-stranded and double-stranded molecules did not change, and the change in the number of paired bases was minimal. Under these conditions, the standard free energy change (ΔG°) of the displacement reaction was nearly zero for the correct target. The presence of an SNV anywhere within the target sequence could, however, increase ΔG° of the reaction by a few kcal mol^{-1} and make the displacement

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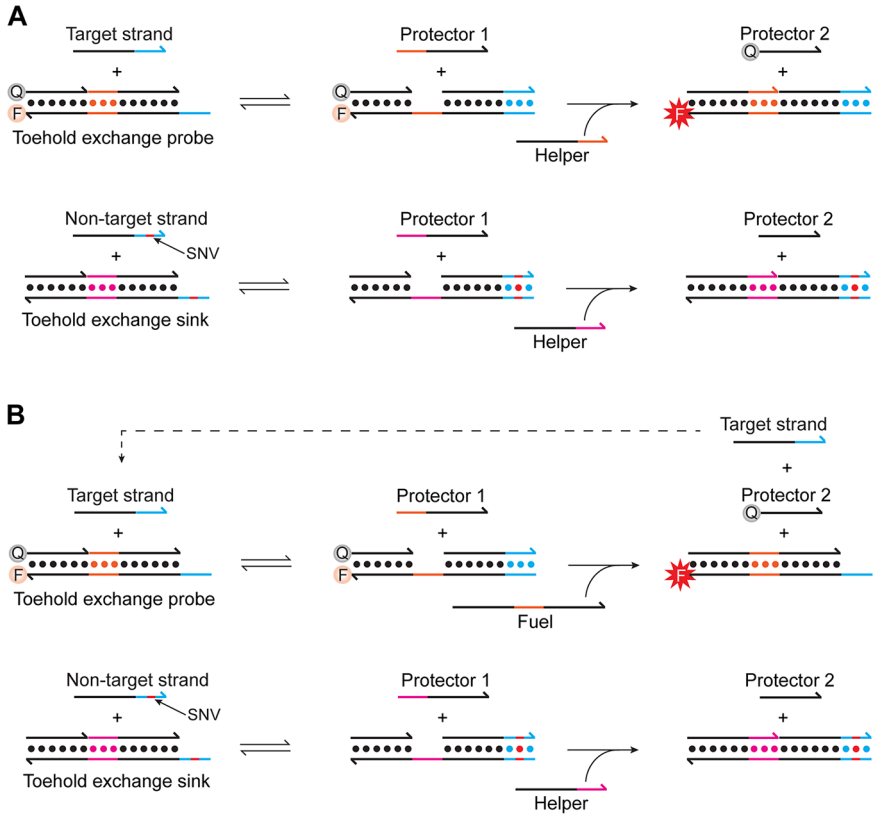

Figure 7. (A) Two-step toehold exchange reactions for the irreversible sequestration of the target and non-target strands. The two-step reaction pathways consist of a reversible toehold exchange reaction and irreversible binding of a helper strand, which makes binding of the target to the probe and the non-target mismatched strand to the sink irreversible. (B) Two-step toehold exchange pathways combined with a target recycling amplification. Target recycling is mediated by a "fuel" strand that can bind to the probe–target complex and release both the quencher and the target strand. "F" denotes fluorophore, and "Q" denotes quencher. Reproduced with permission from ref 75. Copyright 2016 American Chemical Society.

reaction significantly less favorable. In addition to being insensitive to the mismatch position, toehold exchange probes require no prior knowledge of the SNV identity and can function robustly across a broad range of temperatures, salinities, and oligonucleotide concentrations.[74] There is, however, a price to pay for the high specificity and robustness of toehold exchange probes: their hybridization yield and thus their sensitivity are low due to the ΔG° of the probe–target binding reaction being close to zero. To address this issue, Wu et al. demonstrated an on-the-fly method of tuning probe sensitivity and selectivity by adjusting the relative concentration of the auxiliary oligonucleotides involved in the displacement reaction.[76] Notably, this stoichiometric tuning methodology is applicable to any "double-block" probe that releases an auxiliary protector molecule upon binding of a target strand. Further, when applied to multiplexed assays, the stoichiometric tuning approach permits independent modulation of the hybridization yield for individual probes, which cannot be achieved through temperature and salinity adjustments.

More recently, Chen and Seelig reported a different approach to improve the hybridization yield of toehold exchange probes.[75] In their sensing scheme, the reversible toehold exchange reaction between the probe and target was followed by the irreversible binding of a "helper" strand to the probe–target complex, which resulted in the irreversible sequestration of the target. Combined with competitive inhibitors or "sinks" designed to preferentially bind and inactivate single-base mismatched strands, the competitive two-step reaction mechanisms provided 2 times higher hybridization yield than the one-step toehold exchange reaction (Figure 7A). Further improvement in the sensitivity, as well as the specificity, was achieved through coupling this competitive two-step discrimination assay with a target recycling amplification reaction, a process designed to preferentially amplify the signal in the presence of the correct target strand (Figure 7B). The use of competition in parallel with amplification offered quadratically better specificity and a hybridization yield that was at least 10-fold better than that for the simple toehold exchange probe.

PROTEIN-ASSISTED SNV DETECTION

The sensitivity of some proteins to mismatches within nucleic acid sequences has rendered the emergence of powerful methodologies for SNV detection. In this section, an overview of these methods is presented.

SNV Detection Using Mismatch-Binding Proteins. To prevent mutations, living organisms benefit from different modes of DNA repair processes, including mismatch repair, base excision repair, and nucleotide excision repair.[77] The key initial step of these processes is the recognition of damaged or mismatched DNA bases by particular proteins. In the mismatch repair system, the repair process is initiated by the binding of MutS proteins to mismatched base pairs. These proteins have binding affinities 10- to more than 1500-fold higher for mismatched than for perfectly matched base pairs,

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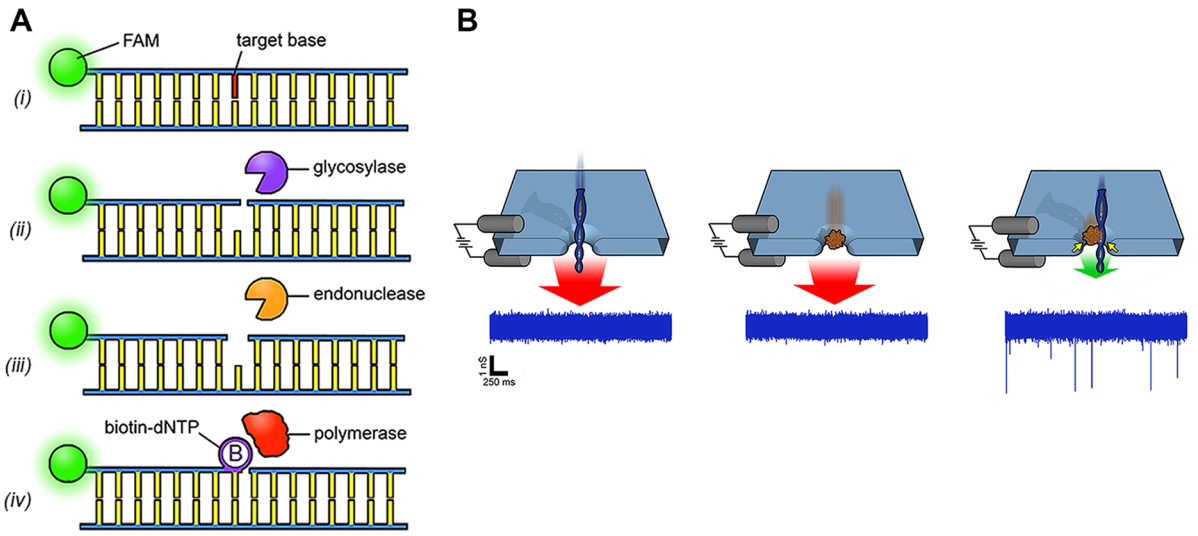

Figure 8. Principles of the base lesion analysis method exploiting the enzymatic machinery of the DNA BER pathway. (A) Schematic representation of the general labeling approach: (i) a dsDNA bearing the target base element (red), (ii) the recognition and excision of the base element by a glycosylase, (iii) the removal of the backbone 5′ to the excision by an abasic endonuclease, and (iv) the incorporation of a single biotinylated nucleotide at the modification position by a gap-filling polymerase. (B) Probing of the labeled DNA with a solid-state nanopore assay. Adapted with permission from ref 80. Copyright 2017 American Chemical Society.

depending on the mismatch type and the local sequence context.[78] Consequently, they have provided a means of detecting SNVs in DNA by acting as mismatch-discriminating elements.[79] Another class of DNA repair proteins is DNA glycosylases, which possess the capability of recognizing base lesions caused by alterations in the chemical structure of DNA bases. Such structural modifications can lead to the generation of base mismatches and are primarily addressed by the base excision repair (BER) mode. In this pathway, the process of DNA repair is initiated by DNA glycosylases identifying and removing aberrant bases from DNA. The process is followed by the activities of multiple enzymes, which eventually restore the original DNA sequence. Inspired by the BER pathway, Wang et al. recently developed a method for the detection of several modified bases, including a T-G mismatch (Figure 8).[80] By recruiting the enzymatic components of the BER pathway, a biotin-conjugated nucleotide was incorporated at the precise location of the identified modified base. The obtained complex was then probed with a solid-state nanopore assay capable of sensing DNA duplexes featuring the biotin tag. The method can reportedly be adjusted for analysis of different types of base lesions by employing the corresponding glycosylases. Even so, the applicability of this approach can be limited by the poor specificity of some glycosylases that recognize more than one type of damaged bases.[77]

SNV Detection through Primer Extension. Primer extension methods rely on DNA polymerases to specifically extend a primer by incorporating one (as in single-base extension) or multiple (as in allele-specific PCR) nucleotides complementary to the nucleotides of the target DNA. In single-base extension (SBE) or minisequencing, primers are designed to anneal to PCR-amplified DNA fragments so that the 3′-end of the primers is placed immediately adjacent to the SNV site on the DNA template.[81,82] A DNA polymerase will then incorporate to the 3′-end of the primer a single 2′,3′-dideoxynucleoside triphosphate (ddNTP) complementary to the targeted SNV. No further chain elongation will occur once the dideoxynucleotide has been added to the primer, as dideoxynucleotide lacks the 3′-OH group essential for polymerase-mediated strand extension. The identity of the incorporated nucleotide, and hence the SNV, can then be determined by analyzing primer extension products using different technologies.[83,84] A commonly used approach for the analysis of primer extension products is based on electrophoresis and fluorescence detection. In this method, ddNTPs complementary to different bases are labeled with different fluorophores, and the SBE products are separated by capillary electrophoresis. The fluorescence color readout reports the incorporated base whereby the SNV can be determined.[84] Notably, such SBE assays possess excellent capabilities for the highly multiplexed detection of various SNVs; a 55plex SBE assay was recently reported by Wang and co-workers.[85]

Allele-specific PCR (ASPCR),[86] also known as amplification refractory mutation system (ARMS),[87] is a modification of PCR that allows preferential amplification and detection of sequences differing by as little as a single nucleotide. The method relies on the higher efficiency of the primer extension process by DNA polymerase when the primer is perfectly complementary to a given DNA template than when there is a 3′-terminal mismatch in the primer−template duplex. In this technique, allele-specific primers (AS primers) are designed so that they complement one allele but form 3′-terminal mismatches at the SNV site of the other allele. Since a single 3′-terminal mismatch may be insufficient to effectively suppress the primer extension, an artificial mismatch is sometimes deliberately introduced within a few nucleotides from the 3′ terminus of the AS primers so as to increase the specificity of the extension reaction.[87] Under appropriate PCR conditions, the extension of the AS primers occurs only in the presence of the target sequences. ASPCR combined with fast and inexpensive detection schemes for the analysis of PCR products (e.g., lateral flow test strips) has provided a useful means for genotyping.[88]

Despite its excellent performance in selectively amplifying and detecting target sequences, the limited multiplexing capability of ASPCR represents a major challenge toward their POC applications. This is largely the result of different optimized conditions (reagent concentration, primer length,

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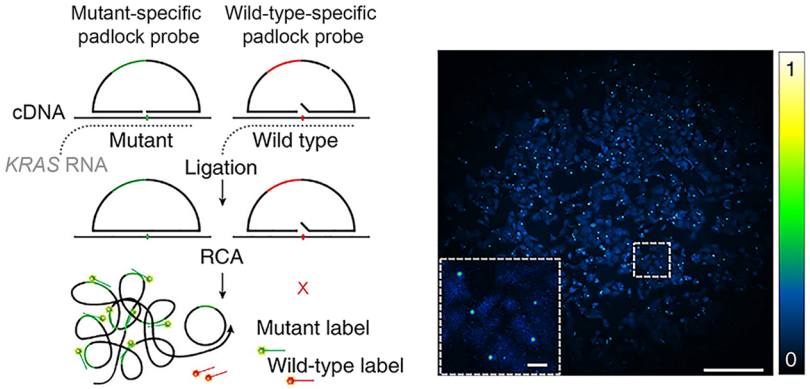

Figure 9. Ligation-based *in situ* analysis of point mutations directly in preserved cells. *KRAS* mRNAs are first copied into complementary DNAs (cDNAs) using reverse transcription reactions, and the obtained cDNAs are then targeted by single-base discriminating padlock probes. Mutant-specific padlock probes are ligated and amplified through RCA whereas wild-type-specific probes are not ligated and, thus, generate no RCA product. Following RCA, the RCA products are specifically labeled with fluorescent hybridization probes and detected with a mobile phone fluorescence microscope. Adapted from ref 102 under the terms of Creative Commons Attribution 4.0 International License.

annealing/extension temperature, etc.) required for each primer sets involved in the multiplex ASPCR.[89] As a result, efficient amplification of all of the expected fragments may not always be achieved under a given set of reaction conditions. In addition, the probability of primer−dimer formation, which can generate amplicons unrelated to the target sequences, is relatively high and this makes primer design for multiplex ASPCR a challenging task. The requirement for expensive thermal cyclers is another weakness of PCR-based methods, which makes them less useful in resource-limited test settings. Isothermal DNA amplification techniques are alternatives to PCR that can amplify target sequences at a constant temperature, thereby obviating the need for thermocyclers.[90] The use of isothermal DNA amplification instead of PCR can therefore effectively reduce the cost of SNV discriminating devices. In this context, Ng and co-workers recently reported a point mutation detection assay in which isothermal DNA amplification with sequence-specific primers was adapted to a three-dimensional-printed device that costs only ∼U.S. $20.[91] It should be noted, however, that many isothermal amplification technologies are more complicated than PCR, particularly in terms of the components of the reaction mixture.[90]

SNV Detection Assisted by Enzymatic Ligation. First introduced by Landergren et al. in 1988,[92] enzymatic ligation-based methods for SNV detection rely on the specificity of ligase enzymes to distinguish between matched and mismatched nucleotides at the ligation site. In these methods, two oligonucleotide probes of which one has a discriminatory base often at its 3′-end are designed to anneal immediately next to one another on a complementary target DNA. A ligase then covalently joins the two adjacent oligonucleotides into one strand, provided that the nucleotides at the junction (the SNV site) are correctly paired with the nucleotides on the target strand. The occurrence of the ligation reaction thus determines the identity of the SNV.[92] An approach to analyzing the products of the ligation reaction is the use of labeled probes, which can generate ligation products bearing specific moieties for detection purposes.[93] Nevertheless, the preparation of labeled probes can be costly and limit practical applications of such detection schemes. Label-free methods, on the other hand, generally provide less expensive means for SNV discrimination. One such label-free method was recently developed by Xu et al. and used for the detection of both known and unknown SNVs.[94] In their approach, the ligation byproduct adenosine 5′-monophosphate was involved in a bioluminescence reaction, producing a measurable signal.

In ligation-based assays, the use of an amplification strategy is crucial to detecting trace amounts of target DNAs. Performing ligation reactions in repeated thermal cycles using thermostable DNA ligases can produce amplified ligation products and, therefore, intense detection signals. In a method known as ligase chain reaction (LCR),[95] ligation products are amplified exponentially much in the same way as in PCR. Two pairs of hybridization probes of which one pair is complementary to one strand of the target DNA duplex and the other pair is complementary to the other strand of the duplex are used. In case of perfect base-pairing of the hybridization probes with the strands of the target, multiple hybridization probes can be joined together per target strand through thermal cycles of annealing, ligation, and melting. This results in the exponential DNA amplification and helps highly sensitive and specific detection of DNA targets.[95] In spite of the high sensitivity and robustness of LCR-based methods for nucleic acid detection, the widespread applications of such methodologies are greatly limited. This is mainly because the LCR products are traditionally analyzed through a gel electrophoresis separation step, which is laborious and time-consuming.[96] Recent advances have provided real-time analysis of LCR products using different assay formats, eliminating any post-reaction analysis.[97−99] In particular, Sun et al. labeled a pair of hybridization probes, one with a fluorophore and the other with a quencher, so that the ligation of the labeled probes in the presence of the target could bring the fluorophore and the quencher close enough to each other for quenching to occur.[97] Through real-time monitoring of the fluorescence intensity during the LCR process, the assay permitted specific analysis of the target DNA (0.1% mutant DNA in the presence of a wild-type DNA background) at low concentrations down to 10 aM.

The SNV discrimination capability of ligation reactions has also been combined with other signal amplification strategies, particularly isothermal rolling circle amplification (RCA).[100−102] The coupling of ligation reactions with RCA is performed by the use of padlock probes, which are linear oligonucleotides that hybridize to a target DNA with their 3′-

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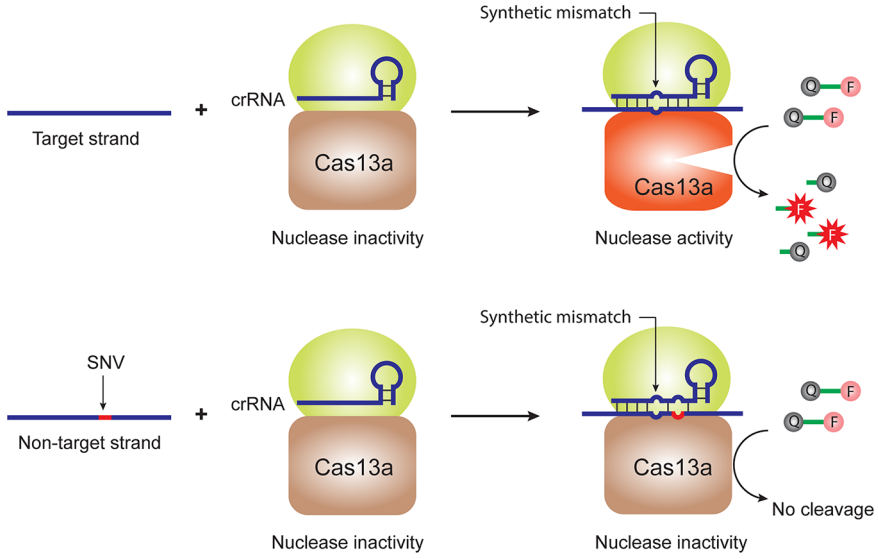

Figure 10. Schematic of CRISPR/Cas13a-based platform for the detection of target nucleic acids with single-base specificity. The introduction of the synthetic mismatched base in the crRNA retains the collateral cleavage activity of Cas13a in the presence of the target sequence while hindering such activity in the presence of a non-target strand containing an additional mismatch. "F" denotes fluorophore, and "Q" denotes quencher.

and 5′-ends lined up adjacent to each other at the SNV site. The two ends of a padlock probe can be joined through a ligation reaction to form a single circularized DNA, provided that there is a perfect match between the probe and the target at the location of the SNV. The circularized probe can then be used for the template synthesis of many linked copies of the target through isothermal RCA, hence amplification of the signal. Recently, a padlock probe-based RCA was combined with lateral flow strips for the visual detection of the most common point mutations causing multi-drug-resistant tuberculosis.[101] This simple and cost-effective molecular diagnostic test is a promising tool for the rapid (~75 min) SNV discrimination at resource-limited clinical laboratories where sophisticated instruments for reading out detection signals are not easily accessible. In another intriguing report, the employment of padlock probes combined with RCA enabled *in situ* analysis of point mutations directly in preserved cells using mobile phone fluorescence microscopy (Figure 9).[102]

SNV Detection with the Help of Enzymatic Cleavage. Enzymatic cleavage-based methods for SNV sensing rely on the ability of certain classes of enzymes to specifically cleave DNA strands. Nicking endonuclease is one of these enzymes that cleaves one strand of a dsDNA substrate when recognizing a specific nucleotide region within the DNA sequence.[103] The cleavage is, however, restricted when there is a single-base change in the recognition region of the enzyme. This discrimination ability of nicking endonucleases has been coupled with signal amplification methodologies and exploited for the sensitive detection of SNVs within target DNAs.[104] Nevertheless, the application of such assays is limited to targets containing the specific sequences recognizable by nicking endonucleases and to SNVs located within these regions, rendering many SNVs undetectable.

Invader assays have provided a solution to the above limitation as they rely on structure-specific cleavage reactions and do not require recognition regions within the target sequence.[82] To identify a particular SNV, an SNV-specific probe and an invasive oligonucleotide are designed to overlap by one nucleobase at the site of the SNV when hybridizing to the target. A structure-specific endonuclease then recognizes the one-base overlapping structure and cleaves the probe at the site of its overlap with the invasive strand. As a result, the 5′ arm (known as "flap") of the probe is released, which serves as a reporter of the SNV type. In contrast, sequences that are not complementary to the probe at the SNV site renders conformations that are unrecognizable by the enzyme and therefore cannot be cleaved. Invader assays can be easily applied to multiplexed SNV analysis by designing and using multiple probes, each targeting a particular SNV and having a specific flap representing its target SNV. A multiplexed assay based on invasive reactions coupled with a universal genotyping microarray was recently proposed by Chen et al. and tested for the analysis of 14 SNVs in the *BRCA* gene.[105]

Performing the invasive reaction at elevated temperatures allows for denaturation of the probe after cleavage followed by hybridization of an intact probe to the target and initiation of a new cycle of cleavage.[106] Consequently, multiple flaps per target sequence can be generated and the signal will be amplified. The produced flaps can even be involved in a second amplification reaction to further increase the detection sensitivity.[106] An example of such a highly sensitive assay was reported by Liu et al., who employed a cascade of two invasive reactions with a low background signal to detect down to 0.07 fM of target sequences with high specificity (as low as 0.02% mutants could be detected in a background of wild-type DNA).[107]

Recently, Gootenberg et al. introduced SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) as a means of detecting target nucleic acids of interest with single-base specificity.[108] SHERLOCK exploits the combined use of (1) the isothermal amplification of target DNA or RNA, (2) the T7 RNA polymerase transcription of amplified DNA to RNA, and (3) the collateral cleavage activity of Cas13a upon recognition of the amplified RNA fragments. Cas13a is a CRISPR (clustered regularly interspaced short palindromic repeats)-associated endoribonuclease, which can be reprogrammed with CRISPR RNAs (crRNAs) to recognize RNAs of specific sequences. Upon target recognition and the formation of the crRNA−target duplex, Cas13a becomes activated and engages in collateral cleavage of nearby self-quenched

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fluorescent RNA reporters. The Cas13a-mediated collateral cleavage of RNA reporters abolishes the quenching, thereby generating an amplified fluorescent signal that correlates with the amount of the target nucleic acid.[108] Importantly, Cas13a-based detection was demonstrated to be able to discriminate between targets that differ by a single nucleotide when loaded with crRNAs containing a synthetic mismatched base placed near the targeted SNV site (Figure 10).[108,109] Whereas the presence of the synthetic mismatch in the crRNA sequence retains the target-triggered cleavage activity of Cas13a, it impedes such collateral cleavage activity in the presence of a non-target sequence with an additional mismatch (two mismatches in total). Highly homologous viral strains and cancer mutations at levels as low as 0.1% of wild-type DNA background were successfully discriminated by SHERLOCK.[108]

Having attomolar sensitivity and single-base specificity toward nucleic acid detection, SHERLOCK is a promising tool for low-cost POC disease diagnosis and monitoring.[110] A SHERLOCK test was reported to cost as low as U.S. $0.61 when the reaction reagents are reconstituted on paper.[108] Recent refinements made to SHERLOCK have enabled quantitative, visual, more sensitive, and multiplexed readouts, which further extend its practical utility to resource-limited environments where instrument-free medical testing is greatly appreciated.[111]

OPPORTUNITIES AND CHALLENGES FOR CLINICAL TRANSLATION OF SNV ASSAYS

SNV discrimination assays are useful tools for the detection of known point variations associated with a clinical phenotype, providing information that can potentially affect clinical decisions. Depending on the type of patient, SNV analysis may be used for risk assessment, screening, diagnosis, prognosis, therapy selection, and monitoring of the response to therapy.[112] An appealing potential application area for SNV assays that is being actively investigated is the diagnosis and monitoring of cancer through the analysis of cell-free circulating tumor DNA (ctDNA).[54,113] ctDNAs are DNA fragments carrying tumor-specific sequence alterations (including somatic point mutations) that are shed into the bloodstream by tumor cells and are considered as potential biomarkers for the assessment of cancer.[114,115] Obtaining such tumor-derived genetic materials from patients' blood, known as "liquid biopsy",[116] offers several important advantages over tissue biopsies: it presents a minimal procedural risk to the patient and may deliver more complete information regarding the patient's entire tumor burden.[117] Moreover, ctDNA analysis may enable early detection of cancer when no solid tumor is detectable, and, because of the short half-life of ctDNAs *in vivo*, it can offer the option of repeated sampling to inspect temporal heterogeneity in mutation profiles (e.g., the development of resistance mutations).[118] The analysis of ctDNA could, therefore, play an important role in the implementation of precision medicine for cancer patients, and advanced SNV assays for the simple, rapid, and targeted ctDNA testing is expected to make headway toward this desirable end.

To be applicable to clinical use, SNV detection tools need to possess several attributes. High analytical specificity is one such characteristic and is particularly important in detecting low-abundance genetic variants, such as somatic mutations. In contrast to germline variants (e.g., single-nucleotide polymorphisms or SNPs), which can comprise up to 50 or 100% allele frequencies, somatic mutations in clinical samples can be outnumbered by a large excess of wild-type sequences differing by as little as a single nucleotide.[52,119] This poses a persistent technical challenge in detecting somatic mutations and demands highly specific detection methods. The analysis can be further complicated by the limited amount of DNA in clinical specimens, especially when interrogating samples extracted from serum or plasma.[118] The total amount of circulating cell-free DNA in plasma is frequently reported to be of the order of 1000 copies/mL,[120] of which only a fraction (sometimes less than 0.01%)[121] may originate from tumor cells. Therefore, besides high specificity, the analysis of such tiny amounts of nucleic acid sequences requires high detection sensitivity, which can be obtained through the use of target and/or signal amplification strategies; see, for example, refs 54, 83, 107, and 108.

Despite the tremendous efforts devoted to developing new SNV assays with promising analytical characteristics (e.g., high analytical sensitivity and specificity), further work is needed to implement these methods outside of academic laboratories. In this context, simplification is one of the most desirable improvements to be made to the new assay formats prior to clinical translation. Indeed, analytical methods that do not require highly trained personnel or the use of complex instrumentation will be more readily adaptable to the majority of clinical laboratories.[122] New test methods also need to offer analysis at a reasonable cost for widespread clinical adoption and should ideally incorporate quality control to ensure the reliability of the test results.[123] Additionally, the exact clinical application may impose specific requirements and restrictions on assay designs that should be taken into account.[118] For example, assays to be used for screening of undiagnosed cancer need to possess a very low false-positive rate to minimize the impact on individuals with no disease. Finally, standardization and validation are essential steps that need to be taken before the clinical utility of the new test methods.

CONCLUSION AND PROSPECTS

Various detection schemes capable of discriminating nucleic acids that differ from one another by as little as a single nucleotide have been reported in recent years. These strategies generally rely on the use of melting profile analysis, enhanced hybridization probes, and mismatch-sensitive proteins, and each comes with its own strengths and weaknesses. Newly developed techniques based on melt profiling, such as HRM, take advantage of the SNV-induced change in duplex stability and have generally shown great potential for the rapid analysis of clinical samples. Even so, unless being integrated with microfluidics at an affordable price, the implementation of these techniques in resource-limited test settings can be hindered by the need for the bulky and often expensive equipment (e.g., temperature controllers). Rationally designed hybridization probes with enhanced single-base specificity have been developed based on optimizing probe's length, modifying probe's chemical structure, and applying constraints to the probes. Limited multiplexing capability is the main drawback of the use of such probe architectures in assays with homogeneous reaction formats. When they are immobilized on the solid support of highly multiplexable two-dimensional microarrays, the relatively long time of hybridization with DNA targets present in the solution is a major concern. Similar to melt profiling and enhanced hybridization probe-based

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assays, methods relying on the specificity of proteins and enzymes for single-base mismatches can provide a high level of SNV discrimination. In spite of being well-recognized and widely used, protein-based methods are potentially more resource intensive than the other two methods, as they involve the production and storage of proteins and require specific assay conditions to maintain protein activity.

An ideal SNV discrimination tool for disease diagnosis and monitoring should be simple, rapid, cost-effective, sensitive, and specific with the capability for multiplexed detection of targeted SNVs. Developing a biosensing platform that satisfies all these criteria without compromise is highly challenging and demands more research efforts in the field of nucleic acid biosensing. Combination of different sensing methodologies is a feasible approach that can help scientists toward attaining more powerful SNV discriminating platforms in the future. When a detection process fails to offer a desirable property (e.g., high specificity), the coupling of two or more SNV-sensitive processes in the design of the assay may be the solution.[124] Designing assays with principles similar to protein-based SNV discrimination assays but without the involvement of proteins should be another area of future research.[125−127] Such research efforts can lead researchers toward the development of advanced protein-free detection schemes of reduced analysis cost and increased compatibility to a wider range of conditions.[125−127] The employment of different nanomaterials in the sensing designs with a view to improving the detection performance, particularly the specificity, sensitivity, and analysis speed, is also an active area of biosensing research and worth further investigation in the field of targeted SNV detection.[128,129]

The desire to better address the needs of patients and physicians in precision medicine has directed SNV sensing research toward the development of disease diagnosis and monitoring tools applicable at POC. Microfluidic technology is expected to pave the way toward producing such practical devices by allowing the incorporation of sample preparation and sensing elements into inexpensive sample-to-answer platforms.[130] Some of the detection methodologies described in this Review were already integrated with microfluidic platforms and showed promising performance.[7,15,17,18,21] Further advancements in the fields of SNV detection and microfluidics are expected to substantially improve the capability of current sensing devices for the automated and multiplex SNV detection. Such molecular diagnostic tools are hoped to provide timely health status information at the POC, thereby bringing about a paradigm shift from the one-size-fits-all treatment approach of traditional medicine to tailored therapy of precision medicine that takes into account the specifics of each person's biology.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: alireza.abi@shirazu.ac.ir (A. Abi).
*E-mail: asafavi@shirazu.ac.ir (A. Safavi).

ORCID

Alireza Abi: 0000-0002-9056-5376
Afsaneh Safavi: 0000-0003-3441-1687

Notes

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ABBREVIATIONS

ARMS, amplification refractory mutation system; AS primers, allele-specific primers; ASPCR, allele-specific polymerase chain reaction; BER, base excision repair; cDNAs, complementary DNAs; CRISPR, clustered regularly interspaced short palindromic repeats; crRNAs, CRISPR RNAs; ctDNA, cell-free circulating tumor DNA; ddNTP, 2′,3′-dideoxynucleoside triphosphate; dsDNA, double-stranded DNA; HRM, high resolution melting; LCR, ligase chain reaction; LNA, locked nucleic acid; MBs, molecular beacons; miRNA, microRNA; PCR, polymerase chain reaction; PNA, peptide nucleic acid; POC, point-of-care; qPCR, quantitative polymerase chain reaction; RCA, rolling circle amplification; SBE, single-base extension; SHERLOCK, specific high sensitivity enzymatic reporter unlocking; SNP, single-nucleotide polymorphism; SNV, single-nucleotide variation; ssDNAs, single-stranded DNAs; TSD, toehold-mediated strand displacement

VOCABULARY

Germline variant, an alteration in the DNA of germ cells which can be passed on to offspring; Liquid biopsy, a minimally invasive or noninvasive test to analyze tumor-derived materials in a patient's blood or other body fluids; Point-of-care testing, medical testing conducted at or near the site of patient care; Precision medicine, an approach to patient care that includes medical treatment tailored to the characteristics and needs of individual patients; Single-nucleotide polymorphism, a single-nucleotide DNA change detectable in >1% of the population; Somatic mutation, a mutation in the DNA of somatic cells which is acquired over the life of an individual and cannot be transmitted to offspring

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