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Emerging technologies for hybridization based single nucleotide polymorphism detection

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Detection of single nucleotide polymorphisms (SNPs) is a crucial challenge in the development of a novel generation of diagnostic tools. Accurate detection of SNPs can prove elusive, as the impact of a single variable nucleotide on the properties of a target sequence is limited, even if this sequence consists of only a few nucleotides. New, accurate and facile strategies for the detection of point mutations are therefore absolutely necessary for the increased adoption of point-of-care molecular diagnostics. Currently, PCR and sequencing are mostly applied for diagnosing SNPs. However these methods have serious drawbacks as routine diagnostic tools because of their labour intensity and cost. Several new, more suitable methods can be applied to enable sensitive detection of mutations based on specially designed hybridization probes, mutation recognizing enzymes and thermal denaturation. Here, an overview is presented of the most recent advances in the field of fast and sensitive SNP detection assays with strong potential for integration in point-of-care tests.

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Introduction

Recent advances in the field of molecular biology have created opportunities for the development of novel diagnostic methods, which are becoming irreplaceable not only in the detection and typing of diseases, but also in steering drug therapy, being either with antibiotics or novel cancer drugs. For instance, numerous genes have been related to various human diseases[1] and as a result genetic screening has been applied to prenatal diagnostics,[2] detection of cancer,[3] cardiovascular disease[4] and many other pathologies.[5,6] Moreover, molecular diagnostics can even be used to enhance cancer therapy through assessing a patient's susceptibility for certain treatments.[7–9] These so-called 'companion diagnostics' are increasingly becoming an essential guide for the successful application of a number of treatments.[10] Furthermore, molecular diagnostic methods are used in microbiology[11] for identifying pathogens and their antibiotic resistance genes, hence guiding treatments towards the correct

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Karel Knez (1986) obtained a MSc in bio-medical science from the KU Leuven (Belgium). At the Inter-university Electronics Centre, Leuven, Belgium he learned the implementation of gold nanoparticles in bioassays. In 2009 he started his PhD at KU Leuven in the group of Prof. Lammertyn; his research is focused on the development of new biosensors for sensitive detection and identification of micro-organisms. His main interest lies in using novel assay designs to enable improved detection and identification of DNA using SPR transducers in combination with gold nanolabels. In 2013 he finalized his PhD in Bioscience engineering.

Dragana Spasic received her MSc and PhD degrees in Medical Sciences from the KU Leuven – University of Leuven in Belgium in 2004 and 2009, respectively. She was a post-doctoral researcher at the Faculty of Medicine (KU Leuven, 2009-2011). Currently she is a postdoc in the Biosensor group at the Faculty of Bioscience Engineering (KU Leuven). She is involved in research and projects related to development of bioassays (e.g. aptamer selection and application as bioreceptors, biofunctionalization of nanomaterials) and biosensors, particularly fiber optic SPR sensors, towards diagnostic tools with food and medical applications.

antibiotic type/dose and preventing further growth in global antibiotic resistance of bacterial pathogens.[12]

Currently, however, the molecular diagnostics field is still limited to laborious and costly methods that require significant infrastructure and skills, only available at specialized laboratory facilities. For human genetic screening the preferable method is sequencing because it can capture the complete spectrum of genetic variation in an individual.[13] Although in recent years sequencing technologies have experienced a dramatic drop in price and time to acquire results,[14,15] some hurdles still severely limit their direct application, in routine diagnostics:[13] (1) reliable sequencing results depend in the first place on careful construction of a sequence library and sample preparation[16] and (2) the amount of data generated by sequencing and the complexity of their processing are not compatible with routine applications.[17,18]

In microbiological applications, the preferred method for pathogen detection and evaluation of antibiotic resistance still relies on actual selective bacterial growth, limiting the speed of these assays to 48 h.[19] Molecular diagnostics such as PCR are able to improve the sample throughput, time to detection and sensitivity for non-cultivable bacteria but introduce new problems such as sample preparation, expertise and facility requirements.[20]

Recently, the trend in developing rapid and cost-effective point-of-care (POC) diagnostics has increased tremendously. Devices that allow detection of genetic changes, such as point mutations, with minimal user intervention and laboratory requirements and which could even be deployed in resource limited regions are being developed.[21,22] Although this next generation of molecular diagnostic devices presents a large improvement compared to classical methods and can process complex samples, such as blood, saliva or urine to ensure DNA extraction for the PCR analysis,[23] they remain linked to the intrinsic limitations of the PCR method such as total analysis time and high sensitivity towards sample contamination. Furthermore the cost per test remains too high for the intended markets such as developing countries.[24]

In recent years, several new methods for detection of genetic mutations have become available that are, currently, at least comparable to PCR in their performance. These new developments have been promoted to drastically improve and simplify present-day POC tools, enabling sensitive detection in minutes' time and at a fraction of the cost of current methods. However not a lot of these new concepts have found their way to commercial applications due to numerous obstacles such as performance, price and applicability,[25] allowing classic PCR to remain the method of choice for molecular diagnostics. In this review a selection is made of new molecular diagnostic methods that could break this spell and live up to the promises of improving molecular diagnostics. The review has a particular focus on single nucleotide polymorphism (SNP) detection for two reasons: one, SNP mutation detection can be taken as a benchmark and two, the impact of a SNP on the overall double strand is limited and strongly depends on multiple factors, making this one of the most challenging mutations to detect. The review will focus on hybridization-based assays, therefore excluding novel sequencing techniques, such as nanopores and nanochannels.[26] Furthermore, the review is limited to assay design and largely excludes detection methods as most of these assays can be applied on multiple detection platforms (*i.e.* optical, electrochemical, mass based, *etc.*).

New assays are categorized in the following three fields based on the applied methodology.

1. Hybridization mediated SNP detection.
2. Protein mediated SNP detection.
3. SNP detection through thermal denaturation of hybrids.

Each of these topics will be discussed elaborately in the review with a particular focus on sensitivity and applicability of the methods.

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Prof. Lammertyn obtained a Master in Bioscience Engineering (KU Leuven) and in Biostatistics (UHasselt) as well as a PhD in Applied Biological Sciences in 2001 (KU Leuven). In 2003 he was awarded the Quetelet biostatistics Prize. From 2002 he worked as postdoctoral researcher at the Postharvest Technology laboratory of Prof. Nicolaï. In 2004 he spent a year at the Pennsylvania State University, USA, as senior researcher. Since 2005 he is Professor in the division Mechatronics, Biostatistics and Sensors (MeBioS) of the Biosystems department at the KU Leuven. He is author of 100+ peer reviewed research papers and acts as reviewer for many international journals.

Direct hybridization mediated SNP detection

Single hybridization probes

Detection of SNPs through direct hybridization remains one of the most popular methods for genotyping. However, the greatest challenge in these assays is the specificity of the hybridization,[27] because the impact of a single variable nucleotide on the overall double strand stability is rather limited (Fig. 1). For example, the influence of a SNP on a 20 bp DNA double helix target comprises only 5% of the total number of basepairs. Several factors can improve the selectivity of hybridization, all being related to the mass transport and kinetics involved in the hybridization process.[28] Therefore, parameters such as temperature, ionic strength, sequence and oligonucleotide concentration, which all have a direct influence on mass transport and kinetics, need to be precisely controlled ensuring optimal hybridization efficiency.[29] However, for every new target, new optimal parameters need to be defined, requiring a substantial investment in assay optimization and making the detection of multiple DNA targets very complex.

These limitations have pushed SNP detection towards a new generation of assays where the influence of specific conditions on the hybridization process is being eliminated as much as possible (Table 1). By introducing a better control over hybridization, new assays have become more sensitive and robust. One example of improved specificity of the hybridization process is the introduction of secondary structures (*e.g.* stem-loop) into reporter oligonucleotides. These reporter molecules can hybridize to a target sequence only after the target strand has displaced the secondary structure through a process called branch migration.[30,31] Branch migration can happen between any single stranded DNA and a double stranded molecule when they share a homologous region for binding the single strand to the duplex. As a result of the homologous regions, the single strand can attack the existing duplex resulting in a three-stranded structure where all strands compete for binding their complementary strands. In the presence of a SNP, the thermodynamic driving force towards breaking up the secondary structure in the reporter sequence and hybridization to the target molecule will be lowered, explaining why these structured probes possess increased specificity and robustness in comparison with simple complementary strands.[32] One of the first implementations of the branch migration process in molecular diagnostics was in Molecular Beacons (MB).

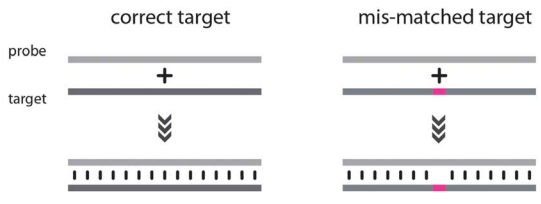

Fig. 1 Basic concept of hybridization based strategies for SNP detection. In this direct hybridization of two complementary strands detection of a mutation is difficult, as the mutation only gives lower hybridization yields in short target sequences. Detection platforms can be of any kind: fluorescent, electrochemical, or SPR.

Molecular beacons

MBs are a particular class of branch migrating molecules that use a self-complementary region as the competing DNA duplex in the process of SNP identification (Fig. 2).[33] The internal structure of MBs forms an oligonucleotide hairpin probe with a fluorophore and a quencher conjugated to the opposite ends of the oligomer. When the complement of the MB is not present, they form a stem-loop structure that brings the quencher in close proximity to the fluorophore, resulting in a low fluorescent signal. From the moment the target sequence is present, the hairpin unfolds in favour of target hybridization, resulting in an increased fluorescence signal.

Hybridization to this hairpin requires, as already discussed earlier, the breakup of the stem duplex and is therefore less favourable than a standard hybridization reaction. MBs can thus be regarded as constrained polymers,[30] which can adopt only one conformation (the hairpin) after they dissociate from their target thereby strongly lowering the entropy (ΔS) of the reaction. Further increasing this constraint and thus the specificity of the hybridization reaction can be achieved by extending the length of the stem. Extension of the MB stem part can make ΔS so small that it lowers the hybridization rate towards keeping MB permanently closed. On the other hand, as a result of this constrained nature of MBs, they can distinguish targets over a wider temperature range in comparison with unstructured probes. Because, the MB has a strong tendency to self-hybridize in a hairpin after dissociation, the process of forming a target–probe complex bearing a mutation at lower temperatures becomes less favourable.[30] Thus, again the increased robustness is found to be a consequence of a reduction in ΔS of the MB target hybridization caused by the stem-loop structure.

Binary DNA probes

An alternative for molecular beacons, known as the binary probe, is based on a simple division of the probe into two individual parts (Fig. 3), where the individual, shorter probes, also known as the analyte binding arms, are used to bind to the target. The individual binding arms can be labelled with any kind of reporter molecule that only gives a signal when both are bound to the target, resulting in a remarkable increase in both the specificity and sensitivity towards SNP detection. The improvements in performance can be explained by the relatively short length of the individual fragments of the binary probe, which consist each of 7–10 bases, emphasising considerably the effect of a single variable base on the DNA hybrid.

Initially, binary probes were mainly used in combination with fluorescence resonance energy transfer (FRET)

Table 1 Comparison of different hybridization probe based assays

Hybridization mediated SNP detection	Transducer	Optimization required	Target length (bp)	Detection limit	Number and/or position of detected mutation	Differentiation of mutations	Expected assay time
Single hybridization probes							
Urakawa *et al.*[28]	Fluorescence (microarray)	For every Target	39	500 nM	All positions	Hybridization spot on the array	1 h
Milkani *et al.*[29]	SPR	For every target	21	100 pM	3′ end – middle – 5′ end	Hybridization speed and yield	10 min
Molecular Beacons							
Tyagi *et al.*[65]	Fluorescence	No	26	100 nM	4 different alleles	Fluorophore emission spectrum	30 min
Binary DNA Probes							
Kolpashchickov[35]	Fluorescence	No	14	2 μM	All 42 possible basepair substitutions	Fluorescence yield	15 min
Kolpashchickov[36]	Fluorescence	No	20	80 nM	19 out of 20 possible SNP positions	Fluorescence yield	2 h
Lu *et al.*[38]	Fluorescence	No	25	10 pM	1 SNP	Fluorescence yield	2 h
Kolpashchickov[39]	Colorimetric	No	29	1 μM	2 SNPs	Optical density	5 min
Deng *et al.*[40]	Colorimetric	No	40	20 nM	SNPs at different positions	Optical density	5 min
Li *et al.*[42]	Colorimetric	No	25	1 nM	1 SNP	Optical density	30 min
Wang *et al.*[43]	Fluorescence	No	47	10 fM	No SNP tested	No differentiation	4–6 h
Shimron *et al.*[44]	Colorimetric	No	19	100 fM	1 SNP	Optical density	4–6 h
Dong *et al.*[45]	Colorimetric	No	29	100 zM	1 SNP	Optical density	40 min
Junction forming probes							
Grimes *et al.*[47]	Fluorescence	No	20	2.5 nM	9 different SNPs in a secondary structure containing target	Fluorescence yield	15 min
Cornett *et al.*[49]	Fluorescence	No	80	250 nM	4 SNPs discriminated in 2 loci	Fluorescence color/yield	15 min
Nakayama *et al.*[52]	Fluorescence	No	24	2 μM	3 SNPs containing targets	No differentiation	5 h
Kong *et al.*[53]	Fluorescence	No	23	5 pM	3 SNPs containing targets	No differentiation	30 min
Zhang *et al.*[57]	Electrochemical	No	24	760 fM	3 SNPs containing targets	Cyclic voltammetry	1 h
Yeh *et al.*[59]	Fluorescence	No	120	2 μM	11 positions with each 4 bp substitutions	Fluorescence emission shift	1 h
Toehold mediated hybridization							
Zhang *et al.*[61]	Atomic force microscopy	No	24	/	4 positions with different bp substitution	Change in origami structure	1–3 h
Subramanian *et al.*[62]	Atomic force microscopy	No	15	5 μM	1 position with all 3 possible bp substitutions	Change in origami structure	3 h
Zhang *et al.*[63]	Fluorescence	No	25	1 nM	16 positions with different substitutions	Fluorescence yield	20 min

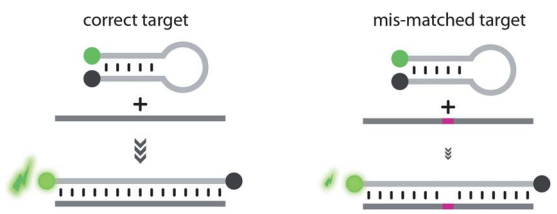

Fig. 2 Molecular beacon strategy for SNP detection. Mutation discrimination is improved by introducing a secondary structure (hairpin) into the DNA reporter strand which drastically lowers the hybridization yield in the case of a mutation, resulting in lower fluorescence. The hairpin also brings together the quencher and fluorophore when the MB is not bound to the DNA target.

detection. Kolpashchikov *et al.* have recently made improvements on the binary probe concept, through integration of an RNA aptamer. Aptamers are RNA or DNA oligonucleotides selected for their binding capacity towards a particular molecule, being in this case the fluorescent dye malachite green (Fig. 4). The aptamer was integrated into the binary probe concept by splitting its sequence into two parts, each linked to a target-binding probe. The aptamer only becomes functional when its two target-binding probes are bound to the target DNA strand, which brings the individual aptamer parts in each other's vicinity. Once functional, the aptamer can bind the malachite green dye stabilizing it in a more fluorescent conformation, which results in a 2000 fold increase in the fluorescence signal.[34] The assay is able to detect 41 out of the possible 42 mutations in a 14 bp

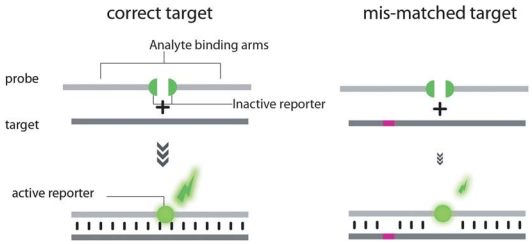

Fig. 3 General concept of binary probes where a probe is split into two halves to increase the impact of a mutation on the hybridization yield. Binary probes always use reporters, which require the presence of the two probes hybridized to the target in order to activate a reporter molecule or reaction.

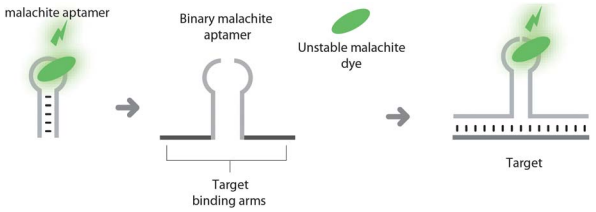

Fig. 4 Design of the binary malachite aptamer. On the left, the structure of the parent malachite aptamer is shown, which stabilizes the dye resulting in an increased fluorescence. This aptamer is split into two halves, each extended with a target binding arm, resulting in the binary malachite aptamer. When the target DNA strand is present, the functional malachite aptamer is formed. Reproduced after Kolpashchikov *et al.*[35]

nucleotide target strand.[35] However the assay is limited in sensitivity by the affinity of the aptamer for the target dye.

Binary probe assays can be further improved with the integration of ribozymes (Fig. 5),[36] which are DNA/RNA sequences with an enzymatic activity. In the first application of this concept, the assay was designed to integrate a deoxyribozyme in DNA probes that binds specifically to a 20 bp target. When bound to this target, the two fragments form the functional ribozyme, which then cleaves a phosphodiester bond in a DNA

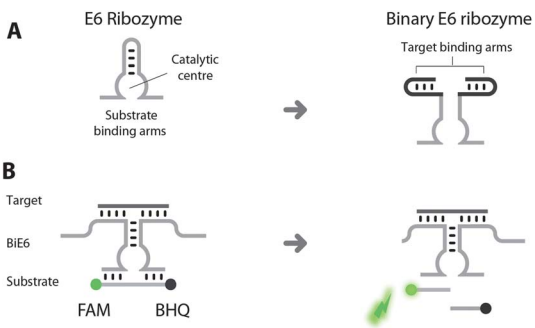

Fig. 5 Design of the binary deoxyribozyme probe. (A) Structure of the parent deoxyribozyme E6, which is transformed into the binary deoxyribozyme biE6 with hairpin forming target binding arms to reduce non-specific binding. (B) Scheme for fluorescent detection of the analyte-dependent catalytic activity of biE6. FAM indicates fluorescein; BHQ is a black hole quencher. Reproduced after Kolpashchikov *et al.*[36]

reporter molecule, resulting in the separation of a fluorescent FAM at the 5′ end from a black hole quencher at the 3′ end. Mutations can in this way be discriminated but not identified, by calculating the ratios between probe fluorescence intensities at 517 nm in the presence of the true target and fluorescence intensities in the presence of each mismatched oligonucleotide, after subtraction of the background fluorescence. The resulting fluorescent signal can easily discriminate 11 out of 20 tested SNPs in the target strand with a high signal to noise ratio compared to MBs. Furthermore, the integration of a ribozyme in a binary probe has the advantage that the formation of one functional ribozyme can be used to activate multiple reporter molecules, which results in a strong signal amplification. As a result, the detection limit of this ribozyme-based assay is 1 nM, which is two orders of magnitude lower than that of the previously described malachite based assay. The use of more efficient DNAzymes in similar assays has shown to further improve the detection limit, with the theoretical detection limit being 10^{-15} M due to the general limitations of enzyme catalysed reactions.[37]

Further improvements in the detection limit are possible with catalytic cascades, where a specific recognition event results in the activation of multiple DNAzymes and thus even stronger signal amplification. The group of Willner proved this concept for the detection of a SNP involved in the Tay–Sachse genetic disorder.[38] They made a combination of a Zn^{2+} dependent ligation DNAzyme and an Mg^{2+} dependent DNAzyme that cuts a fluorophore/quencher DNA substrate, resulting in fluorescence. Both DNAzymes are integrated into the binary probes, and thus depend on highly specific hybridization events before activation. In short, the presence of the target strand opens a loop in the inactive Zn^{2+} ligation enzyme, which catalyses the ligation of a 5′ imidazole modified DNA strand with a 3′ hydroxylated DNA strand. This ligation product assembles the functional Mg^{2+} dependent DNAzyme, which cleaves another sequence, releasing a fluorescent reporter strand from its quencher. The main advantage of this enzyme cascade is that the presence of one target molecule results in the activation of multiple DNAzymes, allowing activation of multiple reporter molecules and thus enabling lower detection limits. With the most optimal assay design it is possible to detect the mutant with an SNP from the wild type 25 bp DNA target at concentrations down to 10 pM.

Similar assays have also been performed with peroxidase like DNAzymes, which are more suited for POC diagnostics, as the colorimetric reaction does not require specialized readout equipment. Peroxidase DNAzymes are characterized by a high density of G triplet repeats, which bind with high affinity to hemine. Hemine is responsible for the catalytic activity of the DNAzyme as it can catalyze the oxidation of either 3,3′-diaminobenzidine (DAB) or 2,2-azino-bus(3-ethylbenzyiothiozoline-6-sulfonic acid) (ABTS2-) by means of H_2O_2 into their respective blue and brown coloured radicals. The first attempt to use this DNAzyme in a binary probe resulted in a somewhat poor detection limit of 1 μM.[39] The assay was further improved by splitting the DNAzyme asymmetrically, leading to a lower background signal due to a decreased tendency of the DNAzyme to self-assemble without the target strand. This method has also

been applied for longer targets (up to 44 bp), by the introduction of competing probes for the target sequence with a detection limit of 5 nM.[40]

Combining DNAzymes with bimolecular beacons leads to even further improvements in the assay detection limit.[41] Bimolecular beacons are two molecular beacons, which have complementary regions. When a target strand is present, it will bind to one of the molecular beacons (MB1), opening the stem-loop of MB1, which can then bind the second MB (MB2). Binding of MB2 with MB1 liberates the target, making it available for opening up another MB1. This process is called 'catalysed hairpin assembly' and will result in a self-propagating chain reaction of molecular beacon hybridization events.[42] The method as such can also be used in combination with DNAzymes to construct nanowires consisting of functional DNAzymes. The first configuration of this method had a detection limit of 10^{-9} M of target molecules[43] when combined with a magnesium DNAzyme that could cut a reporter oligonucleotide, releasing a fluorophore from a quencher. When this concept was combined with repeating horseradish like DNAzymes that can catalyse the formation of a colorimetric substrate, an even more sensitive assay was created, capable of detecting SNPs in a 19 bp target derived from the BRCA1 oncogene at concentrations of 10^{-13} M.[44] Although this assay is extremely sensitive, the long assay time of 4–6 hours necessary to reach this sensitivity is not suited for a POC diagnostic test. Therefore, it is currently combined with PCR for achieving better sensitivity in a shorter time period.[45]

DNA junction forming probes

As an extension to the principles of binary probes, a combination is made between them and MBs, resulting in several advantages over both traditional strategies, foremost in an increased sensitivity and specificity. In general, the assay consists of 4 oligonucleotides that can only assemble in a DNA four-way junction in the presence of the analyte and a molecular beacon. Both binary probes have complementary regions to the analyte and at the same time have binding arms that are complementary to the MB separating the quencher from the fluorophore at the MB ends upon hybridization. However, the binary probes need sufficient conformational freedom to bind both the target oligonucleotide and the MB at the same time, which is ensured through the connection of these binding domains by triethylene glycol linkers. In the absence of analyte, both the molecular beacon and the binary probes are in a stable hairpin form, limiting chances of non-specific hybridization. These junction probes are able to discriminate a SNP at any position in a 20 bp target with a more clear discrimination than a conventional MB.[46] Moreover, the junction probes can be applied on analytes with strong secondary structures directly at room temperature without any loss of sensitivity towards the SNP,[47] showing a clear advantage over MBs on that aspect. The highly specific analytical power of junction probes[48] can also be used to analyse the presence of multiple interesting genetic markers in one target. In recent research, junction probes are applied to detect the presence of both a *Mycobacterium* specific sequence and an antibiotic resistance mutation (towards Rifampin) (Fig. 6).

This application used two junction probe designs to detect both a general locus present in all *Mycobacterium tuberculosis* and a SNP present only in Rifampin resistant strains of *M. tuberculosis*. The combination of these junction probes results in a DNA 'Logic Gate'[50,51] meaning that the presence of none, one or two junction probe signals results in an NO, YES, OR, and AND answer to two questions: 'Is there any *M. tuberculosis* present in the sample?' and 'Is this *M. tuberculosis* Rifampin resistant?'.[49]

A variant of the initial junction probe, known as the three-way junction (Fig. 7), is designed in such a way that the analyte binds together with an assistant probe, probe B, to a reporter sequence, probe A, containing both a quencher and fluorophore, forming thus a three-way motif. In the initial assay design the junction allowed the formation of a double stranded restriction enzyme recognition site in the reporter probe (probe A), resulting in the separation of the fluorescent label from the quencher and a detectable fluorescent signal.[52] The same process is repeated with a MB resulting in a sensitive self-recycling assay that can detect SNPs in short oligonucleotides at low concentrations.[53] This concept is applied successfully using different detection concepts, such as colorimetric, electrochemical and fluorescence detection.[54–58]

Werner and coworkers combined the three-way junction with silver nanoclusters, creating an assay that can identify the exact basepair substitution in the SNP. The assay is based on earlier research of the group, which states that a silver nanocluster can emit a fluorescent signal in the presence of particular basepairs and that the emission wavelength changes when

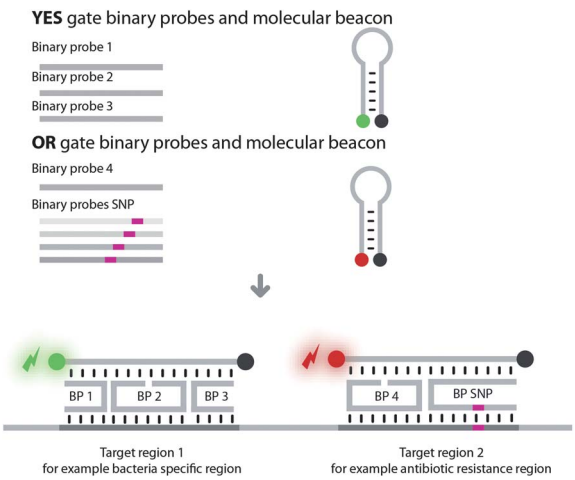

Fig. 6 Design of the logic gate binary probe, designed to detect multiple regions in one target. The YES gate is made of a first molecular beacon (MB1), that is combined with 3 different probes that only activate MB1 in the presence of the target DNA by hybridizing both with the target and the molecular beacon resulting in a highly specific detection. A next logic gate is used to locate mutations in a particular locus, and is therefore named OR as its activation can be the result of multiple mutations. This logic gate uses a different molecular beacon (MB2) labelled with another fluorescent label. Activation is done by only two binary probes, one present in different versions, for all possible target mutations. Adapted from Cornett *et al.*[49]

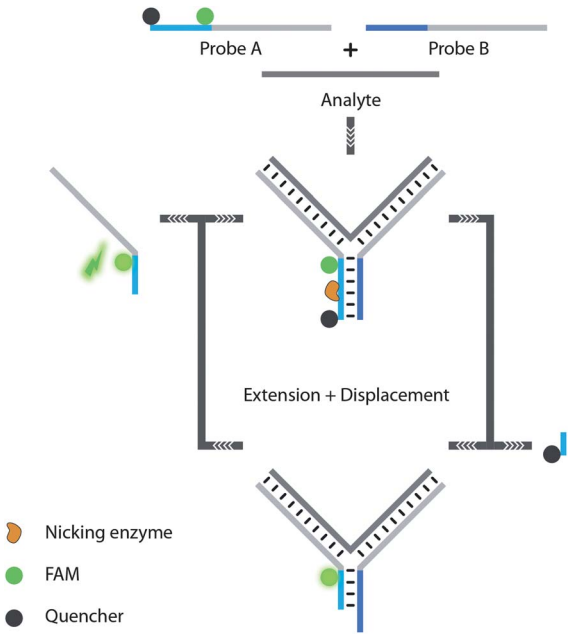

Fig. 7 Three-way junction concept where the target strand and a reporter strand (probe A) can hybridize with the help of an assistant probe (probe B) into a 3-way structure. Only when this structure is formed, a restriction site is generated that liberates the quencher from the reporter. Afterwards the analyte can be recycled by denaturing the 3-way structure to activate a next reporter strand, resulting in signal amplification. Adapted from Nakayama *et al.*[52]

these basepairs are altered. To apply this in a real assay, they labelled a reporter strand with silver nanoclusters (consisting of ±30 silver atoms) and combined this with a complementary 'enhancer' strand, which formed a three-way junction in the presence of the target sequence.[59] Hybridization of both the reporter and the enhancer strand to the target moves the enhancer sequence in close proximity of the nanocluster, which results in a fluorescent signal. When a mutation is present in the target sequence, the enhancer sequence will be shifted, resulting in a change of the sequence in the local vicinity of the silver nanocluster leading to a corresponding shift of the fluorescence emission band of the silver nanocluster of up to 60 nm. The assay proved to be highly sensitive and can even discriminate easily between 3 out of 4 possible base variations for one SNP position. Furthermore the assay is applicable to both short (20 bp) and long oligonucleotides (120 bp). Although no LOD has been determined, the lowest concentration estimated from the calibration curve at which SNPs can be clearly discriminated is 2 μM.

Toehold mediated hybridization

Even though the above-mentioned 'next generation' hybridization probes can be applied on complex targets harnessing secondary structures and are able to discriminate sensitively all types of SNPs in a target, they still rely in general on complex designs and require optimization of performance under particular conditions, such as ionic strength and temperature. Another type of structured probe, namely the toehold probe, tackles these limitations.[60] Toehold probes are DNA duplexes where the reporter strand has a short extension, which is used as a nucleation site for hybridization with a complementary target strand (Fig. 8).

Toeholds were first applied for SNP detection by Z. Zhang *et al.*[61] and were improved by Subramanian *et al.*,[62] where they were used to initiate the formation of a DNA origami structure. The presence of a SNP in the target sequence inhibits the strand displacement of the toehold, resulting in the absence of the DNA origami complex. Each of the four possible A, C, G or T base pair substitutions results in the formation of a different DNA origami that corresponds to the letter of the displaced nucleotide. The origami structure itself is read out using an atomic force microscope.

D. Zhang *et al.* redesigned the original toehold probe into a SNP detection method, which is almost completely insensitive towards changes in temperature, salinity and target concentration.[63] This SNP detection consists of a fluorescently labelled reporter strand and its reverse compliment labelled with a quencher (Fig. 9). The toehold reporter probe is designed in such a way that hybridization to both the quencher sequence and the complementary DNA target sequence is not fully overlapping, resulting in a ssDNA toehold domain. In contrast with an original toehold, in this assay both hybridization events are catalysed with a toehold reporter probe. Without the reverse toehold, probes would discriminate nucleic acids on a single-base level using initial association kinetics, rather than thermodynamics, which would drastically limit the SNP discrimination power.

The presence of the toehold increases the kinetics of the displacement reaction, normally having a half-life of months

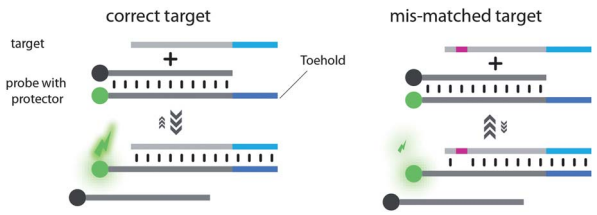

Fig. 8 Toehold concept, where the reporter is extended with a non-overlapping region, complementary to a region on the DNA target, which drastically increases the hybridization speed and specificity when the reporter is hybridized to a protector containing strand (the protector is a fluorophore quenching molecule).

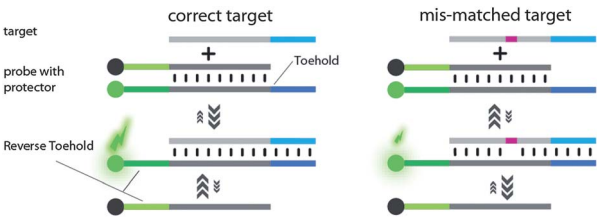

Fig. 9 Toehold exchange assay according to D. Zang *et al.*[63] In this assay, the reporter oligonucleotide contains a toehold domain that speeds up hybridization both to the target strand and the protector strand, resulting in a hybridization assay that is subject to equilibrium hybridization even under stringent conditions, such as high salinity, temperature variability and high DNA concentrations.

under typical conditions to a half-life of 10 s. This equilibrium-based toehold design is able to discriminate SNPs at any position in target sequences with a concentration of down to 1 nM even when secondary structures are present in the target. The hybridization yield does not change when excessive target is added (200×), when the temperature is varied over more than 20 °C or if the Mg^{2+} concentration is increased to several μmoles. However, a downside to this highly robust assay with the increased specificity is the much slower reaction kinetics similar to all assays that rely on conformational constrained probes.[64]

Protein mediated SNP detection

Hybridization based assays have clearly evolved in the last 5 years both in specificity and sensitivity reaching a level where these assays can compete with other means of detection. However, they are still mainly applied to detection of SNPs in short oligonucleotides. Contrary to this, protein enzymes and mismatch binding ligands are not limited to the target oligonucleotide length. This can be explained by the fact that both protein enzymes and protein ligands recognize specifically the uncomplimentary base pairs, thereby not being constrained to hybridization processes for recognition of the SNP and as such have no limitation in the target length. A technical comparison of all discussed protein based SNP assays can be found in Table 2.

Mismatch binding ligands

Mismatch binding ligands are a class of reporters that specifically recognize mismatched base pairs and bind to them with high affinity. One of the best-known mismatch binding ligands is the protein MutS, which is a part of the post replicative mismatch repair system of the *E. coli* bacteria. Recognition of a mismatch triggers structural conformational changes of this protein, which results in MutS strongly binding mismatched base pairs. MutS can be applied for identifying a mismatch in a target directly on microarrays.[66] Another application for MutS can be to enhance the discriminative power of polymerase based assays. For example, Tan and co-workers showed that binding of MutS to a mismatch in a target–primer complex assisted in inhibition of the polymerase reaction upon the presence of a mutation.[67] Furthermore, MutS, in combination with a hairpin probe, is used in electrochemical assays for detection of SNPs. Here, the hairpin is used to detect the target DNA, by opening up in the presence of the target DNA, while in the case of a mutation the electrical resistance is further increased upon binding of MutS to a SNP allowing sensitive and discriminative detection down to 100 pM of the target DNA.[68] The main limitation in the use of MutS is the disability to discriminate among different mutations and to identify multiple mutations in one strand. These limitations can be prevailed by combining MutS with gold nanoparticle (Au NP) labelled oligonucleotides complementary to the target

Table 2 Comparison of different protein mediated hybridization assays

Protein mediated SNP detection	Transducer	Optimization required	Target length (bp)	Detection limit	Number and/or position of detected mutation	Discriminate between mutations	Expected assay time
Mismatch binding ligands							
Jiao *et al.*[67]	Fluorescence	No	21	20 nM	2 SNPs	No discrimination	30 min
Cho *et al.*[69]	Colorimetric	No	21	1 μM	4 SNPs	Shift in melting temperature	80 min
Ligation based SNP detection							
Wang *et al.*[76]	Colorimetric	No	24	300 pM	1 SNP	No discrimination	30 min
Xue *et al.*[77]	Colorimetric	No	24	1 nM	5 positions with different SNPs	Grayscale	10 min
Li *et al.*[79]	SPRI	No	36	1 pM	1 position with all 3 basepair substitutions	Location of SPRI spot	15 min
Toubanaki *et al.*[80]	Colorimetric LFA	No	40	12.5 fM (after PCR)	1 SNP	Position LFA membrane	20 min
Li *et al.*[84]	Colorimetric	No	30	70 fM	1 SNP	No discrimination	3.5 h
Hu *et al.*[85]	Raman spectroscopy	No	32	10 pM	1 SNP	No discrimination	2 h
Bi *et al.*[87]	Chemiluminiscence	No	39	71 aM	1 SNP	No discrimination	1.5 h
Cheng *et al.*[91]	Electrochemical	No	51	1 fM	1 SNP	No discrimination	3 h
Shen *et al.*[95]	Colorimetric	No	45	20 aM	2 SNPs	No discrimination	1.5 h
Nicking enzymes							
Ji *et al.*[98]	Electrochemical	No	23	11 aM	1 SNP	No discrimination	2.5 h
Xu *et al.*[107]	Colorimetric	No	27	500 aM	3 SNPs	No discrimination	30 min
Exonuclease							
Zuo *et al.*[102]	Fluorescence	No	27	7.8 nM	1 SNP	Fluorescence yield	30 min
Liu *et al.*[104]	Electrochemical	No	15	10 fM	1 SNP	Current	2.5 h
Gao *et al.*[106]	Electrochemical	No	/	1 fM	1 SNP	/	2 h

sequence. When a mismatch is present in the target strand, MutS binds to this Au NP–DNA complex. This influences the melting temperature resulting in a shift to higher values in the presence of MutS. The various nucleotide mismatches influence the melting temperature differently allowing discrimination among base pair substitutions.[69]

Another group of non-protein molecules, known as intercalators, with affinity for particular mismatches,[70] can also be used as mismatch binding ligands. Although some of these intercalating molecules have been applied for SNP detection,[71–73] they are not widely used because in general they can detect only a particular subset of base pair substitutions.

Ligation based SNP detection

Ligation enzymes catalyse covalent joining of two adjacent oligonucleotides into one strand. Strands can only be joined when the nick is located between fully adjacent nucleotides and if there is a perfect match between the template and the oligonucleotides at the nick. Landegren and coworkers were the first to exploit these properties of the enzyme in an assay where oligonucleotides were joined in the presence of a SNP.[74] Since then, the ligase enzyme has been a popular tool for SNP detection as the combination of short oligonucleotide hybridization and the selective enzymatic activity ensures high assay specificity.[75] Ligase enzymes can be used in simple assays for SNP detection when combined with Au NPs.[76,77] One of these assays is based on the feature of DNA having a strong tendency to bind to the Au NP surface when in the single stranded form, a feature that is strongly diminished after hybridization into double stranded DNA. In the presence of a 24 bp wild type strand, two ssDNA strands, of which one has a fluorophore, are ligated, allowing the target and the ligated product to form a stable double stranded helix. On the other hand, the presence of a SNP in the target molecule prevents ligation of the two ssDNA oligonucleotides. Because these two oligonucleotides are individually too short to form a stable double helix with the target molecule, they remain in the ssDNA form and thus bind to the Au NP, which results in fluorescence quenching. Not only is this assay highly sensitive towards SNPs, it also has a fairly low LOD of 0.3 nM of the target DNA.

In another ligation assay combined with Au NPs, different SNP mutations are discriminated in a 24 bp target sequence using a colorimetric approach.[77] The assay relies on the hybridization of Au NPs to a glass slide surface, which are both modified with oligonucleotides complementary to the target strand. In the absence of a SNP in the target molecule, the Au NPs are ligated to the surface, followed by a signal enhancement through silver deposition on the Au NPs. This assay allows even discrimination of the SNP position in the target molecule because the presence of mutations at an increasing distance from the ligation site decreases the inhibitory influence on the ligation reaction. As a result, a gradual increase in ligation yield is visible with each shift in the mutation position.

In a similar approach, ligation of Au NPs to a surface is measured in real-time with Surface Plasmon Resonance Imaging (SPRI). Surface plasmon resonance uses partial coupling of the energy of a light wave with a noble metal, in order to measure refractive index changes at the noble metal surface,[78] thereby enabling detection of surface events such as the binding of biomolecules. In SPRI, a Charge Coupled Device (CCD) camera is used to image the noble metal surface, which is prepared in an array format where each active spot is used as an SPR active measuring point. This layout greatly increases the measurement throughput. For SNP detection, each spot of the array is functionalized with different short oligonucleotides complementary to half of the target strand. The other half of the target strand is complementary to the oligonucleotide immobilized on the Au NP surface. These captured oligonucleotides bare a different base at a known mutation site (A–T–C–G) of the target strand. Hybridization of target strands to the capture oligonucleotides on the SPRI spot triggers further hybridization with an oligonucleotide attached to Au NPs. Next, the Au NP-oligonucleotide and the capture oligonucleotide are ligated, but ligation will only occur on spots where a perfect sequence match is present at the ligation site, resulting thus in a covalent coupling of Au NPs to the corresponding array spot. The subsequent chemical denaturation removes all non-ligated Au NPs, which results in a very specific signal present only at the patch of the array where a 100% complementary capture oligonucleotide is hybridized.[79] Although this method is innovative as mutations can be detected at the nucleotide level, its applicability is limited as it is only applicable to known mutation sites.

Even more interesting is a POC concept that results in a highly sensitive assay, requiring only a couple of minutes without the need for any specialized instruments. Here, the highly specific ligation reaction is integrated into a lateral flow test and combined with the colorimetric properties of Au NPs.[80,81] As described in the previous assay, the presence of the target DNA results in the ligation of two oligonucleotides. However this assay uses oligonucleotides labelled with digoxin and the other one labelled with biotin. Antibodies specific for digoxin are immobilized on a detector zone of a nitrocellulose membrane (test zone), while molecules with high affinity towards biotin (being either an antibody or streptavidin) are immobilized on Au NPs. In the presence of the target strand, the nanoparticles are captured on the test zone, resulting in a coloured band that can be seen with the naked eye. Although, lateral flow assays are very convenient POC biosensors, reproducibility issues limit their usage. Thus, major variations can be attributed to variations in nitrocellulose membrane fabrication and changes in the surface properties of these membranes over time. Other factors that have been found to influence lateral flow assays are temperature and viscosity of the tested sample.[82]

The detection limit of ligation assays for SNP detection can be further improved when used to create padlock probes, which are circular templates for an isothermal amplification reaction known as rolling circle amplification (RCA) (Fig. 10).

This reaction requires a special polymerase enzyme, phi29, which creates a ssDNA ribbon of repeating padlock sequences. RCA combines the specificity introduced by the ligation step with a strong signal amplification resulting from the high

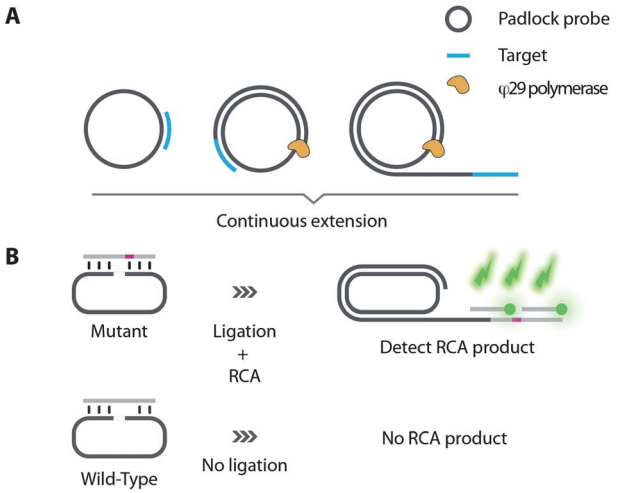

Fig. 10 Rolling circle SNP detection concept: (A) the rolling circle concept where a circular primer is used to repetitively extend a target sequence; (B) the integration of SNP detection in a rolling circle reaction: a padlock probe becomes circular only in the presence of a particular mutation (as indicated in the figure), or alternatively in the absence of SNP, depending on the assay design (not shown). The circular padlock is further extended and detected with a reporter oligonucleotide.

template copy numbers.[83] When further combined with Au NPs, a very sensitive colorimetric assay can be developed for SNP detection. The ligation of a circular primer into a padlock probe is initiated in the presence of the target strand. After the target has been amplified with RCA, its multiple copies can be cut out of the ssDNA ribbon using a restriction enzyme. These cut fragments interlink specifically Au NPs, resulting in a characteristic colour shift of the gold solution, which is visible to the naked eye. Because of the high amplification efficiency of the RCA polymerase, the assay can reach a detection limit of 70 fM even in the presence of high copy numbers of wild type DNA.[84] A similar combination of RCA and Au NPs, but without the restriction step, also allows sensitive surface enhanced Raman spectroscopy (SERS) detection of SNPs in a target strand. This SERS based assay even outperformed PCR for SNP detection.[85]

Zhang and co-workers[85] further exploited the circular ligation concept, for developing an ultrasensitive SNP detection assay.[86] Here, the presence of a SNP in the target strand triggers the formation of a circular ligation product on which multiple other circular templates can interlock by means of a complementary sequence. The interlocking circular primers contain DNAzymes, which are amplified with an RCA amplification, resulting in thousands of copies of the DNAzyme. This strategy allows creation of 1000 active enzymes, even if only a single SNP containing target strand is present, resulting in an ultrasensitive assay also when SNP expression is low. The assay was applied to detect the K-ras SNP and reached a detection limit of 71 aM even when applied directly on blood serum.

Although RCA offers an elegant target amplification strategy for ligation products,[87] the ligase reaction on its own can also be used to amplify the target sequence. In a process called ligation chain reaction (LCR), a thermophilic ligase from *Thermus thermophilus* is used (tth ligase[88]) that enables multiple sequential ligation cycles. After each ligation reaction, the temperature is increased to denature the ligation product from the target sequence, making place for two new oligonucleotides to be ligated. The main advantage of this strategy is the high specificity of the reaction due to the ligation enzyme catalysing the reaction only if the sequences at the nick are perfectly complementary and because hybridization at elevated temperatures is more specific. Nonetheless, this technology has never been very popular because the LCR products cannot be easily detected, except for laborious methods such as gel-electrophoresis.[89,90] However, recent developments have implemented the LCR reaction on new platforms allowing more straightforward detection of LCR products. For instance, Cheng *et al.* designed an assay to fluorescently detect LCR products by using a cationic conjugated polymer (CCP).[91] The assay uses two sets of probes, one complementary to the target DNA and another to the reverse complement. In the presence of the target, the first set of probes, complementary to the WT target, is ligated creating a new template for the ligation reaction of the second set of probes. This enables an exponential amplification of the target sequence. Subsequently, the CCP is added and binds electrostatically to the negatively charged DNA. Because CCP has unique light absorbing properties,[92] it is used to transfer excitation energy to a 5′ FAM label on the ligation probes, resulting in a strong fluorescence of LCR products. Non-ligated products are degraded with exonuclease I and exonuclease III. The resulting FAM labelled monomer by-products have only a very weak affinity for CCP leading to a low background signal. The assay allowed detection of targets down to 1 fM and could be applied on mixtures of WT and mutated DNA.

Trau and co-workers used a somewhat similar approach to allow electrochemical detection of LCR products.[93] In short, they showed that a successful LCR could both be detected by the intercalator methylene blue that changes the redox current or by a HRP labelled probe through electrocatalytic reduction of hydrogen peroxide. The second approach proved to be more useful as it was able to detect the target strand at 100 times lower concentrations and with higher specificity than the methylene blue approach. Albeit, HRP remains a bit unpractical for POC integration as the reaction requires a continuous stream of H_2O_2, which is not a stable component.

Finally, a method was developed to monitor LCR in real-time[94,95] ligating, in the presence of a target sequence, probes immobilized on Au NPs. Therefore, in every cycle of the LCR reaction, an increasing fraction of the Au NPs is ligated, leading to an increasing amount of closely linked Au NPs. Their interlinking results in a characteristic shift in the absorption band of the Au NP solution, which is monitored in real time using a standard spectrophotometer. The resulting assay has a very wide dynamic range spanning 6 orders of magnitude with a detection limit of 20 aM and strong discriminative power, even in the presence of 1500× excess of mutant target DNA.

Next to ligases, another class of enzymes, the so-called nicking enzymes, can be used for recognition of specific sequences, which is followed by cutting one of the strands in the double stranded DNA. Although they can only recognize a particular subset of sequence motives, which limits their

universal use, they can be used in combination with a three-way junction for specific target recycling.[96,97] This strategy was applied for developing an ultrasensitive and specific assay in combination with MBs.[98] In this assay, a MB is nicked when forming a Y junction, which can only be created in the presence of a target molecule. The opened MB serves afterwards as a primer for an RCA reaction, while the target molecule is recycled. The RCA product allows specific hybridization of multiple quantum dots, which are measured with voltammetry after being dissolved in acid. The assay has a dynamic range of 6 orders and a detection limit of 11 aM. Similar strategies for target recycling using nicking enzymes but without RCA are applied and can also reach sub-femtomolar detection limits without losing specificity, thus proving the versatility of the method.[99–101]

Target recycling can also be achieved with exonucleases, which independently catalyse the degradation of the target reporter duplex starting at the single stranded part of a blunt or at the recessed 3′ end. They were first used in combination with MBs in order to digest the MBs that get opened upon target binding, freeing thus the target-sequence (Fig. 11).[102] This strategy has two advantages; the target is released from the MB and can bind to another one, while the fluorophore is separated permanently from the quencher of the degraded MB. This approach allows a much lower detection limit to be reached than a classic MB assay with the same amount of target DNA without any loss in specificity. A similar target recycling strategy is applied for SNP detection by Xuan *et al.* on an electrochemical detection platform.[103] In their assay, target binding opens the stem of a MB resulting in its enzymatic digestion and the release of a methylene blue labelled nucleotide. This nucleotide can bind with a much higher efficiency to a negatively charged indium tin oxide (ITO) electrode than the MB, because the repulsive negative charges between ITO and the single nucleotide are lower than the ones between ITO and MB. The assay can detect label free, pM concentrations of the target sequence while differentiating SNPs with a high specificity. The sensitivity of this strategy is further improved by Tang and co-workers, who used the liberation of a DNAzyme upon exonuclease III digestion[104] instead of a labelled nucleotide. This led to an improvement of one order of magnitude in the detection limit to 10 fM, while the specificity towards SNP detection was unaffected.

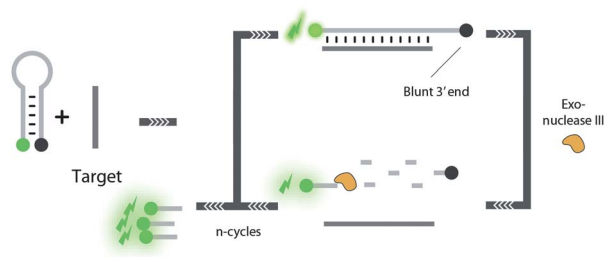

Fig. 11 Target recycling concept combining a MB with exonuclease III to liberate the target after binding with the MB. Because a blunt end is created at the 3′ end of the MB, only the MB will be degraded, separating the quencher from the fluorophore, while the target can open a new MB in a next round of exonuclease III degradation. According to Zuo *et al.*[102]

A new type of nuclease, known as the CEL1 endonuclease, combines the mismatch specificity of mismatch binding enzymes with exonuclease activity. The enzyme is therefore used to increase the sensitivity of SNP analysis on a microarray, as the enzyme can detect mismatches, inserts and deletions of up to 12 bp, initiating the degradation of mismatched oligonucleotides. Performing SNP analysis on a microarray requires highly specific hybridization, which can be achieved by lowering the salt concentrations and adjusting the hybridization temperature close to the target melting temperature. Because this approach lowers the sensitivity of the assay severely, the CEL1 endonuclease is used to digest all mismatched DNA on the microarray before readout of the fluorescent signal. One of the first applications of the CEL1 endonuclease was to improve microarray screening for antibiotic resistance in *Klebsiella pneumoniae*.[105] The same strategy can be used on DNA biosensor platforms to increase the specificity and sensitivity towards a DNA target of interest as has been shown recently.[106]

SNP detection through thermal denaturation of hybrids

Enzymes are effective tools for detection of SNPs even in the presence of complex media such as blood. However, most of the enzymatic SNP assays are only applicable to known mutations, which is just a fraction of those causing diseases.[108] Moreover, the capability of detecting unknown mutations allows acquisition of new mutations to be followed up.[109,110] Therefore, methods such as DNA melting that allow screening of mutations in a much more generic way are appreciated alternatives.

When a DNA duplex is thermally denatured, the temperature at which half of the strands are in the fully hybridized state, while the other half is in the single stranded state, is known as the melting point (T_m) and is highly sequence dependent. Changes in the DNA sequence have a direct effect on the thermodynamic stability of the DNA duplex resulting in a predictable change in T_m.[111–113] However, DNA melting was initially not easily applicable to identifying SNPs routinely as the minimal thermodynamic effect of a SNP on a DNA double strand could not be detected with then standard fluorescent intercalating dyes. These intercalating dyes are fluorescent molecules that increase their fluorescence upon binding to double stranded DNA. However, because intercalating dyes inhibit PCR, they are used at non-saturating concentrations, at which the dye can redistribute during the DNA melting process to the remaining double stranded part of the DNA complex, resulting in a hampered resolution of the melting curve. Initially, this problem was solved using specially designed fluoresence resonance energy transfer (FRET) hybridization probes, which even allowed multiplex melting analysis of different targets using multiple fluorescent dye molecules.[114] Later on, the technology was made generally applicable by introducing saturating dyes. These dyes can be used at a much higher concentration without PCR inhibition. As a result, monitoring of DNA melting is made available at an unmet resolution without the use of labelled detection probes, providing a universal SNP detection platform.[115] The technology

has been commercialized and is gaining popularity as a worthy alternative for differential gel electrophoresis,[116] the golden standard technique for mutation detection, which is based on gel separation of PCR products with mutations.

DNA melting is also being applied as a first line fast mutation screening tool to identify possible mutational hotspots.[117,118] Nonetheless, the technology is not perfect and requires optimization for detection of some types of mutations such as base pair neutral mutations, where for example a G–C from C–G is mutated or A–T from T–A base-pair.[119] Recently, new developments have been made in the field of DNA melting technology increasing both the sensitivity and applicability of the method. In general, one can distinguish two approaches in DNA melting analysis, liquid and solid phase DNA melting methods. An overview of all DNA melting assays discussed in this section can be found in Table 3.

Liquid phase DNA melting

Liquid phase DNA melting monitors the denaturation of DNA free in solution and, in comparison with solid surface melting, the DNA does not need to be chemically modified for surface immobilization. Furthermore, the method can be applied immediately after PCR as the saturating intercalating dye does not inhibit amplification, which even allows monitoring of the amplification process, thereby resulting in both quantification and direct identification of amplicons. As the technology has mostly been used in combination with a traditional PCR sample preparation and on bulky high resolution melting (HRM) enabled qPCR devices, the technology was for a long time considered as not being fit for POC concepts. In an attempt to solve this problem, Crews *et al.* integrated DNA melting analysis in a microfluidic chip.[120] Here, the liquid flows through a microfluidic channel over a temperature gradient. While the sample is slowly heated along this gradient, the DNA double strand is melted, which is visible by a decrease in the fluorescence intensity of the intercalating dye. As the position along the microfluidic channel, where the fluorescence signal is switched off, represents the melting point, this method is called spatial DNA melting. Spatial DNA melting allows monitoring of both DNA denaturation and renaturation along the temperature gradient since the fluorescence intensity increases when leaving the temperature gradient in the opposite direction as a result of gradual re-annealing of the DNA duplex. The method proved to be highly sensitive towards SNPs and allowed discrimination of heterozygous mixtures of WT and mutant DNA in real clinical samples even when targets exceeded 150 bp. The concept was further developed and integrated in a chip for direct genotyping from a human saliva sample[121] proving the applicability of the concept.

Another label free DNA melting approach is to monitor the change in movement of the molecule rather than the change in fluorescence. This method is called thermophoresis and has some particular advantages over traditional DNA melting. The movement of molecules in a temperature gradient depends on their size, charge, ionic shielding and hydration, implying that change in any of these parameters can be monitored by the changes in thermophoresis. Using this method, Wienken *et al.* were able to detect SNPs of target sequences in very low sample volumes (250 nl). Although thermophoresis has only been applied on short oligonucleotides (22 bp) at high concentrations (1 μM), the method is very sensitive towards secondary structures and nucleotide modifications such as methylation.[122]

Currently, one of the major limitations of DNA melting is that the small free energy difference caused by the polymorphisms results in only a minimal variation of the melting profile measured as a function of change in the fluorescence intensity, requiring careful experimental design and data analysis. Lee *et al.* designed a strategy to amplify the thermal dynamic difference allowing easy discrimination of mutations. By monitoring DNA melting inside an optofluidic cavity that is excited with a laser, a gain in laser signal intensity is realized that is dependent on the hybridization state of the DNA in the cavity.[123] As can be noted in this assay, the generated laser

Table 3 Comparison of different solid phase hybridization assays

SNP detection through thermal denaturation of hybrids	Transducer	Optimization required	Target length (bp)	Detection limit	Number and/or position of detected mutation	Discriminate between mutations	Expected assay time
Liquid phase DNA melting							
Crews *et al.*[120]	Fluorescence	No	190	PCR amplified	Different SNPs, heterozygous	Melt profile	2–90 min
Wienken *et al.*[122]	Thermophoresis	No	22	1 μM	SNPs, but especially sensitive to methylation	No discrimination	1 h
Lee *et al.*[123]	Laser intensity	No	100	250 μM	1 SNP	Laser intensity	/
Liao *et al.*[124]	Fluorescence	No	100	PCR	48 SNPs	2D label	2 h
Solid phase DNA melting							
Stehr *et al.*[143]	Colorimetric	No	30	1 μM	1 SNP	Extinction time	Milliseconds
Van grinsven *et al.*[146]	Thermal resistance	No	29	600 pM	3 SNPs	Melting temperature	2 h
Knez *et al.*[151]	SPR	No	80	1 nM	9 SNPs	Melting temperature	20 min

output of the optical cavity is monitored instead of fluorescence, which results in a strong amplification of the signal. The method is highly sensitive and can detect a SNP in a 100 bp target with a 25× signal intensity difference between the WT and the mutant DNA, allowing more easy discrimination of SNPs in comparison with standard HRM. However the method is only proven to work at very high DNA concentration (250 μM), limiting this technique to post PCR DNA melting analysis.

DNA melting technologies are also limited in their ability to detect multiple targets in parallel in one sample. This is due to the fact that only one intercalating dye can be used at a time, resulting in the detection of only a single target. In recent work, Li and co-workers increased the potential for multiplexing both DNA melting assays and PCR by combining different fluorescent labels with T_m labels, creating a two-dimensional label (Fig. 12).[124] The assay uses two different oligonucleotides that are ligated together in the presence of a specific target sequence. The two oligonucleotides consist of different domains necessary for multiplexing.

Both right and left ligating probes have a genomic DNA-specific binding site and primers, whereas the left ligating oligonucleotide also contains a T_m tag. The T_m tag is a sequence not complementary to the target. For each target, a unique set of T_m tags is made which are ligated in the presence of particular local SNPs. The specificity of the ligation reaction allows introduction of a different tag if the target contains a particular SNP. Because each T_m tag comes with a different sequence variation, their melting temperatures are diverse. After the tag is ligated, the ligation product containing the temperature tag is amplified with an asymmetric PCR and probes are used to hybridize to the amplified tags. Finally a melting analysis is performed to identify the amplified tags. Performing simultaneous genotyping of 48 forensic SNPs occurring on 23 different human chromosomes proved the usefulness of the assay. Therefore three different reactions using each a unique T_m tag allowed identification of 32 possible alleles per reaction, resulting in a total of 96 identified alleles. The robustness of the method was proven by its direct application on clinical samples.

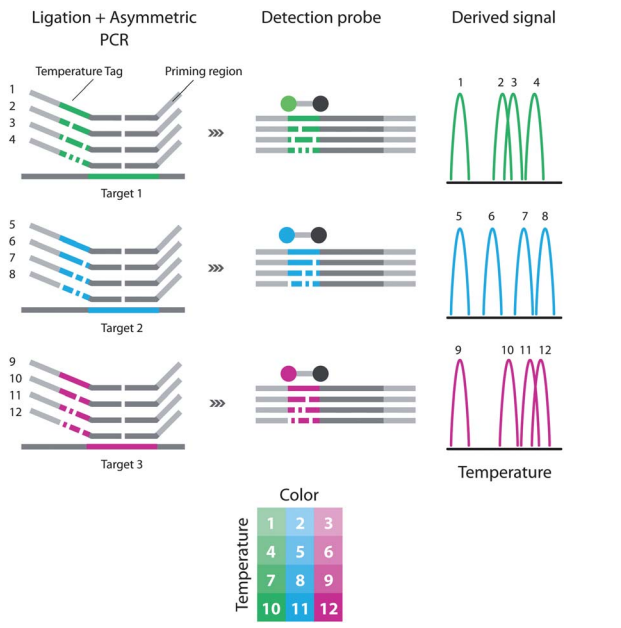

Fig. 12 Combination of fluorescent labels and ligation labels that affect melting temperature in order to increase the multiplexibility of SNP assays. Each target has its own fluorescent label, while internal target variation (SNPs) results in ligation of an extra tag (T_m tag), which is incorporated into the asymmetric PCR product. The amplified tags are used to identify the target and the resulting change of melting temperature is used to generate a 2D label. Reproduced according to Liao *et al.*[124]

Surface-immobilized DNA melting

DNA melting performed on a surface has some advantages over liquid phase melting, such as extra options to increase the sensitivity and parallelization of DNA analysis[125,126] that compensate for the possible downsides, mostly being slow hybridization kinetics due to steric hindrance and electrostatic repulsion,[127,128] as well as the need for labelled oligonucleotides for surface immobilization.[129] To overcome the first limitation, extensive research has been done on surface immobilization methods. It has become clear that the density and conformation of the DNA on the sensor surface are critical for hybridization efficiency.[130–132] These two parameters can be carefully controlled using small alkane thiol molecules or extending the oligonucleotides with adenine repeats, known to have high affinity for gold surfaces. Both approaches helps in organizing the DNA strands on the sensor surface while also diluting the strands on the surface, which results in better accessibility of the target strands and improved hybridization kinetics.[133–137] Other options include the use of small internal complementary DNA strands that can boost hybridization kinetics on surfaces by assisting in DNA organization and presentation, a strategy that can result in a 5-fold increase in the hybridization rate.[138] Moreover, new strategies allow DNA surface immobilization within minutes,[139] making it an easy procedure. Lastly, it has even been shown that labelling is not a necessity for surface immobilization.[140]

Mirkin and co-workers were the first to show the great potential for DNA melting assays on surfaces.[141] They discovered enhanced resolution in melting analysis when DNA is immobilized on Au NPs, the so-called spherical nucleic acids. In comparison with DNA melting in solution, where the melting phase occurs over a range of approximately 20 °C, gold labelled DNA has a melting transition over a very narrow temperature range (*ca.* 2–8 °C) which is shifted to a higher temperature range. One explanation for this melting behaviour is the number of interparticle connections at the nanoparticle surface, which results in a collectively stronger bond that melts at higher temperatures. Together with a higher local salt concentration at the NP surface, this cooperative binding results in a more narrow melting transition. This was proven by a broader melting transition when a number of strands, and consequently a number of interparticle connections on the nanoparticle surface, were diluted.[141,142] Using this enhanced melting resolution, Stehr *et al.* designed a SNP detection assay, in which Au NPs acted both as carriers of reporters for the target

DNA and local heating elements.[143,144] As stated earlier, Au NPs have a strong light adsorption wavelength, which varies with the size of the nanoparticle. When a pulsed laser source is used at this specific adsorption band of the Au NPs, an optothermal conversion can be realized. Au NPs transform light into thermal energy, resulting in a fast temperature increase in the local nanoparticle surrounding. This highly sensitive and fast SNP assay is capable of performing DNA melting analysis for SNPs in a 30 bp target strand in less than a millisecond.

Although DNA melting on nanoparticle surfaces allows better melting transitions, their monitoring is limited to either the use of laser absorption or scattering techniques such as dynamic light scattering (DLS) or classic spectrometry based methods.[145] These techniques are all somewhat limited in sensitivity as they measure a bulk effect and do not support easy real-time monitoring of the melting process. Other options include electrochemical sensors such as the one recently described to monitor the transition from single stranded to double stranded DNA.[146] By immobilizing the complementary DNA strand of a 29 bp target on a diamond surface, which allows excellent thermal conduction, the biosensor is used to monitor DNA binding during temperature cycling. Because the solution was heated from below the diamond sensor surface, the authors observed a strong heat transfer resistance when DNA was single stranded, while the resistance was not present when the DNA was in a double stranded state. Using this principle of heat resistance, the authors can derive the melting point of the DNA duplex from the temperature measurement at the sensor surface corrected for the heating power input. This method made SNP detection readily available on a cheap platform, with a sub-nanomolar (600 pM) sensitivity, although the concept was only able to detect SNPs in short oligonucleotides.

As discussed earlier, SPRI allows direct measurement of DNA behaviour at a surface in real-time and with high precision, making this technology ideally suited for DNA melting. Fiche *et al.* were one of the first to implement continuous real-time DNA melting monitoring on an SPRI chip.[147] This strategy allowed detection of SNPs in short oligonucleotides, albeit at high DNA concentrations (1 μM). The assay was further optimized towards a universal HRM protocol on microarrays for detection of low abundant DNA with mutations.[148] By using temperature cycling, they were able to concentrate the target DNA on the array surface. Hybridization at the optimal temperature of the target sequence allowed a 10-fold increase in hybridization of mutated DNA on the surface in comparison with the sample content. This approach lead to ultrasensitive detection of mutated DNA in heterozygous mixtures even when the DNA of interest was present at a fraction (1%) of the total DNA concentration. Nonetheless, in general, these melting transitions did not show the same narrow melting transitions as observed on the nanoparticles surfaces (<10 °C) because the assay monitors the melting behaviour of unlabelled DNA strands.

Knez *et al.* addressed this by using SPR to monitor melting of DNA immobilized on Au NPs.[149] Here, the narrow melting transitions of Au NPs were combined with SPR real-time monitoring of the melting process, enabling great possibilities for SNP detection (Fig. 13). This was possible due to an in-house developed SPR platform integrated in an optical-fiber dipstick (FO-SPR),[150] which is more easily combined with nanoparticle solutions than microfluidic based SPR layouts that require extensive cleaning of their small channels after each run with NPs.

Furthermore, using a modified thermocycler, high precision temperature control is enabled allowing HRM on the FO-SPR device. The assay monitors binding of Au NPs onto a capture probe, immobilized on the FO-SPR sensor surface, through hybridization with the target DNA. Binding of Au NPs results in a signal increase, both for WT and mutant DNA. Subsequently, with increasing temperatures, the SPR signal shows a gradual decrease due to a decrease of the sample density (*i.e.* the refractive index decreases as a result of temperature increase). However, when the melting point of the captured DNA is reached, the SPR signal decrease becomes superimposed by the drop in signal due to the Au NP detachment from the surface. Because the WT DNA forms a thermodynamically more stable complex on the sensor surface with the Au NPs, dissociation of this complex will occur at a higher temperature than that in the case of mutant DNA, increasing the sensitivity of SPR strongly for SNP detection. The width of the derived melting peak is comparable to the melting of DNA complexes on Au NPs (<5 °C). The FO-SPR assay is that sensitive to mutations, that it can even resolve their position in the DNA duplex, enabling location awareness of the mutation.[151] Furthermore, the method could also detect low abundant SNPs, outperforming in a direct comparison both HRM and classical Sanger sequencing for detecting these mutations.[149] In a proof of concept study, the FO-SPR assay was applied to identify SNPs in PCR amplicons of a locus found in a gene of the bacteria *L. pneumophila*.[152] The FO-SPR SNP assay had a good sensitivity in the low nanomolar range and could detect mutations in oligonucleotides of up to 80 bp. However, in order to make this assay a true asset for POC diagnostics, the detection of multiple targets is necessary, requiring further developments in multiplexing.

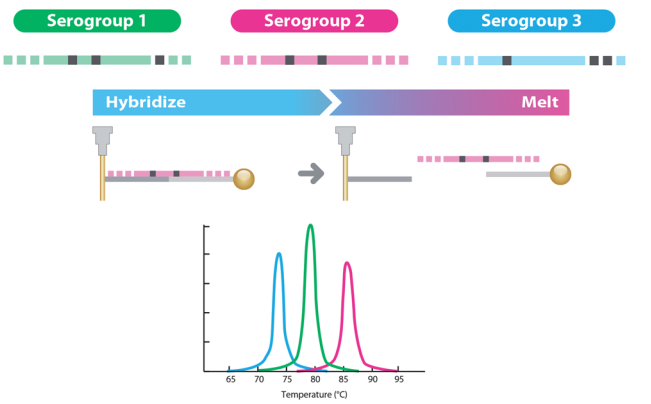

Fig. 13 FO-SPR SNP detection concept, where Au NPs are bound to the sensor in the presence of a target. When mutations are present in the target DNA, the melting temperature of nanoparticle–DNA complex will shift according to the type and position of mutation. Reproduced according to Knez *et al.*[149]

Conclusions

The last 3 years have seen a remarkable evolution in SNP detection techniques, leading to an unmet resolution by employing albeit simple but clever techniques. This evolution has made classic labour intensive assays such as DGGE, PCR, FISH and others obsolete and allowed the design of simple and fast SNP assays that can be integrated in POC tests. In general, the improvements in SNP detection have been achieved with technological advances in one of the following three domains.

1. Hybridization sensitivity.
2. Enzymatic cascades.
3. Thermal DNA denaturation.

Better understanding of processes that determine the speed and specificity of hybridization have enabled the design of target specific DNA probes for detecting SNPs at room temperature. The employment of specific short hybridization oligonucleotides resulted in more sensitive and robust SNP detection. Furthermore, the integration of binary and ternary probes, which rely on independent hybridization events, resulted in both the high specificity and sensitivity within the same assay. This was achieved by activation of a label only when all probes are hybridized to the target strand. Although these advanced hybridization probes are ideally suited for SNP detection, they still rely on complex DNA binding probe designs. This problem has been addressed by the reversible toehold, a universally applicable concept that allows us to make hybridization probes insensitive to external factors, such as temperature and ionic concentration, for their specificity and sensitivity. This enabled SNP identification under almost any condition.

A lot of effort has been made to make SNP assays work at low target concentrations. Therefore several strategies have been employed to strongly amplify SNP detection signals, such as the use of multiple enzymes for SNP detection. Also, some enzymes only work in the case of a perfect sequence match, so enzymes not only increase sensitivity but can also be used to enhance the reaction specificity. One of the most important findings in the last decade are so-called enzymatic cascades, which rely on the activation of multiple enzymes in the presence of the target sequence resulting in detection limits that can compete or even outperform PCR DNA amplification. This is very important, as PCR is known to introduce errors while amplifying target sequences, which decreases the sensitivity of the method.[153] Another important concept introduced through this approach is enzymatic target recycling. In this concept specific enzymes degrade the reporter probe while bound to the target sequence allowing the next reporter to bind to the target. These self-amplifying strategies result in highly sensitive assays, whilst maintaining specificity.

Lastly, a lot of research has been done on the improvement of DNA analysis by monitoring thermal denaturation, as this assay allows screening for unknown mutations. Of all new techniques, HRM is the only one that is becoming a standard procedure both in research and in clinical laboratories.[154] New developments have made the technology even more versatile, especially the application of the technology on Au NP supports, resulting in improved SNP sensitivity. Furthermore the first microfluidic integrated POC test based on the DNA melting assays has seen the light, proving that bulky thermocyclers are not a necessity for HRM.

All the summarized techniques contribute individually to the general applicability of SNP assays by introducing increased assay robustness, sensitivity and specificity. Nonetheless, some limitations are not yet resolved and require special attention in the coming years to push the development of molecular diagnostics. The main limitation of the state of the art technologies is sample throughput, multiplexing and mutation discrimination. Most of these assays are not able to detect more than 4 targets at the same time, nor allow fast processing of multiple samples. Also, they are not capable of discriminating a standard polymorphism from a pathologic mutation. Current screening tools that are being commercialized are limited by these problems.[155] Therefore, future research should focus on the combination of different technologies to address these limitations. For example the assay Liao *et al.* developed combines ligation SNP detection with DNA melting which allows multiplexing of up to 50 different SNPs.[124] If this assay would be integrated on a micro-array and combined with Au NP enhanced melting, a large number of mutations could be detected in one run. Furthermore recent advances in microfluidics can address the throughput of these assays by automating array-based systems.[156] If these needs can be met, SNP diagnostics will become an indispensable tool in development of personalized medicine.

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