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Novel biosensing methodologies for improving the detection of single nucleotide polymorphism

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ABSTRACT

The growing volume of sequence data confirm more and more candidate single nucleotide polymorphisms (SNPs), which are believed to reveal the genetic basis of individual susceptibility to disease and the diverse responses to treatment. There is therefore an urgent demand for developing the sensitive, rapid, easy-to-use, and cost-effective method to identify SNPs. During the last two decades, biosensing techniques have been developed by integrating the unique specificity of biological reactions and the high sensitivity of physical sensors, which provided significant advantages for the detection of SNPs. In this feature article, we focused attention on the strategies of SNP genotyping based on biosensors, including nucleic acid analogs, surface ligation reaction, single base extension, mismatch binding protein, molecular beacon, rolling circle amplification, and strand-displacement amplification. In addition, the perspectives on their advantages, current limitations, and future trends were also discussed. The biosensing technique would provide a promising alternative for the detection of SNPs, and pave the way for the diagnosis of genetic diseases and the design of appropriate treatments.

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Contents

1. Introduction

With the completion of human genome sequencing, the analysis of variations among individual genomes has become a focus in recent research. One of the most common forms of genetic variation is the single nucleotide polymorphism (SNP), which is the single nucleotide variation in a given and defined genetic location and occurs in human genome at a frequency of approximately 1 in every 1000 bases (Collins et al., 1998). Currently, about 1.42 million SNPs have been identified by the SNP consortium

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(Sachidanandam et al., 2001). SNPs are highly conserved and widely distributed in the genome, so the map of SNPs proves to be a high-resolution genetic marker (Hood et al., 2004; Jorde, 2000). In addition, these benign sequence variations in gene coding regions may alter the amino acid sequence and in turn influence the function of the corresponding protein (Collins et al., 1997; Kim and Misra, 2007). All these properties collectively suggest that SNPs could be used as a new generation of genetic markers and valuable indicators for clinical diagnosis and prognosis.

Various techniques for SNP genotyping have been reported in recent years. Classic methods such as DNA sequencing could be used for the detection of new and unknown SNPs (Syvanen, 2001), but the complex procedures and lengthy operation times make direct sequencing sub-optimal. The alternative approaches include conformation changes (Salimullah et al., 2005; Tahira et al., 2009), mass spectroscopy (Millis, 2011), polymerase chain reaction (PCR) (Beaudet et al., 2001; Fujii et al., 2000), and DNA hybridization (Barreiro et al., 2009; Russom et al., 2006). These approaches could specifically detect SNP-containing regions to avoid complicated DNA sequencing, but the intrinsic shortcomings, such as low throughput and specificity, limit their applications. Recently, DNA microarray and denaturing high performance liquid chromatography have been proposed for fast, efficient, and large-scale analysis of SNPs (Deulvot et al., 2010; Ding and Jin, 2009). However, these methods require expensive facilities and radioactive/fluorescent tags. In this context, the rapid, simple, and specific technology is urgently needed for high throughput SNP analysis in both basic research and clinical diagnosis.

At present, biosensing techniques have been developed and adopted to SNP detection for their advantages of high sensitivity, good reproducibility, and short detection time. Moreover, in the use of biosensing techniques, it is possible to detect multiple SNPs in the same biosensor by constructing a microarray, which will increase the fluxes of detection and decrease the cost. This review focuses only on the latest trends in biosensor-based SNP genotyping. Firstly, the basic knowledge on the properties of biosensors is presented. Subsequently, the evolution of biosensor research is introduced. Then, the strategies for SNP genotyping based on biosensors are covered. The following conclusions provide the future trends for development of novel, cost-effective, and high-throughput approaches for SNP genotyping with biosensors.

2. Biosensors

Biosensors are the devices that use biochemical reactions mediated by isolated enzymes, organelles, or whole cells to detect the effects of chemical compounds by electrical, thermal or optical signals, as defined by the International Union of Pure and Applied Chemistry (Thevenot et al., 2001). The biosensor has received considerable attentions due to its sensitivity, selection, and specificity with detection of the concentration of a specific substance (Murugaiyan et al., 2014; Turner, 2013). In general, the biosensor essentially consists of a biological sensing element and a physicochemical transducer (Fracchiolla et al., 2013). The biological sensing element contributes to transforming the analyte of interest into chemical signal or physical signal. And then, the physicochemical transducer, as the key component of biosensors, could effectively convert the chemical or physical signal into a thermal, electrical or optical signal (Bohunicky and Mousa, 2010). Biosensors could be divided into four classes based on the biological sensing elements: enzyme-based biosensors, antigen/antibody-based biosensors, cell/virus-based biosensors, and nucleic acid-based biosensors. Alternatively, biosensors are also classified based on the type of transducers: optical biosensors, electrochemical biosensors, mass sensitive biosensors, and calorimetric biosensors (Chang et al., 2014; Chen et al., 2005; Mehrvar and Abdi, 2004; Monošík and Šturdík, 2012; Zhang et al., 2013b). The classification of biosensors is shown in Table 1.

Table 1
The classification of biosensors.

Transducers	Biological sensing elements
Optical	Enzyme-based biosensors
SPR biosensors	Antigen/antibody-based biosensors
Colorimetric biosensors	Cell/virus-based biosensors
BRET	Nucleic acid-based biosensors
FRET	
Electrochemical	
Amperometric biosensors	
Impedimetric biosensors	
Conductometric biosensors	
Mass sensitive	
QCM	
Acoustic wave biosensors	
Calorimetric	

Abbreviations: SPR, surface plasmon resonance; BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; and QCM, quartz crystal microbalance.

The basic concept of biosensors was proposed by Leyland C. Clark in 1962 (Newman and Setford, 2006). The first published paper concerning the biosensor dated back to 1982, but the development in the field of biosensors was phenomenal. More than 4000 papers concerning the biosensor published annually in the last five-year period (ISI Web of Knowledge database, Thomson Reuters) (Fig. 1). The huge development of biosensors has expanded considerably the range of applications which included biochemistry, clinical analysis, drug screening, and environmental monitoring (Long et al., 2013; Shankaran et al., 2007). Currently, About 37% of articles dealing with biosensors have been published in Chemistry, 32% in Biochemistry molecular biology, while the rest were published in the fields such as Instruments instrumentation, Material sciences, Medical laboratory technology, and Electrochemistry. The classification of published scientific papers by research areas is shown in Fig. 1.

3. Strategies for SNP genotyping based on biosensors

3.1. Strategy 1: nucleic acid analogs-based biosensors

Nucleic acid analogs, as the research tools and diagnostic agents, have been synthesized by interspersing natural nucleobases with artificial nucleobases or modifying internucleoside linkages. The use of nucleic acid analogs has experienced a significant upsurge of interest during the past decade. The nucleic acid analogs are becoming increasingly important in the field of biosensors because of their high selectivity, chemical stability, and affinity toward complementary DNA/RNA.

3.1.1. Peptide nucleic acid-based methods

Peptide nucleic acid (PNA) is a neutral DNA mimic in which the sugar phosphate backbone of natural nucleic acid is replaced by the pseudopeptide backbone composed of N-(2-amino-ethyl)-glycine units (Wittung et al., 1994). Since PNA was first introduced by Nielsen et al. in 1991, it has been confirmed that PNA could efficiently bind to complementary DNA or RNA strand in a Watson–Crick hydrogen bonding rule, with high specificity and affinity (Nielsen et al., 1991). The better thermal stability and higher association constant have been revealed in the

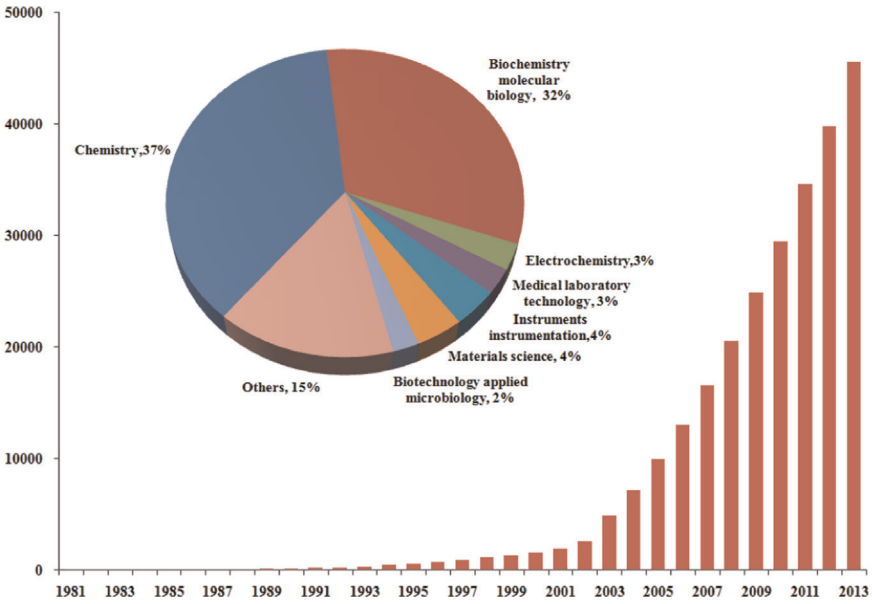

Fig. 1. The number of published papers during the period 1981–2013 (ISI Web of Knowledge database, Thomson Reuters). Inset: distribution of published papers by main application fields.

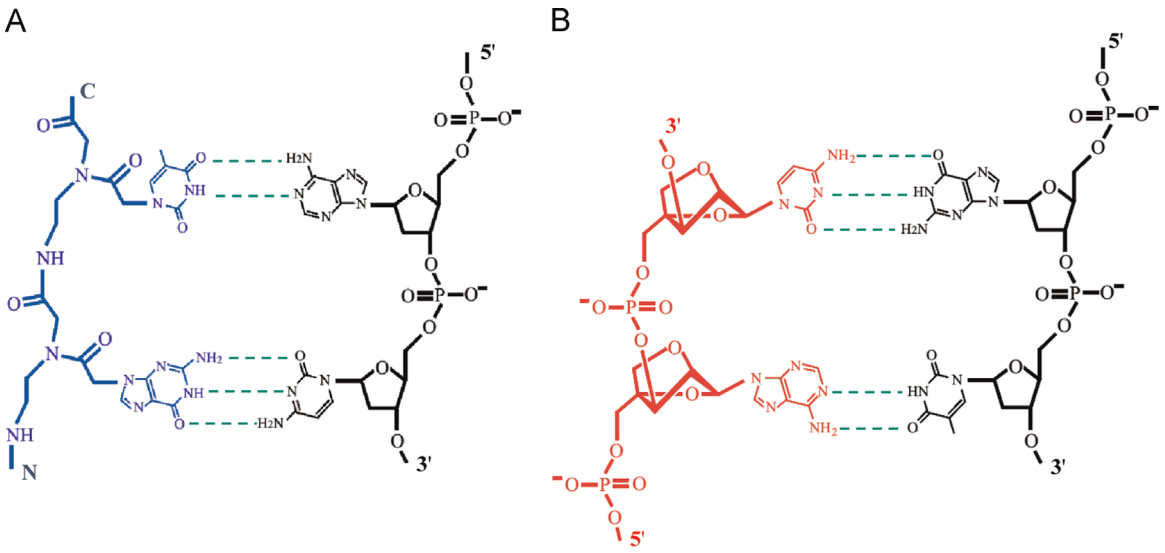

Fig. 2. Schematic chemical model of nucleic acid analogs hybridized with DNA. (A) PNA/DNA duplex. (B) LNA/DNA duplex. PNA depicted by blue. LNA depicted by red. DNA depicted by black. The hydrogen bonding between complementary nucleobases depicted by dotted lines. Modified from Briones and Moreno (2012). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

order: DNA–DNA < PNA–DNA < PNA–RNA < PNA–PNA (Ratilainen et al., 2000). Hence, the melting temperature (T_m) of PNA–DNA duplex is greatly affected by the presence of a single-base mismatch. The T_m of 9- and 12-mer PNA–DNA duplexes with a variety of single-base mismatches could decrease 15–20 °C in comparison to that of the perfectly matched sequences (Ratilainen et al., 2000). In addition, the PNA-based hybridization is no electrostatic repulsion and independent from the secondary structure of target DNA, therefore, PNA could efficiently bind to the single-stranded DNA (Endo et al., 2005). All these properties make the PNA as an optimum probe of biosensors for SNP detection. The schematic chemical model of PNA/DNA duplex is illustrated in Fig. 2A.

The advantages of using PNA as recognition elements in biosensors were first introduced by Wang et al. (1997). In their experiments, the 15-mer thiol-derivatized PNA probe was immobilized on the surface of quartz crystal microbalance (QCM), and then the remarkable mismatch discrimination associated with the PNA/DNA hybrid was illustrated in connection with the detection of a single-base mismatch in the p53 gene. A detection limit of about 1 μg/mL was achieved on the basis of the signal-to-noise characteristics ($S/N=3$). Following this achievement, Hu et al. constructed an electrochemical DNA biosensor by employing covalently immobilized PNA probes. The electrochemical DNA biosensor presented a good linear range from 0.1 fmol/L to 0.1 nmol/L with a detection limit of 62.41 amol/L (Hu et al., 2015). Gao et al. designed a label-free strategy for surface-enhanced Raman spectroscopic detection of target DNA on a PNA modified glass slide. The method showed a detection limit of 3.4 pmol/L (Gao et al., 2013a). Although effective discrimination against a single-base mismatch in DNA target was achieved, these papers did not test their biosensors with PCR-generated targets or real samples. Nguyen and Sim (2014) demonstrated the possible use of PNA to efficiently identify a single-base mismatch in real samples. In their work, a PNA-based biosensor could enrich and detect the

tumor-specific mutations of circulating tumor DNA based on surface plasmon resonance (SPR) and the coupling plasmon mode of gold nanoparticles. Another biosensor based on leaky surface acoustic wave, developed by Wang et al., was able to detect a single-base mismatch in human papilloma virus genomic DNA without PCR amplification (Wang et al., 2009).

Nevertheless, although the detection scheme is simple and specific, the general sequence recognition by invasion of the DNA double helix exploiting just the Watson–Crick hydrogen bonding rule needs be achieved by helix invasion using duplex-forming PNAs (Lohse et al., 1999). In this case, mixed pyrimidine–purine sequence PNAs do not provide sufficient free energy for hybridization. Thus, the longer hybridization time is required to increase the hybridization efficiency in SNP detection (Briones and Moreno, 2012). Recently, a new modification of PNA has been introduced for sequence-unrestricted targeting of double-stranded DNA (Kim et al., 2007; Protozanova et al., 2003). The dubbed pseudo-complementary PNAs (pcPNAs) carry 2, 6-diaminopurines and 2-thiouracils instead of adenines and thymines, respectively. The pcPNAs are able to more efficiently bind to complementary DNA sequences by the formation of double duplex-invasion complex (Demidov et al., 2002). Thus, the pcPNAs may provide a rapid strategy for SNP genotyping based on biosensors in the future.

3.1.2. Locked nucleic acid-based methods

Locked nucleic acid (LNA) was described by the Imanishi and Wengel groups in 1997 and 1998, respectively (Koshkin and Wengel, 1998b; Obika et al., 1997). Since then, LNA has attracted people's widespread attention and been used as a novel oligonucleotide tool. LNA is an oligonucleotide analog in which the 2′-oxygen and the 4′-carbon of ribose moiety are joined via an O-methylene (oxy-LNA) bridge, S-methylene (thio-LNA) bridge, or amino-methylene (amino-LNA) bridge. The covalent bridge can effectively lock bicyclic furanose unit in the N-type (3′-endo) conformation, increasing the degree of local organization of phosphate backbone and reducing the conformational flexibility of ribose (Koshkin et al., 1998a). Due to this chemical difference, LNA induces a more efficient stacking of nucleobases, which leads to twist the B-form dsDNA helix structure into A-form helix structure (Nielsen et al., 1999). Hence, the LNA is capable of sequence-specific binding to complementary single stranded DNA or RNA with a higher affinity. The schematic chemical model of LNA/DNA duplex is illustrated in Fig. 2B. The T_m increase for LNA/DNA duplex ranges from 2.0 to 6.0 °C with each LNA substitution, and the increase of T_m is up to 3.0–9.6 °C for LNA/RNA duplex (Briones and Moreno, 2012). In contrast to DNA/DNA duplex, the LNA/DNA duplex is more destabilized in the presence of a single-base mismatch. In addition, LNA has several peculiar properties, including commercial availability, nuclease resistance, and enhanced triplex formation. These advantages of LNA make it a very flexible alternative in nucleic acid based detection technologies.

An early example of the usefulness of LNA oligonucleotides in biosensing is the detection of factor V Leiden mutation by direct allele-specific hybridization of PCR amplicons in 1999 (Orum et al., 1999). In their experiments, LNA octamer probes (complementary to either the wild type or mutated sequence) were self-assembled on the individual wells of a microtiter plate. Then, LNA probes hybridized with the biotinylated PCR amplicons, which were tested by the chromogenic reaction between horseradish peroxidase and tetramethylbenzidine substrate. Soon after, electrochemical biosensors benefited from LNA potential for the recognition of single-base mismatch. Wang et al. designed a LNA probe for the detection of promyelocytic leukemia/retinoic acid receptor alpha (PML/RARα) fusion gene based on a sandwich-mode amperometric biosensor (Wang et al., 2011). The results exhibited a detection limit of 74 fmol/L, and a linear range from 0.1 pmol/L to 10 pmol/L. Later, the combination of strand displacement reactions and LNA modified probes was used to detect the mutation of p53 gene (Gao et al., 2014). This method exhibited a remarkable discrimination capability of SNPs among 6000-fold (m/m) excess of genomic DNA. In a different approach, combined use of the plasmonic nanowire interstice biosensors and LNA modified probes was reported to detect the diverse single-base mismatches in various microRNAs (Kang et al., 2014). In addition, Ho et al. reported a sensitive and specific platform for SNP detection using short LNA probes and total internal reflection fluorescence microscopy (Ho et al., 2014).

As summarized in the previous sections, PNA and LNA-based biosensors exhibit many advantages in SNP genotyping. However, the shortcomings cannot be ignored. The most common disadvantage of PNA and LNA-based biosensors is sequence limitations in design of PNA and LNA probes, such as probe length, GC content (between 30% and 60%), and consecutive Gs (less than 3).

3.2. Strategy 2: surface ligation reaction-based biosensors

The ligase-mediated gene detection techniques were first introduced in 1988 (Landegren et al., 1988), and have been widely used for SNP genotyping. The principle of surface ligation reaction-based biosensors is illustrated in Fig. 3. Capture probes are self-assembled on the gold electrode surface. The 3′s terminus of the capture probe completely matches with wild-type target. Therefore, after hybridization, the capture probe could be further ligated to the biotin-tagged detection probe by using DNA ligase. Then, the surface-terminated detection probe is conjugated with streptavidin labeled enzyme via biotin-streptavidin binding. Finally, the enzyme catalyzes substrates to form the biosensor signal, which indicates the presence of wild-type target. In comparison, no ligation occurs for the juxtaposed probes associated with a single-base mismatched target.

The surface ligation reaction has been proposed for SNP analysis with optical biosensors, electrochemical biosensors, and mass sensitive biosensors. Knez et al. demonstrated a highly sensitive fiber optic SPR biosensor for SNP detection based on surface ligation reaction (Knez et al., 2014). In addition, Liu et al. described a 16-electrode of electrochemical biosensor array for high-throughput SNP analysis using the surface ligation reaction (Liu et al., 2013). The 16-electrode of biosensor array offered a multiplexed platform for the simultaneous detection of SNPs. Although the surface ligation reaction provides a strategy for SNP genotyping, it is still difficult to identify SNPs without further amplification. Effective signal amplification is significant for enhancing the sensitivities of SNP detection. Yin et al. demonstrated a DNA biosensor based on the color and size changes originating from surface ligation reaction-based gold nanoparticle assembly as an indicator (Yin et al., 2014). The detection limit was 1.5 amol/L with a linear range from 0.01 fmol/L to 1000 fmol/L. Our laboratory also described a technique for the detection of SNPs by integrating a novel leaky surface acoustic wave biosensor, surface ligation reaction, and enzymatic signal amplification (Xu et al., 2012). The leaky surface acoustic wave biosensors showed a detection limit of 1 pmol/L, and a linear range from 1 pmol/L to 1 μmol/L. Furthermore, tagging probes with other markers, such as fluorescence (Hashimoto et al., 2006), quantum dots (Song et al., 2013), and magnetic beads (Chen et al., 2012), have also been used for signal amplification. Based on the above mentioned, the surface ligation reaction-based biosensor offers a simple, sensitive, and specific approach for SNP detection. Nevertheless, it's challenging to apply the method in practice because of the complicated detection schemes and unknown SNPs or mutations in sequence.

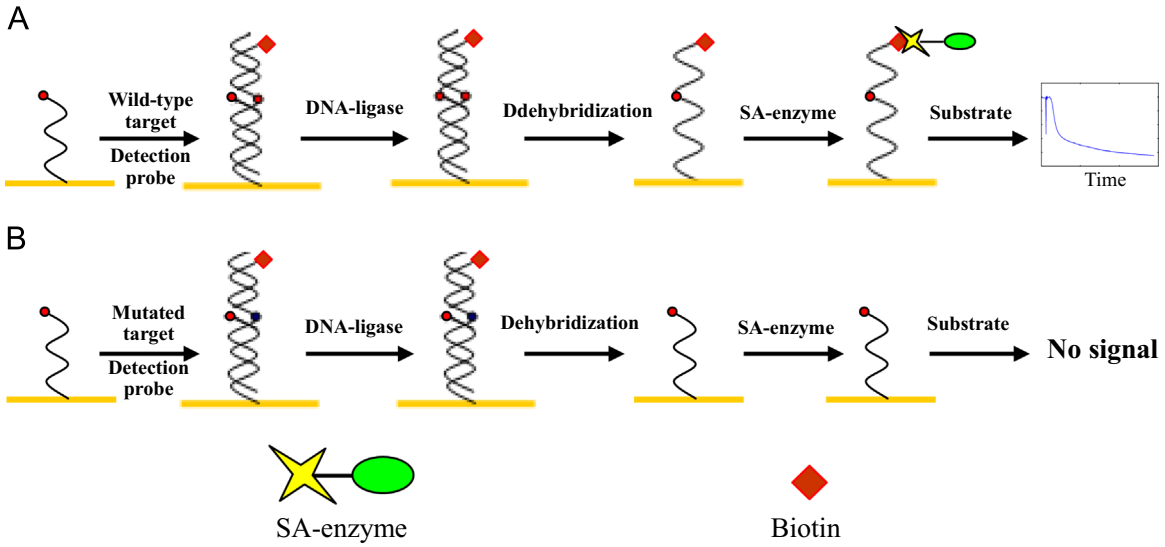

Fig. 3. Schematic illustrating the principle of surface ligation reaction-based biosensors for SNP detection. (A) The capture probe and detection probe hybridize with wide-type target with a perfect match. DNA ligase forms a phosphate diester bond between capture probes and detection probes. After dehybridization, the detection probe is further conjugated with SA-enzyme. The enzyme catalyzes substrates to form the biosensor signal; (B) if the capture probe and detection probe hybridize with mutated target with a single-base mismatch, no ligation occurs for the juxtaposed probes. Abbreviations: SA, streptavidin.

3.3. *Strategy 3: single base extension-based biosensors*

Single base extension is also known as mini-sequencing or single template-dependent nucleotide extension. The single base extension is based on the combination of primer extension reaction (Sokolov, 1990) and dideoxy sequencing method. This technique was first reported to detect SNPs by Lee and Anvret (1991). In their approach, a labeled sequencing primer was designed to lie proximal to the SNP loci. The primer then annealed to the PCR-amplified templates. Subsequently, a proper dideoxynucleotide (ddNTP), which lacked the 3′ hydroxide functionality necessary for elongation, obtained the first chain termination at the SNP loci. Finally, the SNP could be located by electrophoresis. The single base extension is more robust, simple, and flexible than allele-specific oligonucleotide, because the distinction of SNPs is depend on single template-dependent nucleotide extension reaction in the presence of DNA polymerase and ddNTP. Hence, single base extension is becoming the most widely used method for SNP analysis, such as the SNP genotyping array of Orchid Biosciences and Affymetrix.

Based on the principle of single base extension, a novel method referred to as “single base extension-based biosensors” for identifying SNPs has been developed in the past few years (Ito et al., 2007; Patolsky et al., 2001). The principle of single base extension-based biosensors is shown in Fig. 4. This method involves a capture probe with its 3′s terminus close to the SNP loci. The capture probe is first immobilized on the electrode surface. Subsequently, the hybridization of capture probe with target DNA triggers a single base extension in the presence of biotin labeled ddNTP and DNA polymerase. Then, streptavidin-enzyme binds to the capture

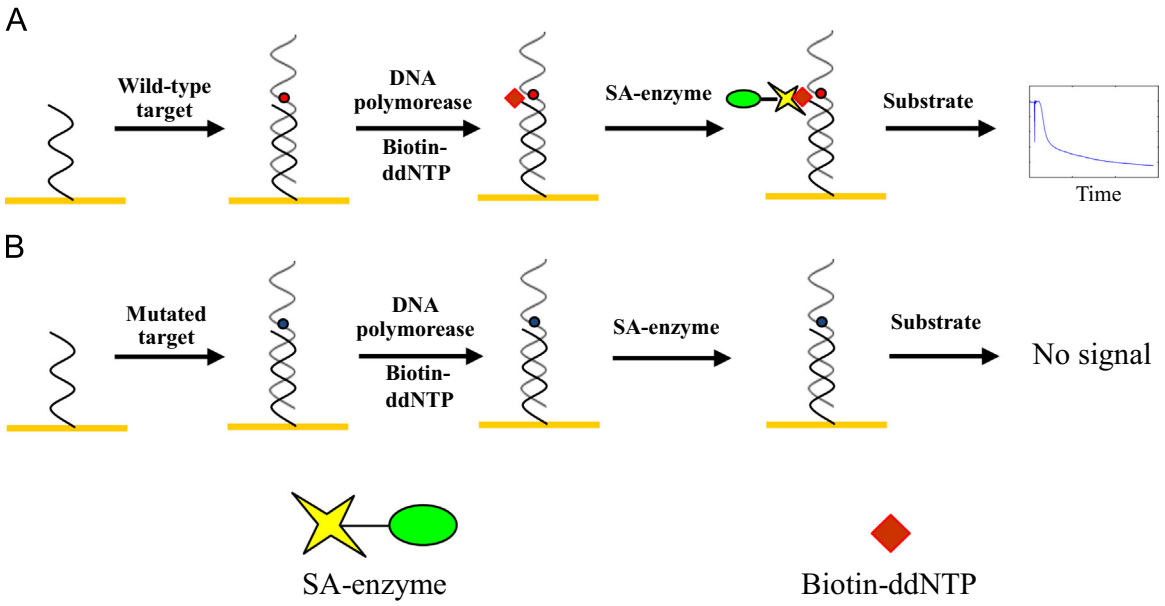

Fig. 4. Schematic illustrating the principle of single base extension-based biosensors for SNP detection. (A) The capture probe with its 3′s terminus close to the SNP loci binds to wide-type target and triggers a single base extension in the presence of biotin labeled ddNTP and DNA polymerase. Biotin labeled ddNTP is further conjugated with SA-enzyme. The enzyme catalyzes substrates to form the biosensor signal. (B) If the capture probe hybridizes with mutated target with a single-base mismatch, the single base extension could not occur. Abbreviations: SA, streptavidin; ddNTP, dideoxynucleotide.

probe if extension has occurred denoting the presence of the corresponding SNP loci. As a result, the enzyme catalyzes substrates to form the biosensor signal. Currently, a series of biosensors have been developed to distinguish the SNPs based on the single base extension. Patolsky et al. (2001) described a method for SNP analysis by faradaic impedance spectroscopy and QCM. In their methods, DNA polymerase (Klenow fragment) was used to catalyze the single base extension. Because of the exonuclease activity of Klenow fragment for 3′-5′ single-stranded DNA, the 5′s terminus of the capture probe was immobilized on the electrode surface. The method enabled the quantitative analysis of single-base mismatch in Tay-Sachs genetic disorder without PCR amplification. The lower limit of sensitivity was 10 fmol/L. Most recently, a label-free biosensing technique was developed for the point mutation detection using SPR biosensor coupled with single base extension. The approach was able to detect mutant sequences with a detection limit as low as 100 pmol/L (Li et al., 2014). However, the above methods are limited to detection of flux, because they can only identify one of the four possible base polymorphisms on a single electrode.

To fulfill the increasing need for high-throughput screening of SNPs, Elenis et al. constructed a solid-phase single base extension protocol on dipstick-type biosensors for multiplex SNP detection (Elenis et al., 2011). Ten SNP loci could be simultaneously genotyped in a single biosensor. Song et al. combined the single base extension with the superior features of PNA to develop an efficient PNA zip-code microarray for the detection of hepatocyte nuclear factor-1α (HNF-1α) mutations (Song et al., 2005). Ten selected HNF-1α mutation sites were interrogated using the PNA zip-code microarray. In addition, Shan et al. demonstrated a tag-extension-based method to identify SNPs using microarray (Shan et al., 2009). After fluorescent nucleotide extension, 89 samples for rs8025575 locus in γ-aminobutyric acid A receptor were accurately discriminated. To sum up, the main advantage of single base extension-based biosensors is simplicity, together with the fact that large numbers of SNPs can be distinguished in parallel. However, the approach only analyzes the known SNPs or mutations in sequence.

3.4. Strategy 4: mismatch binding protein-based biosensors

DNA mismatch repair system plays a crucial role in maintaining the integrity of the genome in all organisms (Hsieh, 2001). In *Escherichia coli* (*E. coli*), the methyl-directed DNA mismatch repair system employs a family of proteins to recognize and repair the mismatch-containing DNA (Lahue et al., 1989). In this process, the MutS protein binding to premutational changes in genomic DNA is the first signal. Then, together with MutL, the MutS activates MutH to cleave the daughter strand within a hemimethylated GATC sequence by exonucleases. In vitro, MutS can specifically bind to mispaired bases, unpaired bases, and short insertion or deletion loops, but not recognize perfectly matched or single-stranded DNA (Stanislawska-Sachadyn and Sachadyn, 2005). In the five domains of MutS monomer, domain I (N-terminal domain) in the minor groove side and domain IV (clamp domain) in the opposite major groove side are involved in the DNA binding (Lamers et al., 2000). Thus, the specificity of biological interaction of MutS with mismatched DNA may provide a strategy for SNP genotyping.

By using MutS as a recognition element, MutS-based biosensors have been exploited to distinguish the SNPs. Fig. 5 illustrates two principles of MutS-based biosensors for SNP detection. In the first scheme (Fig. 5A), the MutS is first immobilized on the electrode surface by coordinate bond formation. Then, MutS binds to the SNP loci by the dimerization of two MutS protein monomers. The interaction of MutS with SNP loci forms the biosensor signal. Based on the above scheme, Wagner et al. first reported the utilizing of immobilized MutS protein for SNP detection (Wagner et al., 1995). A detection limit of 100 pmol/L was achieved by the nitrocellulose membrane-based chemiluminescence mode. Han et al. (2006) and Chen et al. (2009) separately combined the MutS-based mismatch recognition with electrochemical biosensors for scanning the unknown SNPs. In the second scheme (Fig. 5B), the method involves the immobilization of single-stranded probes on the surface of biosensors. Then, the target DNA anneals to form homoduplex or heteroduplex DNA, and finally binding of MutS forms the biosensor signal. Based on the strategy, Su et al. developed a QCM for determination of mismatches in target DNA (Su et al., 2004). The T:G mismatch and one to four unpaired base (s) could be detected by measuring the MutS binding signals. Moreover, Gotoh et al. reported a label-free SPR biosensor for the detection of MutS protein-mismatched DNA interaction (Gotoh et al., 1997).

Thermus aquaticus (*Taq*) MutS, another mismatch binding protein, could maintain its activity under a wide range of temperatures (0–70 °C), which allows the biosensor to work at a broader condition. The other advantage of *Taq* MutS is additional cysteine residue at which the labeled probe could be introduced site-specifically. Hence, *Taq* MutS seems to be a promising probe for SNP detection. Cho et al. developed a fluorescent method for the direct detection of mismatched and unpaired DNAs based on a *Taq* MutS-fluorophore conjugate (Cho et al., 2007). A detection limit of 5 nmol/L was achieved in this method. Moreover, multiple MutS homologs (MSH1, MSH2, MSH3, MSH4, MSH5, MSH6) have been identified in eukaryotes (Buermeyer et al., 1999). The heterodimers of MSH2/MSH3 and MSH2/MSH6 could also bind to mismatches and insertion or deletion loops. These MutS homologs may be expanded as the potential sensing layer of biosensors for SNP recognition. Taken together, the mismatch binding protein-based

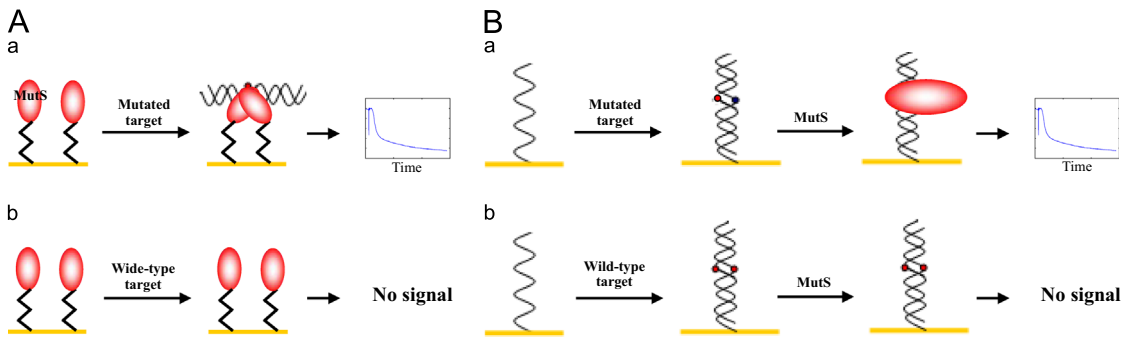

Fig. 5. Schematic illustrating the principle of MutS-based biosensors for SNP detection. (A) Schematic representation of the first scheme immobilizes the MutS on the electrode surface. MutS protein-immobilized biosensor only binds to the mutated target DNA. The interaction of MutS with mismatched DNA forms the biosensor signal. (B) Schematic representation of the second scheme immobilizes the single-stranded probe on the electrode surface. After target DNA hybridization, the MutS only recognizes the mismatched DNA and forms the biosensor signal.

biosensor provides a useful tool for unknown SNPs or mutations in sequence. However, MutS does not recognize mismatches in a sequence-specific manner, and has variable affinities to different mismatches. *E. coli* MutS was reported to form the strongest complexes with G:T mismatch and single T unpaired, while C:C mismatch was observed as a poor binding level (Nakahara et al., 2000). *Taq* MutS would bind better to T:T and C:T mismatches than to other six possible mismatches (Whitehouse et al., 1997). It is not clear that why MutS has varying affinities to each mismatch type. These results indicate that the specificity of mismatch binding protein-based biosensors is poor.

3.5. Strategy 5: molecular beacon-based biosensors

Molecular beacon (MB) is a single-stranded, stem-loop folded oligonucleotide chain, with a donor (fluorophore) and an acceptor (quencher) conjugated to the opposite ends (Huang and Marti, 2012; Li et al., 2008). The common MB has 25–35 bases, and contains two distinctive parts: (1) loop portion is complementary to a target sequence which is typically 15–30 bases in length. (2) Stem portion forms two self-complementary regions at ends of the loop which generally contains 5–8 base pairs. In the absence of target DNA, the self-complementary stem is normally in the closed or "fluorescence off" state. However, after hybridization with target DNA, the MB switches to the open or "fluorescence on" state by undergoing a spontaneous conformational reorganization. One such promising probe was first established by Tyagi and Kramer, and discussed to detect the specific nucleic acid by real-time PCR (Tyagi and Kramer, 1996). Due to the thermodynamic equilibrium relation between self-complementary stem and double-stranded structure of loop/target, the MB demonstrates the more specific towards single-base mismatch compared to the linear probe (Riccelli et al., 2001). For this reason, MB can be used as an advanced biomolecular recognition probe in biosensors. Fig. 6 schematically illustrates the principle of MB-based biosensors for SNP detection. The MB modified with acceptor (quencher) at its 5′ terminus and a donor (fluorophore) at its 3′ terminus is first immobilized on the electrode surface by self-assembly process. The hybridization between MB and target DNA leads to the conformational change of MB, and triggers the attached donor (fluorophore) group on the surface of biosensors. Then, the donor obtains fluorescent signal or catalyzes the substrate to form the biosensor signal. Based on switching from "off" to "on", Mao et al. reported a strategy for SNP detection based on conformational change of MB and enzymatic amplification protocol (Mao et al., 2008). The detection limit was obtained as low as 0.1 nmol/L. Xiang et al. developed a multiplex SNP detection method using a composite MB probe. The composite MB probe included two loops and two stems, and two fluorophores were connected to the two ends of MB (Xiang et al., 2013a). Two SNPs could be simultaneously detected by measuring fluorescence signals with a detection limit of 40 pmol/L.

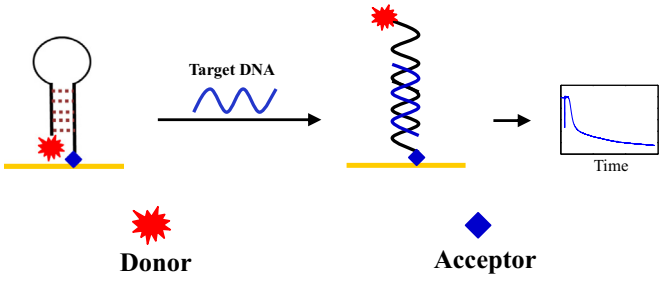

Fig. 6. Schematic illustrating the principle of molecular beacon-based biosensors for SNP detection. The molecular beacon, with an acceptor at its 5′ terminus and a donor at its 3′ terminus, hybridizes with the target DNA with a perfect match. The hybridization leads to the conformational change of molecular beacon and triggers the donor group on the surface of biosensors. Then, the donor forms the biosensor signal.

Nevertheless, although the MB exhibits better stability and specificity than similar assays using the linear probe, false-positive signal is offered in many cases owing to incomplete quenching in the closed form, nonspecific binding to DNA-binding protein, and degradation of endogenous nuclease. In order to overcome the interference of false-positive signal, He et al. developed an universal MB (T_7–MB–T_7) for the detection of SNPs based on a fluorescence assay (He and Ma, 2014). Due to the T–Hg^{2+}–T interactions in stem, the Hg^{2+}–DNA complexes in MB were more resistant to nuclease degradation and less binding to DNA-binding protein. Moreover, He et al. established a MB-functionalized gold nanoparticle to improve the quenching efficiency of MB (He et al., 2010). Another limitation of the MB is the fact that the target-specific recognition depends primarily on the selectivity of MB. New strategy which combined LNA with MB has been established for the identification of SNPs (Zhang et al., 2013a). The LNA-MB showed excellent selectivity and affinity, and was capable of detecting SNPs with a detection limit of 1.7 fmol/L. Thus, the nucleic acid analogs substitution may provide an effective way to improve the selectivity of MB. To sum up, the MB is extensively used as a recognition element of biosensors in SNP detection. However, the new ways to overcome the interference of false-positive signal are still needed in the future.

3.6. Strategy 6: rolling circle amplification-based biosensors

The amplification strategy is significant to detect trace amounts of DNA. Although PCR provides the most versatile method for nucleic acid amplification, the sophisticated procedure and expensive equipment limit the applications of PCR-based amplification strategy. Rolling circle amplification (RCA), an isothermal DNA replication technique, has been demonstrated to be a novel tool for the amplified assay. In nature, the viruses and plasmids replicate their genomes by the RCA mechanism. Inspired by this phenomenon, RCA was first reported to detect the DNA target by Nilsson et al. (1994), and was first demonstrated to detect the SNPs by Lizardi et al. (1998). In their methods, the padlock probe was especially designed with 80–90 nucleotides in length, comprising of target complementary sequence and primer recognition sequence. The 3′- and 5′- terminal sequences of padlock probe firstly annealed to the target DNA. Subsequently, the two termini of padlock probe were joined by a DNA ligase. Then, the DNA polymerase extended primer along the closed padlock probe. In this RCA process, the generated single-stranded DNA sequence contained thousands of repeated segments which were complementary with the closed padlock probe. The long single-stranded DNA sequence could be served as the detection target. Compared with the PCR, RCA exhibits the isothermal amplification in a linear fashion. Therefore, RCA requires the minimal manipulation, and offers an efficient signal enhancement in the detection of nucleic acids.

The RCA provides a promising strategy for SNP genotyping based on biosensors. Fig. 7 illustrates the scheme of RCA-based biosensors for SNP detection (Hatch et al., 1999). A primer anchors the 5′ terminus to the electrode surface, and the padlock probe hybridizes with the primer. Subsequently, the 3′- and 5′-terminal sequences of padlock probe anneal to the target DNA and then are joined by DNA ligase. Here, the DNA ligase only circularizes the perfectly matched padlock probe. Afterwards, a phi29 DNA polymerase with strand displacement activity extends the anchored primer. The amplified single stranded DNA remains on the surface of biosensors, and provides the detection signal. However, if the target DNA contains a single-base mismatch, the ligation fails, and

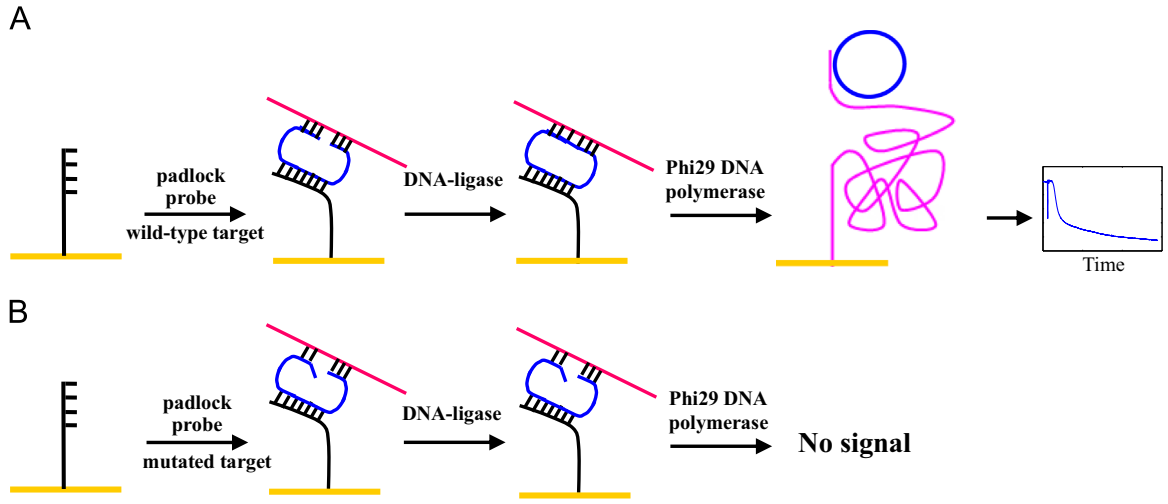

Fig. 7. Schematic illustrating the principle of rolling circle amplification-based biosensors for SNP detection. (A) Padlock probe is perfectly complementary at the 3′ and 5′ termini to the wide-type target. DNA ligase circularizes the padlock probe, and phi29 DNA polymerase extends the primer in rolling circle replication reaction. The amplified single stranded DNA forms the biosensor signal. (B) Padlock probe is not perfectly complementary at the 3′ and 5′ termini to the mutated target. Ligation fails, and rolling circle amplification could not occur. Modified from Hatch et al. (1999).

minimal extension provides a weak biosensor signal. Based on the strategy, Zhang et al. developed a label-free and sensitive method for SNP detection by the combination of RCA and electrochemical biosensors (Zhang et al., 2009). Using methylene blue as the electro-active indicator, the method enabled the detection of SNPs with a detection limit corresponding to 40 amol/L. Moreover, combining the magnetic beads based electrochemiluminescence (ECL) technique with RCA, an ECL assay for highly sensitive point mutation detection was constructed (Su et al., 2010). In this technique, the detection limit was calculated to be 2 amol/L. Since the ligation exhibits considerably less cross reaction, the RCA is more suitable for high-throughput screening of SNPs. Xiang et al. developed a new SPR method for multiplex mutation detection based on RCA (Xiang et al., 2013b). Five point mutations associated with drug-resistance in multidrug-resistant *Mycobacterium tuberculosis* were detected in parallel. Although RCA is widely applied in SNP analysis, nonspecific RCA products cannot be completely eliminated, particularly in instances where very low amounts of DNA are detected. This may influence the analytical performance of RCA-based biosensors.

Aiming at further improving the analytical performance of RCA-based biosensing strategies, Wang et al. developed an electrochemical biosensor for the detection of the mutant human p53 gene based on nicking endonuclease-assisted target recycling and RCA (Wang et al., 2014). The nicking endonuclease achieved the dual signal amplification, and increased the sensitivity of biosensors. A detection limit of 0.25 fmol/L was obtained. In addition, Cheng et al. constructed a novel electrochemical biosensor for ultrasensitive and specific detection of SNPs by integrating RCA with MB mediated circular strand displacement (Cheng et al., 2014). The excellent selectivity of MB for discriminating from mismatched sequence ensured the specificity of RCA. The results exhibited a detection limit of 90 fmol/L. In the above studies, the RCA-based biosensor offers an exquisite strategy for the detection of the low-abundance mutation. The high sensitivity of the method may mainly attribute to the remarkable fidelity of DNA ligase and robust amplification potential of RCA. Besides, the padlock probe will topologically hybridize with the target DNA after ligation because of the helical structure of DNA. The topological duplex allows stringent washing conditions after annealing and ligation. However, padlock probe requires relatively long oligonucleotides (80–100 nt), which increase the cost of detection. Meanwhile, the unknown SNPs or mutations could not be analyzed by the RCA-based biosensors.

3.7. *Strategy 7: strand-displacement amplification-based biosensors*

Strand-displacement amplification (SDA) is another isothermal amplification technique that combines the action of a restriction endonuclease to nick the unmodified strand of its target DNA with the ability of an exonuclease-deficient DNA polymerase to initiate the replication process at the nick and displace the downstream DNA strand (Walker, 1993). Since the SDA was first described by Walker et al. in 1992, it has attracted extensive interest in the detection of target DNA (Walker et al., 1992). Due to its excellent property of signal amplification, the SDA provides a useful technique for biosensor-based SNP genotyping. The operation of SDA-based biosensors for SNP genotyping is depicted in Fig. 8. This SDA system consists of hairpin-shaped capture probe, biotin modified primer, and exonuclease-deficient DNA polymorease. The hairpin probe is first immobilized on the electrode surface by self-assembly process. In the presence of target, the hairpin probe undergoes the conformational change to hybridize with target. Following this, the biotin modified primer anneals with the opened hairpin probe, and triggers a polymerization reaction in the presence of dNTPs and DNA polymerase. In the process of primer extension, the target is displaced by the DNA polymerase with strand-displacement activity. The displaced target then binds to another hairpin probe, which triggers next polymerization reaction cycle. After this cyclical process, a large number of biotin-attached duplex DNA complexes are generated in the presence of trace amounts of the target. Subsequently, the biotin-attached duplex DNA is further conjugated with streptavidin-enzyme via biotin-streptavidin binding. Finally, the enzyme catalyzes substrates to form the biosensor signal.

Based on the above principle, Gao et al. combined the isothermal SDA with silver enhancement to develop an electrochemical biosensor for SNP detection (Gao et al., 2013b). Luo et al. described a chemiluminescence imaging method for high throughput SNP detection based on SDA and functionalized magnetic microparticles (Luo et al., 2014). In order to enhance the sensitivity, Wang et al. combined the SDA with hybridization chain reaction (HCR) to establish an ultrasensitive electrochemical biosensor (Wang et al., 2013). The HCR is an enzyme-free process, which would achieve the dual signal amplification. The detection limit of this method was as low as 8 fmol/L. Combined use of SDA

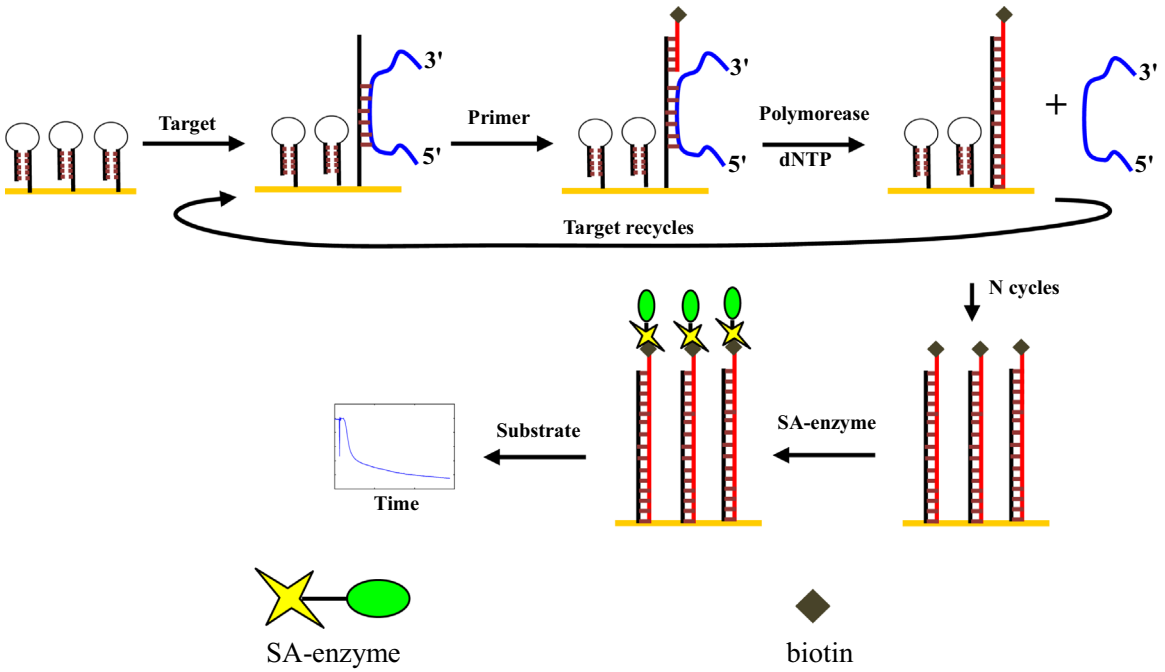

Fig. 8. Schematic illustrating the principle of strand-displacement amplification-based biosensors for SNP detection. The hairpin probe hybridizes with target DNA with a perfect match and undergoes a conformational change. Subsequently, the biotin modified primer anneals to the opened hairpin probe, and triggers a polymerization reaction in the presence of dNTPs and DNA polymerase. The displaced target DNA triggers next polymerization reaction cycle. Finally, a large number of biotin-attached duplex DNA complexes are generated on the surface of biosensors. The biotin-attached duplex DNA is further conjugated with SA-enzyme. The enzyme catalyzes substrates to form the biosensor signal. Abbreviations: SA, streptavidin.

and hyperbranched RCA was reported by Zhang et al., who developed a strategy for label-free detection of the single-base difference among the microRNA. The proposed method exhibited high sensitivity with a detection limit of 0.18 pmol/L (Zhang et al., 2014). The SDA-based biosensor has been used to detect the minute amounts of target due to its isothermality, low cost, and high sensitivity. However, the specificity of SDA is dependent on the thermodynamic equilibrium relation between self-complementary stem and double-stranded structure of hairpin probe/target. In order to improve the specificity, Xiao et al. described a new method by integrating a dipstick-type biosensor, SDA, and T4 DNA ligase (Xiao et al., 2012). The high fidelity of T4 DNA ligase would improve the specificity of recognizing single-base mismatch. The dipstick-type biosensor was performed on a nitrocellulose membrane and offered a promising approach for realizing point of care SNP detection. The most significant disadvantage of SDA-based biosensors is not able to efficiently amplify long target sequences ($>$ 100 nucleotides) owing to the poor processivity of exonuclease-deficient DNA polymorease (Walker, 1993). Meanwhile, the unknown SNPs or mutations could not be analyzed by the approach.

A third isothermal amplification technology is known as loop mediated isothermal amplification (LAMP). The LAMP is also a highly efficient amplification method that relies on auto-cycling strand displacement DNA synthesis. Considering the advantages of rapid amplification, low cost, and high specificity, LAMP-based biosensors have been developed and extensively used to detect SNPs. Zhi et al. fabricated a microfluidic biochip for SNP genotyping by integrating LAMP and a giant magnetoresistive biosensor (Zhi et al., 2014). Focusing on the development of isothermal amplification methods, helicase dependent amplification, recombinase polymerase amplification, and nicking endonuclease signal amplification have been applied to SNP genotyping (Eckert et al., 2014; Hu et al., 2014; Lillis et al., 2014). If these isothermal amplification methods could effectively integrate into the biosensors, it should be possible to develop a powerful innovative technique for SNP detection.

4. Conclusions and future trends

During the last two decades, many efforts have been devoted to the sensitive, rapid, easy-to-use, and cost-effective method for SNP genotyping by worldwide researchers. Among all of the methods, biosensing techniques have attracted extensive interest due to their advantages of high selectivity and sensitivity, short analysis cycle, and real time sensing. In this review, we attempt to give a brief overview of recent progress for the existing strategies of SNP genotyping based on biosensors, including nucleic acid analogs, surface ligation reaction, single base extension, mismatch binding protein, molecular beacon, rolling circle amplification, and strand-displacement amplification. The general working principle is the exclusive properties of biosensors, which combine the unique specificity of biological reactions with the high sensitivity of physical sensors. There is no doubt that these strategies offer the reliable and desirable techniques for SNP detection. However, biosensing techniques also have several challenges before they can be widely used in both basic research and clinical diagnosis. For example, the throughput of biosensing techniques could not meet the requirements of large-scale genotyping. The sample requires the pre-treatment for SNP detection. The existing strategies of SNP detection have an operational complexity in order to acquire the sensitivity and selectivity. Thus, the establishment of new strategy for SNP genotyping and improvement of sensor performance to overcome the above limitations should remain an area of focus in the future.

Recently, more and more sensor technologists turned to the study of single molecule detection. The most widely researched example is DNA nanopore technology for DNA sequencing. The nanopore technology is extremely sensitive to the changes in the size and shape caused by single nucleotides across the pore. Thus, nanopore DNA sequencing has tremendous potential to achieve

label-free, long reads, and high speed with minimal sample preparation and instrumentation. At present, nanopores have been made of various materials to realize the single molecule detection, but protein-based pores have proven the most suitable for sequencing purposes. Laszlo et al. combined the high sensitivity of a mutated form of the protein pore Mycobacterium smegmatis porin A with the phi29 DNA polymerase to read the characteristic temporary change as DNA was drawn through the pore (Laszlo et al., 2014). Similarly, another group combined sub-10 nm nanogap electrodes with solid-state nanopores to detect the translocating DNA molecules (Fanget et al., 2014). These results indicated that the DNA nanopore provided the possibility of DNA sequencing.

Another active area is the development of new type of selective biorecognition elements. The molecular imprinted polymer exhibits high selectivity and affinity for target DNA, and provides a promising technique to develop the new generation of biosensors. In addition, the nanomaterials, such as gold nanoparticles, carbon nanotubes, and graphene, play an increasingly important role in the development of biosensors. Due to their unique nanoscale dimensions, the combination of biosensors and nanomaterials would tremendously improve the sensitivity and overall performance of biosensors. Briefly, the existing strategies of biosensors provide the great potential for SNP genotyping, and yet the futuristic goal of high throughput, highly selective, and easy-to-use approaches has yet to be truly realized. Although there is a long way to go, it is almost certain that the biosensor will play a crucial role in SNP genotyping.

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