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Liquid biopsy of circulating tumor DNA and biosensor applications

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ABSTRACT

Circulating tumor DNA (ctDNA) as a class of liquid biopsy is a type of gene fragment that contains tumor-specific gene changes in body fluids such as human peripheral blood. More and more evidences show that ctDNA is an excellent tumor biomarker for diagnosis, prognosis, tumor heterogeneity and so on. ctDNA is a tumor code in the blood. Liquid biopsy of ctDNA is firstly summarized. Compared with the traditional detection technologies of ctDNA, the biosensor is an excellent choice for the detection of ctDNA because of its portability, sensitivity, specificity and ease of use. This review mainly evaluates various biosensors applied to the detection of ctDNA. We discuss the most commonly used bioreceptors to specifically identify and bind ctDNA, including complementary DNA (cDNA), peptide nucleic acid (PNA) and anti-5 MethylCytosines, and the biotransducers which convert biological signals to analysable signs. The review also discusses signal amplification strategies in biosensors to detect ctDNA.

1. Introduction

A tumor is the new tissue formed by local tissue cell hyperplasia under the influence of all kinds of tumorigenic factors (Capozza et al., 2003; Suzuki et al., 2003). Tumors are divided into benign and malignant tumors. Cancer is a general term for malignant tumors. Cancer is currently a major cause of death all over the world. According to World Health Organization (WHO) statistics, 8.8 million people died of cancer in 2015, accounting for 1/6 of the global death toll. In the next 20 years, the estimated number of cancer patients will increase by 70% from 14 million in 2012 to 22 million. WHO has pointed out that more than 40% of cancers can be cured if they are discovered early. Failure to discover a tumor in the early stages and the appearance of tumor heterogeneity have resulted in the high death rate of cancer patients. Tumor heterogeneity leads to differences in growth rate, invasion ability, sensitivity to drugs (Heppner, 1984; Nguyen et al., 2015). The main diagnostic techniques for cancer are tissue biopsy and imaging techniques including color ultrasound, computed tomography, molybdenum targets, magnetic resonance imaging and so on. These techniques have contributed to the diagnosis and treatment of tumors. Although tissue biopsy as a gold standard has an irreplaceable status in the diagnosis of cancer, there are still some limitations. These techniques are not informative in early diagnosis and in most case of tumor heterogeneity (Gerlinger et al., 2012; De et al., 2014; Zhang et al., 2014). There are other restrictions besides these. Pathological sections are acquired by puncturing, but this is not appropriate for some patients who have had surgery and some lesions. Moreover, puncturing has clinical risks and can cause trauma to patients. Puncturing usually causes tumor cells to fall away from the tumor tissues, consequently leading to tumor metastasis.

Apart from tissue biopsy and imaging techniques, some tumor biomarkers based on liquid biopsy such as oncofetal proteins, tumor-associated antigens, enzymes, circulating tumor cells (CTC), circulating tumor DNA (ctDNA) and so on are also diagnostic tools. Liquid biopsy refers to the use of substances in the blood to obtain information about the tumor. Compared with traditional biopsy techniques, liquid biopsy is safer, less invasive, and sampling of liquid biopsy can be repeated to monitor disease progression and treatment risk. In addition, doctors can

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obtain more comprehensive messages by liquid biopsy because these tumor biomarkers are released into the blood from various niduses around the body (Cai et al., 2015). Moreover, liquid biopsy is well suited to the early detection of a tumor because these tumor biomarkers are released and appear in the blood in the early stages of a tumor (Babayan and Pantel, 2018). Moreover, with the development of molecular diagnostics and individualized treatment, liquid biopsy has attracted the attention of more and more medical personnel. Liquid biopsy is rated as one of the top ten breakthrough technologies of 2015 by MIT Technology Review. ctDNA in liquid biopsy is nowadays one of the preferred tumor biomarkers on account of its high accuracy, sensitivity, and its presence in the blood of early tumor patients.

ctDNA has attracted more and more researchers because of its superior advantages. The detection of ctDNA is a key step in the clinical application of ctDNA. Some traditional techniques for detecting DNA are applied to the detection of ctDNA such as PCR (Zhang et al., 2016) and DNA sequencing (Austin et al., 2015; Liu et al., 2017). Most of the conventional techniques for ctDNA detection require a long testing time, a tedious process, high cost and large instruments that are inconvenient to carry (Leary et al., 2010). Based on these, ctDNA is partly restricted in clinic. Hence, it is necessary to develop a low cost, real-time, handy and portable system to detect ctDNA for screening and diagnosis of cancers. In recent years, the rapid development of biosensing technology has brought a new approach to the detection of ctDNA. Biosensors have been widely used in environmental monitoring (Rogers, 2006), food safety (Amine et al., 2006), disease diagnostic (Torati et al., 2016; Zheng and Li, 2010) and so on due to their unique excellent characteristics such as their short detection time, ease of use, capability for high selectivity and specificity in complex samples like blood, saliva and urine (Gupta and Kakkar, 2018). Biosensors show remarkable features compared to the conventional techniques for the detection of ctDNA. Biosensors can make up for the defects of conventional techniques in detecting ctDNA, and can further promote the clinical application of ctDNA such as point-of-care testing. So we will discuss various biosensing techniques for detection of ctDNA and the benefits of using these over conventional techniques to provide the basis for further application of ctDNA.

2. Liquid biopsy – ctDNA

Liquid biopsy includes protein markers (Cohen et al., 2017), CTC (Alixpanabieres and Pantel, 2014; Lianidou and Markou, 2011), ctDNA (Diaz and Bardelli, 2016), circulating RNA (Sestini et al., 2015) and exosome. Among them, CTC, ctDNA and exosome are the most commonly used test items for liquid biopsy. They have been widely utilized in the diagnosis and treatment of cancer, as they offer real-time tracking, convenience and are minimally invasive. But there are some limitations. Table 1 shows their advantages and limitations.

Although these tumor biomarkers have some defects, ctDNA has better specificity, stability and the capacity to detect tumors earlier over time compared to other tumor biomarkers (Dawson et al., 2013; Bettegowda et al., 2014). The study of Diehl et al. (2008) proved that ctDNA is more suited to monitor colorectal cancer than carcino-embroyonic antigen(CEA) by examining 162 plasma samples from 18 colorectal cancer patients. They found that ctDNA as a tumor biomarker has higher sensitivity, higher accuracy and earlier detection of changes in tumor loading than the other two tumor markers (cancer antigen 15-3 (CA15-3) and CTCs) by comparing the concentrations of three tumor biomarkers. Therefore, ctDNA has greater clinical potential. A magazine published in the Nature Reviews Cancer recently also showed the great potential of ctDNA as a tumor biomarker (Wan et al., 2017).

The origin of ctDNA can be traced back to early studies of circulating DNA (cfDNA). As early as 1948, cfDNA was discovered in human peripheral blood. In subsequent studies, scientists have discovered that cfDNA involves gene fragments that contain tumor-specific genetic changes which are also termed ctDNA (Sorenson et al., 1994; Vasioukhin et al., 2010; Silva et al., 1999). The main biological characteristics of ctDNA are the following: 1. ctDNA is released from tumor cells in many diseased areas into the circulatory system. ctDNA contains tumor-specific genetic changes (single nucleotide variation, microsatellite changes, copy number changes, epigenetic variation of DNA methylation, etc) that guarantee its specificity as a tumor biomarker. The generally accepted release mechanisms include the release of necrotic cells, apoptotic cells or metabolically active tumor cells (Masago et al., 2015). ctDNA is present in peripheral blood which can be excreted in urine, sputum or feces, etc. Therefore, the acquiring procedure of ctDNA is convenient and minimally invasive. 2. The size of ctDNA fragments fluctuates widely, but is usually larger than non-tumor circulating free DNA (Jahr et al., 2001). The ctDNA level of cancer patients is higher than that of healthy people, and it changes with the different stages of the disease (Roschewski et al., 2015; Bettegowda et al., 2014). The detection of ctDNA can easily be perturbed by non-circulating tumor DNA when detecting ctDNA. The ratio of ctDNA to cfDNA in peripheral blood is from as low as 0.01% to as high as 90% (Jahr et al., 2001). 3. The half-life of ctDNA in the blood is short, from about ten minutes to a few hours (Yong, 2014), indicating that the concentration of ctDNA in the blood can reflect the dynamic changes of the tumor in real time.

ctDNA is the password of the tumor in the blood and is closely related to the occurrence and development of the tumor. Cancer is essentially a genetic disease. Although different tumors have different causes, the activation of oncogenes and inactivation of tumor suppressor genes are indispensable in the final analysis. The presence of ctDNA will indicate the presence of a tumor, enabling early diagnosis of the tumor (Tie et al., 2015; Sozzi et al., 2001). Tumor heterogeneity is mainly mediated by genetic changes. ctDNA is released into the blood from different pathological tissues, consequently the analysis of ctDNA can reflect the condition of the tumor and its heterogeneity. Forshew et al. (2012) detected ctDAN (EGFR mutation) in the plasma of ovarian cancer patients by tagged-amplicon deep sequencing (TAm-Seq). No such mutation was found in primary ovariectomy specimens, but the same EGFR mutations were present in the omental samples during tumor surgery. Hence, the heterogeneity of tumors in time and space can be solved to some extent by ctDNA. Oxnard et al. (2014) detected the ctDNA levels of 9 erlotinib hydrochloride tablet-treated patients in the plasma and found that the concentration of the T790M mutation of EGFR in the plasma before erlotinib hydrochloride tablet treatment was not detected, but that the T790M mutation of EGFR concentration began to increase in 6 out of 9 patients after erlotinib hydrochloride tablet treatment. Therefore ctDNA has the ability to identify resistance mutations. In addition, ctDNA also plays an important role in every stage of cancer diagnosis and treatment, including minimally invasive molecular profiling (Siravegna and Bardelli, 2016), treatment monitoring (Fiegl et al., 2005), detection of the progress of disease and so on (Hamakawa et al., 2015). ctDNA has important clinical significances as shown in Fig. 1.

At this stage, the detection of ctDNA can be divided into qualitative detection and quantitative detection (Taniguchi et al., 2011; Newman et al., 2014). The qualitative detection technology is mainly DNA sequencing technology (Poole et al., 2014) and the dideoxy chain-termination method is used most frequently. This technique utilizes a DNA polymerase to extend the primers that are bound to the sequence template to be determined. Nucleotides continue extending until a chain-terminating nucleotide is incorporated, then electrophoresis on urea denatured polyacrylamide gel is carried out, and the DNA base sequence can be obtained. The quantitative detection technology mainly includes methods based on polymerase chain reaction (PCR) (Garciamurillas et al., 2015; Malpeli et al., 2015). PCR requires the precise control of enzymes and temperature. PCR is strictly followed by complicated procedures:Denaturation-Renaturation-Denaturation. Although these technologies have made significant contributions to DNA testing, the further application of these techniques is still limited. The

Table 1
The advantages and limitations of different tumor biomarkers.

Tumor biomarkers	Advantages	Limitations
Protein markers	The detection method is mature enough to allow the detection of large clinical samples. Judging tumor growth before computed tomography, magnetic resonance imaging and other means.	Limitations of cancer screening Poor accuracy Difficult to separate the proteins in the blood Non-specific protein interference False negatives or False positives for other pathological conditions
CTC	Active circulating tumor cells contain a wealth of information Cancer development can be tracked	Difficult to separate circulating tumor cells from other cells Not very high specificity Its genetic phenotype is not necessarily consistent with the primary tumor Rare in peripheral blood
Circulating RNA	Not vulnerable to RNase damage	Poor chemical and biological stability Immune status can affect the results
ctDNA	Short half-life for real-time monitoring of tumor burden	Lack of uniform standards Debris easily formed
Exosome	Many substances including nucleic acid, protein, lipids and so on can be carried. Exosomes can be preserved in blood samples for long periods of time Smaller blood sample required	Incomplete Separation technology Immature detection system

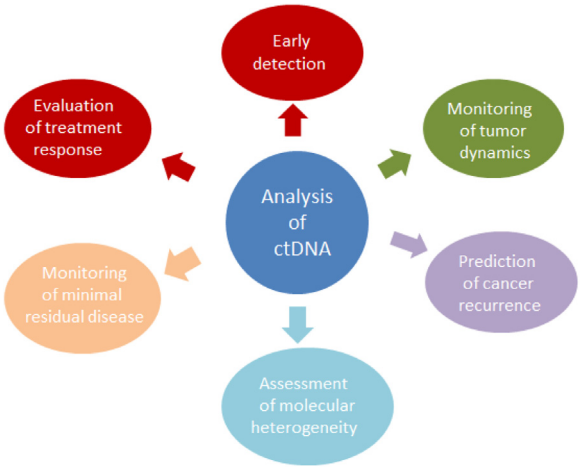


Fig. 1. The clinical values of ctDNA.

Table 2
Conventional techniques for ctDNA and their limitations.

Technique	Limitations
PCR	Susceptible to interference Need to strictly control the temperature Takes too long for the short half-life of ctDNA Analysis of point mutations is not very effective
Quantitative Real-time PCR	Expensive Low amplification efficiency
COLD-PCR	More cumbersome than traditional PCR processes Restrictions on sequences less than 200 bp in analysis Prone to Polymerase Boot Errors
Digital PCR	Accuracy of deformation temperature control requires within ± 0.3 ℃ High cost
DNA sequencing	Long time period (2–3 weeks) Expensive Unnecessary Information False positives

techniques for determining DNA and their limitations are shown in the following Table 2.

3. Biosensing techniques for ctDNA detection

The biosensor as a novel and powerful analytical tool is applied in many fields. It detects target substances by combining biometric elements with physicochemical sensors. The International Union of Pure and Applied Chemistry (IUPAC) defines a biosensor as a device that biochemically reacts and produces a biological signal when detecting enzymes, cells, or whole cells, and then converts the biological signal into an electrical, thermal, or optical signal response (Thipmanee et al., 2016; Xuan et al., 2015).

A bioreceptor and a biotransducer are the main components of a biosensor. Bioreceptors immobilized on the electrode or the optical platform can be enzymes, antibodies, cells, microorganisms, nucleic acid and any substances that have specificity towards analytes. The bioreceptor and the analyte react with each other, producing a biological sign, which is then output as voltage, current or light intensity by the biotransducer as a physicochemical detector (Bueno, 2015). The biosensor is connected to the detector system via a connector, which

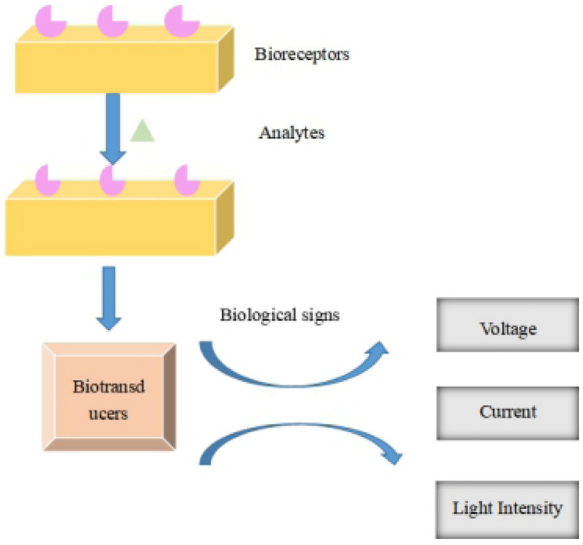


Fig. 2. Schematic diagram of biosensor.

amplifies the signal and converts it after analysis to a concentration unit. This data is then displayed by the device or stored in the device for later recall (Dorothee et al., 2008). Fig. 2 shows the basic components of a biosensor. The bioreceptor and biotransducer determine the technical specifications of the biosensor. This review summarizes the bioreceptors and biotransducers frequently used for ctDNA.

3.1. Bioreceptors

ctDNA can be identified and separated from complex samples by a bioreceptor which is a key to improving detection selectivity. When detecting ctDNA, it can be transformed into a single strand and subsequently identified with the probe, or double-stranded DNA can be directly recognized without distortion. The following description will focus on the bioreceptors extensively used for ctDNA.

3.1.1. Complementary DNA (cDNA)

cDNA is used in biosensors by researchers as a convenient probe. Target ctDNA hybridizes with its cDNA immobilized on the biosensor surface, resulting in changing signs, which is the principle of the biosensor. However, there may be electrostatic repulsion when the cDNA binds to target DNA. In this case, it is often necessary to reduce the repulsive force at a certain ion concentration so that cDNA and target DNA can form a stable dimer. Nonetheless the presence of ions will increase secondary and tertiary structures which increase the proximity of the target molecule to the probe molecule, rendering hybridization impossible.

The simplest recognition mechanism of the bioreceptor in the biosensor is the binding of two single-stranded DNAs (ssDNA-ctDNA and cDNA) by a hydrogen bond between base-pairs. A hairpin DNA probe is used in the process of signal amplification strategies for the detection of ctDNA that depends on the cycle between target DNA and hairpin DNA probes (Fig. 3). This means that a target gene can repeatedly cause hybridization reactions to generate multiple signal molecules.

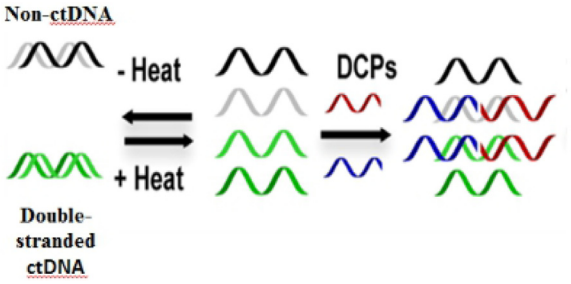
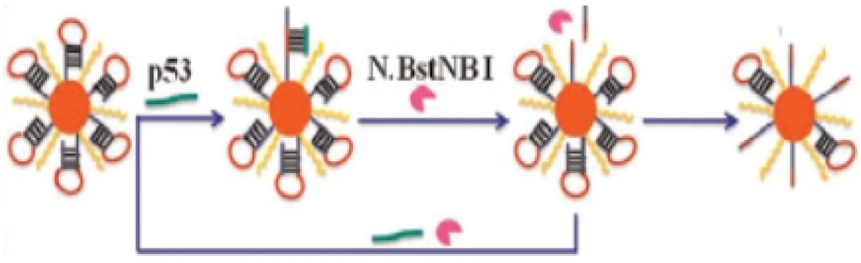
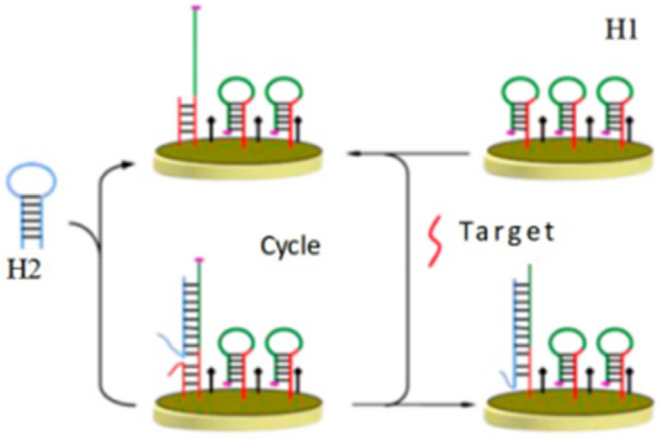


Fig. 4. Schematic illustration of how to prevent reassociation by relying on the use of DCPs:DCP hybridized with one of the two strands of dsDNA and other closely related sequences in the sample (with permission from Das et al., 2016).

The methods discussed above are based on the detection of single-strand ctDNA(ss-ctDNA). In fact, ctDNA exists generally in the form of double-stranded DNA(ds-ctDNA). It is essential to denature to ss-ctDNA. But the denatured DNA strands are easily reassociated, leading to interference with detection. Currently, few strategies have been developed to solve this problem. A DNA clutch probe (DCP) has been designed to prevent the reassociation of denatured DNA strands (Fig. 4) (Das et al., 2016). Researchers have also exploited some methods based on the theory that ssDNA and double-stranded DNA (dsDNA) are capable of forming triple-stranded structures to detect ds-ctDNA directly (Z. Li et al., 2015; Miao et al., 2014). But these strategies are limited to homopyrimidine/homopurine oligonucleotides. In addition, a triple-



(A)



(B)

Fig. 3. Schematic illustration of a signal amplification strategy using hairpin DNA probes: (A) the P53 gene hybridized with hairpin DNA probes immobilized on the electrode, and N.BstNB I, one of the DNAzymes, recognized specific nucleotide sequences in double-stranded DNA and cleaved only one of the two strands. Consequently, the liberated P53 gene again hybridized with other hairpin DNA probes and initiated the cycles (with permission from Wang et al., 2016). (B) The target DNA opened the hairpin structure of H1 which was self-assembled on the electrode by a thiol-gold linkage through a toehold-mediated strand displacement reaction, leading to the combination of H2 with H1. The stability of the H1–H2 duplex is better than the H1- target DNA, resulting in there placement and release of the target DNA. The cycle continued (with permission from Sun et al., 2016).

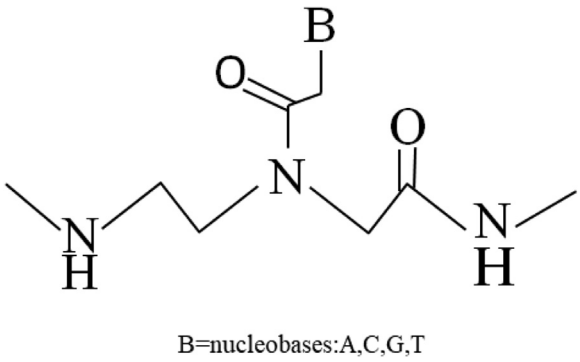


Fig. 5. The structure of PNAs: bases (Adenine, guanine, thymine, cytosine) linked to the main chain via methylene carbonyl. The base is separated from the backbone by 3 bonds and separated from the adjacent base by 6 bonds. Its spatial size is similar to that of natural nucleic acids (Ramani et al., 2018).

chain structure is often unstable, researchers need polyamines and multivalent cations to eliminate electrostatic repulsion.

3.1.2. Peptide nucleic acid (PNA)

PNA, known as a class of DNA analogues, has attracted researchers' interest because of its excellent properties. PNA was first proposed by Nielsen in 1991. It is a third-generation antisense reagent that is designed by computer and it is based on the first and second generation antisense reagents. PNA can form a stable complex with DNA by means of the principle of complementary base pairing or Hoogsteen base pairing (Egholm et al., 1993; Nielsen et al., 2010). The backbone of PNAs which is formed by 2-aminoethyl and glycine under the acting force of amide bonds replaces the electronegative pentose phosphate diester backbone in DNA, and PNAs are electrically neutral. So PNA binds target DNA without electrostatic repulsion and a PNA-DNA complex has good stability. Generally, the melting temperature increases 1 ˚C for each additional base (Fig. 5).

PNA has the ability to distinguish single base mismatches because PNA binding to target DNA is strictly controlled by bases. Because PNA is not easily degraded in complex real samples, a hybridization probe is preferred in the biosensor. Besides, the hybridization of PNA and DNA is not affected at low salt concentrations.

Several PNAs for ctDNA have been developed so far. The most frequent method of using PNA as the bioreceptor in the biosensor is that where single-strand ctDNA can specifically bind to PNA immobilized on an electrode surface or optical platform by complementary base pairing. However, it is subject to the interference of the reassociation of denatured DNA strands. Some studies have shown that PNA has the ability to invade DNA-DNA duplexes (Boffa et al., 1995; Smolina et al., 2005), which means PNA is capable of directly displacing strands of dsDNA. This is because the hydrogen radical at the end of the DNA-DNA is weaker than that in the middle and the affinity of PNA to DNA is much higher than that of DNA-DNA. The schematic diagram is shown in Fig. 6 (Hu et al., 2017). The research of Lee et al. (2014) and Smolina et al. (2005) also confirmed the ability of PNA to replace one of the DNA double strands. This provides a new idea for the direct detection of ctDNA without the need for degeneration. Hu et al. (2017) took advantage of this ability of PNA and the growth of electroactive polymers in situ by surface-activated electrochemically mediated atomic transfer free radical polymerization to carry out direct and highly sensitive detection of ds-ctDNA. This method had strong anti-interference ability. When PNA is used as a hybridization probe, it must be noted that its specificity is good at a certain length; however, hybridization efficiency will be affected if the length of PNA is too great. The purine content of PNA should not be too high; there can be no stretches of continuous purines or consecutive guanines to prevent self-aggregation. At the same time, continuous complementary bases in the chain must be avoided in order to prevent self-complementation.

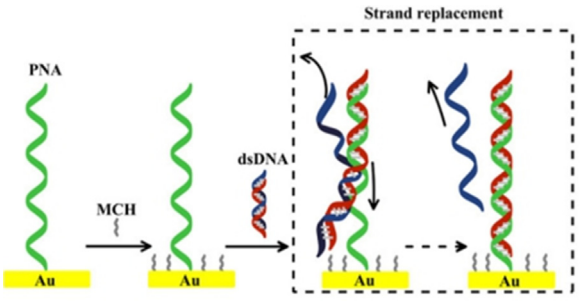


Fig. 6. Scheme of strand displacement of DNA (with permission from Hu et al., 2017).

In addition to biosensors, PNA has also been applied to many fields. Kim et al. (2013) used a PNA-mediated polymerase chain reaction (PCR) to detect EGFR mutations in plasma. Sharifisanjani et al. (2017) utilized quantitative fluorescence in situ hybridization (Q-FISH) based on PNA to evaluate telomere length. Now many researchers have also applied PNA probes to biosensors to detect a causative agent, microorganism or nucleic acid (Xu et al., 2017; Kangkamano et al., 2017; Sato et al., 2016; Xuan et al., 2015; Bertucci et al., 2015). PNA probes labeled with radioactive elements have also been used to treat diseases. The labeled probe binds to the oncogene, whereafter the electrons emitted by the labeled probe can directly destroy the oncogene, thus treating the tumor at the genetic level. Besides, PNA can also be used as an antisense agent to regulate genes to treat cancer. PNA can specifically bind to corresponding tumor-associated genes, thereby regulating gene transcription and translation (Ray and Nordén, 2000; Janowski et al., 2005; Hanvey et al., 1992). Although PNA has limited ability to cross the cell membrane, this limit is solved by using molecules as the carrier that enables PNA to cross the cell membrane more efficiently. Furthermore PNA introduced into the body is also not easily degraded because PNA does not contain nuclease and protease recognition sites.

3.1.3. Anti-5 MethylCytosine

Apart from cDNAs and PNAs, anti-5 MethylCytosine has been designed as a bioreceptor in biosensors. In addition to genetic mutations, genetic changes also include epigenetic changes (DNA methylation, genomic imprinting, maternal effects, gene silencing, RNA editing etc). DNA methylation consists in ctDNA and 5-methylcytosine (5-mc) is the main ending of DNA methylation. cDNA or PNA hybridize with a target ctDNA and an anti-5 MethylCytosine combines 5-mc. The sandwich structure of cDNA/PNA-ctDNA-anti-5 MethylCytosine is the principle for the detection of ctDNA methylation (Cai et al., 2018; Daneshpour et al., 2016) (Fig. 7).

3.2. Biotransducers

Biotransducers can convert biological signs into measurable ones and measure the signals precisely. Electrochemical and optical biotransducers have principally been employed for the detection of ctDNA. The article will mainly discuss these two biotransducers in biosensors. Table 3 shows the advantages of the biotransducers.

3.2.1. Electrochemical transducers

Electrochemical transducers are applied widely in biosensors due to their great sensitivity, specificity, portability, simple use, fast response and low cost. Apart from analyzing the signs with regard to the concentration of analytes, electrochemical biosensors can offer information with respect to the reaction between analytes and bioreceptors. The microstructure of the electrochemical biosensor surface can provide many potential fields that can be used for effective separation and enrichment of the substance to be measured, and the selectivity can be further improved by controlling the electrode potential, and the

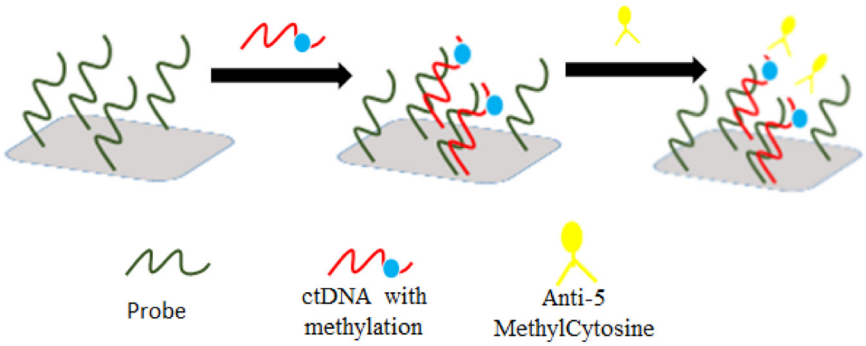


Fig. 7. Construction of a sandwich structure of cDNA/PNA-ctDNA-anti-5 MethylCytosine.

Table 3
The advantages of the biotransducers.

Biotransducers	Advantages
Electrochemical transducers	Easy and simple to handle Fast analysis On-line analysis Living analysis High sensitivity Low cost Real-time unmarked monitoring Pollution free detection
LSPR	Sensitive to the changes of refractivity No temperature control High level of integration No effect of the sample shape on the detection
SERS	Not easy to contaminate and destroy samples because of no direct contact between the len and samples Suitable for testing water phase solvent samples Simple and convenient
Optical transducers	Perceptual intuition Easy to handle Simple interpretation
Colorimetric assays	Excellent commercial potential Wide range of application Crime scene analysis because of portability
Fluorescent biosensors	Short measurement time Easy to carry Small size of the instrument

sensitivity of the method can be measured. The chemical reaction of surface materials combined with the sensitivity of analysis enables high sensitivity, high specificity and rapid detection of target substance. Some electrochemical biosensors have been applied in biotechnology, the food industry, clinical testing, the pharmaceutical industry, biomedicine, environmental analysis and other fields. Electrochemical biosensors use electrodes as transducing elements to convert captured reaction signals into voltage, current or resistance (Bahadır and Sezgintürk, 2015). Nowadays electrochemical methods commonly used to detect ctDNA include Differential Pulse Voltammetry (DPV) and Electrochemical Impedance Spectroscopy (EIS). This article will focus on the progress of these two types of electrochemical methods in ctDNA detection.

3.2.1.1. Electrochemical Impedance Spectroscopy (EIS). EIS can detect DNA hybridization signals and even detect single-base variants or mismatched hybridization signals. The principle is that the DNA molecule has a negative charge and it forms a negative electrode layer on the surface of the electrode. If it is an oxidation-reduction electrode, there will be electrostatic repulsion and the resistance will increase.

Zhang et al. (2018) used poly-xanthurenic acid (PXA)/MoS2 nanocomposites to analyze the ctDNA (PIK3CA gene) based on EIS. The study achieved a limit of detection of the PIK3CA gene as low as 1.8×10^{-17} mol/L, the ΔRet value was linear, with the logarithm of the PIK3CA gene target sequence concentrations in the range from 1.0×10^{-16} mol/L to 1.0×10^{-10} mol/L. MoS2 is a kind of nanometer material similar to layered graphene. It has the superior characteristics of large surface area, large storage space, ease of functionalization and excellent mechanical strength (Kalantar-zadeh, 2016; Singhal et al., 2018). PXA as a novel conducting polymer can immobilize DNA molecules by π–π interaction between their respective nucleobases (Liu et al., 2011). PXA/MoS2 composites increased the binding rate of ssDNA probes and increased the signal. When the target DNA binds to the probe, the resistance changed once the double strand detached from the PNA/MoS2, and the PIK3CA gene was quantified without the presence of any external indicator. When they detected one-base mismatched DNA and two-base mismatched DNA, the Ret values were larger than target DNA, which meant this indicator-free DNA biosensor presented great selectivity for DNA hybridization recognition. A RSD of 5.6% for the signal changes was estimated. This sensor could be stored in the refrigerator at 4 °C for one week. No apparent change of EIS signal was observed after one week, showing that the DNA sensor had good stability.

EIS was used by Wang et al. (2015) to measure and record the electrochemical behavior of BRCA1 which is related to breast cancer in serum samples on the electrode. As the concentration of the target substance increased, the Ret increased accordingly. The detection limit of the experiment was 1.72 fM. In this study, they utilized functional polyethylene glycols and gold nanoparticles which immobilize complementary probes self-assembled on a PEG polymer film. On account of the mixed interface of PEG polymer films and gold nanoparticles, this strategy enabled highly sensitive and specific detection of BRCA1.

3.2.1.2. Differential Pulse Voltammetry(DPV). DPV as an electrochemical detection method can amplify the signal by more than 30 times because its longer pulse time allows full attenuation of charge current and improves the signal-noise ratio (SNR). Meanwhile the lowest limit of detection reached is 10^{-8} mol/L. Hence, DPV can measure trace substances very well. Especially when measuring ctDNA with low abundance, DPV shows great advantages.

Das et al. (2015) designed a chip-based method. In this study, the sensor was set in the chip. Firstly, SiO2-coated silicon wafer was used to form contact pads and electrical leads. Then a layer of Si3N4 was deposited to passivate the surface, after which the 5 μm apertures was opened in the top passivation layer by photolithography to provide a template for the growth of the Au electrodeposition and a thin layer of Pd was used to coated the Au structures. Finally nanostructured microelectrodes (NME) was formed. The average surface area of the sensor was $4.75 \pm 0.3 \times 10^{-4}$ cm^2. In this study, they used NME electrodes to detect ctDNA. The electrocatalytic reporter pair was made of Ru $(NH_3)_6^{3+}$ and $Fe(CN)_6^{3-}$. The signal value was recorded through Differential Pulse Voltammetry (DPV) to improve the sensitivity. As shown in Fig. 8, NME had a three-dimensional structure with a large surface

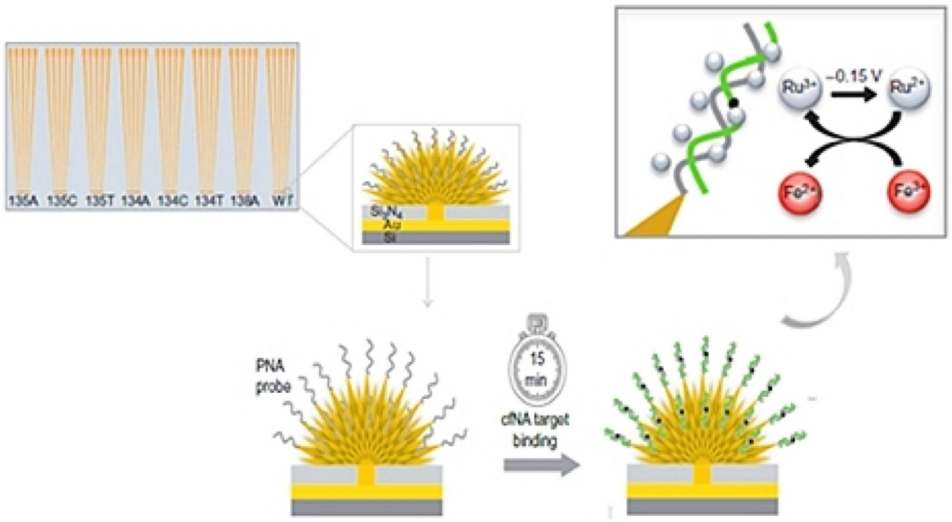


Fig. 8. Schematic of a microfabricated chip and the detection of ctDNA (with permission from Das et al., 2015).

area. And the nanostructure of the NME allowed the probes to have an appropriate alignment direction, thereby increasing hybridization efficiency. The time required for this experiment was only 15 min, which can satisfy the short half-life of ctDNA in peripheral blood. So it makes point-of-care testing possible because of its quick response.

Later, they further improved the experimental scheme by introducing DCP based on previous experiments. This method was successfully applied to lung and melanoma patient samples and could detect 0.01% mutations. The result of the approach could produce comparable results to existing PCR-based methods, which was time-consuming.

An electrochemical biosensor was proposed by Daneshpour et al. (2016) that was capable of detecting RASSF1A DNA promoter methylation in human blood plasma at trace levels by DPV. They used a DNA probe labeled by Fe3O4/TMC/Au nanocomposites as the signal amplification unit. Methylated DNA was separated from non-methylated DNA by highly specific anti-5-methylcytosin monoclonal antibody embedded in a thin Polythiophene (PT) film. Due to the synergistic effect of Au nanoparticles and PT, their conductivity combination resulted in a great signal amplification. The detection limit was approximately 2 fM. The precision and accuracy of the method were acceptable (The RSD values varied from 1.3% to 8.4% and the recovery ranged between 96.37% and 118%). The schematic of this experiment is shown in Fig. 9.

Hui et al. (2016) developed a new electrochemical method based DPV to detect BRCA-1 gene in serum that was responsible for breast cancer with hereditary breast-ovarian cancer syndrome. The electrochemical biosensor was based on hybrid chain reaction(HCR) using RuHex as the active substance (As shown in Fig. 10).

HCR as an isothermal amplification technique, whose principle is two auxiliary nucleic acid probes (H1 and H2), which can stably exist in the solution in the absence of an initiator, and H1 and H2 are alternately opened loop to form dsDNA when the initiator appears. HCR not only does not require complicated variable temperature programs, but also does not require the participation of enzymes, so it has been widely used in food safety testing (Hong et al., 2018), protein detection (Chang et al., 2015) and DNA detection (C. Li et al., 2015). HCR has great significance for the detection of ctDNA. Wang et al. (2016) developed an ultrasensitive and selective electrochemical biosensor and recorded the electrochemical behavior of a tumor gene (P53) by DPV. In their research, they took advantage of a large area of chitosan–graphene (CS–GR) to increase the attachment of gold nanoparticles(AuNPs)-DNA so that electron transfer ability was improved.

3.2.2. Optical transducers

3.2.2.1. Localized Surface Plasmon Resonance (LSPR). LSPR is a class of optical phenomena. When incident photons coincide with the frequency of conduction of electrons by nanoparticles or metal islands, nanoparticles and metal islands produce very strong light absorption, causing peak displacement of LSPR. Anh H (Nguyen and Sang, 2015) presented a biosensor (as shown in Fig. 11) that was able to achieve dual detection for ctDNA (the mutation of E542K and E545K and methylation) based on coupled plasma modes of LSPR and AuNPs. In this study, they used PNA-AuNPs probes to recognize and bind 69 bp PIK3CA ctDNA, and then PNA-AuNPs-target genes were exposed to 200 fM. The initial response took place, which resulted in the change of refractive index and generated an LSPR peak shift of 4.3 nm. They used 5-mc monoclonal antibody (mAb)-AuNPs immunogold colloids to detect methylation of ctDNA. The mAb-AuNPs immunogold colloids were able to make secondary responses to plasmon coupling and amplify the resonant signal. The increased response by approximately 107% resulted in a four-fold increase in sensitivity. This study provided a new platform for the simultaneous detection of hotspot mutations and epigenetic changes of ctDNA.

3.2.2.2. Surface-enhanced Raman scattering (SERS). A SERS biotransducer is another optical phenomenon. The basic principle of the SERS-based sensor is that if the sample is adsorbed on the surface of some metal conductors or sols in the excitation region, the enhancement signal of the adsorbed molecule enhances, which is caused by the increase of the electromagnetic field on or near the surface of the sample (Kang et al., 2010; Du et al., 2014). Based on this, methylated DNA (5-mC) and its oxidation derivatives, namely 5-hydroxymethylcytosine (5-hmC) and 5-carboxylcytosine (5-caC) which are important indicators for cancers, were detected by a SERS sensor. By this method, the time taken was less than 60 min and methylation levels from a mixture at 0.1% could be distinguished (Ouyang et al., 2016). SERS overcomes the shortcomings of the low sensitivity of Raman spectroscopy, becoming promising for ctDNA detection. Wee et al. applied SERS technology to simultaneously detect BRAF V600E, c-Kit L576P and NRAS Q61K in the serum samples. As shown in Fig. 12. the target gene sequences were amplified by PCR. Then amplicons were tagged with SERS nanotags (reporter-modified gold nanoparticles) and enriched by magnetic beads. After that, the signal value changed under the illumination of the laser (Wee et al., 2016). Zhou et al. developed a new SERS sensor, combining with nanometer materials, to successfully detect ctDNA. Obvious SERS enhancement was observed because the presence of

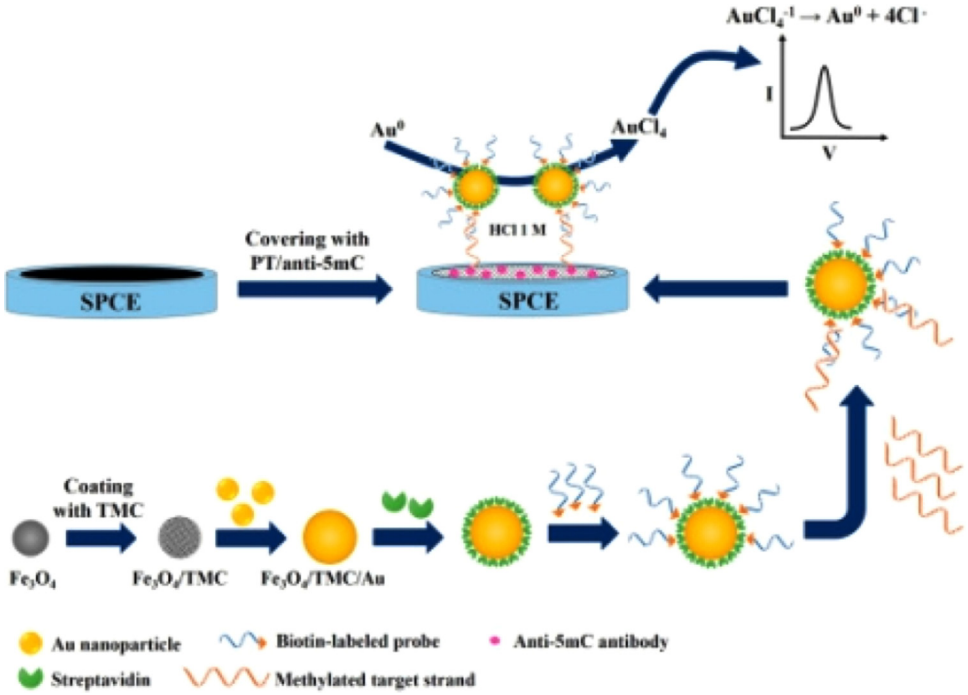


Fig. 9. Schematic representation of the electrochemical methylation detection mechanism (with permission from Daneshpour et al., 2016).

nanomaterials resulted in the electromagnetic enhancement effect (Zhou et al., 2016).

3.2.2.3. Colorimetric assays. Colorimetric biosensors work on the principle of the change in color intensity proportional to the concentration of the target substance. Based on this, Li et al. described a new strategy to detect ctDNA by combining with HCR and G-Quadruplex DNAzymes. As show in Fig. 13, HCR products were specially recognized by triplex forming oligonucleotides (TFOs). The asymmetrically split G-Quadruplex structure was formed and then the structure bound with hemin, which could catalyze the 2,2′-azino-bis (3-ethylbenzothiozoline-6-sulfonic acid) ($ABTS^{2-}$) and H_2O_2, yielding the green radical anion ($ABTS^{\cdot-}$) and H_2O. Quantify ctDNA by a spectrophotometer of small size and portability. This method could detect PIK3CA with a 0.1 pM detection limit (Li et al., 2017). This method has a potential for point-of-care testing in clinical analysis because of its enzyme-free and visualization. What's more, with the introduction of nanomaterials it has broad application prospects for the detection of DNA by colorimetric biosensors (Liu et al., 2018; Qu et al., 2018; Bhattacharjee et al., 2018; Ganareal et al., 2018). For example, Baetsenyoung et al. proposed a colorimetric detection of DNA by dextrin-capped AuNPs (d-AuNPs). They took advantage of the dispersion and aggregation characteristics of d-AuNPs for sequence-specific detection and successfully detected as little as 2.94 fM of the target DNA (Baetsenyoung et al., 2018).

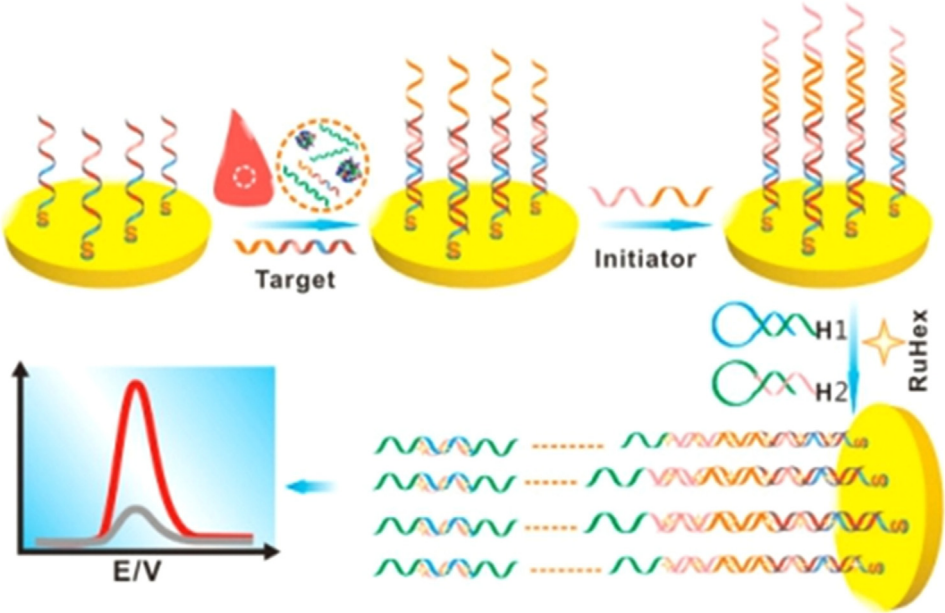


Fig. 10. Schematic illustration of hybridization chain reaction(HCR) mediated ultrasensitive genosensor of BRCA-1 gene (with permission from Hui et al., 2016).

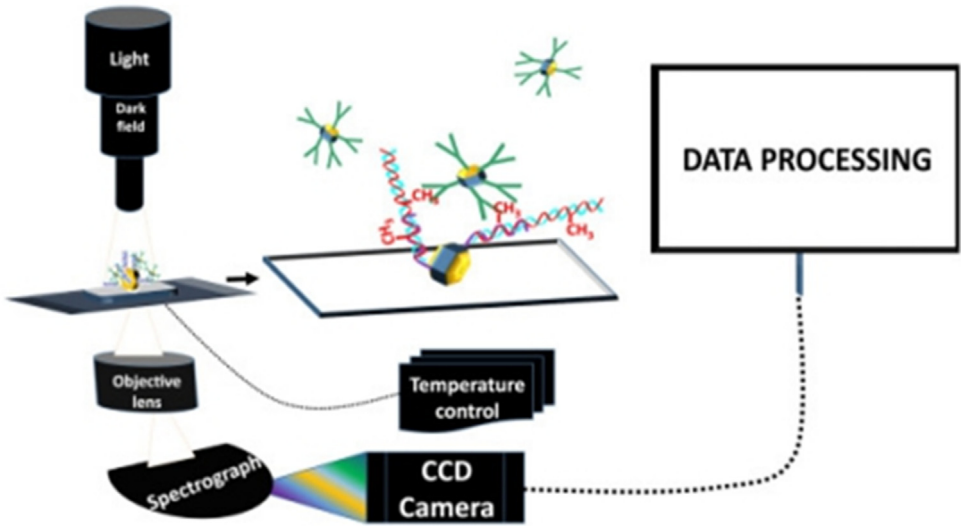


Fig. 11. Nanoplasmonic biosensor: detection and amplification of dual bio-signatures of circulating tumor DNA (with permission from Nguyen and Sang, 2015).

3.2.2.4. Fluorescent biosensors. Fluorescent biosensors rely on the change of fluorescent signals to detect the targets because the fluorescent signals are proportional to the targets concentration. Takalkar et al. (2017) proposed a portable fluorescent biosensor (as shown in Fig. 14) based on fluorescent carbon nanoparticle (FCN). In this study, there was a pair of DNA probes which were complementary with the target DNA at different portions (detection DNA probe and capture DNA probe). The FCN-detection DNA complexes which was formed by the covalent bonding between the carboxyl groups of FCN and the amino groups of DNA were dispensed on the conjugate pad of the biosensor. And the streptavidin-biotin-capture DNA conjugates was immobilized on a test line. In the presence of the target DNA, FCN-detection DNA probe-target DNA complexes were formed and migrated along the strip. When arriving at the test line, the sandwich-type complex was formed. Another probe was the control DNA probe, which was dispensed on the control line. The function of the control DNA probe was to determine whether FCN was working well. Quantify the target DNA by a portable ESE-Quant lateral flow reader (as shown in Fig. 14(B)). The detection limit of the method was 0.4 fM and the detection time was around 20 min. N. Li et al. (2016) proposed a fluorescent method. They successfully detected the target DNA in human serum based on HCR as the signal amplification and core–shell Fe3O4 polydopamine nanoparticles (Fe3O4@PDA NPs) as separation. The detection limit of this study was 0.05 nM. These methods holds promising potential to detect ctDNA.

4. Signal amplification strategies

In order to obtain a satisfactory output signal, choosing the suitable bioreceptor to improve the sensitivity of the biosensor is the key. In addition, signal amplification strategies are also necessary to improve the detection limit when detecting very small amounts of substances. Several signal amplification strategies applied to biosensors have been developed by investigators. In biosensor technology, nanomaterials are often used as signal amplification strategies because of their special optical, electrical, magnetic, mechanical properties, and other chemical properties such as small size effect, surface effect, quantum size effect and macroscopic quantum tunneling effect. Nanomaterials can be used as carriers of biomolecules to increase the adsorption of biomolecules. For instance, AuNPs were used as the carrier to absorb onto a large number of probes because of their large specific surface area, good biocompatibility and rich functional groups on the surface, resulting in the capture and enrichment of more target ctDNA (Nguyen and Sang, 2015; Wee et al., 2016). Due to the surface effect, the surface contains many dangling bonds and has high chemical activity, so nanomaterials can modify a large number of electroactive substances to realize the signal amplification. What's more, conductive nanomaterials are often used as the electrode or electrode modification materials, which not only promote electron transport at heterogeneous interfaces but also increase the reversibility of the redox species reaction at the electrode surface. For example, a nanostructured microelectrode successfully detected mutated sequences in patient serum with a limit of detection

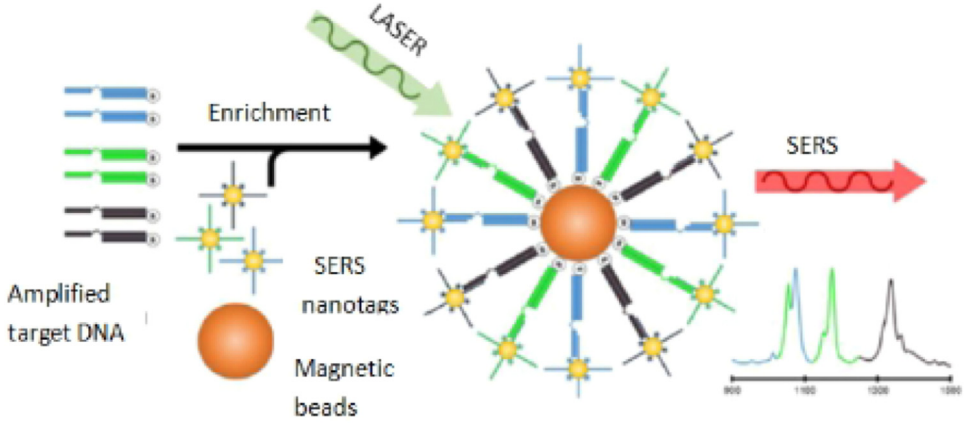


Fig. 12. Schematic illustration of the multiplex PCR/SERS assay and SERS nanotags (with permission from Wee et al., 2016).

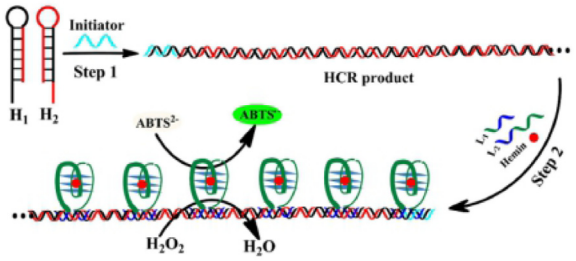


Fig. 13. Schematic illustration of the colorimetric biosensors (with permission from Li et al., 2017).

of 1 fg μL^{-1} (Das et al., 2015). A PXA/MoS_2/carbon paste electrode (CPE) was applied to detect ctDNA. A PXA/MoS2 nanocomposite served as the substrate for DNA immobilization and MoS2 promoted the electropolymerization of XA (Zhang et al., 2018).

In addition to nano materials, nucleic acids have their own means of signal amplification—the amplification reaction because nucleic acids have the property of self-replication. For example, PCR is a commonly used technique in-vitro amplification. Wee et al. (2016) proposed a new method for hypersensitized analysis of ctDNA by a multiplex PCR/SERS method combined with gold nanoparticles as SERS nanotags. 0.1% (10 copies, CV < 9%) of target sequences could be detected using this strategy. However, PCR often requires cumbersome temperature-changing procedure that is not easily controlled. Isothermal amplification is another kind of in vitro amplification technology. The process is always maintained at a constant temperature, and the target of rapid nucleic acid amplification is achieved by adding different active enzymes and their respective specific primers. Isothermal amplification mainly includes loop-mediated isothermal amplification (LAMP), Rolling Circle Amplification (RCA), Single Primer Isothermal Amplification (SPIA), Helicase-dependent Isothermal DNA Amplification (HDA), strand displacement amplification (SDA), Crossing Priming Amplification (CPA), Exponential amplification reaction (EXPAR) and HCR. Some biosensor technologies based on isothermal amplification to detect DNA have been developed by various researchers (Soraya et al., 2018; Zhao et al., 2018; Chen et al., 2018). The Wnt7B gene related to breast cancer, gastric cancer, esophageal cancer and pancreatic cancer was detected by an electrochemical biosensor based on enzyme-based exponential amplification. The schematic illustration of this strategy is shown in Fig. 15. This method was first developed in dsDNA using nicking enzymes. Then, long ssDNA was generated from the cleavage site by a polymerase extension reaction. Thereafter, long ssDNA triggered the hairpin self-assembly cycle reaction. Firstly, nicking enzymes were used to cut the dsDNA. Then, long ssDNA was released from the cutting site through the polymerase extension reaction. Thereafter, long ssDNA triggered a hairpin self-assembly cycle reaction. Finally, capture probes immobilized on an Au electrode could hybridize with the short ssDNA which was produced from this amplification process and the signal value changed. The linear range was 2 fM to 500 pM and the detection limit was 1.6 fM (S/N = 3). The isothermal amplification

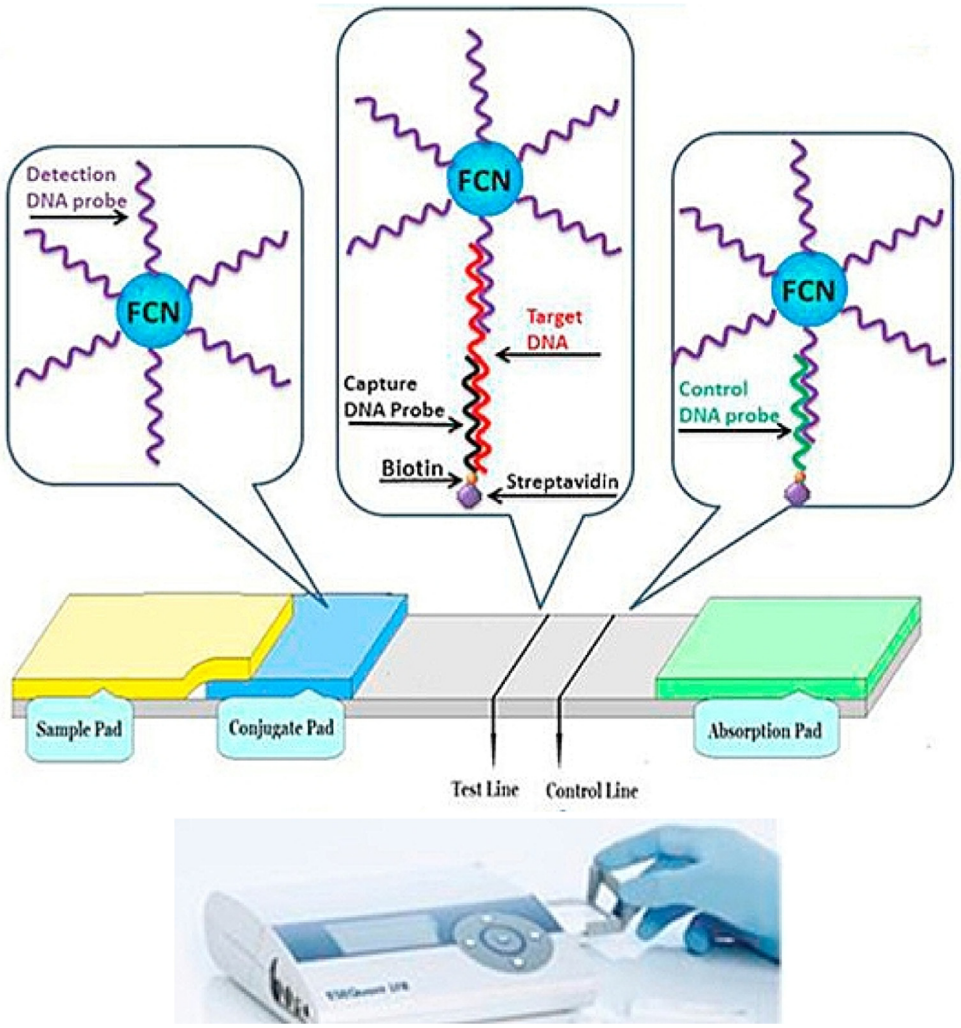


Fig. 14. (A) Schematic illustration of the biosensor. (B) The portable ESE-Quant lateral flow reader (with permission from Takalkar et al., 2017).

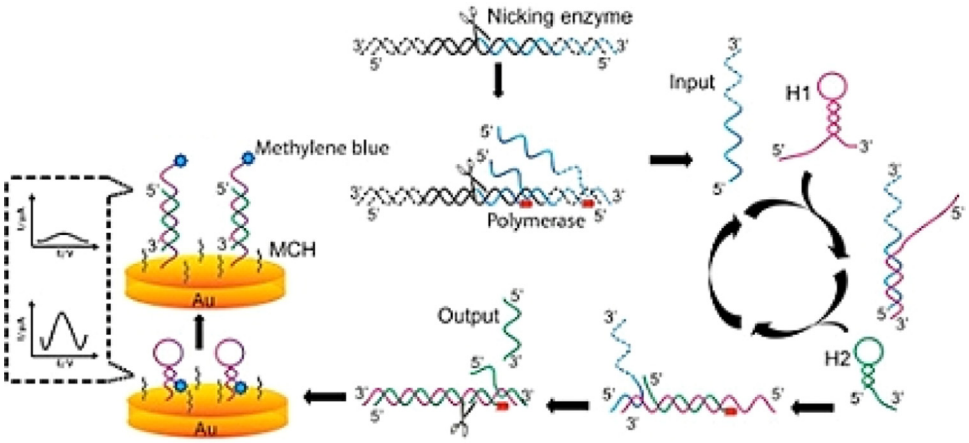


Fig. 15. Schematic illustration of electrochemical biosensor for Wnt7B gene detection based on enzymatic isothermal amplification (with permission from J. Li et al., 2016).

process lowered the detection limits so that a low concentration of the target gene could be detect. The specific recognition of the nicking enzyme and hairpin self-assembly-triggered recycling improved selectivity (J. Li et al., 2016).

Compared with PCR, isothermal amplification does not require such a complicated temperature change procedure and greatly simplifies the process. Isothermal amplification brings a new idea into the detection of ctDNA. Based on this, Q. Zhou et.al applied SERS to detect KRAS G12DM ctDNA by combining single-walled carbon nanotubes (SWNTs) and CuNPs. A lot of T-rich ssDNAs were released after isothermal amplification of the target ctDNA, hybridizing with the triple-helix molecular switch and RNase HII enzyme-aided amplification. Then a large number of T-rich ssDNAs were bound to the SWNTs because of the Π-Π stacking interactions and the large specific surface area of SWNTs. By this strategy, ctDNA could be measured with a sensitivity of 0.3 Fm (Zhou et al., 2016). Li et al. developed a colorimetric biosensor for analysis of ctDNA (PIK3CA) by combining G-Quadruplex DNAzyme activity through triplex DNA formation. The colorimetric sensing platform was successfully applied to detect ctDNA with a detection limit of 0.1 pM (Li et al., 2017).

5. Summary and prospects

In this review, recent advances in the liquid biopsy of ctDNA and biosensor applications are discussed. Compared with other types of tumor biomarkers, ctDNA has a unique advantage. In addition to the basic advantages of liquid biopsy such as convenient sampling, low invasiveness and real-time monitoring, it has better specificity. What's more important is that it can solve the problem of tumor heterogeneity. ctDNA has great clinical value. Although common detection techniques such as PCR and DNA sequencing contribute to the detection of ctDNA, their detection process is long, complicated and high cost. Therefore, their practical application has been limited. Biosensors have been developed to detect ctDNA because of they are simple, fast, sensitive and specific. Electrochemical biosensors are more widely used in the detection of ctDNA than optical biosensors because electrochemical biosensors not only have the ability to be used in clinical diagnosis, but also have commercial potential due to their miniaturization and low cost. Among bioreceptors, PNAs have received more attention. Because PNAs are electrically neutral, they are not affected by electrostatic repulsion when they hybridize with complementary targeting nucleic acid sequences. PNA-ctDNA can form a stable complex with higher stability than natural hybrid molecules. PNAs can greatly increase the sensitivity of genetic testing as hybridization probes. In recent years, some amplification strategies have been developed. Nanomaterials are commonly-used signal amplification methods due to their excellent properties. The amplification reaction also has the ability to amplify the signal because DNA can replicate itself. Among these methods, isothermal amplification is not like PCR which requires complicated experimental procedures. It has great application prospects for the detection of ctDNA. However, there are still few biosensor studies based on isothermal amplification. How to properly apply isothermal amplification to biosensors or how to combine the isothermal amplification with nanomaterials to achieve signal amplification is the direction we need to work on hard. In addition, the currently known biosensors for detecting ctDNA are limited to detecting ctDNA having known mutation sites. In future research, the challenge is to use biosensors to detect ctDNA at unknown mutation sites.

6. Future perspectives

Biosensors not only have the ability to be used for clinical diagnosis, but also have commercial potential because biosensors are simple to operate and easy to make into portable devices. Hence, biosensors can be used for the screening of cancer patients in the general population and by family doctors. Recently, the rapid development of microfluidics has been applied to various fields such as chemistry and life sciences. The biosensor associated with microfluidics technology, which combines the operation of different units in an integrated lab on a single chip, has advanced automation and integration. The microfluidic chip has great potential for ctDNA detection and analysis. In recent years, researchers have used paper as the detection base of the biosensor which has the advantages of low cost and harmlessness and used mobile phones as the workstation to detect and analyze target objects. If this innovative detection method is designed as a type of test strip to analyze ctDNA, the detection process can be easier to operate and commercialize for doctors and patients.

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Conflicts of interest

The authors declare no conflict of interest.

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