

Received: 2019.10.29

Accepted: 2019.12.23

Available online: 2020.01.29

Published: 2020.03.22

Nanotechnology Strategies for the Analysis of Circulating Tumor DNA: A Review

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Data Collection B

Statistical Analysis C

Data Interpretation D

Manuscript Preparation E

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Source of support:

This work was supported by the Shanghai Jiao Tong University Fund (No. YG2015ZD11), and the Innovation Group Project of Shanghai Municipal Health Commission (No. 2019CXJQ03)


Circulating tumor DNA (ctDNA) describes the fragmented DNA released from tumor cells into the blood. The ctDNA may have the same genetic changes as the primary tumor. Currently, ctDNA has become a popular biomarker for diagnosis, treatment, real-time clinical response monitoring, and prognosis, for solid tumors. Detection of ctDNA is minimally invasive, and repeat sampling can easily be performed. However, due to its low quality and short DNA fragment length, ctDNA detection still faces challenges and requires highly sensitive analytical techniques. Recently, liquid biopsies for the analysis of circulating tumor cells (CTCs) and circulating tumor-derived exosomes have been studied, and nanotechnology techniques have rapidly developed. Compared to traditional analytical methods, these nanotechnology-based platforms have the advantages of sensitivity, multiplex detection, simplicity, miniaturization, and automation, which support their potential use in clinical practice. This review aims to discuss the recent nanotechnological strategies for ctDNA analysis and the design of reliable techniques for ctDNA detection and to identify the potential clinical applications.

MeSH Keywords:

Circulating Tumor DNA • Liquid Biopsy • Nanomedicine • Nanotechnology • Nanomaterials

Full-text PDF:

<https://www.medscimonit.com/abstract/index/idArt/921040>

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Background

The detection and analysis of tumor-derived biomarkers is a key factor in current patient management and treatment decisions. Recently, blood-based liquid biopsies, which sample circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), circulating tumor-derived exosomes, and tumor-educated platelets (TEPs), have attracted increasing attention due to their non-invasive sampling and ability to serially assess disease progression as an alternative to conventional tissue biopsy or cytology [1,2].

Circulating-free DNA (cfDNA) is fragmented DNA present in the blood, released through cell apoptosis, necrosis, and autophagy [3,4]. The cfDNA from tumor cells, also known as ctDNA, carries tumor-specific genomic alterations, including mutations, methylation, loss of heterozygosity, and microsatellite instabilities [5,6]. Due to their short half-life in the circulation, from between 16 minutes to 2.5 hours [7], ctDNA analysis is applicable to the real-time monitoring of tumor burden. Also, tumor genotyping based on the detection of ctDNA has advantages, particularly in precision medicine, as a safe and minimally invasive alternative to tissue biopsy. For example, there are activating and resistant mutations of the epidermal growth factor receptor (*EGFR*) gene, which is an important driving gene in non-small cell lung cancer (NSCLC) that responds to *EGFR* tyrosine kinase inhibitors (*EGFR*-TKIs) [8]. Specifically, resistant *EGFR* T790M mutation in NSCLC occurs less commonly in primary tumors compared with activating mutations, such as the exon 19 deletion and *EGFR* L858R [9]. In 2016, liquid biopsy detection of *EGFR* mutations was approved for the assessment of treatment response following therapy with *EGFR*-TKIs in patients with NSCLC [10].

Currently, ctDNA analysis remains challenging. The challenges include large variability in the concentration of plasma cfDNA varying from 0 to more than 1,000 ng/mL in human samples [11], where ctDNA often representing a small fraction of the total cfDNA, which can be as low as 0.01% [12], and the most common ctDNA fragment length is less than 167 bp [13–15]. Therefore, ctDNA detection requires high-sensitive techniques. More importantly, the analytical methods used for the detection of mutant ctDNA must have a high specificity to avoid interference from wild-type genes that are released from normal cells. Therefore, multiplexed detection approaches have significant advantages in a small sample volume and for precision therapy.

Currently, the main techniques for ctDNA analysis are mainly based on sequencing, quantitative real-time polymerase chain reaction (qRT-PCR), or digital PCR (dPCR) platforms. Each one of these techniques has its own advantages and limitations in terms of sensitivity, specificity, and multiplexed

detection (Table 1). For example, next-generation sequencing (NGS) can offer high-throughput analysis with modest sensitivity, but is time-consuming [16,17]. The qRT-PCR-based platforms, such as amplification refractory mutation system (ARMS) [18,19] and peptide nucleic acid (PNA) clamping PCR [20,21], have high specificity and wide application due to the rapidity and ease of implementation, but their sensitivity varies greatly in plasma ctDNA analysis. Currently, droplet digital PCR (ddPCR) is an ultra-sensitive and accurate method for the detection of trace amounts of ctDNA and single-molecule analysis [22,23]. However, conventional ddPCR and qRT-PCR are only suitable for a specific type of molecular detection in a single reaction system, which means there is a need to perform multiplex ctDNA analysis by increasing or diluting the required cfDNA sample. Therefore, the key drawbacks of current methods limit their application and partly restrict the application of ctDNA in clinical practice.

Nanomedicine is a new branch of medicine that has contributed to breakthroughs in both the diagnosis and treatment of human disease. Recently, the rapid development of nanotechnology has led to technological progress in the use of liquid biopsies, particularly in the isolation of CTCs or circulating tumor-derived exosomes [24,25]. Nanometer-sized particles, such as iron oxide nanocrystals, gold (Au) nanoparticles (AuNPs), and quantum dots (QDs) have novel optical, electronic, magnetic, or structural properties that differ from solid compounds [24,26]. Several nanomaterials-based approaches to DNA analysis have been reported, but few have been applied to ctDNA detection. Therefore, this review aims to discuss the recent nanotechnological strategies for ctDNA analysis (Figure 1), the design of reliable techniques for ctDNA detection, and to identify the potential clinical applications.

Nanomaterials-Based Techniques for ctDNA Analysis

Beads, emulsion, amplification, and magnetics (BEAMing)

BEAMing is a digital PCR-based platform that combines magnetic beads-based emulsion PCR and flow-cytometry to identify and quantify mutant DNA [27]. The principal process of the commercial BEAMing (OncoBEAM™) (Sysmex Inostics, San Diego, CA, USA) involves pre-amplification of the genetic regions of interest, the formation of a water/oil emulsion and PCR amplification (emulsion PCR), breaking of the emulsions and hybridization to fluorescent probes, and the flow-cytometry readout [12,27]. A key feature unique to BEAMing is that each created microscopic emulsion droplet contains a single DNA molecule and a primer-coated magnetic bead. Following a conventional PCR amplification, the bead is coated with thousands of target DNA fragments, to provide a digital readout of

Table 1. Overview of the conventional and nanomaterials-based methods for the detection of circulating tumor DNA (ctDNA).

Method	Features	Detection limit	Advantages	Limitations
NGS	PCR-based sequencing	0.1–5% [17]	High-throughput analysis, detection of unknown genetic alterations	Time-consuming and expensive detection; low sensitivity
ARMS	qRT-PCR based on the 3'-end of the primer targeting the mutant sequence for extension	0.2% [18]	Ease of use, lower cost	Detection of known mutations, lower sensitivity
PNA-PCR	qRT-PCR based on PNA inhibiting the extension of the wild-type sequence	0.1% [20]	High specificity, ease of use	Detection of known mutations, lower sensitivity
ddPCR	DNA templates amplified separately in water-oil droplets and quantified	0.001% (20/200,000 copies) [22], 0.04% [23]	High sensitivity, a single molecule analysis, absolute quantitation	Detection of known mutations
BEAMing	DNA templates amplified on beads and quantified	0.01% (1/10,000 copies) [28]	High sensitivity, a single molecule analysis, absolute quantitation	Detection of known mutations, pre-amplification of the target sequence
PCR-SERS	PCR products analyzed by SERS detection	0.1% (10/10,000 copies) [42]; 9.24–59.7 pM [40,41].	Multiplexed analysis	Detection of known mutations.
PAPL	DNA templates amplified by PNA-aPCR and detected by suspension array	0.02–0.05% (2/10,000–5/10,000 copies) [47]	Multiplexed analysis, high specificity, high sensitivity	Detection of known mutations, qualitative analysis
Fe-Au nanoparticle-coupling strategy	Hybridization-based detection	0.12% [55]	PCR-independent detection	Lower sensitivity, detection of known mutations
Electrochemical DNA biosensor	Detection based on generated electronic signal	1 aM–1.72 fM (mutations) [60,62]; 2 fM–42 pM (DNA methylation) [59,63,70,71]	High sensitivity, PCR-independent analysis, detection of methylated DNA without sample pretreatment	Detection of known genetic alterations
IC3D ddPCR	ddPCR analysis based on the microfluidic device	0.00125–0.005% [76]	High sensitivity, a single molecule analysis, miniaturization	Detection of known mutations

NGS – next-generation sequencing; ARMS – amplification refractory mutation system; qRT-PCR – quantitative real-time polymerase chain reaction; PNA-PCR – peptide nucleic acid clamping polymerase chain reaction; ddPCR – droplet digital polymerase chain reaction; BEAMing – beads, emulsion, amplification, magnetics; SERS – surface-enhanced Raman spectroscopy; PNA – peptide nucleic acid; aPCR – asymmetric polymerase chain reaction; PAPL – peptide nucleic acid clamping asymmetric polymerase chain reaction and liquidchip; IC3D – integrated comprehensive droplet.

copy number for further flow-cytometry analysis. BEAMing delivers a high-sensitive detection for rare mutant DNA against a large background of wild-type genes (1/10,000) [28]. Until recently, BEAMing has been used to detect multiple ctDNA mutations, for example, *PIK3CA*, *BRAF*, *EGFR*, *KRAS*, *NRAS*, *IDH1*, and *ESR1*, which have demonstrated a high concordance between ctDNA in patient blood samples and somatic mutations

detected in tumor tissue [29–36]. As a digital PCR-based analysis platform, BEAMing, like ddPCR, is more sensitive than other assays for target sequence detection and is suitable for monitoring disease progression according to the absolute ctDNA quantitation [22,28].

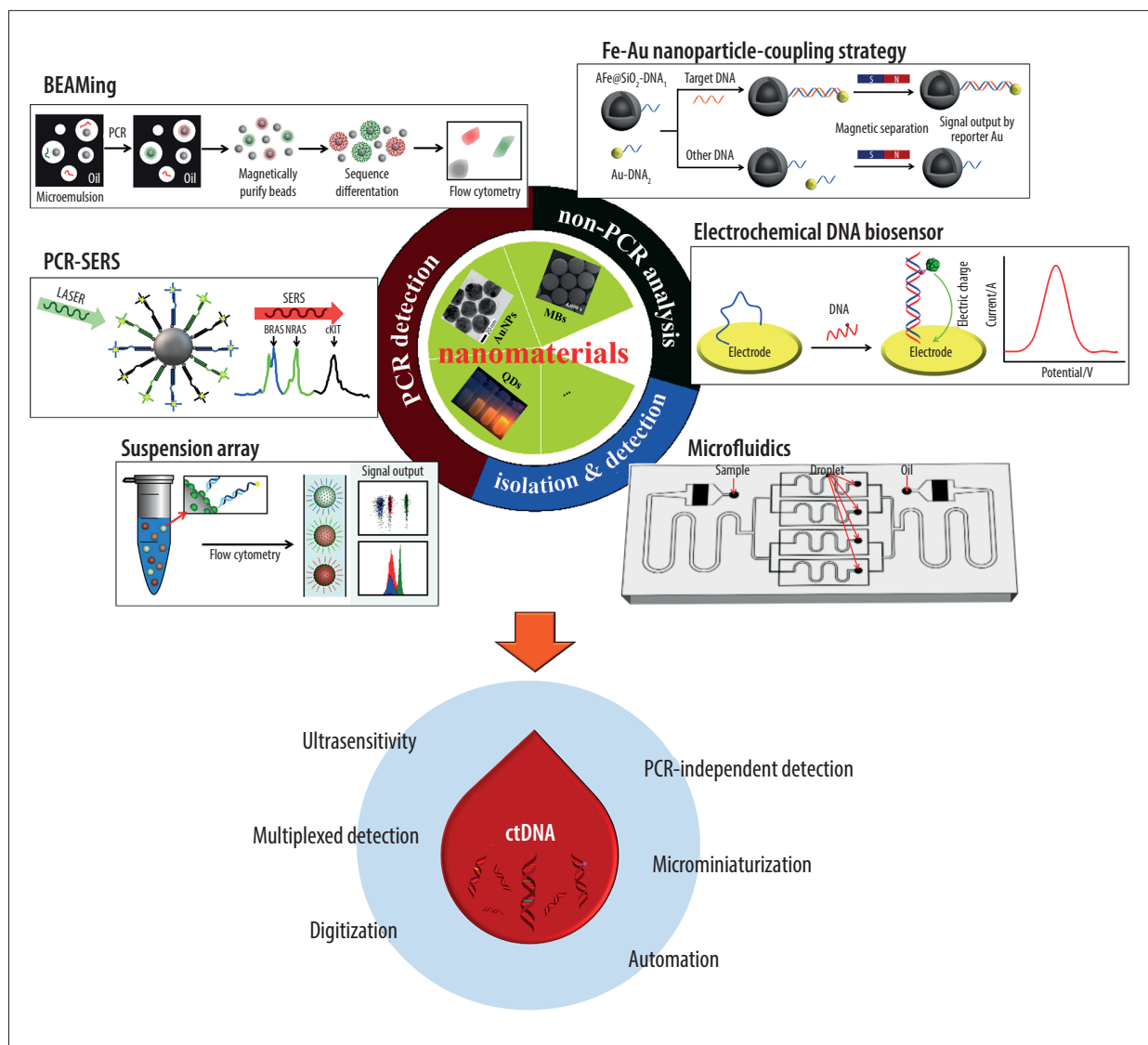


Figure 1. Nanomaterials-based strategies for detection and analysis of circulating tumor DNA (ctDNA). This figure shows the beads, emulsion, amplification, magnetics (BEAMing) method, modified from Dressman et al., 2003 [27]; the polymerase chain reaction and surface-enhanced Raman spectroscopy (PCR-SERS) method, modified from Wee et al., 2016 [42]; the suspension array method, modified from Chen et al., 2019 [47]; Fe-Au nanoparticle-coupling strategy, modified from Hu et al., 2018 [55]; the electrochemical DNA biosensor, modified from Campuzano et al., 2019 [57]; and microfluidics method, modified from Ou et al., 2019 [76].

PCR-surface enhanced Raman spectroscopy (SERS)

Raman scattering is a phenomenon that the scattered light has frequency change due to the interaction between oscillation of light and molecular vibration [37,38]. Because a Raman spectrum contains unique molecular fingerprint vibrational information on the sample, Raman spectroscopy contributes to the analysis of the chemical components, the molecular structure, and the interaction between molecules [38]. Although the Raman scattering signal is intrinsically weak, it can be significantly enhanced when the analyte is placed on or near a roughened

noble-metal substrate, which is either Au (gold) or Ag (silver), known as SERS [39]. The main SERS mechanisms include electromagnetic and chemical enhancements [38,39]. Electromagnetic enhancement relies on the excitation of the localized surface plasmon resonance (LSPR) of nanostructured or nanoparticle metal surface, and it contributes an average enhancement factor of more than 10,000 [38,39]. In the chemical enhancement, which is now believed to contribute an average enhancement factor of 100, a charge-transfer state is created between the metal and the adsorbate molecules [38,39]. The SERS-based PCR method is suitable for multiplexed ctDNA detection. Li [40,41]

and coworkers employed two different SERS-PCR strategies to detect multiple mutant genes. In the study of *EGFR* mutations, they used multiplexed PCR to amplify target mutant genes with primers labeled with the different fluorescence tags R6G and Cy5, and analyzed the purified PCR products via Ag colloid-based SERS detection [40]. The results showed that the limit of detection (LOD) was 5.97×10^{-11} M and 9.24×10^{-12} M for *EGFR* exon 19 and *EGFR* exon 21, respectively [40]. A further study used PCR without any modification of primers to amplify *BRAF*, *KRAS*, and *PIK3CA* mutations in colorectal cancer patients, and employed dye-labeled probes to tag mutant sequences for the following SERS detection [41]. The results showed that the LOD of this method was 5.15×10^{-11} M [41].

In a different approach, in 2016, Wee et al. [42] developed a nanotag-based PCR-SERS assay for multiplexed point mutations detection. In this assay, multiple mutant PCR amplicons were enriched using streptavidin-coated magnetic beads (SMB) in combination with a biotin molecular at the 5'-end of the reverse primer, and traced with specific SERS nanotags complementary to a unique barcode sequence of the forward primer. SERS nanotags are AuNPs modified with DNA probes and Raman reporters such as 4-mercaptobenzoic (MBA), 2,7-mercapto-4-methyl coumarin (MMC), or 4-mercapto-3-nitrobenzoic acid (MNBA). After enrichment with the SMB to remove the excess SERS nanotags, the mutation status was evaluated using Raman spectroscopy, where unique spectral peaks indicate the presence of the mutation of interest. Using this technique, the investigators identified *BRAF* V600E, *c-Kit* L576P, and *NRAS* Q61K mutations in melanoma with a sensitivity of 0.1% (10/10,000) [42].

Recently, in 2019, Lin et al. [43] developed a dual signal amplification SERS method based on the use of metal carbonyls (^{Re-5}CO and ^{Os-5}CO) as probes and substrates to detect low concentrations of Epstein-Barr virus (EBV) DNA. In this method, the target sequences captured by biotinylated DNA probes were first combined with the prepared SERS substrate (Au- ^{Os-5}CO -Au) via biotin-streptavidin interaction, and chelated by daunorubicin (DNR)- $SiO_2@Au-^{Re-5}CO$. After irradiation with an 808 nm laser, a number of ^{Re-5}CO labels were released from mesoporous silica-coated Au nanorods ($SiO_2@Au$) and bound to the surface of the SERS substrate. By measuring the SERS signal intensity of ^{Re-5}CO labels at 2113 cm^{-1} , the target DNA was able to be quantified. Their PCR-free SERS results demonstrated that the LOD was 57.74 nM (5.774×10^{-8} M) [43]. Compared to PCR-free SERS analysis, this method might detect much lower concentrations of DNA if PCR were performed to enrich target sequences.

Suspension array (liquidchip)

Suspension array, or liquidchip, is a high-throughput, large-scale, and multiplexed screening platform utilized to evaluate

biomolecules. This technical platform uses encoded microspheres and permits the parallel testing of multiple gene variants, as each type of microsphere has a single identification based on variations in the optical properties [44]. The first commercial suspension array (Luminex xMAP Technology) incorporates $5.6\ \mu\text{m}$ polystyrene microspheres loaded with two organic dyes with different wavelengths to create a library of barcodes using flow cytometry [45]. Using these encoded microspheres, Li et al. [46] developed a SurPlex[®]-xTAG70plex-EGFR liquidchip platform to detect 70 alleles of *EGFR*, *KRAS*, *BRAF*, and *PIK3CA* mutations from formalin-fixed and paraffin-embedded tissue sections. The method involves multiplex PCR, allele-specific primer extension, hybridization, and flow-cytometry. However, this high-throughput analysis method only detects 1,000 copies of mutant DNA against a background of 20,000–100,000 copies of wild-type DNA (sensitivity, 1–5%). Compared with other high-throughput DNA analysis platforms such as sequencing and microarray, suspension assay is more rapid and less costly. However, the lower sensitivity restricts its clinical application.

Suspension array for nucleic acid analysis is a hybridization-based platform. A useful measure to increase its sensitivity is to enhance the special enrichment of target sequences. From this perspective, our group developed PNA-aPCR-liquidchip (PAPL) as a unique method for multiple mutant ctDNA detection consisting of multiplex PNA clamping asymmetric PCR (PNA-aPCR) amplification and liquidchip test [47]. PNA is a DNA mimic wherein the deoxyribose-phosphate backbone is replaced by an oligoamide formed of N-(2-aminoethyl)-glycine units [48]. The complementary PNA/DNA heteroduplex has higher thermal stability than the corresponding DNA/DNA duplex, and even a base-pair mismatch could result in the unstable binding between PNA and DNA [49,50]. Consequently, there is a unique amplification of the target Cy5-labeled mutant sequences in PNA-aPCR process due to the PNAs as well as special primers blocking their wild-type sequences. Without purification or enzyme digestion, the whole multiplex PCR products directly hybridize with oligonucleotide-coupled dual-encoded magnetic beads (DEMBs). The formed Cy5-DNA/DEMBs complexes are detected by flow cytometer to perform multiplexed analysis according to high fluorescent signals on beads. Different from the commercial Luminex xTAG platform, DEMBs developed in our lab consist of QDs-encoded host particle and fluorescein-5-isothiocyanate (FITC)-labelled guest nanoparticles [51,52]. QDs, also known as nanoscale semiconductor crystals, are generally composed of elements from groups II-VI and III-V in the periodic table, including Ag, Cd, Hg, Ln, P, Pb, Se, Te, and Zn [53]. Compared with conventional organic dyes, QDs have narrow and symmetrical emission spectra, broad excitation wavelength, emission wavelengths that can be tuned, and high brightness [44,54]. These characteristics make QDs ideal candidates for the creation of a diverse barcode array. When QDs and organic dyes are used

together, the potential fluorescence interference can be identified using a barcode library. In addition to two separate fluorescent building blocks, DEMBs are assembled by simple and rapid magnetic separation, rather than centrifugation, due to their excellent magnetic host particles. We applied the PAPL assay to detect three high-frequent *EGFR* mutations, exon 19 deletion, L858R, and T790M, in patients with NSCLC. We identified that 2–5 copies of mutant *EGFR* were distinguished from 10,000 copies of wild-type *EGFR*, with a sensitivity of between 0.02–0.05%. Also, there was a high concordance rate of 88–95% between the *EGFR* mutational state in plasma cfDNA by PAPL and that by ddPCR. These findings show that the PAPL assay can be an effective analytical technique for multiplexed detection of mutant ctDNA.

Fe-Au nanoparticle-coupling strategy

Currently, almost all DNA analysis methods involve PCR amplification to enrich target DNA molecules to achieve highly sensitive detection. PCR is the most commonly used technique for DNA analysis, which has undergone three main technological advances that include qualitative analysis, relative quantitation (qRT-PCR), and absolute quantitation (dPCR), respectively. However, these methods have limitations, such as contamination of the PCR sample and non-specific amplification. With the rapid development in molecular technology, there are now many PCR-independent approaches to DNA analysis. For example, in 2018, Hu et al. [55] used AFe@SiO₂ and AuNPs to develop a Fe-Au nanoparticle-coupling strategy to identify the *KRAS* mutations in serum samples from lung cancer patients. In this technique, target single-stranded DNA (ssDNA) hybridizes to capture both DNA-coupling AFe@SiO₂ (AFe@SiO₂-DNA₁) and AuNPs (Au-DNA₂) [55]. AFe@SiO₂ magnetic nanoparticles are used to separate and enrich the formed AFe@SiO₂-DNA₁/target DNA/Au-DNA₂ complex from the solution [55]. Since AuNPs serve as reporters, the quantity in the separated products enables the quantification of the target DNA. However, although the Fe-Au nanoparticle-coupling strategy is a PCR-independent and hybridization-based platform, it exhibited high specificity and sensitivity for the mutant *KRAS* detection at a low concentration (0.1 pg/mL) [55]. To enhance the detection of trace mutant ctDNA in serum samples, PNA-functionalized magnetic nanoparticles can be used to remove wild-type sequences and complementary strands of the target *KRAS* mutant sequences before the nanoparticle-coupling step [55]. The results showed that Fe-Au nanoparticle-coupling technology detected *KRAS* mutations down to 0.12%, with the findings in agreement with those obtained with the ddPCR assay [55].

Electrochemical DNA biosensors

The electrochemical DNA biosensor is an analytical device that converts a target DNA response into an electronic signal [56].

Based on the linear target DNA concentrations, electrochemical biosensors not only assess the LOD, but also quantify target DNA molecules. The electrochemical DNA sensing platform consists mainly of capture probes immobilized on a surface of an electrode to capture target sequences and signal probes using electrochemical tags for signal generation. The high specificity of electrochemical biosensors relies on a special combination between capture probes and target DNA molecules. Currently, the reported capture probes in electrochemical biosensors involve ssDNA, DNA tetrahedron, and PNA [57]. The PNA probe has a higher specificity due to its increased thermal stability compared with that of the DNA probe [58–61]. The high sensitivity of electrochemical biosensors is due to signal amplification. One of the popular amplification strategies is the introduction of nanoparticles, such as AuNPs, carbon nanomaterials, and MoS₂ nanosheets [58,62–64]. Functional nanomaterials can not only produce a synergic effect in catalytic activity, conductivity, and biocompatibility to accelerate the signal transduction as electrode materials, but also amplify biorecognition events with specifically designed signal tags, leading to high sensitivity detection [65]. Until recently, electrochemical biosensors could detect DNA mutations at 1×10^{-18} M with a combination of nanoparticles and biological amplification, such as the hybridization chain reaction (HCR), and the dual enzyme (ribonuclease H II and terminal deoxynucleotidyl transferase) assay [60,66].

DNA methylation is an important epigenetic modification that governs gene expression and usually occurs in the cytosine within the CpG dinucleotide to form 5-methylcytosine (5-mC) [67]. Appropriate DNA methylation is essential for cell development and function. Therefore, any abnormalities in this process may lead to disease, including cancer [67,68]. Conventional methods for DNA methylation analysis include qRT-PCR, sequencing, and microarray, which require DNA sample pretreatment using bisulfite, methylation-sensitive restriction enzymes, or affinity purification [69]. Without sample pretreatment, electrochemical biosensors use a monoclonal antibody to 5-mC as the biorecognition element to detect methylated DNA. In 2018, Povedano et al. [63] presented two electrochemical biosensing platforms, the DNA sensor and the immunosensor, for the detection of methylated DNA using functionalized magnetic beads (MBs), the antibody to 5-mC as affinity bioreceptor, and amperometric detection of screen-printed carbon electrodes (SPCE) using the hydrogen peroxide/hydroquinone (H₂O₂/HQ) system. The DNA sensor is formed from DNA probes on the MB surface to capture the special methylated sequences, and the methylation in the captured target DNA is recognized by the specific antibody to 5-mC tagged with a secondary antibody, the horseradish peroxidase (HRP)-conjugated anti-mouse IgG [63]. The LOD of the DNA sensor was reported to be 26 pM (2.6×10^{-11} M) for the O-6-methylguanine-DNA methyltransferase (*MGMT*) gene and

42 pM (4.2×10^{-11} M) for Ras association domain family 1 isoform A (*RASSF1A*), respectively [63]. Different from the DNA sensor, the immunosensor uses two different antibodies that include the antibody to 5-mC immobilized on the MB surface to capture any methylated ssDNA sequence, and a second antibody conjugated with horseradish peroxidase (HRP) anti-ssDNA or HRP anti-FITC Fab, used as the detector [63,70]. Also, the immunosensor assay can detect global methylation using the HRP-anti-ssDNA as the detection antibody and identifies the locus-specific methylation with the HRP-anti-FITC Fab antibody. In 2019, Povedano et al. reported that methylated *MGMT* analysis indicated that the LOD of their immunosensor was 1.2 pM (1.2×10^{-12} M) [70]. Daneshpour et al. [71] described a previously developed nanobiosensor to identify target DNA methylation using the antibody to 5-mC immobilized on a SPCE surface and DNA probe-labeled Fe_3O_4 /N-trimethyl/gold (Fe_3O_4 /TMC/Au) nanocomposites as the gene-trapping and signal amplification unit. Based on the linear range of *RASSF1A* concentrations (1×10^{-14} to 5×10^{-9} M), the estimated LOD of this DNA nanobiosensor was reported to be 2×10^{-15} M [71]. Cai et al. developed another assay in 2018 [59], which uses gold PNA probe nanoparticles (PNA-AuNPs) and a lead phosphate apoferritin (LPA)-based dual biomarker detection platform to identify tumor-specific mutations and methylation of *PIK3CA*. The PNA probe and the antibody to 5-mC are used to recognize the different parts of ctDNA [59]. However, the AuNPs and the LPA are introduced for dual signal amplification in a biosensor, and the DNA biosensor yielded a linear current response to ctDNA concentrations (5×10^{-14} to 1×10^{-11} M) with a LOD of 1×10^{-14} M [59]. Therefore, electrochemical biosensing is an interesting approach to mutations and methylated ctDNA detection due to its speed, simplicity, low sample requirement, sensitivity, miniaturization, low cost, and PCR-independent detection method.

Microfluidics

Microfluidics is a technique for handling small volumes of liquids in picoliters to nanoliters [72]. The overall reaction within the microfluidic system involves increased heat transfer, higher surface-to-volume ratio, and controllable mixing efficiency and diffusion rates, resulting in higher energy transfer to reactive species [73]. Microfluidic devices are miniaturized devices that integrate multiple processes. There have been some recently published studies that have reported cfDNA purification on microfluidic devices through MBs-mediated enrichment. For example, in 2018, Kim et al. [74] developed a centrifugal lab-on-a-disc system equipped with electromagnetically-actuated and reversible diaphragm valves to isolate cfDNA from whole blood (3 mL) in a fully automated manner with the entire process of cfDNA purification including plasma separation, protein lysis, cfDNA binding, multiple washing steps, and elution of the cfDNA in less than 30 minutes [74].

Also, in 2019, Gwak et al. [75] developed a microfluidic chip with a gradient magnetic-activated cfDNA sorter (the vortex-GMACS) to spontaneously isolate cfDNA using silica magnetic particles in a method that took 19 minutes without any limitation of the sample volume.

In addition to ctDNA isolation, microfluidic devices demonstrate enormous potential for ctDNA detection. Also, in 2019, Ou et al. [76] developed an ultrasensitive IC3D droplet digital detection PCR (IC3D ddPCR) system that combines droplet microfluidics, fluorescence multiplex PCR amplification, and a high-throughput 3D particle counting system on a microfluidic chip to detect ctDNA. The results from this recent methodological study showed that the IC3D ddPCR system could identify *KRAS* G12D mutant alleles against a background of wild-type genomes at the high sensitivity of 0.00125–0.005% [76]. Compared to the commercial ddPCR kit (Bio-Rad, Hercules, CA, USA), this ddPCR platform accommodates a larger sample volume (20 $\mu\text{g}/\text{mL}$) and greater numbers of partitions (18 million reactions per mL) [76]. Microfluidic devices have attracted increasing attention in liquid biopsies due to their automation, microminiaturization, portability, high sensitivity, low-sample requirement, and speed of use.

Conclusions

Currently, ctDNA is an attractive substitute for conventional early tumor tissue molecular diagnosis, the evaluation of treatment efficacy, monitoring of tumor dynamics, monitoring of tumor progression, and prediction of cancer recurrence. There are different diagnostic methods to detect tumor-specific DNA in carcinoma patients. However, only a few approaches are suitable for ctDNA detection. Due to its short fragment length and low quantity, ctDNA analysis requires more sensitive techniques to ensure its reliability compared to methods used in tissue biopsies. It is worth noting that the existing techniques for ctDNA analysis focus mainly on the identification of mutations in advanced cancer treatment, but rarely on early diagnosis at a low concentration of ctDNA. Other genetic alterations, for example, methylation, loss of heterozygosity, and microsatellite instability are not currently analyzed. Therefore, ctDNA analysis is facing significant technological challenges, which may ultimately determine the potential clinical application. The nanotechnological platforms for ctDNA analysis discussed in this review have highlighted their advantages in terms of analytical sensitivity, multiplexed detection, simplicity, and cost benefits (Table 1). Generally, nanomaterials are used to enhance traditional analytical methods and develop new detection platforms. However, current nanomaterial-based ctDNA analysis is in its early stages and faces technical challenges in terms of stability, sensitivity, and specificity. In addition to BEAMing, PCR-SERS, the suspension assay, the

Fe-Au nanoparticle-coupling strategy, electrochemical DNA biosensors, and microfluidics remain in use in academic research studies and have yet to be evaluated with a large number of clinical specimens. Therefore, there is great potential for nanotechnology-based approaches to ctDNA analysis, and it is only a matter of time before they are approved for clinical use. With the rapid development of this technology, we anticipate that there will be some novel platforms for ctDNA

analysis with unique characteristics, including the non-PCR amplification, multiplexed detection, simplicity, rapidity, micro miniaturization, automation, and digital detection to meet future clinical demands.

Conflict of interest

None.

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