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REVIEW

Biosensors for liquid biopsy: circulating nucleic acids to diagnose and treat cancer

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Abstract The detection of cancer biomarkers freely circulating in blood offers new opportunities for cancer early diagnosis, patient follow-up, and therapy efficacy assessment based on liquid biopsy. In particular, circulating cell-free nucleic acids released from tumor cells have recently attracted great attention also because they become detectable in blood before the appearance of other circulating biomarkers, such as circulating tumor cells. The detection of circulating nucleic acids poses several technical challenges that arise from their low concentration and relatively small size. Here, possibilities offered by innovative biosensing approaches for the detection of circulating DNA in peripheral blood and blood-derived products such as plasma and serum blood are discussed. Different transduction principles are used to detect circulating DNAs and great advantages are derived from the combined use of nanostructured materials.

Keywords Biosensor · DNA · Electrochemistry · Surface plasmon resonance · Liquid biopsy

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Introduction

Cancer is a leading cause of death and accounted for about 8.2 million deaths in 2012 worldwide [1]. Its origin is linked to genetic mutations that accumulate stepwise, thus triggering a network of processes responsible for carcinogenesis [2]. For this reason, the analysis of the tumor-linked genetic mutations is increasingly used for diagnostic and prognostic purposes. Patient follow-up and therapy efficacy assessment are also based on the detection of oncogenic mutations, the presence of which is linked to the reduced efficacy of specific drugs able to slow the tumor progression. Frequent monitoring of tumor-linked genetic mutations is thus requested for an effective patient treatment plan.

Standard clinical protocols for the evaluation of oncogenic mutations are usually based on tissue biopsy, which consists of sampling cells from the human body by puncturing organs with the use of a small-gauge needle [3]. This procedure constitutes a significant barrier for easy and frequent monitoring of oncogenic mutations. In addition, it can potentially introduce clinical risks for the patient, is costly, and patient compliance with this procedure is variable, given its invasive nature as well as discomfort. A number of technical limitations have also been associated with this approach [4], the most important of which is the difficulty in accounting for tumor cells heterogeneity. In fact, individual tumors consist of different sub-populations that are individually sampled with great difficulty by needle biopsy [5]. Other important technical limitations include the possible seeding of tumor cells to other sites and that tumors are sometimes not accessible for tissue biopsy.

The above-mentioned limitations have prompted the development of new methods for the harvesting of cancer biomarkers in a simpler and more convenient way compared with tissue biopsy. Liquid biopsy has emerged as a potential

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complement of traditional biopsy for early cancer diagnosis [4]. Specifically, biological fluids are sampled to monitor the level of cancer biomarkers available in the chosen medium. Peripheral blood and blood-derived products such as plasma and serum have been, so far, the most widely investigated media for liquid biopsy.

Different analytes with potential implications for cancer diagnosis and treatment are present in blood. These include circulating tumor cells (CTCs) [6], circulating membranous structures (microvesicles, exosomes) containing molecular biomarkers [7], circulating free nucleic acids [4] (cfNAs. i.e., circulating cell-free DNA, RNA, microRNA [8]), and proteins [9, 10] (Fig. 1). In this context, circulating cell-free DNAs (ccfDNAs, i.e., DNA released from both normal and tumor cells) and circulating tumor DNA (ctDNA, i.e., DNA released only from tumor cells) offer a number of advantages that have attracted enormous attention over the last 5 years and resulted in CE-IVD tests available on the market starting from 2015 [11]. In addition, a blood-based test for ctDNA detection was marketed by Pathway Genomics in September 2015 [12]. The assay reached the U.S. market with no previous U.S. Food and Drug Administration (FDA) clearance or approval, a procedure that caused concern at FDA.

Gaining regulatory approvals from national and international agencies represents an important step toward the application of the liquid biopsy in clinical practice. So far only one assay detecting CTCs in blood (CellSearch CTC, Veridex, LLC) was FDA-cleared in 2004 for the monitoring of patients with metastatic breast, colorectal, or prostate cancer on the basis of a liquid biopsy approach. The only FDA approved noninvasive genomic tumor test is ColoGuard (Exact Sciences). It spots colon cancer-linked DNA from patient stool and received FDA approval in 2014 [13].

The very limited number of FDA-cleared or -approved assays for liquid biopsy testifies that the new approach for cancer early diagnosis and patient monitoring is mostly confined to basic and clinical research settings. However, efforts are made to enter the liquid biopsy IVD market, which is expected to gross U.S.$ 1 billion mark by the end of 2020 [14], as testified by both the $100 million funded GRAIL company launched by Illumina in January 2016 as well as the commitment of other large companies such as Johnson and Johnson, Qiagen, and Roche.

ctDNAs become detectable in blood before other investigated circulating free biomarkers, such as CTCs, and can be used for longitudinal disease monitoring (patient monitoring over time) [15]. In fact, studies comparing ctDNA and CTC levels in bladder, breast, and colorectal cancer patients have found that ctDNA is often present in patients with no detectable CTC levels [16, 17].

Extracellular nucleic acids released from both normal and tumor cells and freely circulating in the human plasma were discovered in 1948 [18], but it was only in 1977 that an increased concentration of ccfDNA was found in the serum of cancer patients compared with healthy individuals [19], while evidences that tumor cells can release their DNA (ctDNA) into the blood were provided in the 1990s [20, 21]. Different studies demonstrate that the detection of ccfDNA and ctDNA in body fluids can complement tissue biopsy and hopefully will replace it in the future [22].

Properties of ccfDNA and ctDNA in plasma and serum pose several technical challenges for cancer detection and

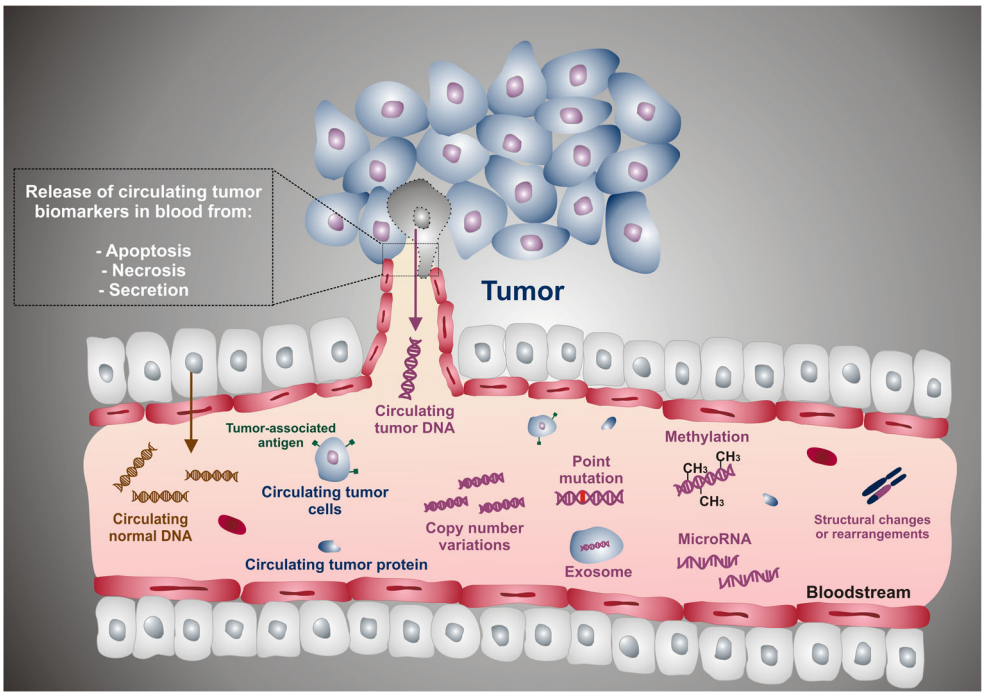

Fig. 1 Schematic representation of tumor-linked molecular markers, cells, and membranous structures circulating in the blood. Cancer cells release tumor biomarkers in blood through various physiological events, such as apoptosis, necrosis, and secretion

monitoring. In particular, challenges arise from ccfDNA and ctDNA low concentration and relatively small size. ccfDNA concentration in cancer patients is highly variable and is often increased compared with healthy individuals. ccfDNA concentration is dependent on the sample pretreatment protocol and nucleic acids extraction kit [23, 24]. Typically, median ccfDNA concentrations lower than 20 ng mL^{-1} are found in plasma obtained from cancer patients, whereas healthy individuals show lower median concentrations (lower than 7 ng mL^{-1} in plasma) with overlaps with concentration ranges for cancer patients [25–27]. ctDNA is a fraction of the total ccfDNA in cancer patients with percentages depending on cancer type and progression stage [16]. In general, mean number of mutated DNA fragments (ctDNA) per milliliter ranging from few units to 10^4 have been found in plasma of cancer patients, accounting for few units percent of ccfDNA. Interestingly, a ctDNA trend showing better concordance to clinical progression than CTCs has been reported for metastatic breast cancer patients [16]. This evidence, although not conclusive [28], might suggest that diagnostic methods involving both ctDNAs and CTCs could be used to analyze tumor status and changes [29].

Even though the mechanism leading to ccfDNA and ctDNA release in blood is still not fully understood, cell deaths through apoptosis and necrosis are claimed as the main processes responsible for the release of cellular DNA fragments into blood. Fragment size ranges in length from few hundreds to few thousands base-pairs. ctDNA fragments of about 145–180 bp are supposed to be generated from cell apoptosis, whereas longer fragments (up to 10 kbp) are generated from cell necrosis [30].

The mentioned ccfDNA and ctDNA properties depict limitations for sequencing approaches such as Sanger sequencing and pyrosequencing that can only distinguish mutant from normal alleles in ccfDNA down to 15 % and 5 %, respectively. Such detection capabilities do not suffice to detect tumor-derived mutant fragments in early stage cancer [31, 32]. By contrast, next-generation sequencing (NGS) [33–35] or innovative polymerase chain reaction (PCR)-based detection approaches such as scorpion amplification refractory mutation systems (ARMS-Scorpion) [36], digital-droplet PCR [16], pyrophosphorolysis-activated polymerization (PAP) assays [37], beads, emulsion, amplification and magnetics (BEAMing) [31], and tagged-amplicon deep sequencing (TAM-Seq) [38] provide good possibilities for the quantification of mutant to normal circulating DNA ratio, with capabilities in discriminating mutant allele of at least 2 % (Table 1).

The above mentioned technologies suffer from intrinsic limitations, such as a slow turnaround time (days for DNA sequencing, hours for PCR-based approaches) [15], relatively large input volume (1 mL of serum or plasma is typically needed for ctDNA extraction) [52], and biases arising from sample contamination and PCR-induced artifacts [53, 54].

The complexity of the analytical protocol and not affordable cost per test represent other important barriers preventing the wide use of some of the above mentioned technologies in daily clinical practice. To overcome these limitations, efforts are made in developing new technologies for the monitoring of ccfDNA and ctDNA.

In this context, biosensing platforms offer attractive alternatives to currently available systems, thanks to currently available advanced possibilities for the sensitive, rapid, and inexpensive target biosensing [55]. In particular, progress has been recently made in nucleic acid biosensing with improvements in biosensor fabrication and production quality that benefit from the availability of new methods for the design of optimized surface chemistry [56], highly efficient probes for nucleic acids detection [57, 58], and protocols for the enhancement of the detected signal [59, 60].

In this review, we will summarize recent advances in biosensing for ccfDNA and ctDNA detection. While a few excellent reviews have given comprehensive summaries of PCR-based processes for ccfDNA and ctDNA detection [23, 61], here we will focus on some recent advances on PCR-free biosensing methods that are expected to benefit from the absence of PCR intrinsic biases and limitations [54].

Over the last 10 years, PCR-free biosensing technologies have been investigated with the aim to obtain more stable, reliable, and sensitive detection platforms to be used for clinical diagnostic purposes [54]. In particular, PCR-free detection of nucleic acids has been attained by putting efforts in optimizing biosensor fabrication procedures and assay design.

Table 2 summarizes PCR-free biosensing technologies that demonstrate the ability to detect circulating DNA and that will be discussed here. They exploit the electrochemical or optical signal transduction, and most of them integrate nanostructured materials for the enhancement of the detected signal.

Electrochemical methods for the detection of circulating nucleic acids

Electrochemical biosensors have great potential for the production of low cost and multiplexed biosensing platforms for the sensitive detection of nucleic acids [62, 63]. Even though electrochemical biosensors have been effectively applied to the detection of a variety of different cancer biomarkers [64–66], the development of electrochemical biochip for ccfDNA and ctDNA quantification is still facing great challenges.

An important improvement has recently been achieved in this domain by combining surface nanostructuring and advanced DNA probe design with a specific electrochemical clamp assay. The assay has been designed to detect BRAF and KRAS mutations in serum samples taken from lung and melanoma cancer patients [67]. It benefited from the use of

Table 1 Overview of PCR-based methods for the detection of circulating tumor DNA in body fluids of cancer patients

Technique[a]	Tumor-specific aberration	Source	Detected mutant to wild type ratio (%)	Ref.
Conventional PCR	KRAS, BRAF	Tissue	10–25	[39, 40]
COLD-PCR	KRAS, BRAF	Tissue	2.5–12.5	[39–41]
ME-PCR	KRAS	Tissue	1.0	[42]
BEAMing	APC, PIK3CA, EGFR	Plasma	≥0.01	[31, 43–45]
Next-generation sequencing	Chromosomal alterations	Plasma	≥0.75	[46]
WGS				
dPCR	TP53, EGFR, BRAF, KRAS	Plasma	≤1.0	[38]
ddPCR	EGFR, KRAS, BRAF / PIK3CA	Plasma	0.001– 0.5 / 0.01–2.99	[47–50]
TAM-Seq	TP53, EGFR, BRAF, KRAS	Plasma	≤2	[38]
dPCR/TAM-Seq	PIK3CA/TP53	Plasma	0.1 / 0.14	[17]
SNPase-AMRS-quantitative PCR	BRAF, PTEN, NRAS	Plasma	0.0005	[51]

[a] COLD-PCR: co-amplification at lower denaturation temperature-PCR; ME-PCR: mutant enriched PCR; BEAMing: beads, emulsion, amplification, magnetics; WGS: whole-genome sequencing; dPCR: digital PCR; ddPCR: droplet digitial PCR; TAM-Seq: tagged-amplicon deep sequencing; ARMS: amplification refractory mutation system

peptide nucleic acids (PNA), the sequences of which were selected in order to hybridize (to clamp) wild-type DNA and all mutants except the mutated sequence to be target (Fig. 2). PNA clamped sequences cannot interact with PNA probes complementary to the detection target that was immobilized on the nanostructured microelectrode surface. This specific procedure improves the accuracy of the detection of the selected mutants that are targeted with a rapid (15 min) and PCR-free approach.

The device was based on an array of 40 sensing areas obtained by growing 3D Au nanostructures. In order to increase the assay sensitivity, Au nanostructures were coated with a thin layer of Pd to obtain finely nanostructured microelectrodes (NMEs) (Fig. 2) [68]. The differential pulse voltammetry electrochemical readout was based on the use of $Ru(NH_3)_6^{3+}$ and $Fe(CN)_6^{3-}$ electrocatalytic reporters after the target binding to surface immobilized PNA probes. $Ru(NH_3)_6^{3+}$ is adsorbed by the negatively charged phosphate backbone of the surface immobilized target sequence and then is electrochemically reduced by applying a reduction potential. $Fe(CN)_6^{3-}$ oxidized $Ru(NH_3)_6^{2+}$, thus restoring the original $Ru(NH_3)_6^{3+}$

Table 2 Use and performances in circulating nucleic acid detection of different sensing platforms

Biomarker	Tumor type	Sample	Biosensor	Surface ligand	Detected target conc.	Detection limit	Reference
KRAS and BRAF mutations	Lung and melanoma cancers	Serum	Electrochemical	PNA	1 fg μL^{-1} –100 pg μL^{-1} (RNA target)	1 fg μl^{-1} (RNA target)	[67]
ccfDNA	-	Serum	Electrochemical	N-doped MGA/ GNS	1.0×10^{-21} – 1.0×10^{-16} g mL^{-1} (dsDNA)	0.42 (7) ng mL^{-1} (ccfDNA)	[75]
ccfDNA	Lung cancer	Serum	Confocal spectroscopy	-	-	320 bp–1000 bp (target size)	[78]
DNA methylation	Prostate, breast, lung, leukemia/ lymphomas, colon cancers	-	SPR	ssDNA	100–400 nM (synthetic methylated target)	100 nM (synthetic methylated target)	[92]
PIK3CA gene mutations and methylation	Colon, breast, liver, stomach, and lung cancers	Serum	LSPR	PNA	50–3200 fM (dsDNA)	50 fM (dsDNA)	[98]
EGFR gene mutation	Lung cancer	Saliva/ Plasma	EFIRM	ssDNA	0.1 % detected mutant to WT ratio-	-	[100]

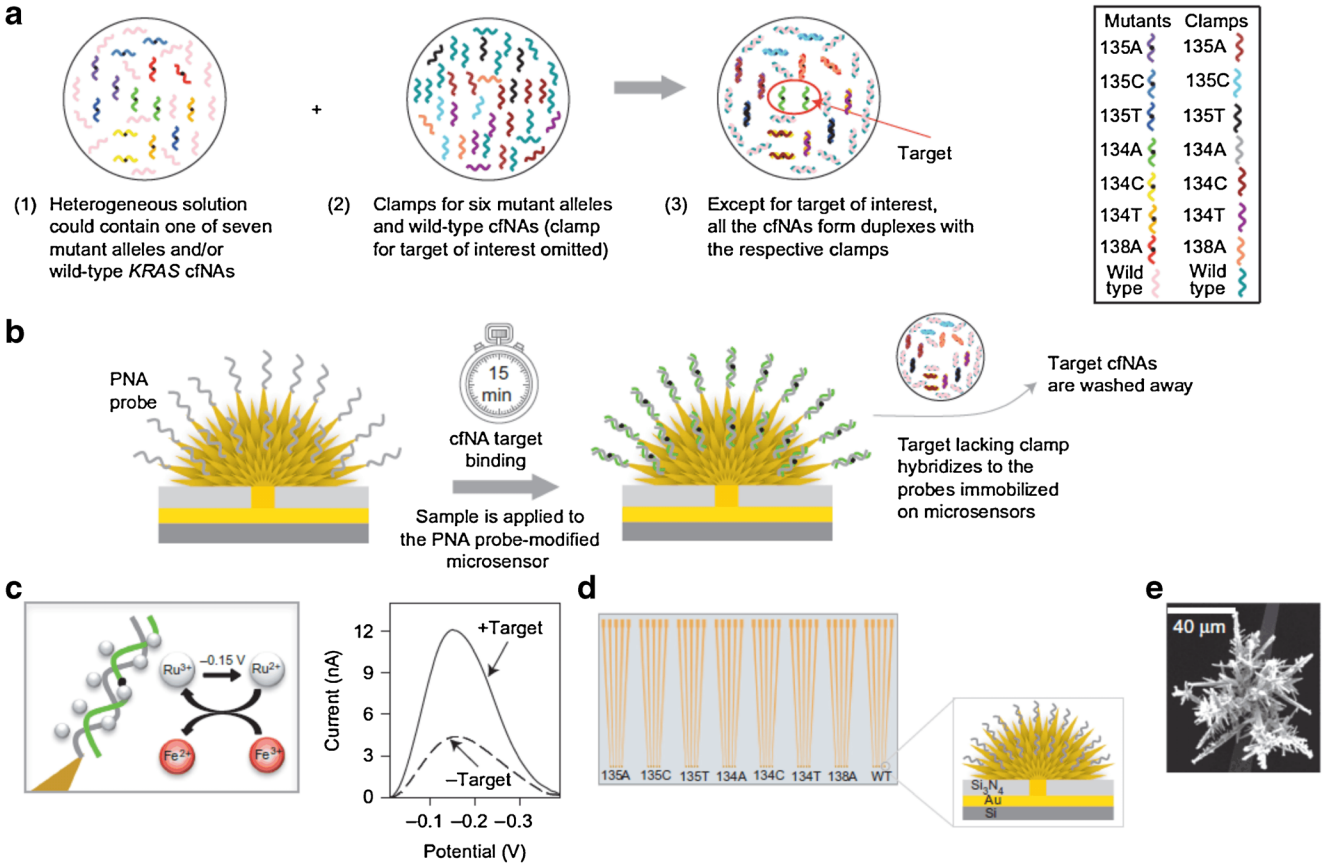

Fig. 2 Schematic representation of the electrochemical clamp assay. (**a**) ctDNA isolated from serum samples was mixed with PNA clamps. (**b**) The mixture was deposited onto the PNA-modified nanostructured surface. (**c**) Differential pulse voltammetry detection. (**d**) Microfabricated chip. (**e**) SEM image of the Pd nanostructured microelectrode. Reproduced with permission from reference [67]

species. The multiple turnover of electroactive species generates a large electrocatalytic current, thus enhancing biosensing performances.

The assay was tested against serum samples from lung and melanoma cancer patients, and cfNA KRAS and BRAF mutations were successfully detected in the undiluted serum. In order to assess the limit of detection (LoD), RNA isolated from A549 cells carrying the 134A mutation was used and a 1 fg μL^{-1} LoD was calculated.

The above discussed electrochemical clamp assay demonstrates the important role played by nanostructured material in improving performances of biomolecular assays. In particular, nanomaterial-based electrochemical signal amplifications have been widely investigated with the aim of improving both sensitivity and selectivity of biosensors [69]. In this context, also the detection of cfNAs takes advantage of nanostructured materials such as graphene aerogel (GA) [70, 71]. Properties of GA have recently been investigated and potential applications in different fields have been envisaged. Unfortunately, GA suffers from poor electrical conductivity and limited mechanical strength. Therefore, great efforts have been exerted to build innovative GA-based materials with improved electromechanical properties [72, 73]. More recently, high-density GA with better mechanical strength and electrical conductivity compared with conventional GA has been produced through a simple multiple gel method. However, the material is highly hydrophobic and it may lead to a relatively slow electrochemical response towards DNA target sequences. To overcome such limitation, high-density GA has been functionalized with gold nanostars (GNS), thus improving its electrical conductivity [74]. The enhanced differential pulse voltammetric response provided by nitrogen-doped multiple GA/GNS (N-doped MGA/GNS) is useful to directly detect ccfDNA in human serum [75]. In fact, after the extraction of ccfDNA from human serum, a facile, rapid, and sensitive detection of ccfDNA can be performed. N-doped MGA/GNS holds excellent electrocatalytic activity towards $Fe(CN)_6^{3-/4-}$ redox pair providing a linear correlation between the current measured in differential pulse voltammetry experiments and dsDNA concentrations in the 1.0×10^{-21} g mL^{-1} to 1.0×10^{-16} g mL^{-1} range with a detection limit of 3.9×10^{-22} g mL^{-1}. The method has been applied to the detection of ccfDNA extracted from human serum and a concentration of

ccfDNA of 0.42 ± 0.07 ng mL^{-1} was found with a recovery ranging between 96.7 % and 102.4 % [75].

Optical methods for the detection of circulating nucleic acids

The detection of nucleic acids by using optical transducers has been extensively investigated [76]. Also in this case, applications to circulating nucleic acids not involving nucleic acid amplification steps have been demonstrated only recently. These involve ultrasensitive approaches for the detection of specific sequences or mutations in ccfDNA and ctDNA as well as new methods for the sizing of ccfDNA fragments.

The fragment size is an important property of ccfDNAs linked to processes leading to the fragmentation of somatic DNA. Interestingly, ctDNA has been consistently found in fragments shorter than somatic ccfDNA [77].

Confocal spectroscopy combined with properly designed microfluidic devices for 1-D detection of fluorescence generated by a TOTO-3 dye interacting ccfDNA can be used to size ccfDNA fragments [78]. The approach, called microfluidic cylindrical illumination confocal spectroscopy (μCICS) (Fig. 3), can be applied for sizing and quantifying ccfDNA from serum samples from cancer patients.

The sizing of ccfDNA fragments can be achieved by calibrating fluorescent burst sizes with λ Hind III digest DNA. A linear correlation has been found between burst size and DNA fragment size in the 564–27,500 bp range. When the same approach is applied to diluted serum samples from lung cancer patients, a greater proportion of larger fragments in the 320–1000 bp range is found for stage IV patients compared with stage I patients. Interestingly, μCICS is able to size non-amplified ccfDNA fragments from diluted serum samples of cancer patients, thus testifying that microfluidics single molecule spectroscopy can be a rapid, facile, and inexpensive alternative to established PCR-based methods.

Plasmonic biosensors have emerged over the last decade as promising platforms for advanced nucleic acids detection [79–82]. In particular, surface plasmon resonance (SPR) biosensors have been developed for the highly sensitive detection

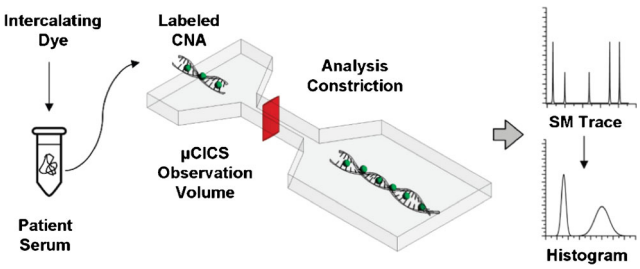

Fig. 3 Schematic illustration of the PCR-free microfluidics single molecule confocal spectroscopy method for ccfDNA sizing. Reproduced with permission from reference [78]

of DNA sequences [83–85] with specific applications demonstrating the attomolar detection of point-mutations in non-amplified human genomic DNA [86].

SPR-based biosensing can detect different cancer biomarkers [87, 88] including point mutations in KRAS gene [89]. In the latter case, the detection benefit of the use of surface immobilized PNA probes, which have been consistently used to improve the selectivity of the hybridization reaction with the target complementary sequence [90, 91]. Advantages offered by PNAs in detecting complementary target sequences are counterbalanced by their higher cost compared with synthetic oligonucleotides (about a factor of 20). However, the cost of PNA probes per assay is often negligible compared with the overall production cost of the biosensing device per assay.

DNA methylation can also be detected by SPR. A single-stranded oligonucleotide capable of hybridizing to methylated DNA target by two inverted recognition ends (molecular inversion probe, MIP) can be used in this case, thus allowing the real time and label-free detection of regional DNA methylation [92]. DNA methylation results from the addition of a methyl group to the carbon-5 position of cytosine in a CpG dinucleotide. Methylation level of circulating DNA has been linked to tumor progression and, for this reason, the quantification of the DNA methylation level is proposed as a cancer diagnostic method [93].

Localized SPR (LSPR) offers a powerful tool for biosensing [94, 95], which is sometimes used to complement detection approaches based on propagating SPR [96]. For LSPR biosensing, metallic nanostructures with plasmonic properties are usually interfaced with specifically designed probes and surface architectures in order to serve in ultrasensitive assays. In fact, metallic nanostructures such as gold nanoparticles exhibit unique optical features, related to localized SPs, which derive from coupled collective oscillations of charge density and electromagnetic field [97]. The resonant excitation of localized SPs depends on the size, shape, and surrounding dielectric environment of the plasmonic nanostructure.

LSPR has been used for the ultrasensitive and simultaneous detection of different cancer biomarkers, namely tumor-specific mutations (E542K and E545K) and ctDNA methylation (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene, PIK3CA) [98]. PNA sequences have been used as the probe for ctDNA mutations, whereas a specific anti-5-methylcytosine monoclonal antibody (mAb) has been immobilized onto gold nanoparticles for the targeting of the ctDNA methylation. The detection apparatus comprises a dark-field microscopy combined with a simple spectroscopic system to collect Rayleigh scattered light. The exposure of functionalized nanoparticles to human serum samples spiked with 50 f. of point mutated

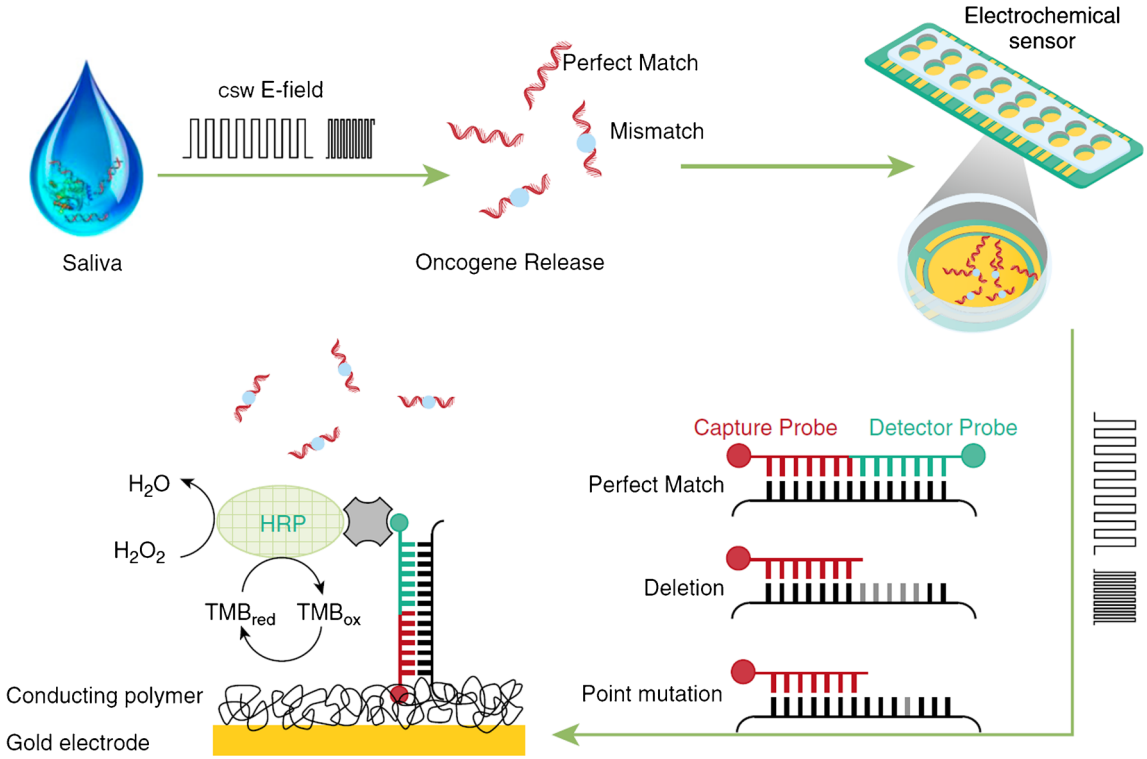

Fig. 4 Schematic representation of EFIRM detection of ccfDNA from plasma or saliva samples. Reproduced with permission from reference [100]

ctDNAs generated a 4.3 nm LSPR-peak shift. The shift is enhanced by using mAb functionalized gold nanoparticles able to target methylcytosine sites in ctDNA.

Other transduction principles for circulating nucleic acids biosensing

Unconventional transduction principles have also been investigated for the detection of circulating nucleic acids biomarkers. In particular, two different approaches have so far been investigated with the direct analysis of ccfDNA in body fluids.

An array of 30 magnetoresistive (MR) sensors integrated in a portable biochip platform has been developed for the multiplex detection magnetically labeled DNA targets [99]. The approach has been applied to detect DNA fragments (ALU115 and ALU247) used to evaluate ccfDNA integrity and obtained after the extraction of ccfDNA from plasma. Key features of the proposed MR sensor are portability, versatility for manufacturing, and capability for integration with various electronic and microfluidic components. However, sensitivity is still not good enough to avoid the PCR amplification of sequences to be detected.

The electric field induced release and measurement method (EFIRM) represents another innovative approach recently proposed for the detection of epidermal growth factor receptor (EGFR) gene mutations present in ccfDNA in saliva or plasma of lung cancer patients [100]. The same approach has also been used for the detection of exosomal RNA/proteins biomarkers transported in blood and saliva [101]. The assay is based on an amperometric detection from a conducting polymeric-based electrochemical chip with an array of gold electrodes (Fig. 4). However, the peculiar aspect of the approach is represented by the cyclic-square wave electric-field (csw E-field) applied during the hybridization between the capture probe copolymerized with the polymeric chip and the target sequence in saliva or plasma. The cyclic wave electric field favors the hybridization of target sequences perfectly matching the capture probe, thus making possible the detection of EGFR mutations in ccfDNA in body fluids with no need for PCR amplification.

Conclusions

The detection of ccfDNA and ctDNA opens new perspectives for cancer early diagnosis and patient follow-up based on liquid biopsy. Over the last few years, few bioanalytical methods have been shown effective for this purpose (Table 1). All of them require PCR amplification of the genetic sample and take several steps to achieve the final detection. PCR-based approaches benefit both simplified quantification methods (digital PCR) and advanced capabilities in discriminating mutated and wild-type alleles. However, they suffer from limitations coming from artifacts and amplification

biases, relatively long analysis time, and relatively high cost per analysis.

A number of innovative PCR-free biosensing approaches have been investigated, which hold promise for an easy, inexpensive, and reliable circulating nucleic acids detection (Table 2). The aim of this review is to provide an overview of currently available possibilities for circulating nucleic acid biosensing with specific attention to highlight possibilities for the analysis of serum or plasma samples.

Both electrochemical and optical transductions, when used in combination with nanostructured-materials, show great potential for the development of PCR-free biosensing approaches. Particularly if detected signal enhancement methods, mostly exploiting properties of nanostructured materials, are used.

Future perspectives are linked to the ability to produce low cost biosensing devices and to develop simplified assays to detect circulating tumor biomarkers in less than 1 hour. In addition, performances in terms of discrimination between mutated and normal alleles should be specifically investigated. In fact, among the different examples here discussed only one reported on the detected mutant to wild-type ratio (0.1 %) [100].

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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