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Review

Recent Advances and Emerging Trends in Cancer Biomarker Detection Technologies

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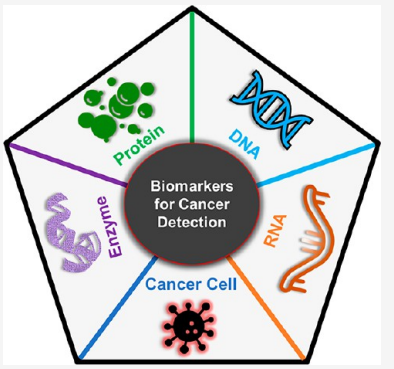
ABSTRACT: Cancer is a major global health burden and poor survival rates can be attributed to lack of early diagnosis and limited access to timely and standard treatments. Significant progress has been made in recent years to establish reliable, cost-effective, and powerful cancer diagnostics. This review presents the recent advances (mostly after 2018) in cancer diagnostic technologies with a prime focus on the various types of biomarkers investigated, such as nucleic acids, proteins, enzymes, and even entire cancer cells, typically known as circulating tumor cells (CTCs). It elucidates several seminal works that utilize a multidisciplinary approach to cancer diagnostics as an alternative to traditional screening methods. Different detection techniques used for each biomarker type have been comprehensively reviewed and our goal is to provide the reader with a critical overview of the current sense and sensibility in the field of cancer biosensing. Furthermore, we discuss emerging trends in cancer biomarker detection using novel multiplexed and integrated platforms for accurate and easy readout, while also shedding light on their technical limitations and existing challenges in achieving high sensitivity and selectivity. We hope that this work will promote collaborative research among different disciplines with an ultimate goal of achieving personalized and user-friendly point-of-care technologies that enable early cancer diagnosis and significantly reduced cancer mortality.



1. INTRODUCTION

The term "cancer" refers to a sizable range of diseases marked by the uncontrolled growth of aberrant cells beyond their normal limits. If not treated at an early stage, these cancerous cells can spread from the primary tumor and invade surrounding tissues and eventually distant organs via a process termed as metastasis, which is the primary reason for cancer mortality. Cancer has been established as an important barrier that needs to be alleviated to ensure increased life expectancy in all nations.[1] According to a 2019 World Health Organization (WHO) report, cancer is the third or fourth leading cause of death before the age of 70 in 23 nations and the first or second leading cause in 112 countries.[2] An estimated 18.1 million new cases of cancer and 9.6 million cancer-related deaths were reported in 2018, according to the GLOBOCAN database for statistics on cancer worldwide,[3] and these numbers quickly increased to an estimated 19.3 million new cases and nearly 10 million cancer-related deaths in 2020. In 2040, there will likely be 28.4 million new instances of cancer worldwide, depicting a 47% increase from 2020.[4] Being a major health problem, cancer has devastating effects on patients and their families while also incurring tremendous economic costs either through direct medical assistance or by the loss of human capital as a result of early mortality. The lack of early and accurate cancer diagnosis translates to poor survival rates as the visible symptoms only start to show up at later stages when widespread metastasis has already occurred. Cancer treatment and recovery are extremely difficult due to late-stage diagnosis and the generally limited availability to prompt and effective therapy.

Consequently, it is established that early detection is essential for appropriate toxicity monitoring and eventually for the treatment of malignancies. Cancers can be detected by monitoring various cancer related "biomarkers", which are substances or activities that can be detected and evaluated. These biomarkers serve as an indicator for a tumor development, metastasis, or pharmacological responses of the cancer to a therapeutic intervention and are generally found in serum or tissues. The ultimate purpose of investigating these cancer biomarkers is to design and develop techniques that can cost-effectively and accurately detect and classify cancers at very early stages so as to enable appropriate and timely treatment to the patients for higher survival rates. Additionally, these biomarkers can be monitored to evaluate disease progression, regression, and recurrence. Researchers have made significant strides in developing promising powerful

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detection techniques based on the specific recognition of cancer biomarkers. Over the past few years, various methods have been investigated including enzyme-linked immunosorbent assay (ELISA),[5,6] colorimetric assay,[7] electrochemical assay,[8] polymerase chain reaction (PCR),[9,10] surface plasmon resonance (SPR), surface enhanced Raman spectroscopy (SERS),[11] and fluorescence methods,[12] among others (Figure 1). Despite sizable efforts, cancer biosensors are yet to achieve clinical diagnostic standards due to lack of accuracy, sensitivity, and specificity.

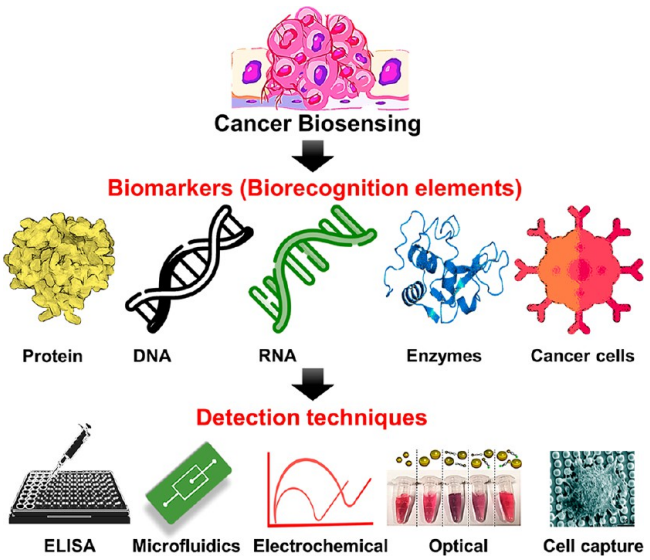

Figure 1. Schematic illustration of the various types of biomarkers and detection techniques employed for cancer biosensing.

These challenges root from two important factors pertaining to cancer biomarkers: first, the term cancer is an umbrella term encompassing a multitude of diseases in various parts of the body and thus a single biomarker cannot and is not viable for the detection of cancer. It thus establishes that the existence of "a single ideal biomarker" for detection of all cancers has not been found yet. Additionally, existing literature suggests that diagnostic, prognostic, and predictive evaluation requires the monitoring of different biomarkers, which further strengthens the former stated argument. Therefore, it is crucial and challenging to investigate new biomarkers for clinically relevant cancer diagnostics. Second, the inherent properties of these biomarkers including low specificity to current assays and presence in minute quantities in diagnostic fluids with substantial amounts of nontarget species poses challenges of high background signals and false positives. Consequently, researchers have devoted efforts to develop powerful detection methods including multiplexed platforms capable of specifically detecting multiple cancer biomarkers to effectively ensure accuracy and eliminate false positives.[11,13−15] Another representative example is liquid biopsy which has gained tremendous interest due to its noninvasive nature and excellent repeatability, and convenience.[16,17] These emerging trends in biosensor design, detection methods, signal amplification, and noise elimination for enhanced analytical performance have been summarized and should be further updated and explored.[18] Additionally, rapid developments in nanotechnology and advanced fabrication techniques are facilitating novel diagnostic technologies capable of integrating multiplexed detection with user-friendly readouts.

In this review, we present a comprehensive discussion on the recent advances in the field of cancer diagnostics. To ensure readability and better understanding of the current status of this research field, cancer detection is presented and discussed based on the type of biomarker employed. Several pivotal works have also been presented in a concise tabular form in Tables 1, 2, 3, and 4 to highlight the current progress and existing challenges. We hope that this work will spark interest across various disciplines to enable collaborative research for realization of technologies that enable reliable early stage cancer diagnosis.

2. COMMON BIOMARKERS FOR CANCER DIAGNOSIS

2.1. Protein Biomarkers. Proteins are made up of amino acids and are often termed as the building blocks of life. They belong to a class of biomolecules that are necessary for living organisms to function properly. Proteins serves as the working unit for numerous key aspects of life processes, including regulation of cellular functions, energy metabolization and storage. Cancer diagnosis and prognosis are often associated with abnormal or misexpression of certain proteins generated either directly by the cancerous cells or in response to their presence.[19] Such protein biomarkers are generally present in blood and occasionally in urine.[20] These proteins serve as vital biomarkers for both the clinical diagnosis of cancer during early or late stages or for post treatment monitoring and/or to identify future recurrence or progression. However, these critical protein biomarkers are present in minute quantities alongside other background proteins making it a challenging effort to selectively and accurately quantify the biomarker of interest. Moreover, proteins unlike nucleic acids cannot be subjected to self-replication and thus are bereft of any possible amplification methods to exponentially increase in concentration to aid in detection. In addition, proteins are highly sensitive to environmental factors like temperature, ionic strength, and pH. While it is difficult to directly detect cancer-related proteins in complex or untreated biological samples, great strides have been made in designing versatile biosensors capable of demonstrating relevant sensitivity, specificity, and acceptable accuracy using novel approaches.

2.1.1. ELISA Detection. ELISA, or enzyme-linked immunosorbent assay, is still regarded as the gold standard for detecting proteins and is extensively implemented in clinical diagnostics.[21,22] Traditionally, ELISA based detection of protein biomarkers is optical in nature, whereby the fluorescence or chemiluminescence associated with an enzymatic conversion of the antibody tagged substrate is directly correlated to the biomarker concentration on the basis of the absorbance value using a plate reader. In essence, the plate reader detects a signal as a result of the chemical transformation of the enzyme substrates 3,3′,5,5′-tetramethylbenzidine (TMB), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and *o*-phenylenediamine (OPD), which is proportional to the number of enzymes attached to the immunocomplex by means of the immunoreaction (Figure 2A). Though highly specific, ELISA methods have yet to overcome several challenges to achieve suitability for point-of-care settings. Unfortunately, conventional ELISA is labor intensive in nature, is inherently dependent on centralized laboratory equipment, and typically requires a larger amount of samples. Also, due to the low extinction coefficients of the

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Table 1. Analytical Achievements and Limitations of Protein Biomarker Based Cancer Biosensors[a]

ref	biomarker	detection method	LOD	achievements	limitations
13	CEA, AFP	pump-free microfluidic assay	0.89 ng/mL 1.72 ng/mL	multiplexed flux-adaptable microfluidic	extensive steps in sensing protocol, unsuitable for point-of-care testing
14	sPD-1, sPD-L1, sEGFR	SERS	6.17 pg/mL, 0.68 pg/mL, 69.86 pg/mL	multiplexed, nanoyeast-scFvs as antibody alternative	potential size dependent dislodging of nanoyeast-scFvs from biosensor surface
29	PSA	fluorescent ELISA	0.032 pg/mL	enhanced fluorescence recovery	large sample volume, questionable specificity of cysteamine induced fluorescence recovery
30	PSA	ALP based ELISA	3 pg/mL	naked eye detection	inherent sensitivity of enzymes to environmental conditions
31	PSA	NECA based ELISA	0.165 pg/mL	nonenzyme-based amplification	potential denaturation of antibody by Ag/Ag^+
32	hcG, CEA	microfluidic ELISA	5 ng/mL	multiplexed volumetric bar-chart chip (V-Chip)	sensitivity limited by V-Chip channels design
50	PSA	eATRP	3.2 fM	eATRP	operational complexities of ATRP-mediated surface-initiated grafting
51	PSA	DPV,CA (dual mode)	0.76 pg/mL 0.42 pg/mL	dual antifouling peptides and an internal reference	the redox tag was not covalently bonded and are susceptible to leakage during biosensing
54	AFP	ECL immunoassay	25 ng/mL	dual antibody probes for one target protein	stringent temperature and pH conditions, spatial/orientational limitations of proximity hybridization
55	AFP	ECL immunoassay	0.03 pg/mL	10-fold increased sensitivity	antibody immobilization on electrode via physical adsorption
58	GPC1	FET	33 particles/μL	noninvasive diagnostic	lower accuracy of GPC1 as a single standalone marker for cancer exosome
65	EpCAM (CD326)	microfluidic (eLoaD) platform	2.6×10^6 cells/mL	multiple assays directly in whole blood	centrifugal speeds of up to 3300 rpm for extended time periods
68	CEA	SPR	0.3 ng/mL	tunable nanostructures of MOFs and NCs	physically loaded aptamers; noncovalent interactions are weak and unstable
69	CEA	colorimetric	160 pg/mL	antigen dependent and label-free catalytic colorimetric sensor	nonspecific interactions and stringent sample preparation for real sample testing

[a]PSA = prostate-specific antigen, ALP = alkaline phosphatase NECA = nonenzyme cascade amplification, hcG = human chorionic gonadotropin, CEA = carcinoembryonic antigen, eATRP = electrochemically controlled atom transfer radical polymerization, ECL = electrochemiluminescence, AFP = α-fetoprotein, GPC1= glypican-1, EpCAM = epithelial cell adhesion molecule, eLoaD = electrochemical lab-on-a-disc, CEA = carcinoembryonic antigen, CA125 = carbohydrate antigen 125, CA19−9carbohydrate antigen 19−9, sPD-1 = soluble programmed death 1, sPD-L1 = soluble programmed death ligand-1, sEGFR = soluble epithermal growth factor receptor, SERS= surface-enhanced Raman scattering, scFvs = single-chain variable fragment, SPR= surface plasmon resonance spectroscopy, MOFS = metal−organic frameworks, and NSC = nanocluster.

enzyme substrates, standard ELISA's detection limit is in the nanomolar range, which is insufficient to identify many protein biomarkers that are clinically relevant, especially during early stage cancer. To address the ongoing need for faster and simpler detection methods, recent developments in nanomaterials have provided various promising tools for improving the performance and expanding the applicability of ELISA.

Nanomaterials play a vital role in the design of ultrasensitive sensors[23−26] primarily owing to their enhanced surface area to volume ratio which results in high density of capture antibodies and improved antibody orientation.[27,28] In the past few decades, noble metal nanoparticles of gold, silver and platinum have been extensively employed to design platforms that deliver promising ultrasensitive probes for biomarkers. Lui et al. presented the use of an activatable probe to improve the sensitivity of fluorescence ELISA tests for prostate-specific antigen (PSA) in serum samples.[29] It was based on the ability of Au NPs to quench the fluorescence of Rhodamine B isothiocyanate (RBITC) in the presence of PSA (Figure 2B). The addition of cysteamine into the detection system facilitates the detachment of the Au NPs-RBITC conjugates, thereby demonstrating significant fluorescence recovery that translates to a detection limit of 0.032 pg/mL which is more than 2 orders of magnitude lower than that of traditional fluorescent probes. Plasmonic ELISA is also a well-studied approach for achieving ultrasensitivity where the surface plasmon resonance (SPR) of noble metal nanoparticles are exploited to demonstrate rapid detectable color change due to intensive changes in morphology in the presence/absence of the analyte. Yanyan Li et al. developed an alkaline phosphatase (ALP) based ELISA for the visual detection of PSA using enzyme-triggered growth of gold nanorods (Au NRs).[30] In this investigation, conjugated ALP releases ascorbic acid by removing the phosphate group from ascorbic acid 2-phosphate in the presence of the enzyme substrate. The ascorbic acid then reduces Au(I) to Au(0) in the reaction solution which contains Au NP seeds and the surfactant CTAB. The reduced Au(0) gets deposited on the surface of the Au NP seeds which initiates the growth of Au NRs. Depending on the growth of Au NP seeds proportional to the concentration of PSA, the test solutions demonstrated vivid colors ranging from colorless to brownish red.

Enzyme cascade amplification is one of the most widely studied amplification methods, in which the end products of one enzyme action catalyzes the activation of another, which in turn exponentially enhances the generation of yet another quantifiable product. However, enzymes are susceptible to several environmental conditions which limits the applicability of enzyme cascade amplification techniques. Another class of novel materials, termed "nanozymes" or artificial enzymes, are catalytically active and can be used as an alternative to establish a nonenzyme cascade amplification method for colorimetric cancer biomarker testing. Xia and group developed a nonenzyme cascade amplification strategy for detection of PSA with a detection limit of 0.165 $pgmL^{-1}$. The group utilized two cascade processes—dissolution of antibody conjugated Ag nanospheres to individual Ag^+ ions via H_2O_2 etching which efficiently blocks Pt nanotube's catalytic activity,

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Table 2. Analytical Achievements and Limitations of Nucleic Acid Biomarker Based Cancer Biosensors[a]

ref	biomarker	detection method	LOD	achievements	limitations
8	NSCLC-related RNAs	DPV	50 aM	reusable electrochemical biosensor with small sample volume and short readout time	separate off-chip and on-chip steps are required
12	BRCA-1	MEF	1 fM	naked eye detection without nucleic acid amplification step	metal-fluorescent tag distance variation causes deviation in MEF intensity
87	methylated DNA	DPV	450 aM	supersandwich DNA structure for one-to-multiple amplification, internal reference	stability of Au−S bond affected by the abundant biothiols in test samples, irreversible changes of the target sequence
88	methylated DNA	CA	0.93 aM	three combinatory signal amplification steps	stringent and lengthy detection steps fit only for clinical laboratories
95	miRNA-141	PEC assays	17 aM	near-zero background noise with THD nanocarriers	interference due to the presence of sophisticated pseudoknots or kissing loops in typical hairpin DNAs
96	miRNA-196a	PEC assays	3.1 aM	superparamagnetic nanostructure coupled enzyme- and hairpin-free EDTD amplification	efficiency of magnetic separation invariable leads to accuracy in analyte quantification
98	miRNA-155	FRET	5.5 fM	a "sandwich" hybridization with aptamer modified La(III)-MOF (fluorophore) and Ag NPs (quencher) pair was developed	pH and temperature variations can severely affect the signal amplification step
103	miRNA-21	EXPAR fluorescent assay	5.2 fM	two reaction circuits generated using polymerase and endonuclease facilitated EXPAR reaction	protein enzymes are inherently susceptible to multiple external factors including temperature, pH, and ionic concentrations
104	miRNA (let-7a)	enzyme-free and label-free fluorescence	7.9 fM	cascade amplification via DNAzyme-powered 3-dimensional DNA walker with hybridization chain reaction	efficiency of magnetic separation, complete turn off fluorescence at bank scans were not observed

[a]BRCA-1 = breast cancer gene-1, MEF = metal-enhanced fluorescence, DPV = differential pulse voltammetry, CA = chronoamperometry, miRNA= microRNA, PEC = photoelectrochemical, THD = three-dimensional tetrahedron DNA, EDTD = entropy-driven tetrahedral DNA, NSCLC = nonsmall-cell lung carcinoma, FRET = fluorescence resonance energy transfer, and EXPAR = exponential amplification reaction.

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Table 3. Analytical Achievements and Limitations of Cancer Biosensors Capable of Direct Cancer Cell Analysis[a]

ref	detected cancer cell	detection method	LOD	achievements	limitations
122	VCaP cells	EC-ELISA	single cell	on chip isolation and ALP based identification of cancer cells	nonspecific capture of white blood cells, elevated error with increasing sample size
123	MCF-7 cells	DPV	8 cells/mL	adenine residue-based immobilization of polydA on Au for label-free electrochemical quantification	inbuilt redox probe absent and relies on external redox solutions for signal generation hence detection in real sample is not achieved
124	MCF-7 cells A549 cells	EIS	5 cells/mL	3D surface array to isolate, release and detection in whole blood	6.7% nonspecific capture was observed
127	SW480 cells	fluorescence assay		SDI-Chip with hydrodynamically optimized immunocoated micropillar surfaces for efficient cell capture	18% nonspecific capture was observed, immunofluorescence staining calls for multiple steps, slow flow rate (1 mL/h)
128	MCF-7, SKBR-3, LM2 cells	immunomagnetic staining	single cell	95% efficiency using specialized microstructures for immunostaining and cytokine secretion analysis	Stringent and extensive detection protocols including multiple washing steps
129	MCF-7, SKBR-3, COLO-205 cells	NIR fluorescence	1 cell/mL	magnetically flattened close proximity NIR fluorescence enhancement (50−120 fold) for microscope-free analysis	diminished capture efficiency in whole blood (5%), cell and plasmonic gold chip distance greatly affect the fluorescence intensity

[a]VCaP cell = vertebral-cancer of the prostate cells, EC-ELISA = enzyme-linked immunosorbent assay, ALP = alkaline phosphatase, MCF-7 = metastatic adenocarcinoma epithelial cell, DPV = differential pulse voltammetry, polydA = polyadenine aptamer, A549 cell = adenocarcinomic human alveolar basal epithelial cells, EIS = electrochemical impedance spectroscopy, SDI-chip = size-dictated immunocapture chip, SW480 = cells from intestine of a Dukes C colorectal cancer patient, SKBR-3 cells = cells isolated from pleural effusion cells of adenocarcinoma patient, LM2 cells = lung metastatic cells, NIR = near infrared, and COLO-205 cells = epithelial cells isolated from ascitic fluid from colon cancer patient.

Table 4. Analytical Achievements and Limitations of Enzyme Biomarker Based Cancer Detection[a]

ref	enzymic biomarker used	detection method	LOD	achievements	limitations
134	telomerase	DPV	8.20 cells/mL (normalized to HeLa cells)	enzyme-free electroanalytical strategy via AuNRs based electrocatalytic oxidation of MB	extended presampling steps, electrochemical response signal due to nonspecific adsorption of AuNRs
135	telomerase	fluorescence assay	280 cells/mL	mimic hybridization chain reaction based signal amplification	size based endocytosis limitations, long incubation time
136	telomerase	anodic stripping voltammetry	0.37 cells/mL	PCR-free and enzyme-free telomerase detection at single cell level	highly pH dependence of the acidic dissolution based Cd(II) release
138	caspase-3	MEF	10 pg/mL	caspase-3-mediated apoptotic cell detection system	specificity issues against caspase-1
140	ADAMs	fluorescence	38.9 ng/mL	multiplexed sensing platform	solution pH affects the enzyme activity and fluorescence intensities. nonspecific fluorescence signal due to HSA

[a]MB = methylene blue, MEF = metal enhanced fluorescence, ADAMs = a disintegrin and metalloproteinases, and HSA = human serum albumin.

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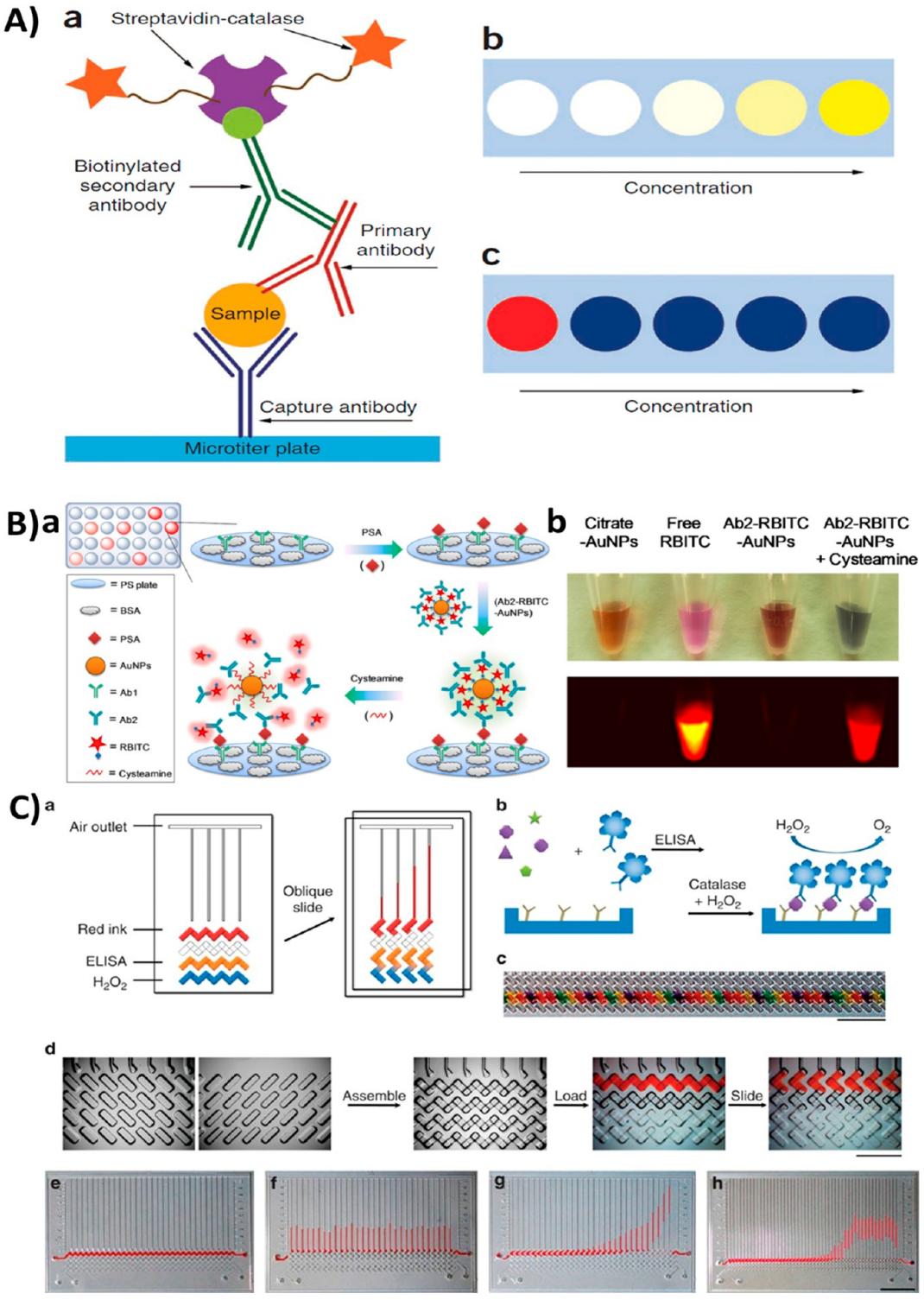

Figure 2. (A) Schematic illustration of enzyme linked immunosorbent assay (ELISA) contrasting the results observed using conventional and plasmonic ELISA. (a) Biotin−streptavidin interactions-based sandwich immunoassay. Detection using (b) Conventional colorimetric ELISA and (c) Plasmonic ELISA. Reproduced with permission from ref 28, Copyright 2013 Nature Publications. (B) Fluorescence-activatable immunoassay for prostate-specific antigen (PSA). (a) The immunoassay performed in 96-well PS plates. (b) Bright-field and fluorescence images of various reaction solutions post treatment with 1 mM cysteamine. Reproduced with permission from ref 29, Copyright 2013 ACS Publications. (C) Illustration of the ELISA based volumetric V-chip for protein biomarker detection. (a) Design of the V-chip. (b) Reaction schematic for oxygen generation during the ELISA reaction. (c) Image showcasing easy loading capability of the V-chip using swab tips. (d) Magnified microscopic images of the fabricated plates of the V-chip. (e) V-chip loaded with red ink. (f−h) Analytical performance of the V-chip showing advancement of the red ink during catalase diffusion. Reproduced with permission from ref 32, Copyright 2012 Nature Publications.

suppressing the production of blue-colored oxidized TMB catalyzed by Pt nanocubes as synthetic peroxidases.[31] Additionally, a 10 pgmL^{-1} PSA standard was used for the assay and neither the detection sensitivity nor reproducibility signifi-

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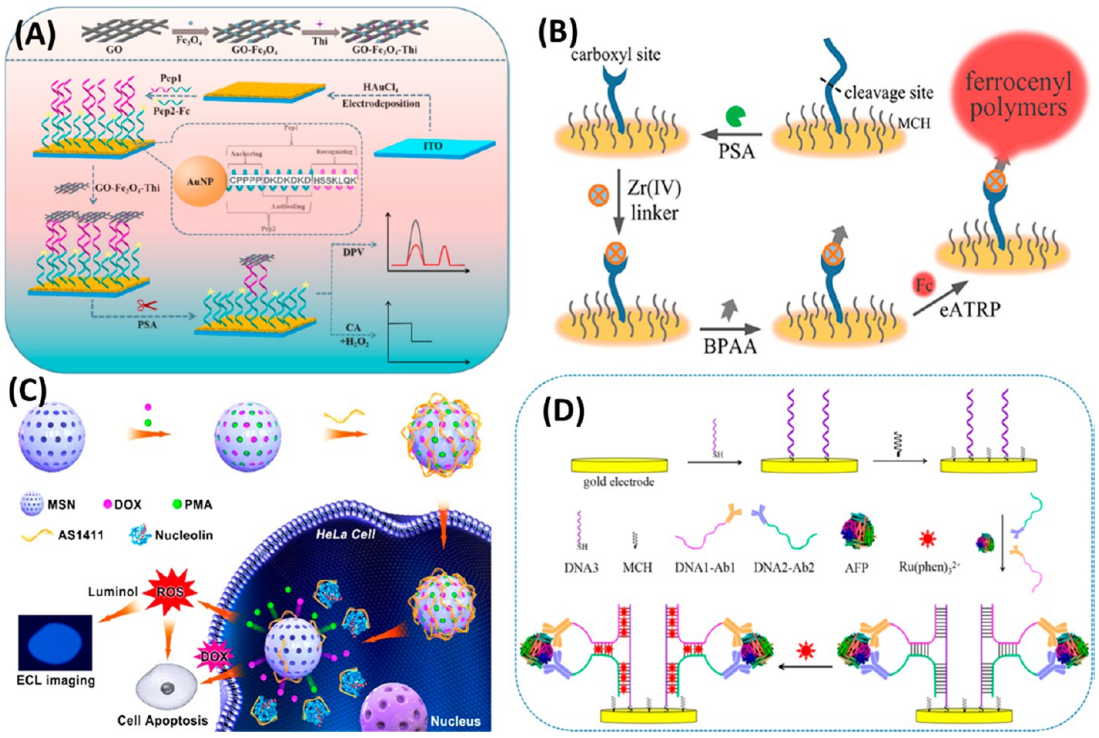

Figure 3. (A) Working schematic of the dual-mode (differential pulse voltammetry and chronoamperometry) PSA biosensor with internal reference ferrocene and peptide grafted graphene oxide-Fe_3O_4-thionine (GO-Fe_3O_4-Thi) probe. Reproduced with permission from ref 51, Copyright 2019 ACS Publications. (B) Signal enhancement utilizing electrochemically mediated atom transfer radical polymerization (eATRP) signal amplification to enable femtomolar level detection of PSA. Reproduced with permission from ref 50, Copyright 2020 ACS Publications. (C) Electrochemiluminescence (ECL) imaging of Nucleolin in a single tumor cell and synergetic apoptosis of the tumor. Reproduced with permission from ref 52, Copyright 2020 ACS Publications. (D) Schematic of an electrochemiluminescence (ECL) immunoassay based on the proximity hybridization regulated ECL for α-fetoprotein (AFP). Reproduced with permission from ref 54, Copyright 2018 ACS Publications.

cantly changed after the reaction solution containing Ag nanospheres and Pt nanocubes was heated to a high temperature (up to 90 °C) for 6 h. This demonstrates the inherent inertness of noble metals and its potential use in harsh detection environments as compared to enzyme-based signal amplification techniques.

Simultaneous detection of multiple protein biomarkers has also clinically proven to provide relatively higher accuracy diagnostic results. A novel volumetric V-chip developed by Song et al. allows for instantaneous real-time visual quantification of one or more target biomarkers without the use of optical tools or further data processing procedures.[32] The catalase reaction was initiated by a straightforward sliding motion of the upper plate over the lower plate, which caused the ink to advance in each of the separate channels (Figure 2C). This is visible to the unaided eye and inversely correlated with the concentration of the relevant ELISA target protein. The quick quantification of chronic gonadotropin (hcG) protein, a routinely used biomarker for various cancer types, including breast and ovarian, proved the efficacy of this approach. By introducing nanozymes like Pt NPs to the V-Chip platform as an artificial enzyme alternative for catalase, the sensitivity could be further enhanced.[33]

2.1.2. Electrochemical Detection. Label-free quantification of protein-based biomarkers poises a fundamental challenge due to the relatively small change in properties during the biorecognition step that is, target binding and the corresponding impediment in retaining the responses acquired with high specificity. Therefore, a sensitive and specific readout technique is highly necessary. Such a need can be fulfilled via electrochemical/electrical detection strategies, which inherently possess high sensitivity and simplicity while also being effectively integrated to miniaturized hardware for amenability in point-of-care settings.[34,35] It can be argued that electrochemical methods are in fact the most practical and scalable diagnostic method owing to its capability for miniaturized, sensitive, and portable biomarker detection.[36]

Numerous electrochemical/electrical strategies have been explored in the recent past for the development of highly sensitive protein biosensors, including voltammetric techniques[37−39] (such as cyclic voltammetry, stripping voltammetry, differential pulse voltammetry, linear sweep voltammetry, and square wave voltammetry), impedimetric methods,[40−42] electro chemiluminescent (ECL)[43] assays, amperometry,[44,45] field-effect transistors,[46,47] and nanochannel/nanopore-based electrical techniques.[48,49] Given that protein-based cancer biomarkers are often present in minute quantities in most diagnostic fluids, a signal amplification step is necessary. Innovative strategies to achieve highly facile and effective signal amplifications for electrochemical detection of cancer biomarkers are being investigated to attain femto and attomolar limits of detection. Qiong Hu et al. exploited a target-induced cleavage of a peptide substrate to develop a sensitive PSA biosensor.[50] For signal amplification, the group used the electrochemically controlled atom transfer radical polymerization (eATRP) approach. Target-induced cleavage of PSA peptides produced free carboxyl sites and Zr (IV) linkers were used to attach the alkyl halide initiator (bromophenylacetic acid, BPAA). Finally, substantial amount of Fc tags was successfully recruited through surface-initiated grafting via the

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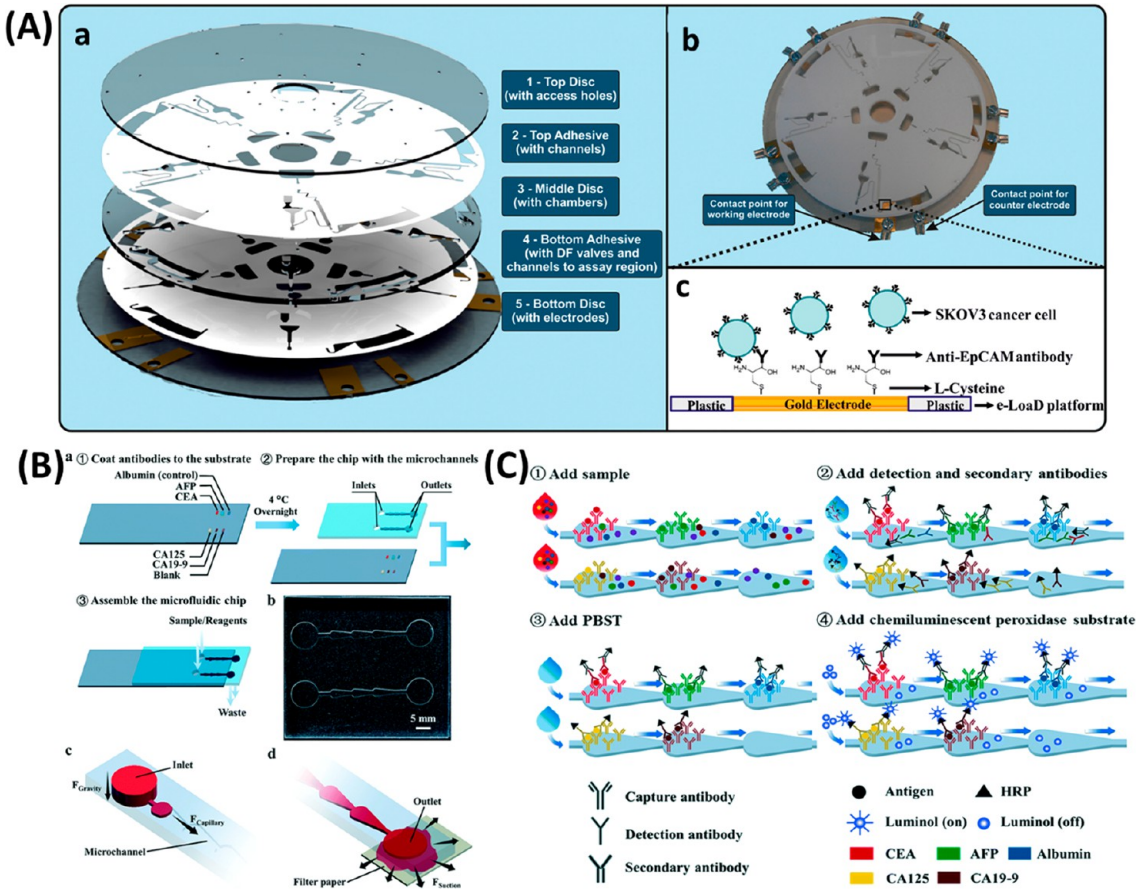

Figure 4. (A) An integrated centrifugal microfluidic device for label-free impedance detection of cancer cells using an electrochemical lab-on-a-disc (eLoaD) platform. (a) A rendered 3-dimensional view of the 5-layer microfluidic disc platform (b) A completely assembled disc displaying the contact sites for the counter and functioning electrodes. (c) Illustration of the electrochemical assay using the polymeric eLoaD platform. Reproduced with permission from ref 65, Copyright 2016 Elsevier Publications. (B) Multiplexed chemiluminescence immunoassay (CLIA) utilizing a paper-based microfluidic chip with adjustable flux. (a) Step by step fabrication procedure of the microfluidic chip. (b) A picture of the fabricated microfluidic chip. (c) Gravitational and capillary forces during the initiation phase. (d) The continuous flow that results from the filter paper's suction during the postinitialization stage. (C) Systematic protocol of the proposed CLIA using paper-based microfluidic chip for detecting multiple cancer biomarkers. Reproduced with permission from ref 13, Copyright 2021 RSC Publications.

potentiostatic eATRP of ferrocenylmethyl methacrylate (FcMMA). A considerably magnified detection signal was produced through the numerous Fc redox tags grafted onto the electrode surface and this resulted in a LOD of 3.2 fM for PSA in spiked normal human serum (NHA).

Caifeng Ding et al. presented an electrochemical biosensor with an internal reference and with capability of performing dual mode PSA quantification using two antifouling peptides (Figure 3A).[51] The group electrodeposited Au on ITO electrodes and immobilized two peptide sequences Pep1 and Pep2 using Au−S bonds. Pep1 was joined to a graphene oxide-Fe_3O_4-thionine (GO-Fe_3O_4-Thi) probe conjugated with a peptide sequence, which can be selectively cleaved in the presence of PSA. The GO-Fe_3O_4-Thi acts both as an electrochemical probe due to the presence of thionine and also as a peroxidase mimic. PSA concentration was measured via two ways: (i) an increase in the differential pulse voltammetry (DPV) signal due to thionine variation as the GO-Fe_3O_4-Thi-Pep1 is cut in the presence of PSA and (ii) the decrease in the chronoamperometry (CA) signal due to GO-Fe_3O_4 mediated electrocatalytic reduction of H_2O_2. Finally, the Fc labelled Pep2 was unaffected by PSA and thus acts as reference to enable reliable and accurate quantifications. The reported dual-mode PSA sensor demonstrated LOD of 0.76 and 0.42 pg/mL using DPV and CA, respectively.

Electrochemiluminescence (ECL), is the ingenious amalgamation of chemiluminescence and electrochemistry. Owing to the simplicity, label-free capabilities, reduced background noise, and superior sensitivity, it has been proven to be an influential analytical tool utilized for disease identification, environmental tests, and biohazard detection, among others. Wanxia Gao et al. developed a new ECL biosensor for imaging nucleolin in a single HeLa cell and also presented a built-in synergetic apoptosis pathway using mesoporous silica nanoparticles (MSN) loaded with doxorubicin (DOX) and phorbol 12-myristate 13-acetate (PMA) that could induce highly specific apoptosis of the HeLa cell (Figure 3C).[52] Nucleolin is one of the most abundant proteins in the nucleolus and has been confirmed to play significant roles in modulating the proliferation, survival, and apoptosis of cancer cells.[53] The group modified MSN with 3-aminopropyltriethoxysilane (APTES) followed by loading with DOX (which is a widely used broad-spectrum antitumor drug) and PMA (which is a phorbol ester that stimulates oxidative stress-induced tumor cell death). The APTES modification induces a positive charge to the probe which then electrostatically attracts the negatively

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charged AS1411 DNA to coat the entirety of the probe. Finally, the probe gets endocytosed by the HeLa cells wherein the AS1411 DNA specifically targets the nucleolin with high affinity. This induces a targeted release of PMA and DOX from the MSN@PMA@DOX that kills the HeLa cells with high efficiency due to the different apoptotic pathways of DOX and PMA. The PMA stimulated tumor cells produces reactive oxygen species (ROS) including H_2O_2 which reacts with luminol to produce an ECL signal that was collected by the electron-multiplying charge-coupled device (EMCCD) for subsequent imaging.

Proximity hybridization-regulated immunoassay is one of the versatile methods that can be effectively implemented with ECL to detect cell surface proteins and protein-overexpressing cancer cells. Xiaofei Wang et al. self-assembled numerous thiolated capture ss-DNA3 (partially hybridized with ss-DNA1 and ss-DNA2) and blocking with 6-mercapto-1-hexanol on a gold electrode surface to develop a "signal on" proximity hybridization regulated ECL immunoassay for α-fetoprotein (AFP) (Figure 3D).[54] The target protein (AFP) was simultaneously bound by two ss-DNA-tagged antibody probes (DNA1-Ab1 and DNA2-Ab2) while DNA1 and DNA2 were brought in sufficient proximity and hybridized with capture DNA3 on the surface of the biosensor. After ECL signal reagent $Ru(phen)_3^{2+}$ was intercalated into the hybridized ds-DNAs, ECL measurement was performed in the coreactant solution and demonstrated a large linear range and excellent sensitivity. The detection limit of the proposed immunoassay satisfactorily met the requirement of the cutoff values of AFP in human serum (25 ng/mL).[55]

Another effective label free detection tool being investigated is the field effect transistor (FET) based biosensor. Field effect transistors work on the basis of detecting micro electrical signals triggered by the interactions between biomolecules on the sensing interface and subsequently transducing into readable electrical signals with high sensitivity and specificity.[56,57] Yi Yu et al. fabricated an RGO FET biosensor modified with specific antibody CD63 for electrical and label-free quantification of exosomes whose concentration is related to the dynamics of certain diseases, especially cancer.[58] The developed RGO FET biosensor exhibited a shift in Dirac point to the left with increasing concentration of exosomes with an impressive limit of detection down to 33 particles/μL. Additionally, the FET biosensor could be easily treated with highly concentrated salt solutions (NaCl, 1 M) to dissociate the binding between exosomes and the anti-CD63 and therefore shows excellent reusability. Various other nanomaterial alternatives to graphene that have a high capability for assaying protein cancer biomarkers have also been investigated for the fabrication of FET devices, including carbon nanotubes[59] and MoS_2 nanosheets.[60] While promising, most of these immunosensors still face challenges in achieving biomarker detection directly in biological fluids mainly because of the lack of specificity which results in potential false positives. However, passivating devices with blocking agents do offer a promising strategy to decrease the false positive signals from nonspecific binding of proteins and other biomaterials to improve the overall sensitivity of the FET biosensor.

Microfluidic point-of-care platforms effectively enabled quantitative, quick, and inexpensive high-throughput measurements and hence shows immense potential to revolutionize clinical proteomic/genomic analyses and personalized cancer diagnostics.[61−64] Additionally, these platforms have a high degree of integration of fluidic handling, specimen processing, and reagent consumption. Nwankire et al. combined the advantages of electrochemical impedance spectroscopy (EIS) and lab-on-a-disc (LoaD) platforms to achieve for the first time, label-free electrochemical detection of ovarian cancer cells (SKOV3) in whole blood. The proposed centrifugal microfluidic system can extract cancer cells from plasma and separate blood in sequence, with targeted capture and accurate detection utilizing label-free electrochemical impedance.[65] This eLoaD platform (Figure 4A) employed rotationally actuated and centrifuge pneumatic dissolvable film (DF) valves to extract and siphon the plasma before transferring it to the five detection chambers which could be run parallelly. L-Cysteine, which was functionalized with anti-EpCAM (CD326) antibodies and self-assembled onto smooth gold electrodes, was utilized to achieve highly specific detection. This resulted in an outstanding 87% capture efficiency with a detection range spanning nearly 3 orders of magnitude was observed by changing the buffer electrolyte concentration and the frequency range of the applied AC current. Although promising for clinical settings, the eLoaD platform still requires accurately maintained centrifugal speeds of up to 3300 rpm for extended time periods, thus questioning its applicability as an effective point-of-care testing platform.

Microfluidic platforms often rely on a variety of accessories to ensure constant and reliable fluid flow and delivery including extensively designed pump or syringe systems that inherently introduce significant additional cost and operational space. Sophisticated fluidic networks additionally restrict the amount of measurements per chip and consequently the device's performance.[66,67] The overall remedy of low cost, portable, and reliable fluid flow with user-friendly analysis of biomarker quantification can be achieved utilizing microfluidic paper-based electrochemical techniques. Multiplex cancer biomarker detection using a flux-adaptable, pump-free microfluidic device was reported by Dai and group (Figure 4B).[13] The four microfluidic units were uniquely designed to resemble a teardrop shape to reduce flux which facilitates the antigen and antibody association and subsequently speeds up the immunoreaction. Four colorectal cancer biomarkers were examined in plasma using a miniature chemiluminescence detector to validate the technique.

2.1.3. Optical Detection. The possibility of rapid naked eye detection makes optical biosensors one of the most promising techniques for POC diagnostics of various clinically relevant biomarkers, including biomarkers for early cancer diagnostics or routine prognosis. Due to its extensive amenability to POC settings, optical biosensors have been of high research interest as a noninvasive alternative to presently used cancer diagnostics. Optical biosensors following the principles of surface plasmon resonance (SPR),[68] surface-enhanced Raman scattering (SERS),[14] colorimetry,[69,70] and fluorescence[71] are being widely investigated. SPR spectroscopy measures variations in the refractive index of metal surfaces during molecular interaction and has been widely applied in biosensing.[72] A multitude of nanostructures have been extensively explored to achieve enhanced sensitivity as compared to traditional optical biosensors and enable multiplexed detection of various cancer protein biomarkers.[73] Li et al. reported a multiplexed cancer protein biomarker detection platform using gold−silver alloy nanoboxes as plasmonic nanostructures with an inherently strong Raman signal enhancement capability to facilitate highly sensitive

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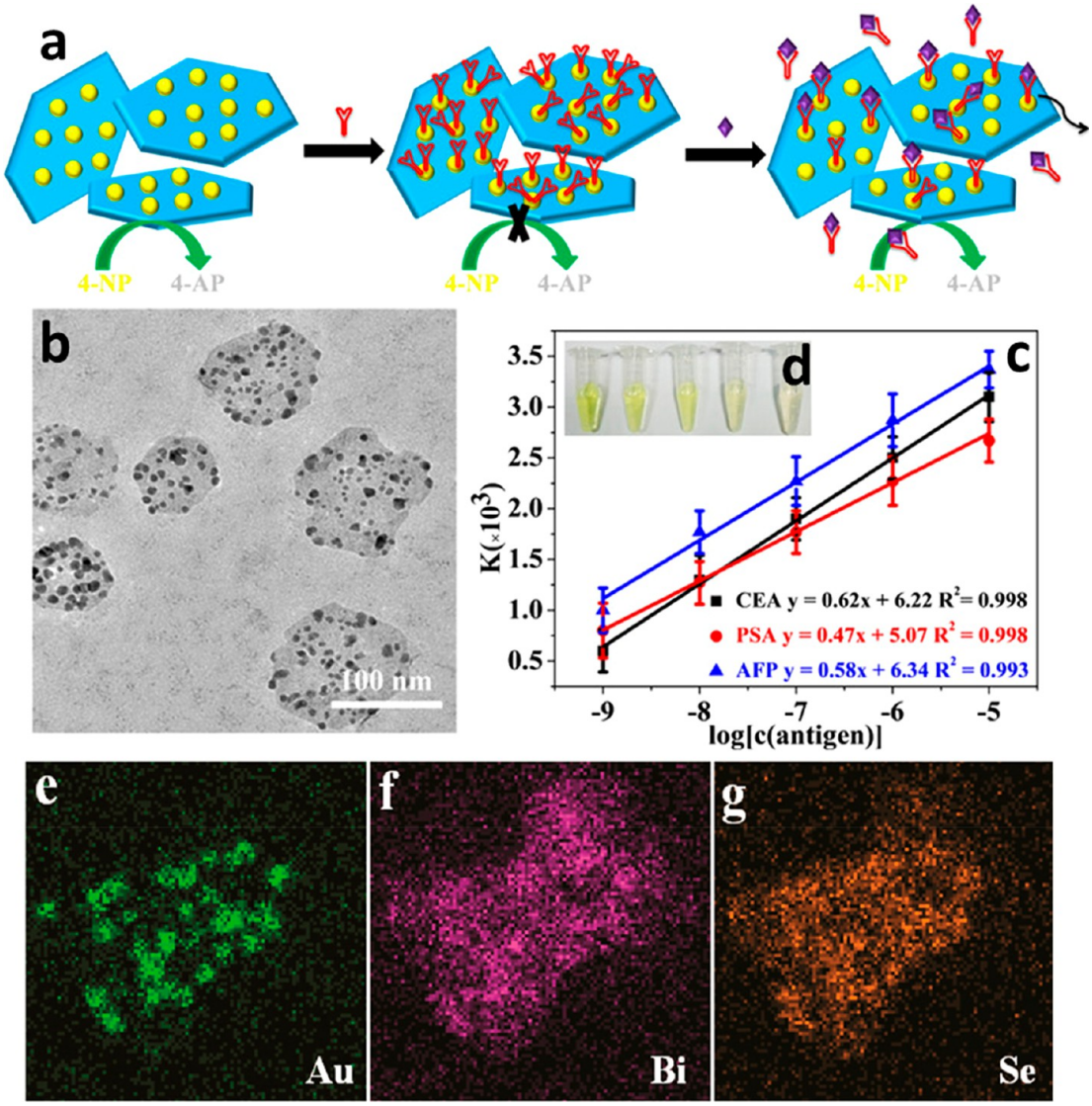

Figure 5. (a) Schematic illustration of the colorimetric biosensor for cancer based on the tunable smart interface of catalytic Au/Bi_2Se_3 nanosheets. (b) TEM micrograph of the Au/Bi_2Se_3 nanosheets. (c) Reaction constant *k* versus the log of concentrations of carcinoembryonic antigen (CEA). (d) An insert that shows the color changes of normal human serum containing the reaction mixture upon addition of different concentration of CEA. (e–g) Elemental mapping of Au/Bi_2Se_3 nanosheets. Reproduced with permission from ref 69, Copyright 2017 ACS Publications.

detection.[14] In order to ensure precise and reliable protein capture, the team utilized nanoyeast single-chain variable fragments as an alternative to monoclonal antibodies (mAbs). They reached detection limits in the pg/mL range for biomarkers (such as such as soluble programmed death 1 (sPD-1), soluble programmed death-ligand 1 (sPD-L1), and soluble epithermal growth factor receptor (sEGFR)) related to cancer progression and treatment efficiency. Metal organic frameworks (MOFs) have high surface areas with well-defined porosities which translates to efficient loading capabilities of various substrates of interests. In a study by Guo et al., silver nanoclusters embedded zirconium metal−organic framework (Zr-MOF, UiO-66) using carcinoembryonic antigen (CEA)-targeted aptamer as template was utilized to develop a two-dimensional nanocomposite with nanoscale thickness.[68] The specific biorecognition event between CEA and the aptamer strands at the sensing layer brings about a change in dielectric constant of the thin adjacent layers which correspondingly leads to variation in the resonance reflectivity of SPR.

Colorimetric biosensors are one of the most straightforward biosensing techniques and have drawn significant interest due to its capability for instrument-free naked eye detection. Yet, significant obstacles such as low target abundance and interference(s) from complex compositions have frequently hindered its practical applicability. Therefore, signal amplification strategies,specific target binding and separation techniques have been widely adopted as a potential remedy to enhance sensitivity and reduce interferences. Miao et al. reported the use of immunomagnetic beads modified with human platelet-derived growth factor-BB (human PDGF-BB) antibody which were subsequently subjected to magnetic separation to prevent possible interferences in a pH-based colorimetric strategy based on glucose oxidase (GOD) enrichment and catalysis for amplified detection of cancer biomarkers.[7] Isothermal DNA signal amplification process with dendritic product and multibranched rolling circle amplification (mb-RCA) were effectively applied to successfully improve the GOD catalysis-based enrichment. Other catalytically active nanostructures

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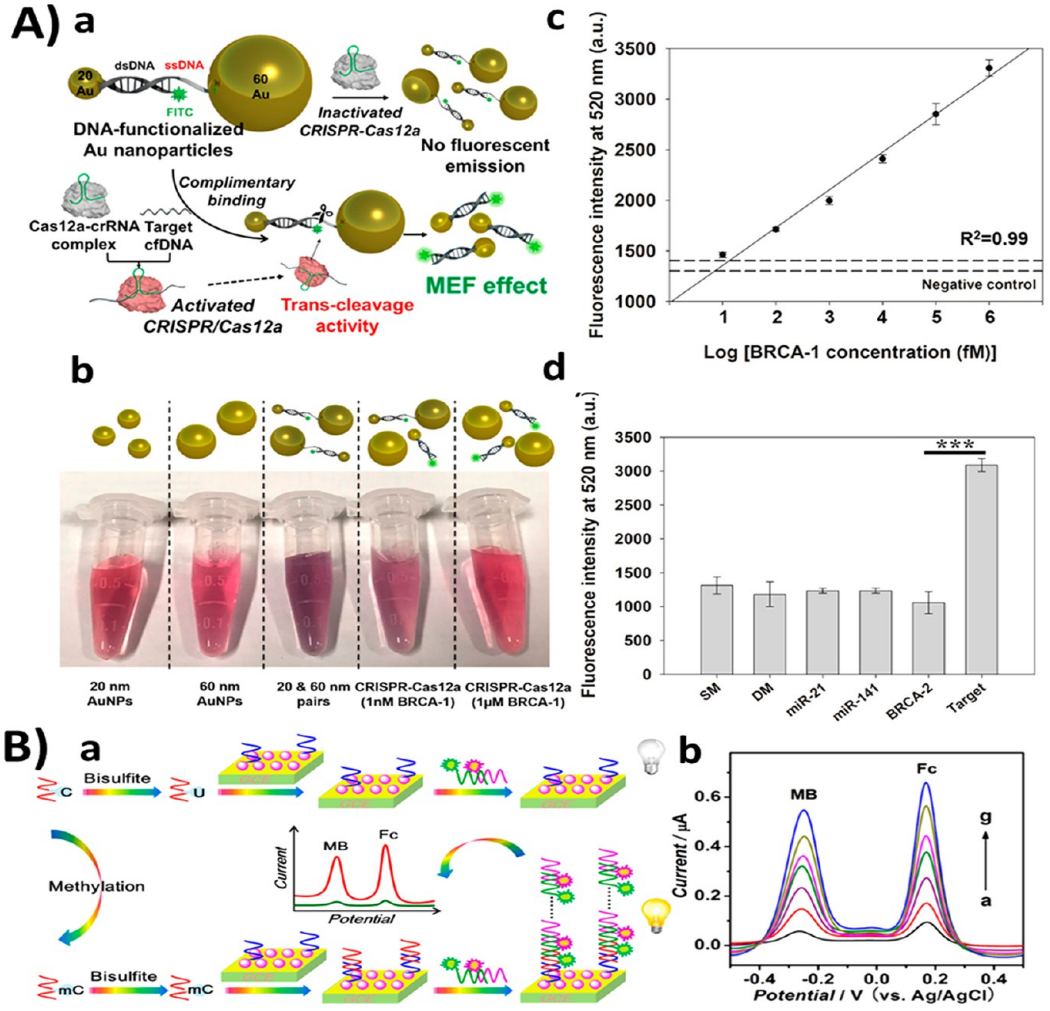

Figure 6. (A) Au NP assisted fluorescence and colorimetric analysis of free DNA via CRISPR-Cas12a-based nucleic acid amplification. (a) Illustration of MEF based CRISPR-Cas12a-mediated cfDNA quantification. (b) The range of color changes of AuNPs under varying detection conditions. (c) Calibration graph exhibiting the relationship between fluorescence intensities and concentrations of cfDNA (1 fM to 100 pM in 20 mM HEPES buffer). (d) Specificity test against miR-21, miR-141, single-mismatched (SM), double-mismatched (DM), and BRCA-1 target DNA. Reproduced with permission from ref 12, Copyright 2020 ACS Publications. (B) Differential pulse voltammetry (DPV) based enzymeless dual-signal readout for monitoring DNA methylation. (a) Schematic illustration of the dual-signal readout showing two electrochemical signals corresponding to Fc-probe1 and MB-probe 2. (b) DPV signals after background corrections generated using the dual-signal readout for quantification of methylated target DNA (a–g: 10 fM^{-1} nM). Reproduced with permission from ref 87, Copyright 2017 ACS Publications.

such as Au NP decorated Bi_2Se_3 have also been reported for the development of a "switch on- switch off" mechanism based colorimetric biosensor.[69] The introduction of anticarcinoembryonic antibody (anti-CEA) can "switch off" the catalytic activity of Au/Bi_2Se_3 while the addition of the appropriate antigen induces the "switch on" state, resulting in an antigen dependent observable color change (Figure 5).

2.2. Nucleic Acid Biomarkers. *2.2.1. DNA-Based Diagnosis.* The development of tumor cells may release DNA into the bloodstream, which causes cancer patients' plasma to have substantially more DNA than that of healthy controls.[50] Hence, cell free DNA (cfDNA) and circulating tumor DNA (ctDNA) serve as essential biomarkers with the potential to advance noninvasive tumor diagnosis and monitoring.

DNA may be introduced into circulation by developing tumor cells which elevates the quantities of DNA in the plasma of cancer patients relative to healthy individuals.[74−76] Hence, cell free DNA (cfDNA) and circulating tumor DNA (ctDNA) serve as essential biomarkers with the potential to advance noninvasive tumor diagnosis and monitoring.[77−80] However, the existing cfDNA and ctDNA analysis methods face several short comings including low sensitivity due to difficulty in designing target-specific primers, false-positive results, and limited patient coverage which hinders the possibility for broad clinical applications. In order to improve the sensitivity of target DNA detection, nucleic acid amplification techniques are being investigated. Clustered regularly interspaced short palindromic repeats (CRISPR) have been successfully utilized as gene-editing tools due to their ability to recognize and degrade target nucleic acid sequences through the direction of guide RNA nucleic acid amplification techniques to increase the sensitivity of target DNA detection.[15,81] When activated by a matching target DNA, CRISPR-Cas12a is renowned for indiscriminately trans-cleaving ambient single-stranded DNA (ssDNA).[53] These characteristics have recently been extensively employed to develop novel biosensing systems based on the aforementioned trans-cleavage phenomenon.[82,83] Choi et al. reported a CRISPR-Cas12a-based nucleic acid amplification-free rapid and highly selective biosensor capable of fast

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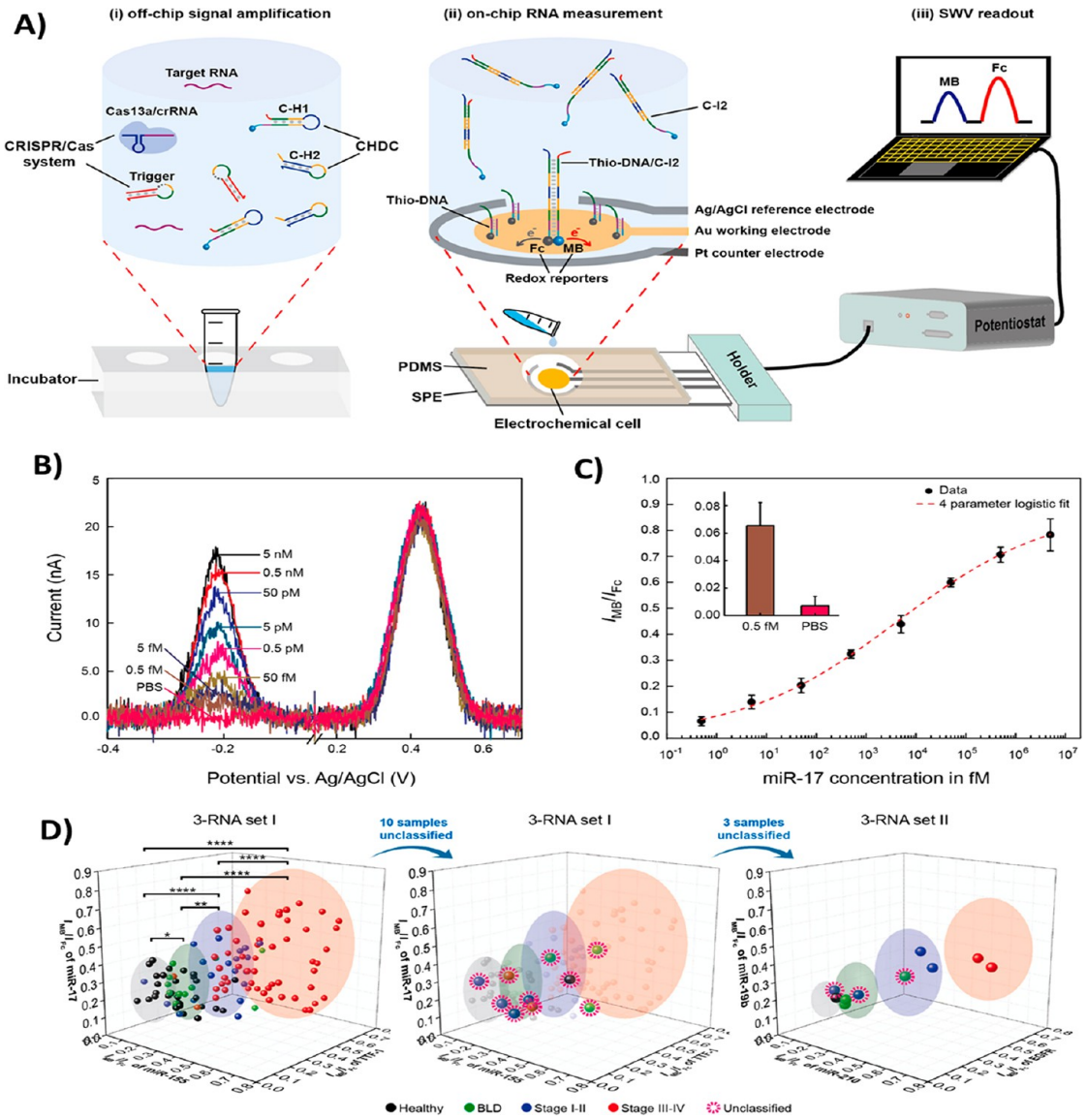

Figure 7. (A) Working principle of the catalytic electrochemical biosensor via the proposed Cas-CHDC-powered electrochemical RNA-sensing technology (COMET-chip) assay. (B) miR-17 quantification in fM to nM range using scanning wave voltammetry. (C) The calibration curves exhibiting a reasonable detection range up to 5 nM with a limit of detection of 50 aM using the COMET chip. (D) 3-Dimensional dot chart of IMB/IFc for miR-17, miR-155 and TTF-1 mRNA (Left to right). Reproduced with permission from ref 8, Copyright 2021 Elsevier Publications.

quantification of breast cancer gene-1 (BRCA-1) under 30 min (Figure 6A).[12] A naked eye detectable color change from purple to red-purple was directed by CRISPR-Cas12a complex activation in the presence of the target cfDNA. This results in a metal-enhanced fluorescence (MEF) via the DNA-functionalized Au nanoparticle (AuNPs).

DNA methylation is a key epigenetic modification associated with the hypermethylation of the tumor suppressor gene and is linked to the weakened biological activity and advancement of malignant transformation. DNA methylation has consequently emerged as a crucial clinical indicator for the molecular diagnosis of malignant tumors.[84−86] Conventional ways for identifying DNA methylation include sequencing analysis and methylation-specific polymerase chain reactions which both call for expensive equipment or time-consuming analysis procedures. Hence, developing a quick, accurate, and affordable solution is required. Electrochemical detection methods exhibit the aforementioned properties and therefore have gained increasing interest. However, majority of existing electrochemical approaches for DNA methylation assessment rely on a single signal response that lacks an identifiable reference. As a result, it is extremely difficult to eliminate false positives from that single signal, which is also easily affected by factors such as experimental efficiency and environmental changes such as temperature, pH, solvent polarity, salt content etc. Feng et al. reported a dual-signal method that exhibited femtomolar level detection with satisfactory analytical parameters (Figure 6B).[87] Bisulfite conversion was employed to translate the status of cystine methylation to nuclei acid sequence via transformation of unmethylated cystine residues into uracil. Two electrochemical signals due to probes modified with ferrocene and methylene blue were recorded using DPV. This resulted from the target triggered cascade of hybridization chain reaction resulting in a supersandwiched DNA structure which translated to a one to multiple signal amplification strategy. DpnI or *Hpa*II are methylation-specific

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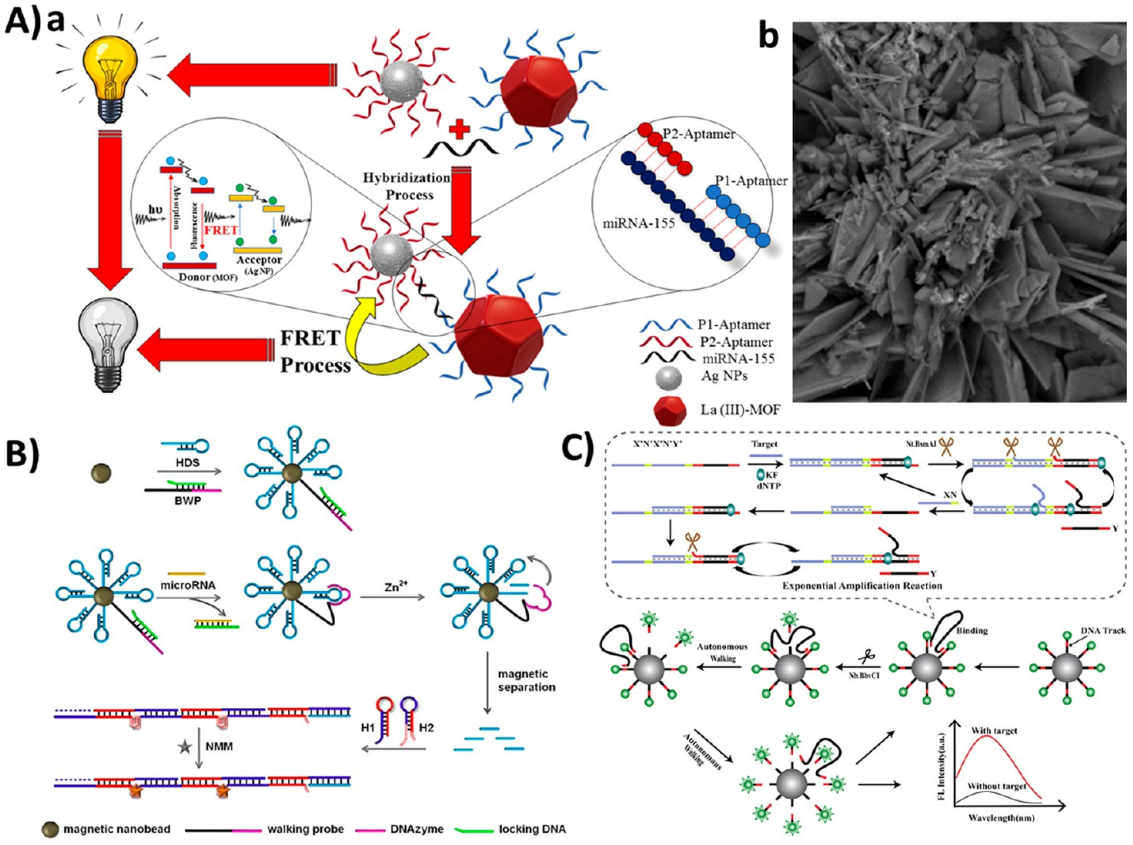

Figure 8. (A) Photoluminescence quenching-based detection of cancer biomarker miRNA-155 by FRET process. (a) Working principle of the fluorescent biosensor showing the FRET based quenching strategy. (b) FESEM image of La (III)-MOF which is part of the donor−acceptor probes. Reproduced with permission from ref 98, Copyright 2020 ACS Publications. (B) Representation of the enzymeless and label-free fluorescence miRNA biosensor with three-dimensional DNA walker and hybridization chain reaction facilitated signal amplification. Reproduced with permission from ref 104, Copyright 2018 Elsevier. (C) Schematic representation of EXPAR-BDW (exponential amplification reaction-triggered three-dimensional bipedal DNA walkers) strategy. Reproduced with permission from ref 103, Copyright 2021 Elsevier.

restriction endonucleases which can specifically cleave unmethylated DNA as an alternative to the bisulfite conversion process (which often has a low conversion efficiency) and therefore can be effectively employed to generate electrochemical signals which can easily distinguish between unmethylated and methylated DNA sequences. Chen et al. reported a hybridization chain reaction-based signal amplification incorporated into the tetrahedral DNA probe anchored onto electrodeposited Au NP and *Hpa*II, with horseradish peroxidase enzymatic catalysis as an effective method for the detection of DNA methylation in attomolar range.[88]

2.2.2. RNA-Based Diagnosis. Irregularity in mRNA metabolism including anomalous processing, export or translation is a well reported cause of several diseases including cancer development and proliferation. These alterations in mRNA metabolism are associated with dysregulation of tumor suppressors that regulate cell proliferation, survival, and differentiation. Mutations in RNA splicing factor genes and 3′ untranslated region shortening frequently accompany the development of cancer. Additionally, current research suggests that other RNA forms, like circular RNAs, can contribute to the development of tumors.[89,90] As such mRNA are widely accepted as a vital clinical biomarker for tumor cell detection and monitoring locally or in the bloodstream. miRNAs are short noncoding RNAs that are known to be related to cancer ever since a study that linked gene producing miRNAs (miR-15 and miR-16) to B cell chronic lymphocytic leukemia.[91] Until now, sufficient data has been collected that associates miRNA mediated regulations to recognized hallmarks of cancer.[92] Hence, these mutations and dysregulations of miRNA in different types and subsets of tumors in addition to its presence in diagnostic fluids such as blood cemented its role as a key cancer biomarker. However, several types of miRNAs are released in highly minute quantities and therefore its low abundance combined with miRNAs having highly similar common secondary structures complicates its sensitive and bias-free quantification.

2.2.2.1. Electrochemical Detection. Electrochemical biosensors have an inherent advantage of high sensitivity, selectivity, portability, and low cost, and have been widely investigated for the quantification of critical biomarkers such as mutated genes or nucleic acid sequences associated with numerous forms of cancer.[93,94] The smart combination of nucleic acid layers with electrochemical transducers results in a simple yet reliable and cost-effective molecular recognition platform for cancer diagnostics. In the past, DNA hairpin based nucleic acid structures have been extensively used to improve specificity and sensitivity. Hybridization chain reaction (HCR), catalytic hairpin assembly (CHA), and rolling circle amplification (RCA), among other two-dimensional DNA hairpin architecture-based signal amplification techniques, all have several limitations, including substantial background noise and false positive signals. Alternatively, three-dimensional tetrahedron DNA (THD) has exhibited desirable properties

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such as effective regulation of DNA hybridization,[10] good cell penetration, supermolecular compact nature via intercalation,[11] and strong resistance to enzymatic degradation. Yuan et al. developed a THD mediated photoelectrochemical (PEC) biosensor for microRNA-141 with virtually zero noise. THD was used as an effective nanocarrier of the signal probe, that is, CdTe quantum dots (QDs)-methylene blue (MB) which demonstrated a low detection limit of 17 aM for miRNA-141.[95] Li et al. reported an hairpin-free and enzymeless PEC based miRNA-196a biosensor using entropy-driven tetrahedral DNA (EDTD) amplifier integrated to superparamagnetic nanostructures (Fe_3O_4@SiO_2@CdS) with CuS functioning as a photocurrent blocker.[96] The presence of the EDTD amplifier drastically improves sensitivity as it reduces potential background noise by effectively eliminating interference from pseudoknots or kissing loops which are inherently present in conventionally used hairpin DNAs. Biorecognition was completed in liquid phase wherein the toehold-mediated strand displacement in the presence of the target initiates the separation and release of the Fe_3O_4@SiO_2@CdS composite from the blocker (CuS). This results in an increase in photocurrent which was detected following magnetic separation and redispersion for the fabrication of the photoanode.

Electrochemical readouts provide an excellent platform for rapid testing. Sheng et al. designed a Cas-CHDC-powered electrochemical RNA sensing technology (COMET) by integrating signal amplification strategy using CRISPR/Cas13a system with a catalytic hairpin DNA circuit (CHDC) (Figure 7). The device was capable of achieving a limit of detection of 50 aM for nonsmall-cell lung carcinoma (NSCLC) related miRNAs such as miR-17, miR-19b, TTF-1 mRNA, and EGFR mRNA using only 10 μL sample under 36 min (readout time of 6 min).[8] The group integrated an off-chip signal amplification and reusable on-chip RNA measurements. The biorecognition of the RNA facilitated the cleavage of a number of "fuse" sites via Cas13a activation initiating the release of the intermediaries. As a result, several C-I2 molecules are produced through multiple cycles of toehold-mediated strand displacement events that catalyze the hybridization of the hairpins C-H1 and C-H2. Thio-DNA/C-I2 complex is generated when C-I2 attaches to the surface of Thio-DNA to augment the electrochemical current between the electrode and the redox reporters (MB and Fc). Post measurement, the Thio-DNA monolayer is regenerated for reuse simply by incubating in an enzyme solution containing endonuclease IV and UDG. This guarantees that the DNA backbone will be cleaved at these specially selected deoxyuridines, thereby spontaneously denaturing and removing the resulting shorter DNA sections during the subsequent buffer washing step.

2.2.2.2. Fluorescent Detection. Fluorescence based biosensors for RNA strands fundamentally employ either a "switch-on" or a "switch-off" strategy coupled with fluorophore-labeled probes consisting of single-stranded DNA (ssDNA) anchored on the support surface (including an extensive variety of nanomaterials).[97] A target induced hybridization or denaturation with/of the probe strand results in the formation or splitting of a double-stranded DNA (ds-DNA) which releases or captures the fluorophore label, thus recovering or quenching the fluorescence via a photomediated electron-transfer mechanism to demonstrate the "switch-on" or "switch-off" strategy, respectively. However, recapture of formed ds-DNAs often leads to false signals. Keeping this in mind, Alzalinia et al. investigated a "sandwich-DNA" hybridization strategy in conjugation with La (III)-metal−organic framework (MOF) and silver nanoparticles (Ag NPs) which function as a pair of energy donor−acceptor probes for the detection of miRNA-155 with LOD as low as 5.5 fM (Figure 8A).[98] Both the donor−acceptor probes were conjugated with different aptamers, which form a "sandwich-type" hybridization of oligonucleotides in the presence of the target while simultaneously quenching the fluorescence through the fluorescence resonance energy transfer (FRET).

In search of effective signal amplification strategies for RNA detection, researchers have developed molecular machine-based assays deriving inspiration from natural machines. These molecular machines are capable of demonstrating quasi-mechanical movements in response to specific stimuli. Among these, the three-dimensional (3-D) DNA walkers have drawn tremendous focus due to their exceptional properties of automaticity and controllability.[99] The construction of the DNA tracks on the surface of nanoparticles with inherently large surface areas results in high loading capacity for enhanced reaction efficiency.[99,100] The driving force is generally derived from the toe hold facilitated strand displacement and/or DNAzyme/exonuclease/endonuclease mediated hydrolysis. Recently, nucleic acid tool enzymes which exhibit exceptional ability to achieve enhanced walking speeds are being investigated. The high catalytic activity of the enzyme in combination with existence of intraparticle interfaces and heightened local substrate concentrations effectively enhances biocatalysis on the nanoparticle surface, leading to accelerated enzymatic reactions.[101,102] With this viewpoint, Yang et al. reported bipedal DNA walkers (BDW) powered using nicking endonuclease for the detection of MicroRNA 21 as a model cancer biomarker. In the presence of the target miRNA, the nicking endonuclease initiates an exponential amplification reaction (EXPAR).[103] This generates a large number of BDW which assembles onto the polystyrene microsphere track which is already functionalized with the fluorescence-labeled DNA strand. Additionally, the endonuclease-based nicking initiates the roboticized and step-by-step movements across the DNA tracks, facilitating the persistence of the produced fluorescence signal output.

However, the functioning of such enzymes is susceptible to multiple external factors including temperature, pH, and ionic concentrations. This limits the reliability of such biosensors due to lack of accuracy and reproducibility. Therefore, an enzyme-free and label-free fluorescence biosensor capable of demonstrating 3-D DNA walking is desirable. Wang et al.[104] reported a DNAzyme-powered 3D DNA walker for enzymeless and label-free miRNA detection. Instead of enzyme-based nicking, the target triggered release of the DNAzyme which catalyzes the cleavage of HDSs, thus releasing abundant triggers which lead to a cascade hybridization chain reaction (Figure 8B). This generated amplified fluorescence signals based on the toehold-mediated strand displacement reaction which guaranteed excellent specificity comparable to that of enzyme-based assays without the drawbacks of being susceptible to sensing conditions.

2.3. Direct Cancer Cell Analysis. Cancer cells can be regarded as persistently proliferating nonfunctioning cells. These cells produce abnormal quantity of proteins, receptors, or specific enzymes on their surfaces or within the cells. These biological components can therefore be detected and captured for effective quantification and the subsequent treatment/

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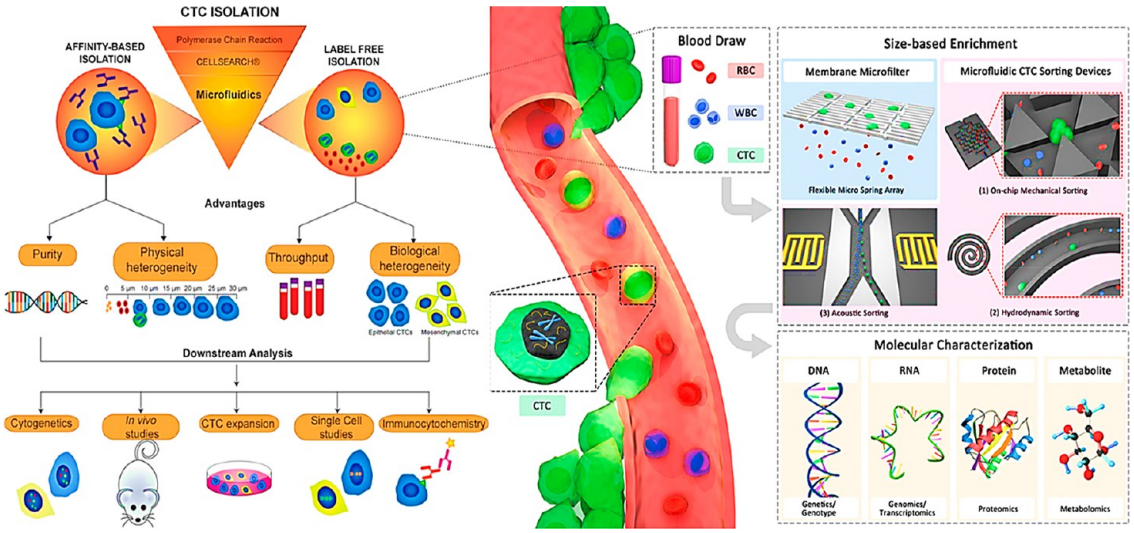

Figure 9. Schematic representation of the two classes of isolation techniques for circulating tumor cells (CTCs). Affinity-based techniques identified markers present at the surface of cancer to capture CTCs while the label-free strategies utilized difference in size between various CTCs and blood cells. Microfluidic CTC isolation is a powerful tool that can also be employed for various downstream applications and molecular characterizations. Reproduced with permission from refs 106,118, Copyright 2016 Wiley Publications and 2018 Elsevier Publications.

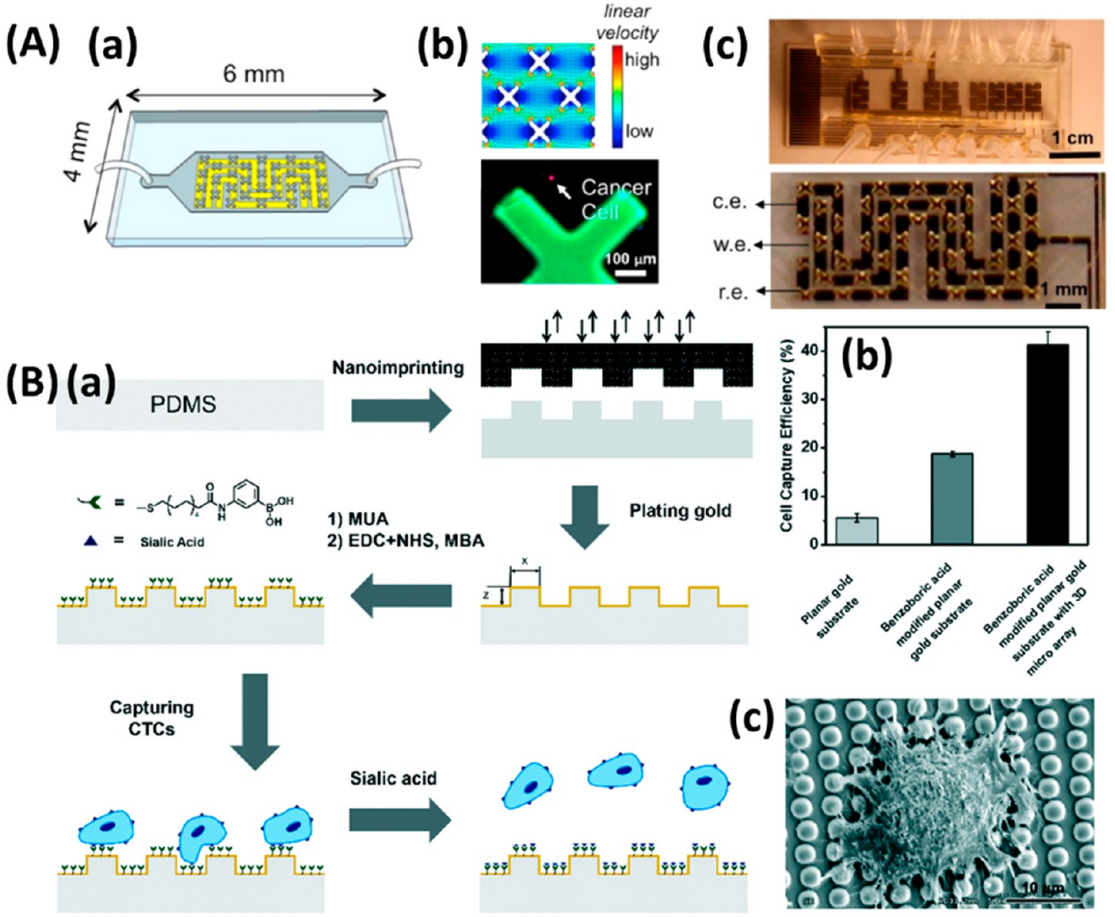

Figure 10. (A) Electrochemical ELISA based cancer cell detection. (a) Illustration of the fabricated chip. (b) The X-shaped post capable of capturing cancer cells. (c) Picture of the chip showcasing eight individual sensors and a magnified image of the single sensor with the reference, counter and working electrodes and the X-shaped posts. Reproduced with permission from ref 122, Copyright 2015 ACS Publications. (B) 3D microarray based cancer cell biosensor. (a) Schematic representation of the design of benzoboric acid modified gold-plated polymeric substrate for effective capture, detection, and release of CTCs. (b) The relative cell capture efficiencies. (c) SEM micrographs of captured MCF-7 cells. Reproduced with permission from ref 124, Copyright 2018 RSC Publications.

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destruction of the tumor. Biopsy is the current gold standard of cancer diagnosis for evaluating the incidence and magnitude of the tumor progression.[105] Nevertheless, this process is invasive and precludes patients from continuous and routine testing. There is an immediate need for new noninvasive methods to detect and capture carcinoma cells which is key to specific and reliable identification and quantification of a plethora of malignancies at an early stage. In particular, the examination of circulating tumor cells (CTCs), also called "liquid biopsy" or "live biopsy", is much less invasive and highly effective especially in monitoring tumor progressions and evaluating response to therapeutic treatments.[106−108]

2.3.1. CTC Isolation. CTCs are biomarkers for noninvasively evaluating the development of various tumor genotypes throughout treatment and tumor advancement. Recent developments have facilitated the effort toward achieving highly sensitive detection and characterizing CTCs even at a single-cell level in complex fluids such as untreated whole blood. The isolation and enrichment of CTCs are based either on the biological properties of tumor cells (which is primarily affinity based isolation) such as surface antigens, cytoplasmic protein expression and invasion capacity or on their physical properties (which is primarily label-free isolation) including size, density, deformability, or electric charges (Figure 9).[109,110] CTC isolation based on physical properties includes gradient centrifugation based on the difference in density of CTCs and other hematopoietic components using density gradient solutions, such as Ficoll (Amersham) and Lymphoprep (Nycomed). Current literature encompasses the physical and biological based separation methods into these widely studied techniques, namely immunomagnetic separation,[11] microfabricated filters,[111] spiral microfluidic systems or microfluidics-enabled immune separation,[112−115] and dielectrophoresis (DEP)[114,116,117] based techniques which are reported for label-free and high throughput CTC isolation for unbiased screening of CTCs from all cancer types, including both mesenchymal and epithelial cell types.

2.3.2. Quantification of Cancer Cell. 2.3.2.1. Electrochemical Detection. Electrochemical biosensors have proven time and again their potential to enable clinically relevant sensitivity and specificity, simplicity, rapid response, cost effectiveness, and most importantly the capacity for miniaturization and integration with existing electronics.[119] Hence, such class of biosensors also represent a particularly attractive method for cancer cell analysis.[93] Furthermore, microfluidic techniques have been immensely successful for the isolation of CTCs.[120,121] In this regard, electrochemical analysis based on microfluidic isolation techniques can potentially exhibit high throughput and improved performance with an easy-to-use readout. Safaei et al. reported a magnetic particle labeling based epithelial cell adhesion molecule (EpCAM) method in association with an on-chip in-situ electrochemical ELISA (EC-ELISA) in TBS buffer for evaluating the signal response to different concentrations of *p*-aminophenol (*p*-AP) (Figure 10A).[122] The chip was microfabricated based on a pre-existing velocity valley (VV) chip design with patterned gold structures on a glass substrate. This included pattered "X" microstructures to facilitate reduction of drag force of the magnetically labeled cancer cells (cells were specifically labeled with magnetic nanobeads coupled with the anti-EpCAM antibody) while passing through the chamber. Finally, the EC-ELISA response was based on the enzymatic conversion of *p*-aminophenyl phosphate (p-APP) to an electrochemically active reagent (*p*-aminophenol) in the presence of alkaline phosphatase labeled anti-CK18 antibody (a universal epithelial cancer marker called cytokeratin) tagged to the captured cancer cells. The electrochemical signal was generated due to the subsequent electrochemical oxidation of *p*-aminophenol (p-AP). The sensor demonstrated high levels of sensitivity for VCaP cells (used as epithelial cancer cell model) and could detect as few as one captured cancer cell. However, natural enzymes are subject to variations in sensing conditions and therefore, nonenzymatic counterparts with robust catalytic stability to harsh environments are highly attractive. As such, metal-based nanoparticles (MNPs) can effectively demonstrate electrochemical signals that allow detection and evaluation of the cancer cells via direct redox chemistry of the MNPs. As a cost-effective alternative to modification of hydrosulfuryl in the traditionally used aptamer functionalization of gold nanoparticles, Wang et al. presented MUC1 aptamer (AptMUC1) modified with a terminal polyadenine (polydA) aptamer which shows high affinity toward gold electrode (GE) and gold nanoparticles (AuNPs).[123] The sensing principle was based on the sandwich formation of the MCF-7 cells self-assembled onto the surface of polydA-aptamer modified GE and the polydA-aptamer functionalized AuNPs/GO hybrid that binds to MCF-7 cells. This sandwich structure causes extensive steric hindrance on the redox couple transfer through GE which translates to an enhanced signal change dependent on the concentration of MCF-7 cells. Furthermore, monitoring of electrical impedance to report the presence of cancer cells based on their dielectric property is also a widely employed strategy. An et al. reported a benzoboric acid modified gold-plated polymeric substrate with 3D micro arrays using nanoimprinting (Figure 10B).[124] The group utilized the conjugation of the substrate (benzoboric acid) with sialic acid present at the cytomembrane of cancer cells for effective capture as low as 5 cancer cells per mL and the consequent changes in impedance with varying human breast carcinoma cells (MCF-7) was monitored.

2.3.2.2. Optical Detection. Optical based detection techniques are highly attractive considering their sensitivity, robustness, and reliability.[125] Furthermore, they can be easily miniaturized, enabling chip-level integration with platform technologies such as microfluidics and realization of lab-on-a-chip devices.[126] Recent advances in optical biosensors integrated with microfluidic devices have proven to be effective for identifying phenotypic characteristics of primary cancers based on the detection of CTCs. As such, microfluidic platforms are extensively integrated with fluorescence detection platform to achieve multiplexed CTCs assay. Such systems typically follow a standard principle, wherein the immunocapture of CTCs (targeted at cancer-related cell surface markers such as $EpCAM^+$, $HER2^+$, and $EGFR^+$) anchors it at the detection site. This is followed by the introduction of fluorescent-dye-labeled antibodies which are then washed through the microfluidic channel resulting in a detectable change in fluorescence signal. Ahmed et al. reported a size-dictated immunocapture chip (SDI-Chip) with immunocoated triangular micropillar-array structures capable of selectively capture and spatially resolve CTCs with different antigen-expressions via deterministic lateral displacement.[127] This eases competitive dislodging of target cells and facilitates improved capture efficiency in the presence of a high cell background. The anti-EpCAM antibody-coated micropillars reportedly captured >90% of CTCs. The presented method

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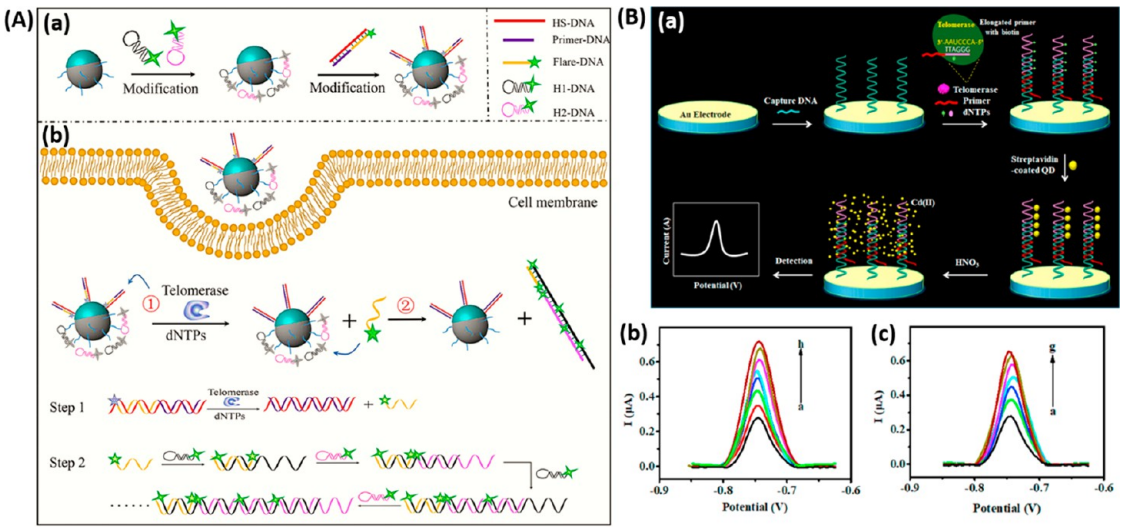

Figure 11. (A) Fluorescence biosensor for intracellular telomerase detection. (a) Schematic of the preparation process of patchy gold/carbon nanosphere (PG/CNS) loaded with a pair of cross complementary DNA hairpins. (b) Schematic representation of intracellular telomerase detection using the PG/CNSs-based mimic-HCR sensor. Reproduced with permission from ref 135, Copyright 2019 Elsevier. (B) Quantum dot-based telomerase identification at the single-cell level using voltammetry. (a) Schematic illustration of design and working of the QD-based electrochemical biosensor. (b) SWASV spectra corresponding to telomerase extracted from 0, 1, 10, 102, 103, 104, 105, and 106 HeLa cells (from a to h). (c) SWASV spectra corresponding to telomerase extracted from 0, 10, 102, 103, 104, 105, and 106 HEK293T cells (from a to g). Reproduced with permission from ref 136, Copyright 2018 Elsevier.

exploited hydrodynamic forces such as shear force effectively to spatially separate CTCs from other nontarget species such as WBCs. However, the immunofluorescence staining calls for multiple steps including several washing steps which can result in loss of captured CTCs, thereby affecting the sensitivity of the developed biosensor. Armbrecht et al. presented a microfluidic device capable of isolating and capturing CTCs from whole blood with an impressive 95% efficiency using specialized microstructures which brings about cocapture of functionalized magnetic beads for the analysis of cytokine secretion in addition to immunostaining.[128] Immunomagnetic methods incorporated with NIR fluorescence enhancement have also been employed for detection and quantification of CTCs. Zhang et al. developed a strategy to magnetically enhanced the capturing of CTCs onto a plasmonic gold (pGOLD) chip integrated with an on-chip near-infrared fluorescence enhanced (NIR-FE) detector.[129] The magnetic forces result in the "squashed" morphology of the trapped CTCs and the presence of the NIR labels on these CTCs which are in close proximity to the plasmonic gold chip translates to a 50−120 fold enhancement in near-infrared (NIR) fluorescence. The effect of the magnetically induced squashing of the captured CTCs toward cell viability was not investigated despite the effective mechanical alteration of the cell for enhanced fluorescence detection of CTCs. Additionally, the study demonstrated that a fast and automated micro scanning of on-chip enriched cancer cells and subsequent digital signal processing of the obtained images is a viable option toward microscope-free analysis.

Raman spectroscopy, unlike fluorescence and SPR based methods, does not suffer from high background noise that results from autofluorescence signals. Surface-enhanced Raman spectroscopy (SERS) techniques enable generation of distinctive nonoverlapping peaks, thus allowing for more precise signal characterization for multiplex imaging in complex fluids. Pallaoro et al. designed a SERS biotag by combining a Raman active reporter label and an affinity biomolecule with a silver nanoparticle dimer core.[130] One of the two cell-targeting silver biotags (SBTs) was designed to bind selectively to cancer cells, while the other was created to bind nonspecifically and thus acted as a noncell-specific control. These biotags were incubated with cancerous and noncancerous prostate cells and allowed to form a single line by injecting into a flow-focused microfluidic channel. Each passing cell was focused onto a laser beam that uses their Raman signatures to distinguish the CTCs from a proportionally greater number of other cells. The developed method could sensitively detect as low as one cancer cell out of a population of 100 noncancerous cells using the laser beam for 20 ms.

2.4. Enzymes. Endogenous or intracellular cancer-associated enzymic biomarkers have also been explored for systemic evaluation of tumor detection and monitoring of cancer progression. The dysregulations of enzyme activity in tissues and body fluids, in addition to abnormal functioning of enzyme variants and/or isoenzymes in cancer patients, invariably indicate that these factors can be utilized as effective biomarkers for cancer detection and monitoring response to therapeutic interventions. Among these enzymic biomarkers, human telomerase activity is the most widely studied.[131,132] Literature suggests that an estimated 85−90% of human tumors indicate the presence of telomerase.[133] Tumor cells show upregulated telomerase activity which is not present in adjacent normal cells. Telomerase has long been associated with cellular senescence, thereby making the cell immortal, which is necessary for tumor cell proliferation. This difference in the telomerase activity between normal somatic cells and tumor cells can be effectively harnessed and utilized as a diagnostic and prognostic tumor marker with the potential for being a therapeutic target for chemotherapy. In recent years, newer signal amplification strategies have been reported which can serve as an alternative to the traditional gel electrophoresis based telomeric repeat amplification protocol (TRAP). The rapid development in nanotechnology brings forth attractive

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solutions to a multitude of challenges including signal amplification strategies for designing highly innovative biosensing devices and ultrasensitive bioassays. The inherent ability to fine-tune high homogeneity/dispersibility of nanoparticles and nonspecific binding to biomolecules or substrates can collectively enhance the signal through extensive surface loading of substantial quantities of amplifying labels or tags, which serves as a foundation for ultrasensitive optical and electrical identification with PCR-like specificity. Efficient telomerase detection platforms using target triggered formation of DNA hairpin structures are gaining interest. Wang et al. used a telomerase triggered extension to from a hairpin structure in one strand of the DNA duplex causing the other to dissociate, which in turn opens up a third hairpin structured DNA to from a duplex with a free 3′ end (Figure 11A).[134] The group then anchored Au nanorods (AuNRs) to the 3′ end, wherein the telomerase activity was proportional to the amount of the adsorbed electrocatalyst. The response was evaluated based on the increased electrochemical signals of methylene blue catalyzed by the AuNRs. In a similar study, Wang et al. reportedly used patchy gold/carbon nanospheres (PG/CNS) preloaded with a pair of cross complementary DNA hairpins, namely hybridized-DNA (HS-DNA/Primer-DNA/Flare-DNA) and H1/H2-DNA, in conjugation with a hybridization chain reaction (HCR)-based signal amplification for in situ intracellular telomerase activity.[135] The telomerase primer produce a telomeric repeated sequence by extending at its 3′ end which leads to release of Flare-DNA accompanied by recovery of the fluorescence. The signal amplification was based on a mimic-HCR system that enhances the fluorescence signal via Flare-DNA mediated cross hybridization of H1 and H2 DNA, thus achieving a limit of detection of 280 A549 cells. Single-cell level detection of telomerase activity has also been investigated. Li et al. employed a similar strategy of telomerase triggered addition of the telomere repeats of (TTAGGG)n to the 3′ end of the primer followed by subsequent hybridization of the extension product with the capture DNA which induces the anchoring of numerous streptavidin coated CdSe/ZnS QDs onto the electrode (Figure 11B).[136] The amount of ODs is thus indicative of the telomerase activity which is detected by quantifying the Cd (II) released following an acidic dissolution of QDs via anodic stripping voltammetry.

Studies corelate high matrix metalloproteinases (MMPs) production with angiogenesis at tumor sites for nutrient and oxygen supply.[137] Consequently, protease associated enzymatic biomarkers have been investigated to design diagnostic tools for identification and enumeration of living cancer cells and their type of differentiation. Fluorescence based analytical methods exploiting signal generation by initiating cleavage of the fluorescence quencher from the probe in the presence of an enzyme have been reported. Choi et al. investigated bifunctional AuNPs loaded fluorophore modified with both ssDNA and a peptide for caspase-3 related preapoptotic cell detection.[138] A simple caspase-3 directed enzymatic cleavage reaction resulted in a metal-enhanced-fluorescence (MEF) signal with an achievable LOD of 10 pg/mL. However, an increased signal was also observed in the presence of caspase-1 and therefore the developed method lacked high specificity. Typically, low accuracy and efficacy are observed for single biomarker-based cancer diagnostics. In order to improve the reliability and eliminate false positives, research efforts have intensified to investigate and identify a panel of cancer markers rather than one specific biomarker. A disintegrin and metalloproteases (ADAMs) are a class of proteins and their implications in metastasis of human cancer cells via protease activities has also been reported.[139] Zhang et al. reported a nonarray multiplexed sensing platform for sensitive profiling of ADAMs using nanographene oxide functionalized with multiple fluorophore-labeled peptide substrates having varying selectivity toward multiple proteases.[140] However, the (protease based) enzymatic cleavage of multiple fluorophore-labeled peptides is highly dependent on the solution pH and therefore prone to significant variations in signal intensities with minute pH alterations. Hence, maintaining stringent sensing conditions is necessary to establish reliability of the signals. Although enzymatic biomarkers like telomerase and protease are promising, there is a scarcity of effective assays to quantify enzyme activity in a sufficiently reliable manner with clinical accuracy. Nonetheless, these challenges continue to be a strong driving force in this research field with a goal to develop accurate cancer diagnostics capable of measuring telomerase and protease activity directly in complex body fluids for potential clinical applications.

3. CONCLUSIONS AND FUTURE OUTLOOK

Clinical cancer diagnosis is typically carried out in centralized laboratories with the aid of expensive instruments and skilled technicians, thereby making routine monitoring highly challenging. Improvements in cancer survival rates is often associated with early stage diagnosis which statistically increases the effectiveness to therapeutic interventions.[141] Substantial progress has been made in recent years toward the development of reliable, cost-effective, and powerful cancer diagnostic and monitoring strategies based on the detection of various cancer biomarkers. This work presents emerging cancer biosensing technologies based on the various biomarkers utilized such as proteins, nucleic acids, enzymes, and entire circulating tumor cells. Despite sizable efforts, cancer biosensors are yet to achieve clinical diagnostic standards due to lack of accuracy, sensitivity, and specificity. Majority of reported literature, although promising, are typically proof-of-concepts and hence require comprehensive investigations to validate their practical applicability toward clinically accepted standards. Therefore, the current challenge is to develop powerful technologies capable of robustly and sensitively transducing recognition events with high specificity in a clinically relevant detection range. For protein and nucleic acid−based biomarkers, ELISA and PCR are the accepted gold standards. Although accurate, both of these techniques have limitations including susceptibility of the enzymes to external environments for ELISA-based methods and the need for time-consuming and costly thermal cycling during PCR. In this regard, research investigations aimed at improving pre-existing methods or developing novel methods are highly needed. Unlike nucleic acids, several naturally occurring cancer biomarkers are incapable of enrichment via replication. Hence, the development of a procedure capable of translating probe-target based biological event recognition into encoded recognition events would result in the capability of indirect enrichment of non-nucleic acid cancer biomarkers via extensive replication of secondary encoded recognition events. Such technologies would bring about significant advances in early cancer diagnosis. The rapidly developing field of nanotechnology also offers attractive opportunities for ultrasensitive cancer biomarker detection due to substantially improved surface areas and introduction of novel nanohybrids with

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highly catalytic and biocompatible properties. This translates to the ability to mimic enzymatic reactions and superior surface loading capabilities to hold immense amounts of signal tags which leads to synergistic effects and effective signal amplifications. It is worth mentioning that nanomaterial-based detection schemes do face limitations due to poor specificity and lethargic binding kinetics in complex fluids. However, given the ease of nanomaterial fabrication and tailoring its morphology as per requirements, it can be safely anticipated that cancer biosensors fabricated with specialized nanomaterials will certainly be investigated to ensure further improvements to meet the requirements of clinical diagnostic applications. Liquid biopsy has also gained tremendous interest due to its noninvasive nature, excellent repeatability, low-cost and convenience. However, cancer detection schemes targeted at CTCs and endogenous dysregulated enzymes generally are subject to variations in sensing conditions and therefore ultrasensitive transduction methods integrated with microfluidic isolation platforms capable of extensive signal enhancement via surface loading of elevated amounts of amplifying labels/tags are necessary. Additionally, integration of automated micro scanning would be crucial to realize multiplexed cancer detection platforms for point of care testing with integrated sample preparation, specialized biorecognition and signal amplification technologies on a single chip. These recent developments in cancer detection technologies point toward the need for achieving high sensitivity, selectivity, and accuracy not only in optimized laboratory environments but also in point-of-care settings. This calls for collaborative research among different disciplines to optimize various aspects pertaining to sensor design and fabrication, elimination of nonspecific interactions, and improvement in signal amplification strategies, among others. The constant endeavors made in this challenging yet exciting research field is expected to invoke great strides toward the development of novel point-of-care technologies with clinically relevant diagnostic accuracy for early cancer diagnosis.

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Notes

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