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Review

대장암 진단용 단백질 바이오마커 측정을 위한 바이오센서 개발의 최신 연구 동향

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Recent Research Trend of Biosensors for Colorectal Cancer Specific Protein Biomarkers

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Abstract

Colorectal cancer (CRC) is one of the most prevalent diseases in modern society, constituting a serious threat to global health. Currently, routine clinical screening and early removal of precancerous polyps are the most successful methods for reducing CRC incidence and mortality. However, the high cost and invasive detection of sigmoidoscopy and colonoscopy limited the CRC-screening participation and prevention. The emergence of biosensors provides an inexpensive, sensitive, less invasive tool for detecting CRC disease biomarkers. This review highlights some of recent efforts made on developing biosensors with electrochemical and optical techniques targeting CRC specific protein biomarkers for early diagnosis and prognosis, potential applications, and future perspectives.

Keywords: *Colorectal cancer, Protein biomarkers, Biosensors, Electrochemical methods, Optical techniques*

1. Introduction

Over the past decades, industrialization and disruptive technology have significantly aided modern civilization and improved the quality of life. However, because of our changing lifestyles, we have developed civilization-wide diseases, such as cancers, cardio-cerebrovascular diseases, and obesity[1]. Cancers remain the second leading cause of death worldwide, with an estimated 9.6 million deaths in 2018, putting a significant strain on global health. Colorectal cancer (CRC) is linked to many factors such as lifestyle, gender, age, and drug use, which is the second and the third most prevalent cancer in females and males, respectively[2,3]. CRC development is a multistep process that begins with genetic alterations and progresses through a series of subsequent changes at the molecule level, such as abnormal transcription, translation, and protein expression, eventually leading to adenomas and metastatic carcinomas at the tissue level[4]. In this process, various CRC related protein biomarkers were reported, such as interleukin-6 (IL-6), interleukin-8 (IL-8), carbohydrate antigen 19-9 (CA19-9), CA11-19, carcinoembryonic antigen (CEA), tumor protein 53 (p53), retinol-binding protein 4 (RBP4), heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), vascular endothelial growth factor (VEGF), thrombospondin (THBS), and epidermal growth factor receptor (EGFR)[4-6].

Currently, fecal immunochemical test (FIT), computed tomography colonography (CTC), sigmoidoscopy, and colonoscopy are used as standard clinical screening tests for CRC to reduce the incidence and mortality[7,8] (see Figure 1). FIT is the most widely used cost-effective approach by testing hemoglobin in feces using an antibody, but still suffers from high false positive and negative results as well as limited sensitivity while CTC, sigmoidoscopy, and colonoscopy provide more accurate direct visualization of lesions but require drastic bowel preparation, higher cost, and low participation rate[3,8]. Thus, there is still a high demand for cost-effective, fast, convenient, sensitive, and specific tools in terms of diagnosis and prognosis of CRC.

Attractively, monitoring of CRC protein biomarker abnormalities using biosensors offers a less invasive way for CRC diagnosis, prognosis, and recurrence due to the simplicity, cost-effectiveness, rapid detection, high selectivity, and sensitivity in addition to the potential for miniaturization[4,9]. Depending on the transducer, recently developed biosensors for CRC protein biomarker include electrochemical, optical, and other signaling devices. The selective detection is based on the biomolecule recognition between the target CRC protein and capture probes such as antibody, aptamer, and receptor immobilized on the

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Table 1. Electrochemical Biosensors Developed for CRC Protein Biomarkers

Biosensor		Biomarker	Electrochemical technique	Linear range	LOD	Ref
Electrode	Configuration					
SPCE	CEA/anti-CEA/Au@Fe_3O_4/MnO_2-graphene nanoplatelets	CEA	Voltammetry Impedometry	0.001~100 ng/mL	0.10, 0.30 pg/mL	[11]
	HRP-SiO_2-Au-biotinylated aptamer-CEA-avidin Fe_3O_4@SiO_2	CEA	Amperometry	1~5,000 ng/mL	210 pg/mL	[12]
	CA19-9/BSA/anti-CA19-9/LbL (CB-PAA/CB-PEI)	CA19-9	Voltammetry	0.01~40 U/mL	0.07 U/mL	[13]
	p53/BSA/anti-p53/LbL (PEI/citrate-$NiFe_2O_4$ NPs)	p53	Voltammetry	1.0~1,000 pg/mL	5.0 fg/mL	[14]
	VEGF/BSA/anti-VEGF/rGO	VEGF	Voltammetry	0.0001~100 ng/mL	0.1 pg/mL	[15]
	CA19-9/anti-CA19-9/GO/CNOs PEI	CA19-9	Capacitance	0.3~100 U/mL	0.12 U/mL	[16]
PCE	RBP4/BSA/anti-RBP4/GA/4-ATP	RBP4	Impedometry	0.1~1,000 pg/mL	0.1 pg/mL	[17]
ITO	IL-6/BSA/IL-6 receptor-/Epx-PPyr	IL-6	Voltammetry Impedometry	0.02~16 pg/mL	6 fg/mL	[18]
	IL-6/BSA/IL-6 receptor/AB/Epx-PPyr	IL-6	Voltammetry Impedometry	0.01~50 pg/mL	3.2 fg/mL	[19]
	IL-8/ethanolamine/anti-IL-8/AuNPs-rGO	IL-8	Voltammetry	0.0005~4 ng/mL	72.73 ± 0.18 pg/mL	[20]
	IL-8/BSA/anti-IL-8/ β -Ag_2MoO_4	IL-8	Voltammetry	1 fg/mL~40 ng/mL	90 pg/mL	[21]
FTO	ALP/trigger aptamer/CEA/capture aptamer/MB/CoOOH/CS/g-C_3N_4-$CuInS_2$	CEA	PEC	0.02~40 ng/mL	5.2 pg/mL	[22]
Paper	EGFR/EGFR aptamer/NH_2-graphene-thionine-AuNPs	EGFR	Voltammetry	0.05~200 ng/mL	5 pg/mL	[23]
	VEGF-C/BSA/anti-VEGF-C/NMB-NH_2-SWCNTs-AuNPs	VEGF-C	Voltammetry	0.01~100 ng/mL	10 pg/mL	[24]

Abbreviations: 4-aminothiophenol, 4-ATP; alkaline phosphatase, ALP; amino functionalized single-walled carbon nanotubes, NH_2-SWCNTs; gold nanoparticles, AuNPs; bovine serum albumin, BSA; carbohydrate antigen, CA; carbon black, CB; carbon nano onions, CNOs; carcinoembryonic antigen, CEA; chitosan, CS; epoxy polypyrrole polymer, Epx-PPyr; fluorine-doped tin oxide, FTO; epidermal growth factor receptor, EGFR; glutaraldehyde, GA; indium tin oxide, ITO; interleukin, IL; layer-by-layer, LbL; magnetic bead, MB; new methylene blue, NMB; photoelectrochemical, PEC; plastic chip electrode, PCE; polyacrylic acid, PAA; polyethylenimine, PEI; reduced graphene oxide, rGO; retinol binding protein 4, RBP4; screen printed carbon electrode, SPCE; vascular endothelial growth factor C, VEGF-C.

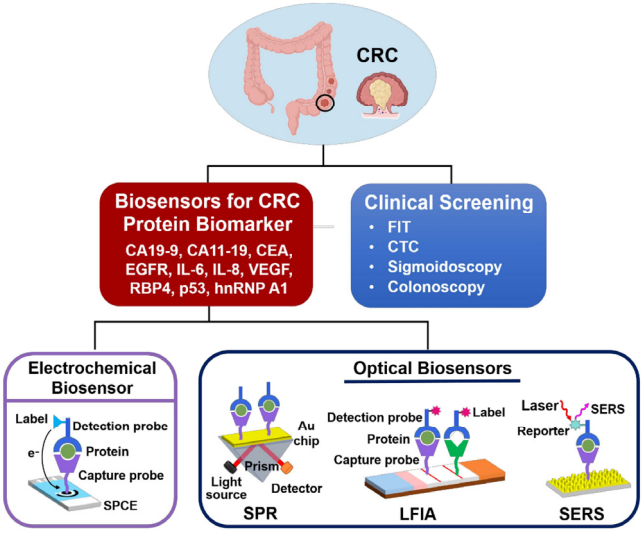

Figure 1. An overview of CRC-screening strategies highlighting different biosensing techniques for CRC protein biomarkers.

transducer surfaces. This mini review will give a quick overview of recent biosensor developments in conjunction with electrochemical and optical methods targeting particularly for CRC protein biomarkers.

2. Electrochemical Biosensing Platforms for CRC Protein Biomarker

Electrochemical biosensors are most extensively used for CRC protein biomarker detection by converting the electrode surface changes caused by biomolecular interactions to electrochemical measurable signals using amperometric, voltammetric, capacitive in addition to impedometric techniques. While significant research efforts have been conducted to detect CRC biomarkers[4,10], this section will only focus on portable electrochemical biosensors recently developed for CRC protein biomarkers, which are summarized in Table 1.

Screen-printed carbon electrodes (SPCEs) are one of the most widely used electrochemical device fabrications with portability for CRC protein biomarker detection[11-16]. Butmee *et al.*[11] reported a label-free sensitive and selective biosensing platform for CEA using anti-CEA antibody immobilized on manganese dioxide decorated graphene nanoplatelets modified SPCE. CEA was detected by monitoring the electrochemical signal of $[Fe(CN)_6]^{3-/4-}$ redox probes associated with biosensor surface changes when incubating with different concentrations of CEA. This method could detect CEA from 0.001~100 ng/mL using linear sweep voltammetry, offering a limit of detection (LOD) of 0.10 pg/mL. Paniagua *et al.*[12] prepared novel Au-SiO_2 Janus nanoparticles (NPs) with the functionalization of horseradish peroxidase (HRP) and biotin thiol-modified DNA aptamer, which enabled CEA to bind specifically with aptamer and was captured by avidin-functionalized magnetic NanoCaptors®. The complex was loaded on SPCE surfaces using magnetic deposition. The label enzyme HRP catalyzing hydrogen peroxide substrate enabled quantitative detection of CEA from 1~5,000 ng/mL and an LOD of 210 pg/mL.

The detection of CRC protein biomarkers was also reported using layer-by-layer (LbL) assembled polyelectrolytes modified SPCE.

Ibáñez-Redín *et al.*[13] described a disposable biosensor for CA19-9 detection from 0.01~40 U/mL and an LOD of 0.07 U/mL. In this work, a multilayer film was constructed on SPCE using LbL assembly of polyacrylic acid (PAA) and polyethylenimine (PEI) coated carbon black (CB) for anti-CA19-9 antibody immobilization. Polyelectrolytes improved not only conductivity but also dispensability of CB. Such an approach was also used to fabricate a sensing platform for p53 protein determination where the authors modified SPCE with LbL assembled PEI and citrate functionalized $NiFe_2O_4$ NPs for subsequent immobilization of anti-p53 antibody and p53 antigen binding. This biosensor offered a detection range of 1.0~1,000 pg/mL and an LOD of 5.0 fg/mL[14].

Meanwhile, an integrated plastic chip electrode (PCE) composed of graphite and polymethylmethacrylate free of exfoliation problem for SPCE printed layers was prepared for RBP4 detection[17]. Gold sputter coating and amino terminated monolayer were used to modify the plastic chip, which enabled the immobilization of anti-RBP4 antibody via glutaraldehyde (GA) crosslinker. The surface resistance caused by the antibody-antigen interaction was monitored by electrochemical impedance spectroscopy using $[Fe(CN)_6]^{3-/4-}$ species for RBP4 quantitation ranging from 0.1 to 1,000 pg/mL.

Another good substrate to fabricate electrochemical biosensors for detecting CRC protein like ILs is the conductive indium tin oxide (ITO) glass-based electrode[18-21]. Aydın[18] demonstrated an impedimetric label-free IL-6 biosensor by immobilizing IL-6 receptor on an ITO glass modified with a conjugated epoxy polypyrrole polymer (Epx-PPyr). This sensor provided a linear detection range from 0.02 to 16 pg/mL with an LOD of 6.0 fg/mL. In another related work, the authors modified ITO electrode with acetylene black (AB) with Epx-PPyr composites to enlarge the surface area and biomolecule-loading capacity, which demonstrated a wider and sensitive detection range for IL-6 from 0.01 to 50 pg/mL with an LOD of 3.2 fg/mL[19]. Besides, both biosensors showed acceptable recovery results for IL-6 analysis in diluted human serum samples.

In addition, photoelectrochemical (PEC) biosensors, a multidisciplinary sensing technique that combines optical and electrochemical approaches, measure electrode photocurrent caused by excitation of photoactive materials exposed to the external light source[25]. The PEC method owes higher sensitivity than the electrochemical and optical ones due to the different energy forms of light excitation source and electrical readout[25,26]. For example, Zhang *et al.*[22] designed a PEC sensing system for CEA using fluorine-doped tin oxide (FTO) glass photoelectrode which is modified with photosensitive graphitic like carbon nitride and copper indium disulfide hybrids ($g\text{-}C_3N_4/CuInS_2$) and cobalt oxyhydroxide (CoOOH) nanosheets for light blocking. CEA sandwich complexes labelled with alkaline phosphatase (ALP) were then constructed on the surface. ALP catalyzed the substrate of ascorbic acid-2-phosphate to produce an ascorbic acid and break down CoOOH nanosheets allowing $g\text{-}C_3N_4/CuInS_2$ exposure to light. This method could detect CEA concentrations from 0.02 to 40 ng/mL in buffer and showed similar sensing results with those detected by an ELISA kit for serum sample analysis.

Paper based microfluidic electrochemical biosensors were also used

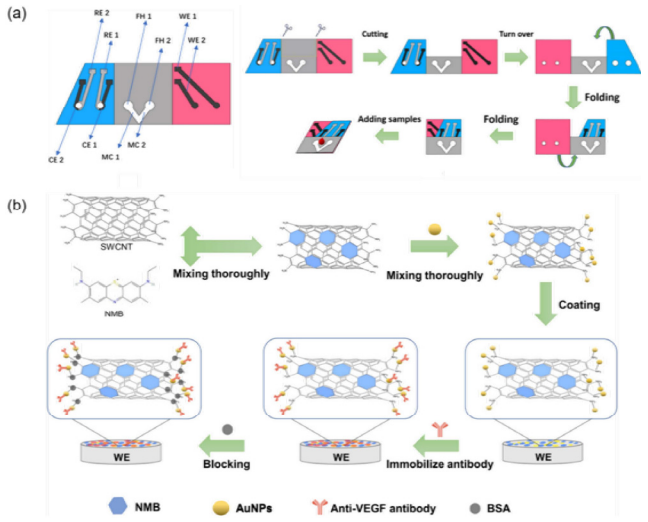

Figure 2. Schematic showing the (a) fabrication process of an origami paper based electrochemical biosensor and (b) modification of the working electrode for VEGF-C detection using SWCNTs, NMB, and AuNPs composites. Reproduced with permission from ref. [24] (Open access under the Creative Commons CC BY license, 2021 Springer Nature).

for CRC biomarkers such as EGFR and vascular endothelial growth factor C (VEGF-C) owing to the advantages of low cost and no need for extra equipment for sample flowing[23,24]. For instance, Sun *et al.* [24] proposed an origami paper-based microfluidic electroanalytical device for real-time sensing of VEGF-C from 0.01 to 100 ng/mL with an LOD of 10 pg/mL (Figure 2). The microfluidic chip was fabricated using wax and screen printing technologies. The modification of working electrodes using new methylene blue (NMB), amino functionalized single-walled carbon nanotubes (NH_2-SWCNTs), and gold NPs (AuNPs) nanocomposites enhanced electrical conductivity and provided sufficient surface area for anti-VEGF-C antibody and antigen loading. Such an inexpensive paper-based electrochemical microfluidic chip was applied for VEGF-C analyses in clinical serum samples and the discrepancy with a commercial instrument was less than 9.81%.

3. Optical Biosensing Platforms for CRC Protein Biomarker

Apart from electrochemical biosensors, this section will describe some of few recent examples of optical biosensing platforms developed for CRC protein biomarker, including colorimetric and fluorescence-based lateral flow immunoassay (LFIA), surface plasmon resonance (SPR), and surface-enhanced Raman scattering (SERS) shown in Table 2. LFIA is a well-established optical sensing technique mainly composed of a sample pad, conjugate pad, nitrocellulose (NC) detection membrane, and absorbent pad[27]. The principle of LFIA is based on the specific recognition of biomolecules and reflected by the colored or fluorescence changes of signal labels on the detection membrane [28,29]. SPR biosensor measures the refractive index changes on the plasmonic metal (e.g., Au) surface in response to molecule biorecog-

Table 2. Some Examples of Optical Biosensors Developed for CRC Protein Biomarkers

Technique	Biosensor configuration	Biomarker	Linear range	LOD	Ref
LFIA	AuNPs/anti-CEA Ab2/CEA/anti-CEA Ab1/protein G/NC membrane	CEA	2~50 ng/mL	0.35 ng/mL	[35]
	EuNPs-anti-IL-6 Ab2/IL-6/anti-IL-6 Ab1/NC membrane	IL-6	2~500 pg/mL	0.37 pg/mL	[36]
	CNTs-Fe_3O_4/anti-CA19-9 Ab1/CA19-9/anti-CA19-9 Ab2/NC membrane	CA19-9	2~200 U/mL	1.75 U/mL	[37]
	EGFR/AuNPs-biotinylated EGFR aptamer/streptavidin/NC membrane	EGFR	0~50 nM	9.8 nM	[38]
SPR	anti-hnRNP A/hnRNP A1/DNA aptamer/Au chip	hnRNP A1	0.1~10 nM	0.22 nM	[6]
	AuNPs/streptavidin/biotin-anti-CEA Ab2/CEA/anti-CEA Ab1/Au coated glass chip	CEA	-	17.8 pg/mL	[39]
SERS	AuNPs/Fe_3O_4 NPs/$Ti_3C_2T_x$ MXenes/anti-CEA Ab2/CEA/ anti-CEA Ab1/MoS_2 nanoflowers@AuNPs/glass slide	CEA	0.0001~100 ng/mL	0.033 pg/mL	[40]

Abbreviations: antibody, Ab; carbohydrate antigen, CA; carbon nanotubes, CNTs; carcinoembryonic antigen, CEA; europium (III) chelate-doped nanoparticles, EuNPs; gold nanoparticles, AuNPs; epidermal growth factor receptor, EGFR; heterogeneous nuclear ribonucleoprotein A1, hnRNP A1; interleukin, IL; lateral flow immunoassay, LFIA; nitrocellulose, NC; surface plasmon resonance, SPR; surface-enhanced Raman scattering, SERS.

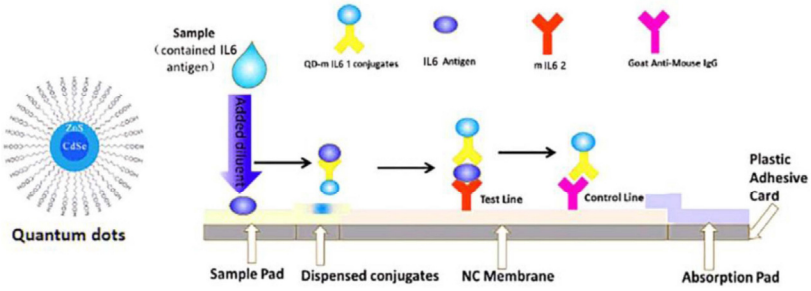

Figure 3. Schematic showing the components of an LFIA strip for CEA detection. Reprinted with permission from ref. [41] (Open access under the Creative Commons CC-BY-NC-ND license, 2021 Wiley Periodicals).

nition[30,31]. It is a highly sensitive label-free technique for real-time detection but still has a limitation, such as nonspecific adsorption[32]. Different from SPR, SERS measures the changes of inelastic scattering on noble metal surfaces induced by biomolecule interaction and used for different malignancy diagnosis[33,34].

Protein detection using LFIA is based on the optical intensity of colored or fluorescent labels such as dyes, quantum dots, colloidal gold, carbon dots, and magnetic NPs[28,29]. For example, Tang *et al.*[41] reported a fluorescent LFIA strip for IL-6 detection using CdSe@ZnS quantum dots as labels, which demonstrated a linear range from 10 to 4000 pg/mL with an LOD of 1.995 pg/mL (Figure 3). This LFIA strip was applied to clinical serum sample analyses and comparable to a commercial chemiluminescence immunoassay kit. In another work, europium (III) chelate-doped NPs (EuNPs) were used as labels in LFIA for IL-6 detection[36]. EuNPs have long fluorescence lifetime, narrow and sharp emission peak, offering a dynamic range from 2 to 500 pg/mL and an LOD of 0.37 pg/mL for IL-6 in the buffer. As for clinical human serum sample application, IL-6 LFIA biosensor showed a high correlation with those from a commercial SIEMENS CLIA IL-6 kit.

Furthermore, Huang *et al.*[37] established a magnetic colorimetric LFIA for CA19-9 detection in whole blood using CNTs decorated with Fe_3O_4 magnetic NPs as labels (CNTs-Fe_3O_4). The CNTs-Fe_3O_4 conjugated anti-CA19-9 could recognize CA19-9 antigen and magnetically separated from the blood matrix. The CA19-9/anti-CA19-9/CNTs-Fe_3O_4 complexes were captured by the probe at test line showing visualized brown band for protein quantitation by measuring the color intensity. This LFIA biosensor showed a linearity ranging from 2 to 200 U/mL with an LOD of 1.75 U/mL in buffer solutions and 30 U/mL in human blood. Remarkably, the magnetic separation of CA19-9/anti-CA19-9/CNTs-Fe_3O_4 complexes from the complex matrix could reduce biofouling and improve the selectivity, which was demonstrated by negligible interfering effects from biomolecules such as CEA and mammaglobin.

Whereas utilizing SPR, Lee *et al.*[6] recently reported a biosensor for detecting hnRNP A1 in human plasma samples of CRC patients (see Figure 4). The DNA aptamers specific to hnRNP A1 were cross-linked on a carboxylic acid-terminated SPR gold chip via (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride/N-hydroxysulfosuccinimide (EDC/Sulfo-NHS) followed by successive specific binding of protein and detection probe to form sandwich complexes. In this work, hnRNP A1 DNA aptamer was used as a capture probe for biosensor fabrication because of its higher binding affinity and robustness compared to the antibody. This biosensor exhibited an LOD of 0.22 nM and was successfully applied to the direct analysis of hnRNP A1 concentrations in both human normal and CRC patient plasma solutions. Also, Ermini *et al.*[39] demonstrated a four-channel SPR immunosensor composed of AuNPs/streptavidin/biotin-anti-CEA Ab2/CEA/anti-CEA Ab1 on a glass chip coated with a thin Au layer, which could detect CEA as low as 17.8 pg/mL. In this work, the authors found that high ligand doses per NP (LDPN) for AuNPs/streptavidin complexes enhanced the absolute zeta potential value as well as the stability, reproducibility, specific sensing response and reduced nonspecific binding. In contrast, the AuNPs/streptavidin biofunctionalization strategy for bio-

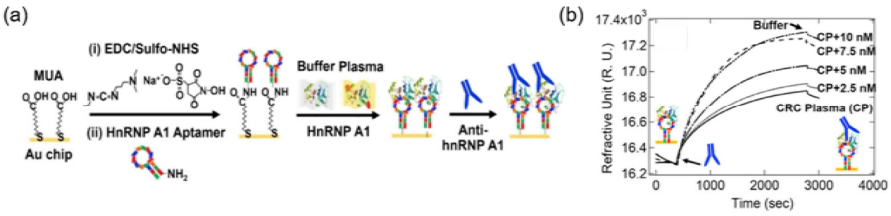

Figure 4. Schematic showing (a) the formation of the sandwich complex on an SPR gold chip for hnRNP A1 detection with an aptamer and anti-hnRNP A1 and (b) real-time analyses in CRC plasma samples. Reproduced with permission from ref. [6]. Copyright, 2020 Elsevier.

tin-Ab2 binding showed lower nonspecific and similar specific responses versus the one using AuNPs-Ab2 via EDC/Sulfo-NHS crosslinking at high LDPN in both buffer and plasma. Overall, both SPR biosensors discussed here showed a diminished response in human plasma samples compared to those from the buffer, which could be attributed to the reduced specific and enhanced nonspecific interactions caused by strong steric problems or energy barriers in the complex matrix[6,39].

There are two types of SERS biosensing methods including direct detection using label-free and indirectly using SERS tags; the label-free one offers molecular information but could suffer from the low signal and interference in the matrix while the SERS tags using different Raman reporters could show stable signal and provide more diversity in biosensor fabrication[42]. For example, Medetalibeyoglu *et al.*[40] reported a SERS-based sandwich assay using 4-mercaptobenzoic acid as Raman reporter for CEA detection in plasma samples. In this work, the composites of d-$Ti_3C_2T_X$ MXene with self-assembled Fe_3O_4@AuNPs were used as magnetic supporting substrate for CEA capture antibody immobilization due to the excellent mechanical strength and large surface area. For the SERS tag, 4-mercaptobenzoic acid conjugated MoS_2 nanoflowers@AuNPs hybrids were used for CEA detection antibody immobilization to form a sandwich assay for CEA biosensing. Thus, CEA quantitation was performed by transferring CEA sandwich complexes on a glass slide, resulting in a linear range from 0.1 pg/mL to 100 ng/mL. In addition, this sensor could detect a standard CEA added in plasma samples with high accuracy.

4. Conclusions and Future Perspectives

This review exhibited some of recent biosensing devices developed for common CRC protein biomarkers and potential applications using electrochemical, LFIA, SPR and SERS techniques. While electrochemical strategies provide inexpensive way of fabricating sensors with facile introduction of different surface modification, LFIA methods generally formed on NC membranes become the most encouraging diagnostic device for point-of-care testing with the advantages of relatively low cost and fast sensing[43,44]. On the other hand, SPR and SERS are capable of label-free and sensitive detection of target protein biomarkers but still requiring expensive plasmonic metal (e.g. Au) materials. Overall, the use of biosensors provides a cost-effective, highly sensitive, less invasive, and user-friendly detection strategy for CRC monitoring compared to some clinically visualizable screening techniques. However, there is no single ideal biomarker for CRC diagnosis making it difficult to identify CRC based on the result of a single biomarker detection. Furthermore, CRC is related to multiple abnormal biomarkers, some of which are multifunctional biomarkers for different diseases, for example, IL-6 for prostate cancer, cardiovascular disease, and diabetes[18]. From our perspective, multiplexed biosensors for CRC biomarkers are needed, as they improve sensing accuracy and reduce cost. However, the application of multiplexed biosensors for CRC diagnosis and analysis in patient samples is still in its infancy. Thus, many efforts are still required to facilitate the transition of CRC biosensors to clinical applications and homecare devices. Multiplexed sensing devices, such as electrode arrays and barcode configurations-based electrochemical biosensors, microfluidic devices, and multiplexed LFIA have made a significant progress on biomarker detection[45-49]. The combined use of multichannel electrodes and multiplexed LFIA for simultaneous detection of different CRC biomarkers could be a promising strategy for improving accuracy and precision of CRC diagnosis. We also envision that the widespread availability of convenient and affordable CRC biosensors could encourage screening participation and reduce the global cancer burden.

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