

Review

A Review: Electrochemical Biosensors for Oral Cancer

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Abstract: Oral cancer poses a serious threat worldwide owing to its soaring case-fatality rate and its metastatic characteristics of spreading to the other parts of the body. Despite the recent breakthroughs in biomedical sciences, the detection of oral cancer at an early stage is still challenging. Conventional diagnosis in clinics and optical techniques to detect oral cancer in the initial stages are quite complicated as well as not completely accurate. To enhance the survival rate of oral cancer patients, it is important to investigate the novel methodologies that can provide faster, simpler, non-invasive, and yet ultraprecise detection of the onset of oral cancer. In this review, we demonstrate the promising aspects of an electrochemical biosensor as an ideal tool for oral cancer detection. We discuss the cutting-edge methodologies utilizing various electrochemical biosensors targeting the different kinds of biomarkers. In particular, we emphasize on electrochemical biosensors working at the molecular levels, which can be classified into mainly three types: DNA biosensors, RNA biosensors and protein biosensors according to the types of the analytes. Furthermore, we focus on the significant electrochemical methods including cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) to analyze the oral cancer biomarkers (such as IL-6, IL-8, CYFRA 21-1, CD 59 and CIP2A) present in body fluids including saliva and serum, using non-invasive manner. Hence, this review provides essential insights into the development of pioneering electrochemical biosensors for the detection of oral cancer at an early stage.

Keywords: electrochemical biosensor; CV; DPV; EIS; oral cancer; interleukin-6 (IL-6); interleukin-8 (IL-8); tumor necrosis factor α (TNF- α); cytokeratin 19 fragments (CYFRA 21-1); CD 59; cancerous inhibitor of PP2A (CIP2A)

1. Introduction

Cancers occurring in the oral cavity are among the most common malignancies in developed countries [1,2]. Each year, about 300,000 new cases of oral cancer are reported and, unfortunately, results in over 140,000 deaths worldwide [3]. In the United States, about 53,000 Americans are diagnosed with oral or oropharyngeal cancer annually, causing over 9750 deaths, killing approximately 1 person every hour [4,5]. Premalignant oral lesions or early-stage cancers are usually asymptomatic, thus usually being ignored at the initial stages, leading to a high mortality rate, especially for male tobacco and alcohol users [6]. It is also reported that oral cancer is associated with betel quid chewing

in various communities [7–9]. Besides the high fatalities, oral cancer is also a critical factor in reducing productivity in developing countries from early deaths [3,10]. Conventional methods for the diagnosis of oral cancer and premalignant lesions (e.g., leukoplakia and erythroplakia) mainly rely on visual inspection and biopsy. Oral cancer includes several types of carcinoma, most often refers to as squamous cell carcinoma (SCC) [11]. To investigate the biomarkers responsible for these malignancies, and to diagnose oral cancer in the early stages, biosensor advancement is vital [12].

At present, the initial diagnosis of oral cancer is made by performing an invasive method such as tissue biopsy of the affected region, followed by further assessment using non-invasive medical imaging techniques including computed tomography (CT), magnetic resonance imaging (MRI) or positron emission tomography (PET) scans which are quite expensive and complicated [1,13]. In comparison to conventional inspection methods, electrochemical biosensors provided with relatively high sensitivity, enhanced specificity, and non-invasive detection methods for biomolecules [14,15]. Furthermore, these biosensors won't be much affected by challenges like optical interference or sample turbidity [16]. In this review, we discuss the cutting-edge methodologies utilizing various electrochemical biosensors for detecting oral cancer. In general, they can be classified into mainly three types: DNA biosensors, RNA biosensors and protein biosensors based on their sensing targets. Furthermore, we focus on the significant electrochemical methods including cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) to analyze the oral cancer biomarkers (Figure 1). Biomarkers can be detected in the body fluids such as serum and saliva, as shown in Table 1. In the following sections, we summarize the fundamental methodologies that are essential for electrochemical biosensors development for monitoring the behavior of numerous biomarkers in oral cancer.

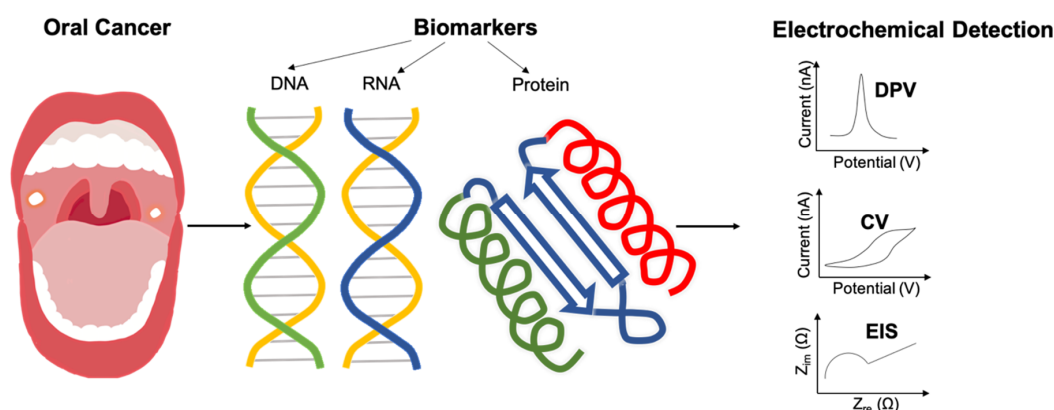


Figure 1. Schematic illustration of an oral cancer-detecting process by using electrochemical biosensors. Abbreviations: Differential pulse voltammetry (DPV); Cyclic voltammetry (CV); Electrochemical impedance spectroscopy (EIS).

2. Electrochemical Methodologies for Biosensing

2.1. DNA Biosensor

In most cases, target DNA collected from human body fluid needed to be centrifuged and purified. DNA biosensors detect the target DNA by modifying the working electrode with complementary sequences [17,18]. Usually, the gold electrode, screen-printed carbon electrode and glassy carbon electrode are utilized as the working electrode for the electrochemical set-up, while indium tin oxide (ITO) can also be used [19]. Ma et al. developed a ratiometric electrochemical DNA sensor for direct analysis of target DNA in artificial saliva specimens, by integrating the exonuclease III-assisted amplification with dual-signal ratiometric output mode [20]. Thus, it enables the detection of a lower concentration of biomarkers. Firstly, oral cancer overexpressed 1 (ORAOV1) DNA is hybridized with a ferrocene-labeled hairpin probe (F_c-Probe), then separated by exonuclease III releasing the target DNA,

which is followed by the next amplification cycle. The amount of the residual F_c probe is quantified by using an electrochemical method. The residual F_c probe is hybridized with the methylene blue-labeled hairpin probe (MB-labeled probe), then a modified MB-labeled probe is fixed on the gold electrode and used as a working electrode. Once the F_c probe combines with the MB-labeled probe, the electric current would decrease. Thus, the more the ORAOV1 DNA presents in saliva, the more F_c probe would be consumed, and the higher current would be detected (as shown in Figure 2a). Chen et al. also developed a DNA biosensor to detect ORAOV1 [21]. They modified signal probes that contain target recognition sequences, nicking the endonuclease recognition sequence and G-rich sequence on a bare gold electrode, forming a cycle after a hybridized signal probe cleaved by the nicking enzyme. Finally, the addition of hemin to bind with a G-rich sequence that is located on signal probes and catalyzed the H_2O_2 -mediated oxidation of 3,3',5,5' tetramethylbenzidine (TMB) to acquire current signal. However, the performance of DNA biosensors is not always very satisfactory along with the metal electrode because of DNA denaturation. To overcome this challenge, Wei et al. introduced a biocompatible DNA dendrimer system, a short nanoscale of DNA modified on the working electrode surface. They adopted a 16-Array electrochemical chip with three electrodes to detect multiple biomarkers simultaneously as shown in Figure 2b [22]. Eventually, the signal level got higher, and the amount of denatured species get lower.

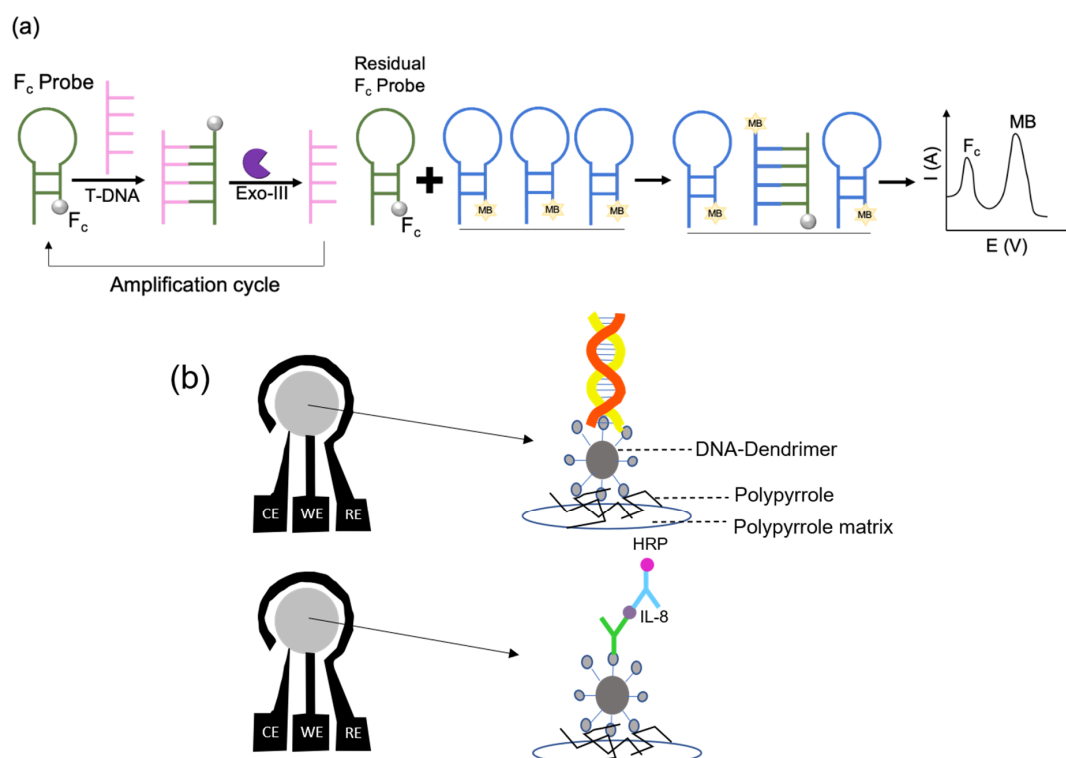


Figure 2. (a) Schematic illustration of the Exo-III involved in the DNA target amplification cycle and the signal readout of electrochemical DNA biosensor. (b) The three-electrode configuration on the electrochemical chip and illustration of biomolecules immobilized on DNA dendrimer. Abbreviations: Ferrocene-labeled hairpin probe (Fc); Exonuclease III (Exo-III); Methylene blue (MB); Counter electrode (CE); Working electrode (WE); Reference electrode (RE); Horseradish peroxidase (HRP); Interleukin 8 (IL-8).

2.2. RNA Biosensor

RNA biosensors are often combined with magnetic beads utilizing the junction strategy to ensure the biosensors achieve better sensitivity and a higher distinction ability. Wang et al. reported the modification of capture probes on magnetic beads. As depicted in Figure 3a, a magnetic bead is capable

of binding numerous capture probes along with the target and the signal probe, getting absorbed on the surface of the electrically magnetic-controllable working electrode on the addition of a voltage on the electric coil [10,11]. Hence, the electrochemical current signal increased because horseradish peroxidase (HRP) catalyzed the H_2O_2 -mediated oxidation of TMB after immersing the working electrode in TMB- H_2O_2 solution. As illustrated in Figure 3b, the magnetic beads were captured on the dual screen-printed carbon electrode (SPdCE) as working electrodes so that target mRNA and target interleukin-8 (IL-8) could be detected simultaneously in the same samples. Furthermore, hydroquinone (HQ) can be used as a redox mediator in the H_2O_2 solution to monitor the current generation and analysis of each target's concentration in artificial saliva.

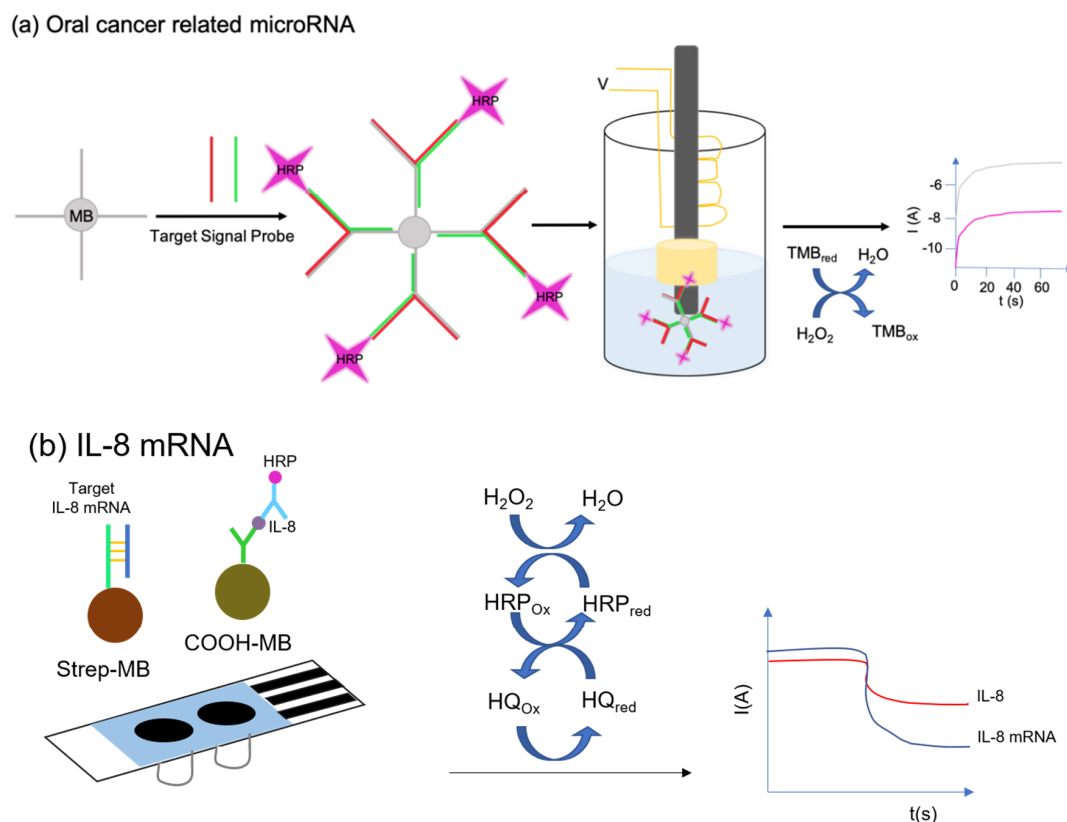


Figure 3. (a) The principle of the magnetic-controllable electrochemical RNA biosensor. (b) The modified MBs on SPdCE for the simultaneous determination of IL-8 protein and IL-8 mRNA. Abbreviations: Magnetic bead (MB); Horseradish peroxidase (HRP); 3,3',5,5' Tetramethylbenzidine (TMB); Interleukin 8 (IL-8); Streptavidin-modified magnetic bead (Strep-MB); Carboxylic acid-modified magnetic bead (HOOC-MB); Hydroquinone (HQ).

2.3. Protein Biosensor

Electrochemical biosensors for measuring protein cancer biomarkers offer a sensitive, quick, and cheap diagnosis framework for point of care (POC) and clinical analysis [23]. In these biosensors, the surface of the electrodes usually is modified with receptors such as antibodies or aptamers. Consequently, the explicit protein analytes are measured with the assistance of quantifiable and electroactive species generated from themselves or a signal transducer that has been added into the solution. Immunosensors are highly selective since the interactions between an antibody and an antigen act like a lock and key binding mechanism [24]. Protein biomarkers for cancer detection are broadly applicable for measuring elements that are considered as indicators of abnormal biological processes, disease processes, or responses to therapeutic intermediation [19,25]. These biomarkers usually can be extracted from serum, tissue, or saliva, and their expression levels can be expressive

of disease states. Several tumor markers can be easily found in saliva which makes the salivary sample in a high level of interest for the non-invasive detection of oral cancer [26,27]. Recent studies have shown both cell-free mRNAs and proteins in saliva display diagnostic values for oral cancer, as previously mentioned [28]. One of the pioneers for the multiplexed electrochemical sensors for the determination of salivary biomarkers for oral cancer detection was reported by Wei et al. as mentioned in Section 2.1 the detection of a certain protein [22]. Their results showed that the multiplex detection of both interleukin-8 (IL-8) mRNA and protein allows an accurate diagnosis for oral cancer detection. In another study, human saliva samples used for the detection of protein IL-8 and IL-8 mRNA by the development of electrochemical magnetobiosensors [29]. In this research, highly sensitive and selective methodologies were reported. Moreover, the detection limits are 0.21 nM for IL-8 mRNA and 72.4 pg mL⁻¹ for IL-8 protein in undiluted saliva samples. The development of reliable immunosensor for POC application for early detection of certain proteins is of interest, especially for an ultra-low concentrated sample without interference from the variety of other non-analyte proteins in serum and saliva [29–34]. The gold standard method for clinical detection of biomarkers is enzyme-linked immunosorbent assay (ELISA) [31]. ELISA has the limits of detection (LOD) of 1–3 pg mL⁻¹ for many protein analytes [19,30]. However, ELISA is limited by costly test kits and equipment, long time of measuring, and difficulties in multiplexing. Consequently, ELISA is not the first choice for the application of POC diagnostics.

Compared with traditional biosensors, aptamer-based biosensors characterized label-free, high sensitivity for electrochemical detection [35,36]. Qureshi et al. developed a capacitive aptasensor to monitor human epidermal growth factor receptor 2 (HER2) protein which was an overexpressed biomarker in many cancers including ovarian, lung, gastric, and oral cancers [37]. Anti-HER2 aptamers (ssDNA) are immobilized on a gold microelectrode surface. Then, dilute human serum was spiked with various concentrations of HER2. Ultimately, the relationship between capacitance and HER2 concentration was measured. The results showed that a dynamic range was between 0.2 and 2 ng mL⁻¹, without adding any redox-chemicals [35]. In another studies, aptamers were utilized for the detection of IL-6 protein. For instance, Kumar et al. reported a nano-aptamer sensor to detect IL-6 in biofluids like sweat using EIS. In this study, aptamers against IL-6 were immobilized on gold nanoparticles deposited on the gold working electrode for capturing the analyte [38]. The detection limit was estimated as 0.02 pg mL⁻¹. Furthermore, the aptasensors had 90% of the original signal of impedance response after 2 weeks which indicates the high stability of the sensor. Another example is that Tertis et al. developed an IL-6 aptasensor based on a glassy carbon electrode modified by p-aminobenzoic acid, p-aminothiophenol and gold nanoparticles [39]. The aptasensor showed the detection limit of 1.6 pg mL⁻¹ and high selectivity in the blood sample.

Another type of protein sensor is based on immunoassays. In a typical sandwich immunoassay, a recognized element, such as primary antibody, is immobilized on an electrode surface to capture the specific analyte. In the next step, secondary antibody conjugated with an enzyme, for instance, horseradish peroxidase (HRP), is added into the solution for binding the antigen (the analyte). HRP can convert its substrate to electrochemically active species, so that the chemical signal can be converted to electrochemical signal through this step [29,40–43]. For instance, Heineman' group pioneered in enzyme-linked electrochemical detection of the analyte using sandwich immunoassays [32]. In this research, two main procedures are being utilized for multiplex electrochemical protein identification. First, the primary antibody was used for binding the analytes, and then the secondary antibodies were attached to nanoparticles. Second, electrodes were utilized by immobilizing with various antibodies and for measuring the electrochemical signals. In addition to their research, the improvement of delicate and precise electrochemical biosensing frameworks relies on bioreceptor, redox mediators and transducer networks. In recent years, nanomaterials are popular, and they are widely applied in the biosensor field owing to their high surface-to-volume ratio. New carbon or metal nanomaterials have been innovated, such as graphene and its derivatives, with special properties including high electron transfer rate, high biocompatibility, and thus many studies combine nanomaterials with immunosensors [29,33,44–46]. Nanostructure based immunosensors increase electrode surface area,

which can provide 10-fold or more antibody coverage compared to flat surfaces, leading to enhanced sensitivity of up to 1000-fold and lower limit of detection [34,47–49].

In addition to oral cancer biosensors, electrochemical biosensors systems for the detection of cancer biomarkers using similar principles have been developed for several types of cancer including lung cancer [47,50], melanoma [51–53] breast cancer [36,54,55] and prostate cancer [56]. Electrochemical signal amplification in these biosensors could be achieved by estimating electroactive chemical responses released or consumed from an enzyme, for example HRP [20,54,57]. Other electrochemical detection methods relied on special nanoparticles labeled on antibodies. This complex could be further dissolved to gain detectable electroactive species [25,32,34,58–61]. Protein sensors for targeting various important biomarkers were listed in the following sections.

Table 1. Biomarkers that have been used for oral cancer electrochemical biosensors and the experimental conditions.

Biomarker	Sample	Electrochemical Method	Detection Limits	The Levels of The Biomarker in Normal Case and Cancer Patient	References
Amylase	Samples spiked in potassium ferrocyanide	Cyclic voltammetry	1.57 pg mL ⁻¹	-	[23]
IL-8 protein, IL-1 β protein and IL-8 mRNA	Samples spiked in buffer	cyclic square-waveform	Protein:100–200 fg mL ⁻¹ mRNA IL-8:10 aM	IL-8 protein, OSCC patient: 720 pg mL ⁻¹ ; Normal: 250 pg mL ⁻¹ IL-8 mRNA, patient: 16 fM; Normal:2 fM	[24]
Interleukin-6 (IL-6)	HNSCC cell lines	Amperometry	2.5 \times 10 ⁻¹⁴ M	IL-6 protein, HNSCC patient: more than 20 pg mL ⁻¹ ; Normal: less than 6 pg mL ⁻¹	[25]
microRNA	Artificial saliva	Cyclic voltammetry Chronoamperometry	2.2 \times 10 ⁻¹⁹ M	-	[30]
IL-6 protein, IL-8 protein	Serum	Amperometry	IL-6:5 fg mL ⁻¹ , IL-8:7 fg mL ⁻¹	IL-6 protein, HNSCC patient: more than 20 pg mL ⁻¹ ; Normal: less than 6 pg mL ⁻¹ IL-8 protein, patient: 720 pg mL ⁻¹ ; Normal: 250 pg mL ⁻¹	[31]
Oral Cancer Overexpressed 1	Human saliva	Differential pulse voltammetry	0.35 pM	-	[19]
IL-8 protein, TNF- α	Artificial saliva	I-V Curve	100 fg mL ⁻¹	IL-8 protein, patient: 720 pg mL ⁻¹ ; Normal: 250 pg mL ⁻¹	[32]
IL-8 mRNA IL-8 protein	Human saliva	Amperometry	IL-8 mRNA: 0.21 nM IL-8: 72.4 pg mL ⁻¹	IL-8 mRNA, patient: 16 fM; Normal:2 fM IL-8 protein, patient: 720 pg mL ⁻¹ ; Normal: 250 pg mL ⁻¹	[29]
CYFRA-21-1	Samples spiked in PBS buffer	Cyclic voltammetry Electrochemical impedance spectroscopy	0.21 ng mL ⁻¹ (calculated)	CYFRA-21-1 protein, normal: 3.8 ng mL ⁻¹ ; patient:17.46 \pm 1.46 ng mL ⁻¹	[34]
CYFRA-21-1	Samples spiked in PBS buffer	Cyclic voltammetry Differential pulse voltammetry	0.122 ng mL ⁻¹ (calculated)	CYFRA-21-1 protein, normal: 3.8 ng mL ⁻¹ ; Patient:17.46 \pm 1.46 ng mL ⁻¹	[33]
CYFRA-21-1	Artificial Saliva	Differential pulse voltammetry	0.001 ng mL ⁻¹	CYFRA-21-1 protein, normal: 3.8 ng mL ⁻¹ ; Patient:17.46 \pm 1.46 ng mL ⁻¹	[47]
CD59	Human saliva	Cyclic voltammetry Electrochemical impedance spectroscopy	Treated Saliva: 0.84 \pm 0.04 fg mL ⁻¹ Raw saliva: 1.46 \pm 0.05 fg mL ⁻¹	-	[48]
IL-8 protein	Human saliva	Cyclic voltammetry Differential pulse voltammetry	72.73 \pm 0.18 pg mL ⁻¹ (calculated)	IL-8 protein, patient: 720 pg mL ⁻¹ ; normal: 250 pg mL ⁻¹	[49]
Oral Cancer Overexpressed 1	Artificial saliva	Alternating current voltammetric Electrochemical impedance spectroscopy	12.8 fM	-	[20]
CIP2A	Human saliva	Cyclic voltammetry Electrochemical impedance spectroscopy	0.24 pg mL ⁻¹	-	[57]
CYFRA-21-1	Human saliva	Differential pulse voltammetry Electrochemical impedance spectroscopy	0.625 pg mL ⁻¹	CYFRA-21-1 protein, normal: 3.8 ng mL ⁻¹ ; Patients: 17.46 \pm 1.46 ng mL ⁻¹	[54]
IL-8 protein	Human serum and saliva	Cyclic voltammetry Electrochemical impedance spectroscopy Single Frequency Impedance	3.3 fg mL ⁻¹	IL-8 protein, patient: 720 pg mL ⁻¹ ; Normal: 250 pg mL ⁻¹	[58]
IL-8 protein	Human saliva	Cyclic voltammetry Differential pulse voltammetry	51.53 \pm 0.43 pg mL ⁻¹ (calculated)	IL-8 protein, patient: 720 pg mL ⁻¹ ; Normal: 250 pg mL ⁻¹	[59]

2.3.1. Proteins Biosensors Targeting IL-6 and IL-8

IL-6 and IL-8 are cellular proteins that are particularly relevant to oral squamous cell carcinoma (OSCC) [62–65]. The uncontrolled expression of these cytokines could be a sign of the development of tumor growth and metastasis [65]. Usually, the normal expression levels of these cytokines in keratinocytes are low. IL-6 is an interleukin that behaves as both a pro-inflammatory cytokine and an anti-inflammatory myokine, that is involved in acute-phase reaction, growth regulation and differentiation of cells [60,66]. Patients with oral cancer have 20 to 1000 pg mL^{-1} of IL-6 in the serum, while, in healthy individuals, it is $<6 \text{ pg mL}^{-1}$ [17]. IL-8 is a 8-kDa cytokine related to the angiogenic and mitotic processes, and inflammatory response. The serum levels in healthy individuals are lower than 13 pg mL^{-1} compared with 20–1000 pg mL^{-1} or more in patients [67]. Figure 4 depicts electrochemical immunosensors based on the detection of biomarkers IL-6 and IL-8 for targeting oral cancer.

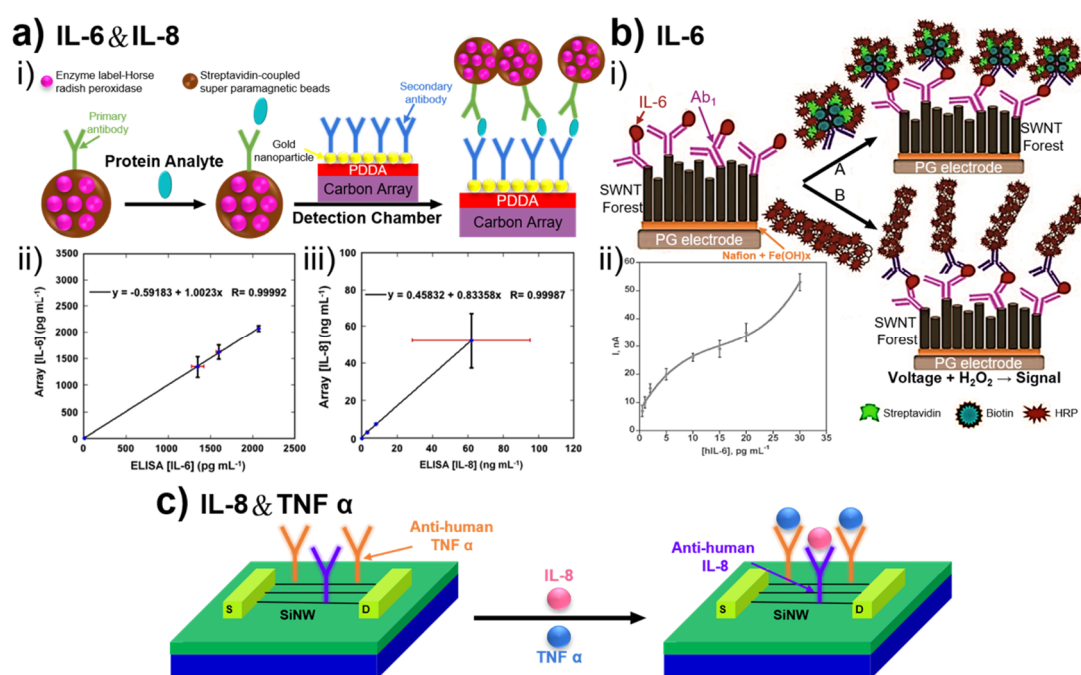


Figure 4. Electrochemical immunosensors based on the detection of biomarkers IL-6 and IL-8 for targeting oral cancer. (a) (i) Conceptual strategy for detection of protein biomarker by micro-fluidic immunoarray. Correlation plots of immunoarray assay results for conditioned media of different cell lines vs. standard ELISA assays for: (ii) IL-6; and (iii) IL-8. (b) (i) Multilabel amperometric immunosensor; and (ii) influence of IL-6 concentration on steady-state current for immunosensor using the bioconjugate. (c) Schematic diagram illustrating SiNW arrays functionalized with two antibodies and two biomarkers. (a) Adapted with permission from ref. [31]. Copyright 2014 Elsevier. (b) Adapted with permission from ref. [25]. Copyright 2010 American Chemical Society. Abbreviations: Poly(diallyldimethylammonium chloride) (PDDA); Interleukin 8 (IL-8); Interleukin 6 (IL-6); Anitbody1 (Ab1); Anitbody2 (Ab2); Single-walled nanotube (SWNT); tumor necrosis factor α (TNF- α).

Otieno and coworkers developed a sensor array coated with gold nanoparticle–antibody conjugates in a poly(dimethylsiloxane) microchannel [31]. In their investigation, protein analytes were captured on Ab₂–MP–HRP bioconjugates from serum or other biological samples. Figure 4a(i) illustrates the principle for fabricating the sensor. The accuracy of the sensor was verified (Figure 4a(ii,iii)). The comparison of the obtained results showed good correlations with standard ELISAs. In another study, Malhotra and coworkers reported an ultrasensitive electrochemical immunosensor for targeting IL-6 in head and neck squamous cell carcinoma (HNSCC) cells [25]. The mean concentration of IL-6 in the serum of patients with HNSCC is higher than 20 pg mL^{-1} compared with 6 pg mL^{-1} in healthy

individuals. In this research, the high sensitivity was achieved by nanostructured single-walled nanotube (SWNT) forest platforms. Two strategies were chosen for the multilabel detection in the amperometric immunosensor, as shown in Figure 4b(i): using 14–16 HRP's labeled Ab₂; or adding active HRP's modified on carboxylated carbon nanotubes.

In another study, Zhang et al. reported a field-effect transistor (FET) sensor for the detection of two biomarkers of OCSS in saliva samples, IL-8 and TNF- α , by using a silicon nanowire (SiNW) (Figure 4c) [32]. The detection of biomarkers in real saliva sample is very tricky since it contains complex components. They overcame this problem by functionalizing with two different antibodies immobilized on the surface of the electrode for capturing IL-8 and tumor necrosis factor α (TNF- α) and avoiding the cross-reactivity of biomarkers carefully (Figure 4c).

In recent years, two-dimensional (2D) graphene-based materials are widely used in biosensors because of their unique properties including high mechanical stability, biocompatibility and high rate of electron transfer [33,68,69]. The advantages of graphene-based biosensors can be roughly concluded into two points. First, graphene oxide (GO) contains certain functional groups that can be easily covalently bonded to biomolecules. Second, the structural defects of GO, reduced graphene oxide (rGO) and graphene-based quantum dots (GQDs) can be used to immobilize the biomolecules onto their surfaces. Graphene-based sensors are successfully used for the incorporation of nanoparticles and/or hydrogel for electrochemical detection of various analytes including glucose [70,71], DNA, cholesterol oxidase, and NADH, etc. [72]. Verma and coworkers fabricated immunosensor based on the gold nanoparticles-reduced graphene oxide (AuNPs-rGO) composites for non-invasive IL-8 detection in the saliva sample [49]. The reported immunosensor has shown fast detection (9 min) of IL-8 and offers high sensitivity with an experimental linear dynamic range of 500 fg mL⁻¹ to 4 ng mL⁻¹ and a detection limit of 72.73 \pm 0.18 pg mL⁻¹. The high sensitivity and rapid detection were due to the high rate of electron transfer through the thin film of AuNPs-rGO nanocomposites.

2.3.2. Protein Biosensors Targeting Other Biomarkers

Besides IL-6 and IL-8, some other biomarkers have also been employed for electrochemical detection of oral cancer. Kumar and coworkers reported a non-invasive, label-free immunosensor based on nanostructured hafnium oxide (hafnia) deposited onto indium tin oxide (ITO) coated glass for detection of CYFRA-21-1 in human saliva [34]. CYFRA-21-1 is a remarkable tumor marker for squamous cell carcinoma (SCC). However, the clinical value of CYFRA-21-1 in OSCC has not been validated [73]. Figure 5a(i) shows the steps of the fabrication of BSA/anti-CYFRA-21-1/APTES/nHfO₂/ITO immunosensor. The BSA/anti-CYFRA-21-1/APTES/nHfO₂/ITO electrode responded according to the concentration of CYFRA-21-1 (2–18 ng mL⁻¹), shown in Figure 5a(ii). In this biosensor, it was found that the CV peak current gradually decreased linearly with increased concentration of CYFRA-21-1. The decreased current was due to the formation of electrically insulating antigen–antibody complex which prevents the electron transfer through redox mediator. The current responses recorded for the real samples (Figure 5a(iii)) were matched with the current signals obtained for standard samples of the same concentration. This result showed an excellent correlation between the current from the real sample.

The first application of vertically aligned carbon nanotube array (VANTA) interdigitated electrodes (IDEs) for electrochemical detection of CIP2A was reported by Ding and coworkers [54]. IDEs worked as electrochemical transducers, consisting of several electrodes which were electrically connected together [74,75]. CIP2A is a cancer biomarker for a variety of human cancers including lung, breast and gastric cancers. However, CIP2A is extensively expressed in OSCC cell lines, and malignant human oral epithelial tissues [76]. The scheme in Figure 5b(i) represents the steps of the fabrication of immunosensor. The dimensions of VANTA IDEs are shown in Figure 5b(ii). Moreover, the highly porous VANTA structures were observed with field-effect scanning electron microscopy (FESEM) (Figure 5b(iii,iv)) in two magnifications. The label-free immunosensor for detecting CIP2A showed a wide linear sensing range (1–100 pg mL⁻¹) with a good detection limit of 0.24 pg mL⁻¹ in saliva.

Furthermore, the electrochemical immunosensor showed higher sensitivity than the corresponding CIP2A ELISA test.

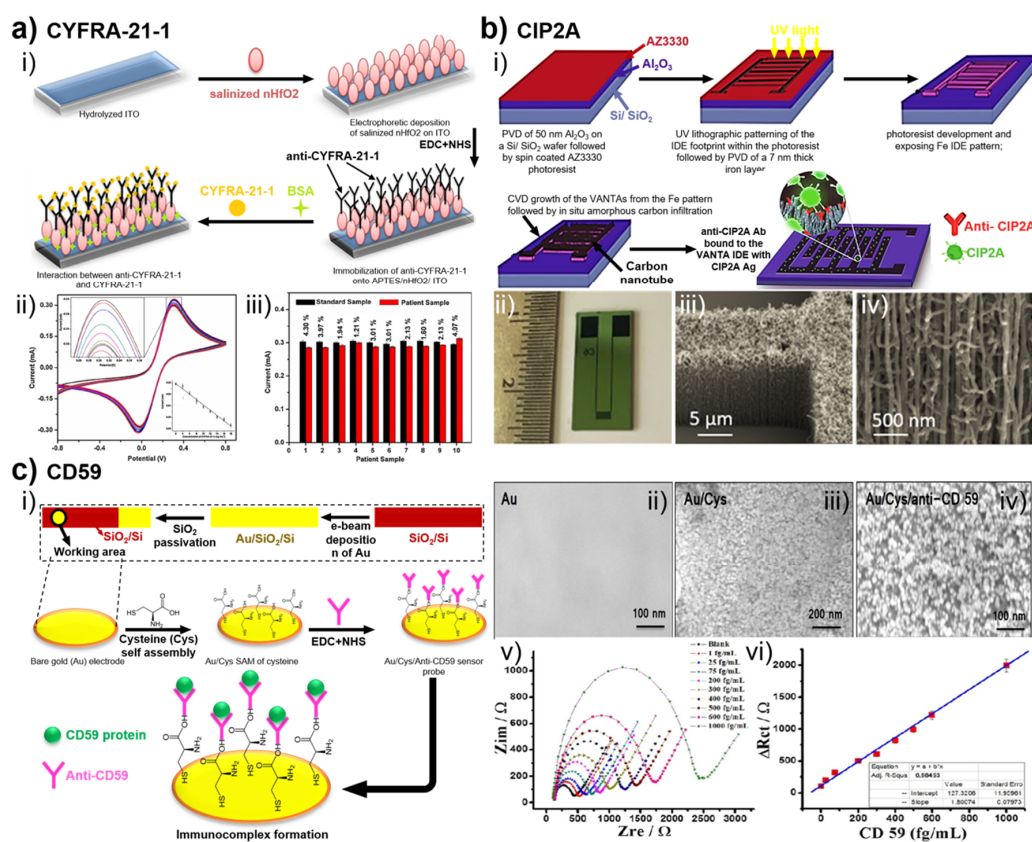


Figure 5. (a) (i) Schematic representation of the fabrication of BSA/anti-CYFRA-21-1/APTES/nHfO₂/ITO immunosensor for oral cancer detection. (ii) The electrochemical response of modified immunoelectrode in different concentrations of CYFRA-21-1. Insets show the calibration curves of peak current and CYFRA-21-1 concentration. (iii) The comparison between the standard sample and patient samples. (b) (i) Schematic representation of the fabrication steps of VANTA IDEs; (ii) optical image of a VANTA IDE (the values of numbers on the ruler are in centimeters); and (iii, iv) FESEM images of VANTAs from the IDE devices in two magnifications. (c) (i) Schematic representation of the fabrication and the detection principle: (top) the stepwise fabrication of immunosensor; and (below) steps of the fabrication of immunocomplex formation. FESEM images of: bare (ii) Au; (iii) Au/Cys; and (iv) Au/Cys/Anti-CD59 surfaces. (v) EIS responses for CD59 detection in 5 mM zobell's solution and (vi) calibration plot from 1 to 1000 fg mL⁻¹ CD59 concentrations. (a) Adapted with permission from ref. [34]. Copyright 2016 Elsevier. (b) Adapted with permission from ref. [57]. Copyright 2018 Elsevier. (c) Adapted with permission from ref. [48]. Copyright 2014 Elsevier. Abbreviations: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC); N-Hydroxysuccinimide (NHS).

Moreover, Choudhary and coworkers developed a label-free impedimetric immunosensor to diagnose oral cancer by detecting CD59 [48]. The immunosensor probe was fabricated by immobilizing CD59 antibodies (anti-CD59) on a self-assembled molecular layer of L-cysteine (Cys) on a gold electrode. The stepwise of fabrication of the immunosensor is shown in Figure 5c(i). The microphotographs for Au, Au/Cys and Au/Cys/anti-CD59 surfaces are shown in Figure 5c(ii–iv), respectively. CD59 is one of the most fundamental and clinically relevant early-stage markers, that can be used to diagnose oral cancer [44]. Figure 5c(v) shows the EIS response of immunosensor in different concentrations of CD59. The R_{ct} value increased with an increase of CD59 concentrations due to the formation of the insulation layer between CD59 and immunosensor. The immunosensor showed a linear range of detection between 1.0 and 1000 fg mL⁻¹ (Figure 5c(vi)).

3. Conclusions

In this review, DNA biosensor, RNA biosensor and protein biosensor for several salivary biomarkers (such as IL-6, IL-8, TNF- α , CYFRA 21-1, CD 59 and CIP2A) have been discussed as promising candidates to provide crucial information for developing non-invasive oral cancer diagnosis. Therefore, the major challenge faced in developing such a diagnostic system accounts for the relatively lower amounts of target analytes present in saliva, which is taken as the diagnostic fluid. However, as the novel and highly sensitive electrochemical biosensor techniques are emerging, the accuracy of analyzing the vital biomarkers in the saliva has been enhanced as well. In addition, these electrochemical sensors meet the requisites, including easy-to-use, non-invasiveness and low cost. The ultrasensitivity and the specificity of biosensors can be improved by combining with MBs, signal probes or nanomaterials on the working electrode so that the electrochemical signal can be more evident. SPdCE and 16-array chips offer the system to detect multiple relevant oral cancer biomarkers simultaneously. However, as oral cancer exhibits some biomarkers that are also corresponding to other types of cancers, their distinctive detection is still an issue. Efforts have been made to differentiate the normal tissues from the different cancerous tissues at various stages by utilizing electrochemical sensing. In the future, the development of electrochemical biosensors on-chip will be one of the main non-invasive detection methodologies of oral cancer at earlier stages in a relatively more precise manner.

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