Review

Electrochemical Sensors for Liquid Biopsy and Their Integration into Lab-on-Chip Platforms: Revolutionizing the Approach to Diseases

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Electrochemical Sensors for Liquid Biopsy and Their Integration into Lab-on-Chip Platforms: Revolutionizing the Approach to Diseases

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Abstract: Screening and early diagnosis of diseases are crucial for a patient's treatment to be successful and to improve their survival rate, especially in cancer. The development of non-invasive analytical methods able to detect biomarkers of pathologies is a critical point to define a successful treatment and a good outcome. This study extensively reviews electrochemical methods used for the development of biosensors in liquid biopsy, owing to their ability to provide rapid response, precise detection, and low detection limits. We also discuss new developments in electrochemical biosensors, which can improve the specificity and sensitivity of standard analytical procedures. Electrochemical biosensors demonstrate remarkable sensitivity in detecting minute quantities of analytes, encompassing proteins, nucleic acids, and circulating tumor cells, even within challenging matrices such as urine, serum, blood, and various other body fluids. Among the various detection techniques used for the detection of cancer biomarkers, even in the picogram range, voltammetric sensors are deeply discussed in this review because of their advantages and technical characteristics. This widespread utilization stems from their ability to facilitate quantitative detection of ions and molecules with exceptional precision. The comparison of each electrochemical technique is discussed to provide the selection of appropriate analytical methods.

Keywords: liquid biopsy; electrochemical sensors; lab on chip; miniaturization; sensors integration; microfabrication

1. Introduction

Biopsy is a technique in which tissue samples are taken from the body and examined under a microscope to see if cancer (but the concept is applicable to many other diseases) or abnormal cells are present. Biopsies can be classified into the following categories based on the sample taken:

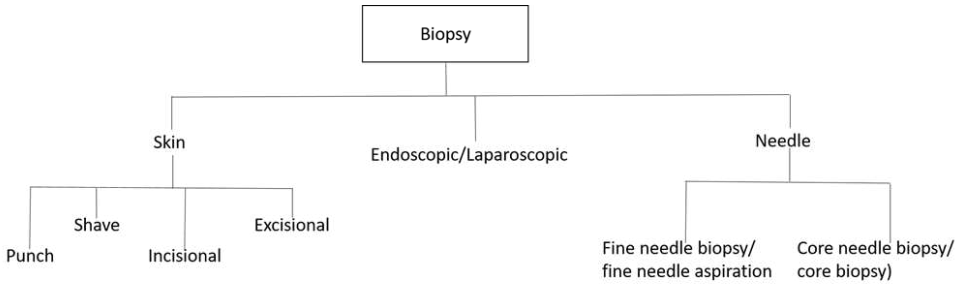

Figure 1. Classification of Biopsies: Understanding the Different Categories.

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In the last decades, liquid biopsy, namely the possibility to have a diagnosis from body fluids without recurring to tissue biopsy has been increasingly investigated. The possibility to detect and classify tumours or other diseases even at a very early stage in a minimally invasive and repeatable way could have significant clinical impact, and significant progresses have been made in the development of devices able to do this in a smarter manner compared to standard analytical methods. Despite the great advantages in patients' compliance and the minimally invasive features, approach has not yet attained the status of a conventional tool in the armoury of clinical oncologists [1].

Electrochemical biosensors were recommended by the International Union of Pure and Applied Chemistry (IUPAC). Which states that "An electrochemical biosensor is a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is kept in direct spatial contact with an electrochemical transduction element".

Biosensors are considered to be promising tools for the quantitative or semi-quantitative detection of analytes. [2] In this type of sensor, a biological molecule interacts with the analyte, previously immobilized on the biosensor, producing a physicochemical signal that is detected by the transducer. Biosensors can be divided into two categories: catalytic-based, which produce a substance starting from substrates compounds, and affinity-based, which binds the analyte directly. According to the type of signal transmitted, biosensors can be classified as electrochemical, optical, [3] magnetic, or piezoelectric, just to name a few [4].

Electrochemical techniques excel among these methods by offering rapid, sensitive, selective, and cost-effective detection and monitoring of a wide range of biological molecules associated with diverse diseases. Additionally, their seamless integration into portable systems enables the implementation of Point-of-Care diagnostic approaches [5]. An electrochemical biosensor is a compact device that utilizes both biorecognition processes and electrochemical transducers to convert biological information into electrical signals. This conversion provides either quantitative or semi-quantitative information about the analyte being detected [6].

Electrochemical methods, such as electrochemical impedance spectroscopy (EIS), differential pulse voltammetry (DPV), and cyclic voltammetry (CV), play a crucial role in both the development of biosensors and the evaluation of their performance. These methods are highly valuable approaches in the field [7]. For research purposes, CV technique is widely used in biosensor development because it provides valuable information such as the types of redox processes present in the analysis and the reversibility of reactions. A sensing system that can identify a cell's location within a microfluidic channel was designed by Rapier et al. Results from electrochemical impedance spectroscopy (EIS) show that cells in microfluidic channels can be positioned between different pairs of electrodes at varied locations along the device's length. Impedance spectra distinguish between confluent, sparse, and empty microfluidic channels. A huge boost to the development of this kind of sensors as well as to their application for liquid biopsy has been given by the high suitability to miniaturization. Electrodes architecture, indeed, can be easily achieved through micro- and nano-fabrication methods, increasing the number of sensing elements per area and allowing high-throughput performances [8].

Also, Lab-On-Chip integration is directly linked to miniaturization. The possibility to perform multiple assays given by lowering dimensions of sensing elements has led to the necessity to differently functionalize and use them for detection of different biomarkers [9,10].

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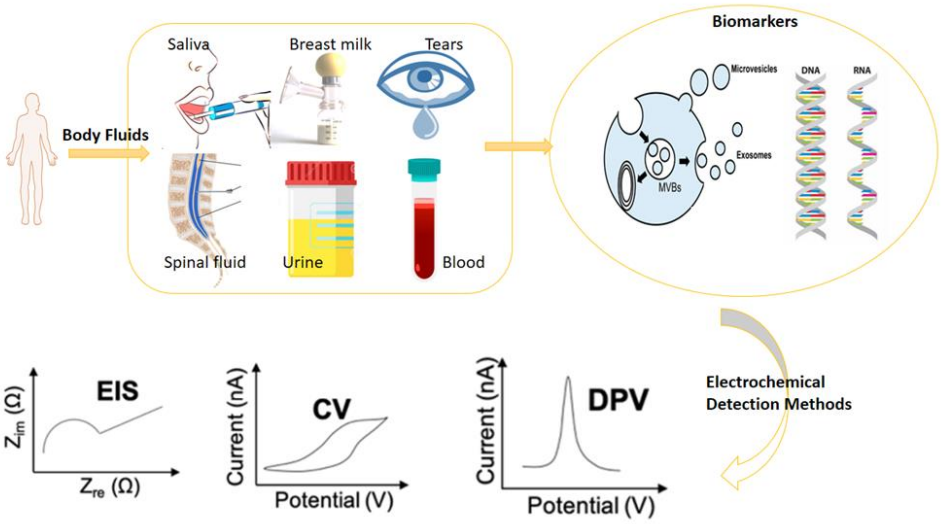

Figure 2. Overview of the Electrochemical Biosensors Operation.

The utilization of impedance-based methods has significantly facilitated the simplification of cellular assays, providing quantitative and highly sensitive results that are amenable to automation and scalability in multi-well formats. Extensive review by W. Gamal et al. has shed light on their efficacy. Moreover, in the context of real-time monitoring of three-dimensional cell culture, dielectric spectroscopy and electrical impedance tomography have emerged as promising alternatives to two-dimensional impedance sensing [11]. These impedance-based cellular assays (IBCAs) serve as label-free phenotyping assays, gaining increasing interest in the field of regenerative medicine applications [12]. To determine the flow in a microfluidic chip, E. Skotadis et al. developed a strain-sensing module based on microfluidic and Lab on Chip systems that offers simple integration with most microfluidic-systems. The sensor consists of interconnected platinum nanoparticles that self-assemble on flexible polyolefin substrates, which also serve as the sealing layer for the microfluidic channels. These nanoparticle networks are formed using a modified sputtering approach, implemented on printed circuit board substrates (PCBs) through milling and computer numerical control machining. The resulting module exhibits competitive limit of detection (LOD), cost-effectiveness, low power requirements, and seamless integration with existing microfluidic systems. It can be utilized as an independent unit or integrated onto the sealing material, enabling detection of flow rates as low as 5 μL/min (equivalent to a strain of 0.00337%). The sensor demonstrates a sensitivity of 0.021 μL per minute [12].

Lucile et al. reported another novel microfluidic method that can selectively extract, preconcentrate, and fluorescently detect IL-6 directly on the chip by the fluidization of magnetic beads. The ability to switch between packed and fluidized states allowed authors to evaluate how the physical characteristics of the beads could be altered to increase mass transport, lessen non-specific binding, and triple the detection signal. A high dynamic range (10 pg/mL to 2 ng/mL) and a twofold reduction in LOD compared to traditional approaches were demonstrated by integrating the entire ELISA protocol into a single microfluidic chamber [14].

In this review, an overview about electrochemical methods and their applications as transduction techniques in the development of biosensors will be provided. A particular attention will be paid to liquid biopsy and to the use of miniaturized platform allowing the spreading of Point-Of-Care devices in this field.

1.1. Essential Biomarkers for Liquid Biopsy Detection and Monitoring

Liquid biopsy is increasingly used for the detection, analysis and monitoring of circulating tumor cells (CTCs), circulating tumor DNA and circulating extracellular nucleic acids [15], in blood or other body fluids such as urine, with the main advantage of diagnosing cancer at an early stage.

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In contrast to traditional invasive biopsy, which includes cells or tissues from the lesion, liquid biopsy is a non-invasive procedure that can also be used during the treatment planning, as well as in the follow-up of the disease. In addition to blood, other body fluids are under consideration for liquid biopsy: urine [16] is already used as a source of biomarkers (PCA3 in prostate and pancreatic cancer detection) and several example of state of art detection from saliva or seminal fluid [17] or stool have been described [18].

The liquid biopsy is a non-invasive method for detecting, analyzing and monitoring cancer cells, DNA and other nucleic acids in body fluids such as blood and urine. It offers several advantages over traditional biopsy methods, including the ability to diagnose cancer at an early stage without the need for invasive procedures. This technique can be used not only for the initial diagnosis of cancer but also during treatment planning and in follow-up procedures. Other body fluids such as urine, saliva, seminal fluid, and stool are also being studied as potential sources of biomarkers for liquid biopsy. Urine is already being used to detect PCA3 in prostate and pancreatic cancer, and several state-of-the-art detection methods have been developed for other body fluids.

Characteristics of An Ideal Biomarker

An ideal cancer biomarker would have high clinical sensitivity and specificity, rapid release in the blood for early detection, high concentration in the blood for prolonged periods of time, and the possibility to be quantified.

Biomarkers are recognized to have a critical role in the early detection of cancer, the creation of personalized treatments, and the identification of disease-related underlying processes.

The detection targets of liquid biopsy primarily encompass cells and nucleic acids found in body fluids, including circulating tumor cells (CTCs) in blood, cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), exosomes, micro-RNA (miRNA), and proteins. An innovative way of using liquid biopsy, going in the direction of point-of-care medicine is the integration of these kind of assays into portable devices, which need a small amount of sample, low power to work and minimal equipment [19]. Electrochemical methods are particularly suitable for integration into near-the-bed platform, since the most of them are label-free methods and all the components can be easily miniaturized into lab-on-chip platforms.

Table 1. Some common biomarkers used for Liquid Biopsy and electrochemical method used.

Biomarker	Biofluid/Sample	Electrochemical method	LOD	Ref.
Exosomes	Plasma	Potentiometric	20pM	[20]
			10^6 mL^{-1}	[21]
			43 particles μL^{-1}	[22]
			<10^5 vesicles/ 10 μL	[23]

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Circulating Nucleic acids • **Circulating tumor DNA (ctDNA)** • **Circulating microRNA (miRNA)**	Human serum Serum	DPV DPV and EIS	3.9 x10^{-22} g/ml 0.45 fM	[24] [25] [26] [27]
Circulating tumor cells (CTCs)	Blood Blood Peripheral blood	Amperometry DPV DPV	5 cells/ml 27 cells/ml 3 cells/ml	[28] [29] [30]
Proteins	Human serum, saliva	CV and EIS	3.3 fg m/L	[31]

2. Miniaturization Strategies for Biosensing

Miniaturization is the key challenge and research trend currently pursued in the field of biosensing. Moore's law, a theory in the field of microelectronics, postulated the continuous advancement of the industry by doubling the number of on-chip transistors every two years. This exponential growth in transistor count has led to a reduction in cost per function. Miniaturizing the transducer as well as the biosensing element means a boost in enhancing sensitivity and specificity features. This advancement will contribute to the achievement of three key performance metrics in biosensors: enhancing the limit of detection, reducing response time, and lowering production costs.

One of the practical and technological advantages of miniaturizing the biosensing system, indeed, is the improved sensitivity of electrochemical and electronic sensors by increasing the systems signal-to- noise ratio reaching dimensions of micro-structured electrodes and nanomaterials which are comparable with entities of interest (as example, cells in the case of microstructures, protein and nucleic acids in the case of nanomaterials). Signal enhancement in this condition is achieved by the presence of nanogaps and/or nanostructured electrodes obtaining the noise reduction making available a high-surface-area for biosensing interactions. This holds true for systems based on field-effect transistors as well as nanoscale electrochemical biosensors. Additionally, reducing the interelectrode spacing at the nanoscale can be also considered as a strategy to amplify redox current and in this way to obtain electrochemical single molecule analysis by generating a significate signal-to-noise ratio [32].

The kinetics of transport reactions in biosensing are closely linked to the time needed for biorecognition events to take place. In this context, the background current associated with the charging of the double layer (capacitive current) varies in proportion to the electrode's conductive area. In miniaturized electrochemical cells, the resistive drop is minimized by shortening the ionic current pathway. As a result, the capacitance is reduced, leading to a decreased time constant of the system. This enables faster electron transfer kinetics compared to macroscale systems.

The possibility to reduce dimensions brings several side advantages. Among these, first of all portability and integration into complex platform, allowing to realize multiplexed analysis, including sample treatment tools and parallelize functions. This not only enables the creation of compact yet robust devices, but also has the added benefit of reducing manufacturing costs by minimizing materials and fabrication expenses per device. This efficiency in mass production contributes to overall cost reduction.

2.1. Micro- and Nanofabrication Methods

Several methods for micro- and nanofabrication of electrodes are available, allowing to produce desired geometries of transducers and sensing elements for electrochemical biosensing. Beside the consolidated optical and electron beam lithography technologies, some innovative tools are gaining popularity thanks to the possibility they provide to achieve better resolution and higher customizable processes in relatively short time with respect to standard techniques. These kinds offer wide-range opportunities to innovate alongside Moore scaling without requiring high investment levels, but offering large-feature, low-end production, high-end performances [33].

Among these technologies, maskless lithography methods based both on direct laser writing source (in example femtosecond laser or UV laser) or 2-photon systems have been widely used for the fabrication of microelectrodes, ensuring a very high resolution of lithographic patterns even in a large surface area and in three-dimensional operating mode [34,35]. Recently, several groups have been working on these kinds of processes. Zhu and co-workers dealing with the realization of planar electrodes onto flexible substrates, realized the direct laser writing on stacked graphene multilayer of a large-areal micro-supercapacitors onto a polyaniline substrate, demonstrating the possibility to fabricate pressure/gas sensors with high sensitivity for multiple applications [36]. Dotan et al. implemented a novel approach for the development of soft and flexible microelectrode structures used in electric and electrochemical sensing. Their method involved the combination of supersonic cluster beam deposition (SCBD) of gold nanoparticles onto Polydimethylsiloxane (PDMS), followed by femtosecond (fs) laser processing. Through this technique, they successfully produced a nanocomposite film with mechanical properties comparable to those of the elastomeric substrate [37]. 2-photons lithography is a very versatile and flexible micro- and nanofabrication technology which allows for 3D architecture of lab on chips and integrated platform. The possibility to combine planar and multilevel structures into the same chip thanks to 2-photons lithography has been explored by Luitz and coworkers who realized complex 3D micro and nano-objects using a platinum containing photoresin, which can be structured via direct lithography 2-photon polymerization paving the way for novel applications like production of innovative metamaterials for biomedical applications, where high surface areas and the physicochemical properties of Pt are highly desirable. Moreover, with subsequential steps of lithography, 2-photons lithography method enables the possibility to embed sensors structures into microfluidic devices, thus obtaining monolithic platform for on-chip sample preparation and characterization [38–40].

2.2. Nanomaterials in Electrochemical Sensors Integrated in LOC Device: From 2D to 3D Electrodes

Nanomaterials have emerged as a promising class of materials for sensing applications due to their unique physicochemical properties [41–43]. The exceptional properties exhibited by nanomaterials, including high surface area, excellent electrical and thermal conductivity, as well as unique optical characteristics, make them highly advantageous for seamless integration into lab-on-a-chip (LOC) devices as electrochemical sensors. This integration enables the detection of molecules in body fluids with significantly improved sensitivity and accuracy [44].

Microfluidic devices offer the ability to control the flow of fluids at the microscale, enabling the rapid and precise detection of biomolecules. When combined with nanomaterial-based sensors, real-time monitoring of low concentrations of biomolecules in body fluids such as blood [45], urine [46], tears [47] and saliva [48] can be achieved. The incorporation of nanomaterials in electrochemical biosensors holds the potential to bring about a revolutionary transformation in the field of clinical diagnostics. This advancement facilitates the rapid, sensitive, and highly specific detection of biomolecules in body fluids, paving the way for significant advancements in medical diagnosis and patient care. This enhancement is achieved through either promoting electronic transfers or increasing the volume/surface area ratio [49]. This has significant implications for medical diagnosis and treatment, as it can enable the early detection and monitoring of diseases such as cancer, diabetes, and cardiovascular diseases or timely identification of bacterial infections.

The integration of nanomaterials onto the electrode surface of microfluidic devices plays a crucial role in the advancement of high-performance electrochemical sensors. This is particularly

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important when electrodes are situated in less accessible locations, as often encountered in lab-on-chip systems. By incorporating nanomaterials, the sensitivity and overall performance of the electrochemical sensors can be significantly enhanced, enabling accurate and reliable detection in challenging sample environments. In this kind of device, nanomaterials are typically integrated onto the electrode surface using various techniques, such as in situ synthesis [50], drop-casting [51], spin-coating [52], electrochemical deposition [53], electrospinning [54] and inkjet printing [55]. Drop-casting and spin-coating are simple and cost-effective techniques that involve the deposition of nanomaterials onto the electrode surface using a dropper or a spinning device, respectively. Electrochemical deposition involves the deposition of nanomaterials onto the electrode surface by applying a voltage or a current to the electrode in the presence of the nanomaterials in solution. Inkjet printing involves the precise deposition of nanomaterials onto the electrode surface using a specialized printer. Among them, electrochemical deposition and inkjet printing are the techniques that allow for the most precise and controlled deposition of nanomaterials onto the electrode surface, which is critical for the development of high-performance electrochemical sensors.

Various types of organic and inorganic nanomaterials, including carbon nanotubes, graphene, metal and metal oxide, polymer, quantum dots, Prussian Blue [56], nanorods, and tubes, have been incorporated into electrochemical transducers to enhance electrochemical sensing.

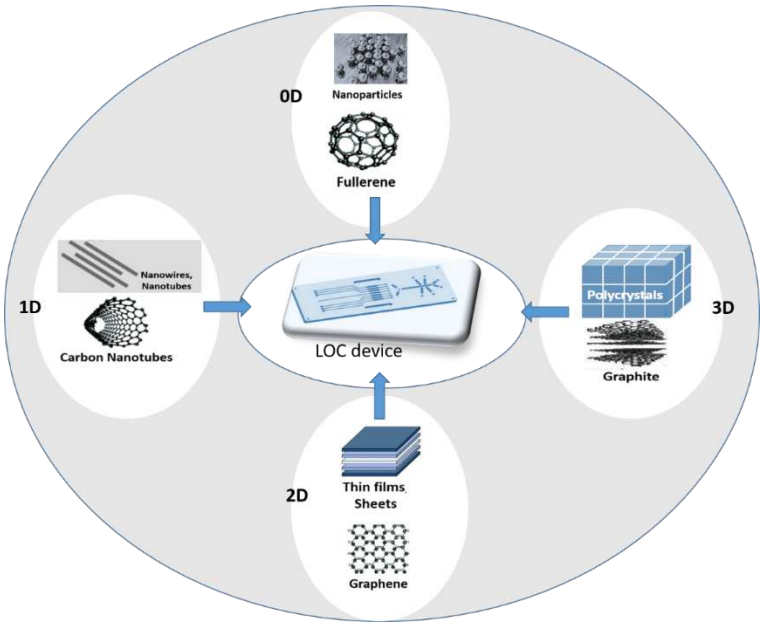

Figure 3. An overview of the nanomaterials used for biosensing.

Metallic nanostructures, such as gold [57], silver [58], and platinum [59], have been widely used in electrochemical sensors integrated into microfluidic devices for the detection of molecules in body fluids. Gold nanoparticles (AuNPs), for example, have been extensively studied due to their unique electronic, optical, and surface properties, which make them ideal for use in biosensing applications [60]. AuNPs have been used in a variety of electrochemical sensors for the detection of different biomolecules, such as glucose, cholesterol [61], and prostate-specific antigen (PSA) [62] .

Magnetic nanoparticles (MNPs), such as iron oxide nanoparticles, have also been used in electrochemical biosensors integrated into microfluidic devices for the detection of biomolecules in bodily fluids. MNPs have unique magnetic and surface properties, which make them ideal for use in biosensing applications [63]. These nanomaterials not only improve the limit of detection of the sensors, but also enable the separation and transportation of bioanalytes inside the microfluidic device, thereby allowing for the miniaturization of analytical methods [64]. For example, MNPs functionalized with iridium oxide nanoparticles and tyrosinase have been used for the detection and quantification of methimazole in microsystems. In the analytical measurements a permanent magnet

was used to immobilize the magnetic complex on the electrode surface. The system was highly sensitive with a low limit of detection (0.004 μM) and demonstrated effectiveness in serum samples. Interestingly, the use of microfluidic device allows for improve limit of detection, reusability, automation, volume of the sample, and response time compared to batch configuration [65].

Among nonmetallic nanomaterials, carbon nanotubes (CNTs) [66], graphene [67], and quantum dots [68,69] (QDs) have shown high sensitivity towards various analytes, including glucose [70], cholesterol [71], and proteins, [72] with detection limits in the sub-nanomolar range. Moreover, carbon-based nanomaterials such as graphene and carbon nanotubes have also been combined with metallic nanoparticles or polymeric layers to produce nanocomposites with improved performance [73–75] in terms of electronic transfer or selectivity.

One of the challenges associated with the use of nanomaterials in electrochemical sensors integrated into microfluidic devices is the reproducibility and stability of the sensors. Due to the small size of nanomaterials, synthesizing and functionalizing these materials can be challenging, leading to variations in sensor performance. Additionally, the stability of nanomaterial-based sensors can be affected by factors such as temperature, pH, and humidity, resulting in reduced sensor performance over time. Efforts are being made to address these challenges, by developing reproducible synthesis and functionalization methods and optimizing sensor design to enhance stability.

Another challenge related to the use of nanomaterials in electrochemical sensors integrated into microfluidic devices is the integration of these sensors into practical clinical applications. While many studies have demonstrated the feasibility of using nanomaterial-based sensors for detecting biomolecules in body fluids, further development and optimization are required before these sensors can be widely adopted in clinical settings. This includes optimizing sensitivity, selectivity, and stability of the sensors, as well as developing user-friendly and cost-effective instrumentation for their use. In fact, despite the enhanced sensor performance offered by nanomaterials implemented on electrode surfaces, the two-dimensional planar electrodes can still limit component and signal transmission when used in vivo, thereby affecting sensor accuracy and sensitivity [76,77]. This limitation is particularly true for complex samples such as blood or plasma used in point-of-care-devices [78,79]. Furthermore, the planar structure of two-dimensional electrodes poses challenges in achieving adequate immobilization of active components and efficient signal transmission, leading to potential issues in sensing accuracy. To address these limitations, the integration of macroscale three-dimensional (3D) porous materials, comprising nanomaterials combined with polymers [80–83] can be employed as electrodes. This approach facilitates expanded microfluidic transport and enables the incorporation of multianalyte detection capabilities, thereby enhancing the overall performance of the sensor system.

The incorporation of porous channels in biosensing systems offers several advantages, including increased surface area, enhanced ion/mass transport pathways, and improved immobilization and stability of active components. In this context, graphene has emerged as a promising avenue for the development of three-dimensional (3D) electrodes. Graphene can be fabricated in the form of aerogel or combined with polymers, providing an excellent opportunity to create highly efficient and versatile 3D electrode structures. Furthermore, the surface of graphene can be easily engineered with other nanomaterials and biorecognition elements. For instance, Xu et al. [84] demonstrated the use of a graphene foam (GF) modified with carbon-doped titanium dioxide nanofibers (nTiO2) as an electrochemical working electrode. The three-dimensional and porous structure of the GF facilitated the penetration and attachment of nTiO2 onto its surface, resulting in enhanced charge transfer resistance, increased surface area, and improved access of the analyte to the sensing surface. The GF-nTiO2 composite was further functionalized with the ErB2 antibody for specific detection of the target ErbB2 antigen, a biomarker for breast cancer. The sensor was employed for quantification of the ErbB2 antigen using differential pulse voltammetry and electrochemical impedance spectroscopy techniques. Remarkably, both methods exhibited high sensitivity across a wide concentration range of the target antigen, demonstrating excellent specificity even in the presence of other members of the EGFR family.

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In another study, Zhang et al. [85] conducted a study where they developed an enzymatic electrochemical microfluidic biosensor for glucose detection. The biosensor incorporated a three-dimensional porous graphene aerogel and glucose oxidase (GOx), taking advantage of the aerogel's high electrical conductivity and specific surface area to enhance the immobilization of GOx. The microfluidic system implemented in the biosensor reduced sample consumption during testing. The biosensor exhibited excellent selectivity and stability and successfully monitored glucose levels in serum samples. This innovative biosensor shows promise for clinical applications in diabetes diagnosis, and the method employed for preparing the graphene aerogel modified electrode holds potential for broader use in diverse electrochemical sensors.

In addition to high sensitivity, nanomaterial-based sensors are highly selective, enabling the detection of specific molecules in complex biological samples. This selectivity is achieved through the functionalization and modification of nanomaterials, by attaching specific ligands (e.g. antibodies, DNA, RNA, aptamers, and enzymes) [86] through covalent or non-covalent interactions to enhance specificity and electronic transfer in sensors. For example, Fan et al. (2022) developed a smartphone-based electrochemical system composed of CNTs functionalized with gold nanoparticles, thionine, and antibodies for the detection of CA125, a biomarker for prostate cancer [87]. The biosensor exhibited high selectivity towards CA125, with no interference from other biomolecules present in human serum.

Another approach to obtaining sensors with high specificity is the creation of molecular imprinted polymers (MIPs). MIPs are polymer-based artificial receptors with the ability to recognize different types of target molecules such as aminoacids, peptides [88], pesticides [89] and drugs [90] but even larger molecules such as proteins [91] or whole cells [92]. The target molecules act as template and interact with functional monomers to form a complex during the polymerization, then the template can be removed and leaving cavities able to rebind template molecules thank to its geometry ant chemical moieties. MIPs have been largerly used as recognition elements in electrochemical sensors offering great advantages such as improved stability, cost-effectiveness and rapid fabrication procedure overcoming limitations of natural receptors (antibodies, nucleic acids, peptides) such as sensitivity to enzymatic digestion, low preservation temperature, etc.

So, combining the advantages of MIPs and electrochemical transducers several sensing platform have been realized joining sensitivity and ease to use of electrochemical sensors with high selectivity and stability of MIPs [93]. To realize a high-performance sensing platform based on MIP as artificial receptor it is necessary to consider at least two key aspect: (i) the choice of polymer and (ii) imprinting processes. Electrochemical sensors are compatible with different imprinting approaches such as in situ bulk polymerization, surface imprinting and electrosynthesis.

The most commonly employed approach for imprinting is bulk imprinting, wherein the transducer surface is coated with a mixture of template and pre-polymer that exhibit mutual interaction. Then after polymerization template molecules are entrapped inside the polymer matrix and can be removed by a washing step creating cavities able to recognize the analyte in the subsequent analytic steps. To apply this technique to larger molecules is necessary to realize a very thin layer of polymer so the imprinted binding sites are near the interface making template removal and rebinding easier. Another possibility for the recognition of large molecules such as proteins is epitope imprinting that consists of imprinting only a portion of the target molecules [94].

An alternative approach is based on electrosynthesis in which polymerization is induced by applying a suitable potential range to a solution containing the monomers with the template molecules without any initiator. The characteristics of resulting films can be tuned by modulating electrochemical parameters. Conductive polymers (CP) and insulators/non-conductive polymers (NCP) can be used with different advantages and disadvantages. Non-conducting MIP films self-limits their growth, so allow a fine control on their thickness, while CPs are more flexible and offer the possibility to tune not only the thickness but even the conductive properties by changing the deposition conditions. The selection of polymers is strictly related to the detection methods: for example capacitive [95] or impedimetric [96] sensors require nonconductive polymers, while for amperometric detection is better to use conductive ones [97].

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Surface imprinting is one of the most used technique for the development of MIPs for large molecules, cells and microorganisms: it consists in the template imprinting only on the MIP surface. Several techniques such as soft lithography, micro-contact imprinting, and sacrificial template support methods have been exploited to confine the imprinted sites on the MIP interface [98].

The analytical performances of electrochemical MIP-based sensors can be furtherly improved by combining MIP technology with nanomaterials and realizing imprinted nanocomposite. Different nanomaterials have been used to this purpose ranging from carbon based materials (e.g. nanotubes [99,100], graphene [101]) to metallic nanoparticles [102]: this transition towards nanoMIP significantly improved the analytical performances of MIP based sensors in terms of detection limits and sensitivity and the nanostructuration of the material allows for better diffusion of the analyte on the transducer surface, resulting in a faster response time for the sensor. Nevertheless further efforts are still necessary to have standardized procedures for industrial applications and medical diagnostics.

3. Electrochemical Biosensors

Electrochemical biosensors are interesting because they are simple to miniaturize and enable low-cost mass production. In the following section the most used techniques in electrochemical biosensors and their working principle, are briefly illustrated and discussed.

3.1. Amperometric Method

In amperometric biosensors the electrode current is measured, and most amperometric electrochemical biosensors are based on an enzyme's redox activity (most often horseradish peroxidase, (HRP)). Enzymes enhance biosensing systems by catalyzing chemical reactions, improving sensitivity [103]. The performance of enzyme-based biosensors relies on factors such as electrode surface, enzyme type, substrate, and mediator usage [104]. HRP is commonly employed as a secondary detection reagent, with the TMB/H2O2 substrate proving most effective. Amperometric biosensors measure current at a constant potential to detect the analyte. This method provides selectivity as the potential used is characteristic of the analyte. The current is measured after directly setting the desired potential, enhancing the accuracy of the analysis [105].

Zhang et al. reported the development of a nonenzymatic immunosensor for the detection of SCC-Ag, utilizing rGO-TEPA and AuAg NCs. [106]. In another study, a competitive RNA/RNA hybridization assay-based biosensor was developed using Streptavidin-Horseradish Peroxidase (SA-HRP) and biotinylated capture probes. The biosensor employed H2O2 as an enzyme-substrate and hydroquinone (HQ) as a RedOx mediator. Two separate platforms, a screen-printed electrode (SPE) with Au NPs and a GCE with tungsten diselenide and Au NPs, were used in the biosensor [107]. Additionally, a microfluidic amperometric immunosensor was developed for the detection of the cancer biomarker CLD7 in circulating extracellular vesicles (EVs). The immunosensor was validated in colorectal cancer (CC) patients [108].

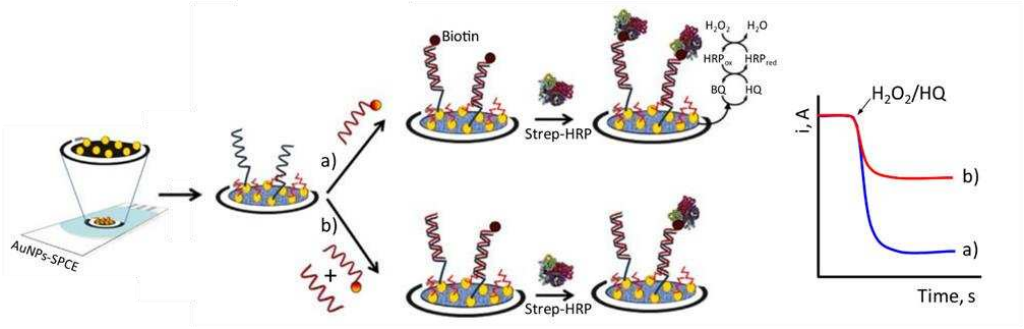

Figure 4. Direct competitive hybridization assay developed for miRNA determination shown schematically (adapted from the paper 98).

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Chronoamperometry is a variation of amperometric techniques that involves monitoring the current generated by the faradaic process at the electrode over time. It involves applying a sufficiently large potential step to the working electrode to initiate a chemical reaction, and then observing the current as a function of time.

3.2. Potentiometric Method

Potentiometric biosensors use a tiny amount of current to monitor the potential of an electrochemical cell [109]. Potentiometric sensors, employing the controlled-current method, utilize an electrochemical cell containing two reference electrodes to measure the potential across an ion-selective membrane. Enzymes are commonly used to facilitate ion production, which is then detected by the supporting electrode. Controlled-current methods offer the advantage of using more affordable measurement instrumentation compared to controlled-potential methods. The Nernst equation relates concentration and potential in potentiometric measurements, offering a low limit of detection (LOD) for early-stage cancer diagnosis [110]. Jia et al. developed a Light Addressable Potentiometric Sensor (LAPS) for the detection of the liver cancer biomarker hPRL-3 [111]. Another study [112] utilized surface molecular imprinted self-assembled monolayers (SAM) for a potentiometric biosensor with a linear range of 2.5-250 ng/ml [113]. Label-free potentiometric detection targeted the HAPLN1 protein biomarker in MPM, achieving pM range LOD. Goda et al. created a hybridization-based potentiometric microarray for exosomal miRNA identification [114].

Potentiometric methods have been employed to target malignant cells, investigating their electrochemistry in microenvironments affected by lactate release and pH fluctuations. Shaibani et al. achieved a LOD of 103 cells per ml, revealing pH flux alterations around cancer cells and their connection to altered cell metabolism [115]. A selective detection of circulating tumor cells (CTCs) in prostate cancer was achieved by utilizing an anti-EpCAM functionalized graphene oxide potentiometric biosensor based on the Light Addressable Potentiometric Sensor (LAPS) approach [116].

Based on pH monitoring, a sensor-integrated microfluidic technique is employed to find cancer cells. In this instance, the CTC's metabolic alteration resulted in a decrease in the pH of the surrounding environment. Potentiometric methods with Ag/AgCl and ZnO electrodes were employed to assess pH variations and cell modifications in vitro, utilizing three cell lines (A549, A7r5, and MDCK) in a microfluidic setup [117].

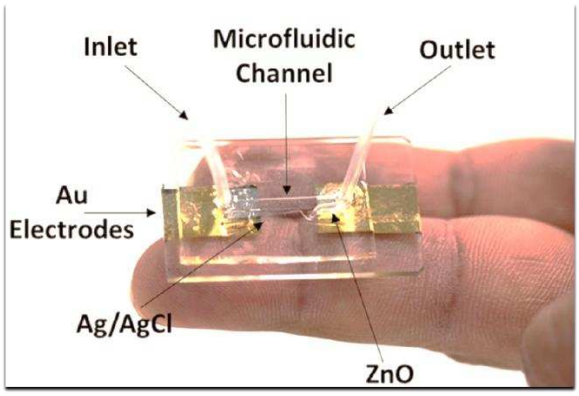

Figure 5. A microfluidics-based pH sensor was successfully created with the use of rf sputtered ZnO thin films and Ag/AgCl ink (Adapted from paper 108).

3.3. Impedimetric Method

Electrochemical impedance spectroscopy (EIS) is a technique that examines the resistive and capacitive characteristics of a system by applying an AC excitation signal with varying frequencies. By analyzing the impedance spectra, it is possible to determine the resistive and capacitive components of the system based on the in-phase and out-of-phase current responses. At higher

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frequencies, the migration rate of redox species can become rate-limiting, resulting in a frequency-dependent phase lag when analytes impede access to the electrode surface.

Electrochemical impedance spectroscopy (EIS) evaluates interfacial characteristics, ion passage, and biomolecule interactions with electrode surfaces. It involves applying AC potential to an electrochemical cell and measuring the resulting current signal. The resulting frequency-dependent impedance is represented on a Nyquist plot using a Randles circuit, showing semicircles at higher frequencies for electron transfer restrictions and a linear line at lower frequencies indicating diffusion-limited electron transfer.

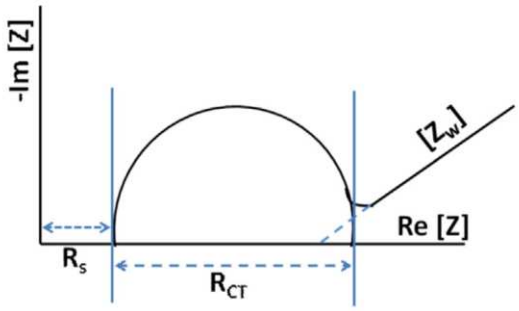

Figure 6. The Nyquist plot exhibits a depressed arc, indicating polarization caused by a combination of kinetic and diffusion processes.

R_{ct} value reflects electron-transfer kinetics, while RS represents bulk electrolyte characteristics and ZW represents diffusion. The Nyquist plot helps calculate ZW, represented by a 45-degree sloped straight line intercept. EIS has been utilized for the label-free detection of cancer cells.

Elshafey et al. developed a label-free impedimetric biosensor for detecting the cancer biomarker EGFR. The biosensor utilized protein G and gold nanoparticles on a modified gold electrode for efficient immobilization. The calibration curve exhibited a wide dynamic range of 1 pg/mL to 1 g/mL, with a low detection limit of 0.34 pg/mL in PBS and 0.88 pg/mL in human plasma. Interference from various substances in human plasma led to slight variations in the electrochemical signal during real-world experiments [118]. Han et al. developed a label-free cytosensor for cancer cell identification using phage display technology and EIS, demonstrating rising Rct values with increasing cell concentration, indicating reduced electron transfer efficiency. The approach employed a specific phage immobilized on a gold electrode, with [Fe(CN)6] as the redox probe indicator, offering high specificity, repeatability, and eliminating the need for complex recognition element purification [119].

Hu et al. utilized EIS for the detection of liver cancer cells. They immobilized a mannose-specific lectin (con A) on a gold electrode, leading to changes in charge transfer resistance that correlated with the concentration of cancer cells (Bel-7404). This label-free approach directly targeted cancer cells, providing a direct, selective, and sensitive method with a detection limit of 234 cells/mL, eliminating the requirement for probe labeling [120]. Azzouzi et al. developed an impedimetric electrochemical biosensor using biotinylated DNA/LNA molecular beacon (MB) probe linked with gold nanoparticles (AuNPs) for miRNA-21 detection in blood samples. The biosensor demonstrated high selectivity, good repeatability, and a wide linear detection range of 1-1000 pM, with a low detection limit of 0.3 pM. The use of neutravidin as a recognition element on the electrode surface enhanced biosensing properties, including sensitivity [121]. Kilic et al. introduced a label-free electrochemical sensor to compare the secretion levels of extracellular vesicles (EVs) in hypoxia and normoxic MCF-7 cells. The sensor utilized functionalized gold electrodes and employed Differential Pulse Voltammetry (DPV) and Electrochemical Impedance Spectroscopy (EIS) for EV detection. The sensor exhibited a linear operating range of 102-109 EVs/ml, with a limit of detection (LOD) of 77 EVs/ml. Selectivity was assessed using RhD protein, and the results were compared with ELISA and Nanoparticle Tracking Analysis (NTA) [122].

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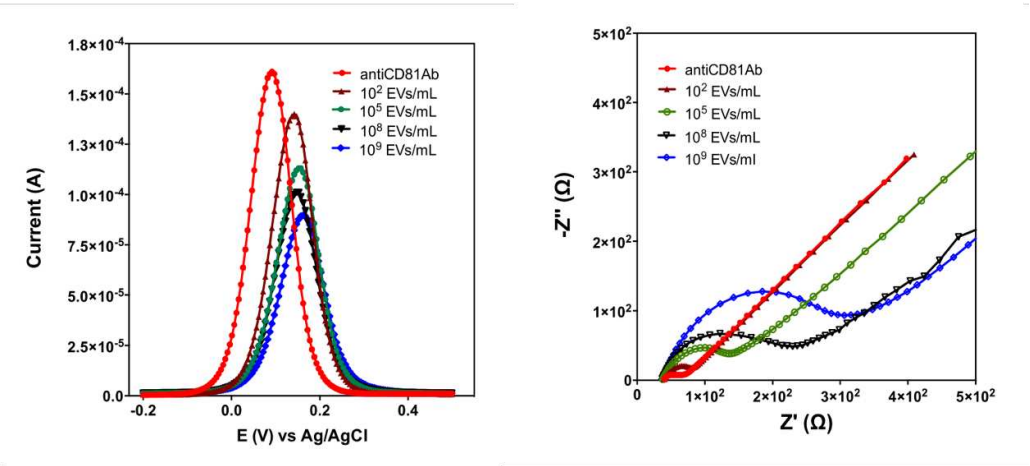

Figure 7. Differential pulse voltammograms recorded for various EVs concentrations (10–1010 EVs/ml) (left) and EIS measurements in the concentration range of 102–5×106 EVs/ml (right) – (adapted from paper 113).

3.4. Conductometric Method

Conductometric biosensors offer exciting possibilities for advanced bioanalytical detection. They measure changes in electrical conductivity during chemical reactions, using enzymes to modify the ionic strength of the sample solution. These biosensors are advantageous due to their miniaturization potential, low voltage requirement, and lack of a reference electrode. They are promising for applications in healthcare, environmental monitoring, and food safety [123]. Capacitive biosensors have the benefit of capacitance measurements being more informative of the biosensor's insulating qualities [124]. Even slight sensor layer desorption generally results in a rise in the capacitance baseline. Furthermore, nonspecific binding is less likely with capacitive sensors.

Liang et al. established a conductometric immunoassay for the detection of alpha-fetoprotein (AFP) in the serum of liver cancer patients. The assay demonstrated robust conductometric responses, achieving a low detection limit of 4.8 pg/mL across a dynamic linear range of 0.01-100 ng/mL [125]. For the quantification of prostate cancer antigen, Bhardwaj et al. [126] introduced a conductometric immunosensing platform utilizing tetracyanoquinodimethane (TCNQ)-doped thin films of copper-MOF, Cu3(BTC)2. The platform exhibited a dynamic linear range of 0.1-100 ng/mL for PSA detection, with a limit of detection as low as 0.06 ng/mL. Lin and co-authors devised conductometric sensors based on silicon nanowires for the detection of apolipoprotein A1, a biomarker associated with bladder cancer. The sensors demonstrated a wide dynamic range spanning from 0.2 ng/mL to 10 µg/mL, with a detection limit of approximately 1 ng/mL [127].

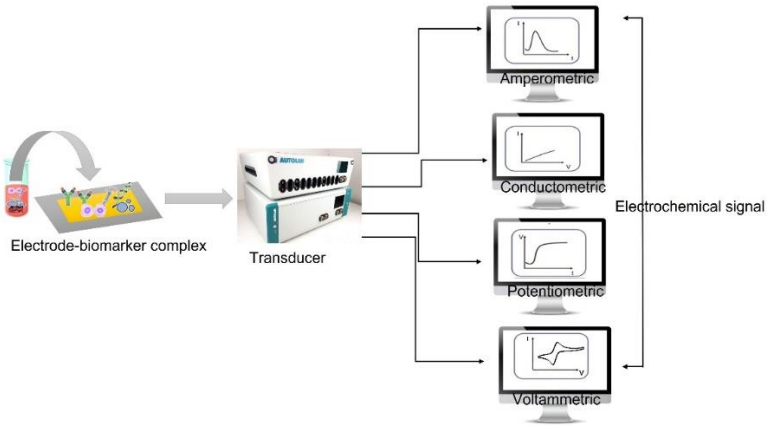

Figure 8. A general presentation illustrating diverse electrochemical techniques employed in biosensing.

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3.5. Voltammetric Method

Voltammetric biosensors measure current intensity by applying potential between a working and reference electrode, detecting analyte concentration. They offer high sensitivity, multiplexed biomarker detection, and serve as valuable point-of-care diagnostic devices. [128].

Voltammetry is widely used for characterizing reaction kinetics and obtaining qualitative and quantitative information about analytes. It provides valuable insights into complex electrode reactions through current measurements. Voltammetry offers technical advantages and fewer limitations compared to other techniques for quantitative determination. It is commonly employed for detecting biomarkers in liquid biopsy. The voltammetric methods broadly used for the detection of biomarkers under liquid biopsy, are described in the following sections.

3.5.1. Cyclic Voltammetry (CV)

Cyclic Voltammetry (CV) is a widely used method for investigating redox processes, monitoring reaction intermediates, and assessing reaction product stability. It involves measuring the potential between the working electrode and reference electrode while measuring the current between the working electrode and counter electrode. CV plots the electrochemical current on the y-axis and the working electrode potential on the x-axis, with the potential cycling back to its starting value. It provides information on oxidation and reduction of redox species. [129]. A partial cycle, a complete cycle, or a series of cycles might be performed depending on the results of the study. The electrons are transferred from the analyte to the WE or from the electrodes to the analyte during the redox reaction.

Kumar et al. utilized nanostructured zirconia (nZrO2) as a transducer surface in a CV-based technique for detecting the oral cancer biomarker (CYFRA). The immobilized receptor antibody (anti-CYFRA) on amine functionalized nZrO2 showed proportional electrochemical current changes, enabling detection in the range of 2-16 ng/ml with a sensitivity of 2.2 mA ml/ng. [130]. Later on, Wang et al. developed an electrochemical sensor using a glassy carbon electrode modified with silver hybridized mesoporous silica nanoparticles (Ag@MSNs) to detect the prostate cancer biomarker PSA. The sensor exhibited improved bioreceptor adsorption and electron-transfer rates, utilizing hydroquinone as a redox probe. PSA detection was achieved in a wide concentration range of 0.05 to 50.0 ng/ml, with a detection limit of 15 pg/ml. [131]. In a study conducted by Kumar et al. based on two-dimensional Ti3C2-MXene nanosheets to detect carcinoembryonic antigen (CEA) biomarker with a detection range of 0.0001–2000 ng/ml [132].

Taleat et al. employed a sandwich technique for detecting MUC1 protein, a key contributor to tumor development in various cancers. They combined MUC1 monoclonal antibody immobilized on a poly-aminobenzoic acid modified graphite screen printed electrode with a methylene blue modified aptamer (specific ss-DNA). [133]. Feng et al. coupled CV and electrochemiluminescence (ECL) techniques to achieve simultaneous detection of AFP and CEA by tagging the detection antibody, using methylene blue as an electrochemical indicator that binds directly to aptamers' G base for MUC1 protein concentration identification. [134].

3.5.2. Differential Pulse Voltammetry (DPV)

DPV (Differential Pulse Voltammetry) involves scanning potential with small amplitude pulses while measuring current at two points before and after each pulse. The difference in current measurements is calculated and plotted as a function of the base potential, allowing for analysis of non-faradaic current decay.

DPV is a widely used electrochemical procedure known for its sensitivity and speed. It involves applying fixed-amplitude electrochemical pulses on a slowly increasing base potential and recording the resulting current differential. DPV has been utilized to detect early cancer and study drug performance in cancer. Lin et al. developed a reusable biosensor using a magnetic graphene oxide modified gold electrode (MGO-Au) to detect VEGF in human plasma for cancer detection [135]. The

DPV-based sensor demonstrated effective sensitivity, rapid reaction time, and a wide linear detection range, outperforming the ELISA approach.

Amjadi et al. studied the influence of doxorubicin (DOX) and Flavonoid modified drug (FMD) on lung cancer cells (A549) using the DPV approach, revealing that FMD had a stronger effect on cancer cells compared to DOX, as demonstrated by the reduction in electrochemical reactivity with increasing drug concentration [136]. Additionally, Pacheco et al. and Wang et al. utilized electrochemical techniques (CV, DPV) with breast cancer cells immobilized on working electrodes to quantify cancer cells in unknown samples using a known cancer cell calibration curve. [137,138].

Fabrication and alteration of electrodes are important in electrochemical measurement. Screen printing technique is widely employed in this context for the manufacturing of portable low-cost electronics, particularly disposable electrodes. Compared to conventional electrode fabrication methods, this technology presents numerous advantages, encompassing precise manipulation of electrode dimensions, a wide range of electrode designs, compact device sizes, reduced production expenses, user-friendly operation, and the capability to create diverse arrays of electrodes [139]. Furthermore, screen printed electrodes allow for additional customization of the electrode surface by altering it with different nanomaterials, resulting in increased surface area, biomolecule immobilization efficiency, and unique electrochemical characteristics.

Using eight disposable screen printed microelectrode arrays as the transducer surface, Zani et al. established a sensitive and easy PSA detection technique. Magnetic beads were employed to collect the main PSA antibody in this experiment. The electrochemical measurements were taken using DPV after the collected beads were washed with antibody-labelled enzyme alkalinephosphatase (AP). The detection range of this biosensor was linear (0–20 ng/mL), with a lower detection limit of 1.4 ng/mL [140]. Erdem et al. described an electrochemical biosensor based on a multichannel screen-printed array of electrodes (MUX-SPE16) for assessing the nucleic acid hybridization of distinct miRNA sequences (miRNA-16, miRNA-15a and miRNA-660). In this study, streptavidin-coated magnetic beads were placed on the electrode surface before a biotinylated DNA probe was immobilized. Following the hybridization procedure, the electrochemical response was measured using the DPV method on the guanine oxidation signal [141].

Furthermore, due to the limited sensitivity and specificity of a single biomarker, measuring or tracking it, is insufficient for reliable cancer diagnosis. Therefore, Researchers are interested in simultaneous detection of numerous tumor markers obtaining more accurate and dependable results. Serum VEGF-C had a specificity of 68 % and a sensitivity of 85 %, whereas MMP-9 had a specificity of 75 % and a sensitivity of 63 %. Similarly, VEGF had a specificity of 59 % and a sensitivity of 80 %, but the combination of these three markers had a greater sensitivity and specificity (83 % and 76 %, respectively) than the single biomarker strategy for lung cancer detection [142]. CEA has also been demonstrated to enhance cancer prediction when combined with other biomarkers. When CEA is combined with CA 15-3, for example, its sensitivity rises from 89 % to 96 % [143].

Wu et al. introduced a novel approach for the concurrent detection of CA 19-9 and CA 125 cancer biomarkers, which involved the utilization of a disposable two-throughput immune-electrode array. The researchers applied a cellulose acetate membrane onto the graphite working electrodes (W1 and W2) of a screen-printed chip, followed by the co-immobilization of thionine/CA 19-9 and thionine/CA 125 on separate electrodes. Antibodies labeled with HRP were then detected on these working electrodes. By establishing an electron shuttle mechanism facilitated by immobilized thionine, the enzymatic reduction of H2O2 by HRP resulted in the generation of electrochemical signals, enabling the detection of both biomarkers simultaneously [144,145].

Two separate cancer biomarkers (CEA and AFP) were identified concurrently utilizing the DPV technology and a metal ions tagged immunocolloidal gold nanocomposite as a signal tag in another manner. Signal antibody was modified with two metal ions (AuNPs/anti-CEA/Cu21 and AuNPs/anti-AFP/Pb21) in this manner. The author leveraged the intrinsic electrochemical characteristics of metal ions in this study to obtain a multiplex detection of cancer biomarkers on a single platform with high sensitivity. The findings were also confirmed using a conventional ELISA, indicating that they might be used in clinical settings [146].

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In the realm of quantitative real-time evaluation and early cancer detection, circulating tumor markers (CTMs) such as extracellular vesicles (microvesicles and exosomes), circulating tumor cells (CTC), and circulating nucleic acids in the blood have emerged as valuable indicators. These CTMs offer a range of advantages, including their potential as cost-effective, reproducible, dynamic, and non-invasive diagnostic tools for both cancer diagnosis and monitoring of disease progression during the early stages. Several detection techniques have been employed to identify CTMs, including quartz crystal measurement (QCM), microcantilevers, colorimetric assays, enzyme-linked immunosorbent assay (ELISA), surface enhanced Raman scattering (SERS), surface plasmon resonance, polymerase chain reaction (PCR), and electrochemical methods [147].

Moscovici et al. developed a microfabricated glass chip with gold apertures for cell counting using DPV, enabling specific detection of 125 prostate cancer cells in 15 minutes, even in complex cell populations [148]. Yang et al. demonstrated a microelectrode-based electrochemical biosensor for micro-RNA detection using nanostructured palladium electrodes, achieving detection as low as 10 aM of target with enhanced signals using the DPV approach and Fe(III) regeneration of Ru(III) for amplification [149].

Zhang et al. utilized DPV to detect capecitabine in serum specimens without the need for labels, using an electrochemical biosensor based on stacked graphene nanofibers (SGNF) and gold nanoparticles (AuNPs). The biosensor showed a wide linear detection range of 0.05 to 80.00 M and an exceptional detection limit of 0.017 M for capecitabine electrochemical reduction [150]. Venu et al. have published an electrochemical biosensor based on a ZrO2/rGO nanocomposite for the detection of an anticancer medication (regorafenib, REG). The fabricated biosensor had a wider linear detection range of 11 to 343 nM, with a remarkable lower detection limit of 59 and a remarkable limit of quantifications of 59 and 17 nM, respectively. For the accurate assessment of REG, the biosensor's effectiveness was also good in both blood samples and pharmaceutical formulations. The biosensor was also useful for detecting REG, uric acid, and ascorbic acid all at the same time [151].

3.5.3. Linear Sweep Voltammetry (LSV)

Throughout the scan, the electrode potential is adjusted at a constant rate, and the resultant current is recorded in LSV. Yan et al., developed an immunosensor of carbon combining screen-printing with the excellent material vegetable parchment. The proposed immunosensor, involving linear sweep voltammetry as electrochemical method and prostate specific antigen (PSA) as a model analyte, showed a limit of detection to 2 pg /mL [152].

In their study, Bo et al. presented an electrochemical immunosensor that employed a double signal amplification strategy utilizing enzyme-encapsulated liposomes and biocatalytic metal deposition. This innovative approach was specifically developed for the detection of human prostate-specific antigen (PSA). Linear sweep voltammetry (LSV) was employed to measure the quantity of deposited silver, which served as an indicator of the target analyte. The experimental findings demonstrated a linear relationship between the anodic stripping peak current and the concentration of PSA within the range of 0.01-100 ng/ml. Impressively, the detection limit achieved by the sensor was as low as 0.007 ng/ml, thereby illustrating its high sensitivity for PSA detection.

3.5.4. Square Wave Voltammetry (SWV)

The excitation signal in SWV consists of a symmetrical square-wave pulse with amplitude Esw superimposed over a staircase waveform with step height E, where the waveform's forward pulse corresponds to the staircase step. The difference between the forward and reverse currents is used to calculate the net current, which is centred on the redox potential. SWV provides various benefits, including high sensitivity, speed, and non-faradic current discrimination.

The combination of autocatalytic deposition and square-wave stripping voltammetry with enlarged gold nanoparticles labeled on goat anti-rabbit immunoglobulin G enabled the detection of rabbit immunoglobulin G (RIgG) analyte with a remarkably low limit of 1.6 fM, highlighting the sensitivity enhancement of the electrochemical immunoassay (GaRIgG-Au) method. [153]. In a subsequent study, Liu et al. conducted a comparative analysis between Square wave voltammetry

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(SWV) and electrochemical impedance spectroscopy (EIS) for the development of a label-free electrochemical immunosensor targeting the hormone estradiol. The researchers aimed to assess the performance of these two techniques in terms of sensitivity. Notably, the results revealed that SWV outperformed EIS, with a lower detection limit of 18 pg/mL for estradiol, as compared to 26 pg/mL achieved by EIS. This comparative evaluation highlighted the superior sensitivity of SWV in the context of estradiol detection, suggesting its potential for enhanced analytical applications in hormone analysis [154]. Zhang at el., detected CTCs using SWV based on catalytic amplification and the limit of detection of was 1 cell/mL.

3.5.5. Stripping Voltammetry (SV)

This technique has the lowest detection limits respect to the commonly used electrochemical techniques. SV is also known as Pre-concentration Technique, and most commonly used stripping voltametric techniques involve anodic stripping voltammetry, cathodic stripping voltammetry, and adsorptive stripping voltammetry.

Despite the fact that each approach has its own distinct characteristics, they all follow the same two procedures. The target analyte is first concentrated onto the working electrode in the sample solution. In the second phase, potential is used to remove the preconcentrated analyte off the electrode surface, which is then measured. In the stripping process, potential waveforms such as linear sweep, differential pulse, and square wave can be employed. Due to their ability to distinguish against charging current, differential pulses and square waves are the most prevalent. In addition, as compared to differential pulse, square wave offers the advantages of a faster scan rate and greater sensitivity.

The Joseph Wang group introduced a novel electrically heated carbon paste electrode designed specifically for conducting adsorptive stripping measurements of trace amounts of nucleic acids. This groundbreaking approach combines the principles of electrochemistry with electrically heated electrodes and adsorptive constant-current stripping chronopotentiometry. The integration of these techniques brings forth notable advantages when it comes to the precise detection and quantification of nucleic acids at trace levels. This innovative coupling of electrochemistry with electrically heated electrodes presents a promising avenue for achieving enhanced accuracy and sensitivity in nucleic acid analysis [155].

4. Sensors Integration

In the advancement of point-of-care and wearable biosensors, miniaturization plays a pivotal role in achieving portability, user-friendliness, cost-effectiveness, as well as maintaining high sensitivity with rapid response times. The downsizing of sensing electrodes or their constituents to the nanoscale, as seen in nanoelectrode, nanotextured, nanogap, and field-effect transistor-based biosensors, can significantly enhance the signal-to-noise ratio. It is worth noting, however, that this enhancement can potentially introduce an undesired increase in the biosensor's response time. Therefore, while nanoscale miniaturization offers various advantages, careful consideration must be given to strike a balance between improved sensitivity and response time to ensure optimal performance of the biosensor.

The above-mentioned electrochemical methods, discussed in the related paragraphs, are well-suited for implementation in miniaturized systems. Achieving miniaturization, along with the features discussed in section 2, is essential for creating compact, user-friendly, and cost-effective point-of-care, portable, and wearable biosensors. These biosensors offer competitive limits-of-detection and rapid response times while maintaining their convenient and accessible nature.

4.1. Lab-on-Chip Platforms: Wearable and Portable Devices for POC

Lab-on-a-chip (LOC) devices notably multitasking devices, show the most attractive advantages of performing several lab procedures on a single chip with little volumes of chemicals and great efficiency. One of the most challenging feature of LOCs, also known as a micro total analysis system

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(μTAS) is to fully integrate microelectromechanical system (MEMS) with automated microfluidic tools to allow their widespread use in medical applications (liquid biopsy, therapeutic follow-ups, health and diseases monitoring). These user-friendly, sensitive and portable LOC sensors for real-time analysis has various benefits over traditional analytical techniques. In this review papers we have listed several examples of sensors suitable for integration into LOC devices. Nevertheless, the step over technological gap to overcome limitations in the widespread diffusion of such devices is still linked to scalability of systems and poor affordability of highly integrated platforms. Only few examples indeed are the commercially available platforms including electrochemical systems and their use for monitoring health parameter is going in the direction of self-diagnostics and fitness applications but not toward becoming a gold standard in clinical practices.

Recently, Atkcakoca and coworkers reported on the realization of an electrochemical biosensor with integrated microheater improving the performance of the nucleic acid hybridisation assay based on Electrochemical Impedance Spectroscopy, paving the way for the development of highly sensitive and specific integrated label-free biosensors [156]. Kasturi and collaborators developed a microfluidic channel with integrated valves and electrochemical biosensor for the detection of beta-amyloid, a biomarker for Alzheimer's disease. Gold microelectrodes are transducer holding the antibody-antigen complexes inside the fluidic chambers governed by pneumatic valves. A linear response of the sensor through DPV measurements was reported for beta-amyloid antigen concentrations from 2.2 pM to 22 uM [157]. One of the most promising applications of integrated LOC for diagnostic purposes is the realization of smartphone-based and wearable devices, which can really realize all the features of portability, low-costs and rapid response needed to fully embody the Point-Of-Care concept.

Flexible electronic devices, making use of specific methods of fabrication and detection, have properties such as flexibility, wearability, conductivity, stretchability, mechanical resistance, and, biocompatibility. In this scenario, a plethora of material suitable as substrates for electrochemical transducers have been considered [158]: cellulose-based, polyaniline (PANI), polyimide (PI) and specific fabrication methods have been demonstrated. Most of them are able to electrochemically detect ions concentrations from skin and sweat as well as monitoring fitness parameters like heart bit, blood pressure, body temperature and oxygen concentration [159,160].

Sempionatto and collaborators, recently developed a non-invasive device for the simultaneous monitoring blood pressure and heart rate via ultrasonic transducers and multiple biomarkers via electrochemical sensors. They optimized the integrated device conformal to curved skin thus ensuring mechanical resistance and reliable sensing of several compounds: glucose in interstitial fluid; lactate, caffeine and alcohol in sweat [161].

Integration of small microfluidic channels into wearable devices is also a challenging topic for the development of such devices, since biological fluids, easily exploitable for liquid biopsy (sweat, interstitial fluid) could be conveyed toward the sensing areas of the devices.

Electrochemical sensors measure the reaction caused by the interaction between the sensing surface and the analytes and the corresponding response is then converted into electric signals that can be monitored by potentiometry, amperometry, and conductometry measurements [162]. The integration of electrochemical biosensors into point-of-care (PoC) platforms, especially when combined with smartphones, has emerging as a powerful tool for personalized health monitoring, thus enhancing the practicality of the diagnosis compared to the traditional laboratory-based diagnostic methods [163]. In the last years, smartphone-based biosensors have been widely employed for human health PoC testing to improve the diagnosis and treatment of several diseases, thanks to their cost-effectiveness, ease of use and portability. Figure 9 illustrates the diagnosis steps in smartphone-based electrochemical biosensors.

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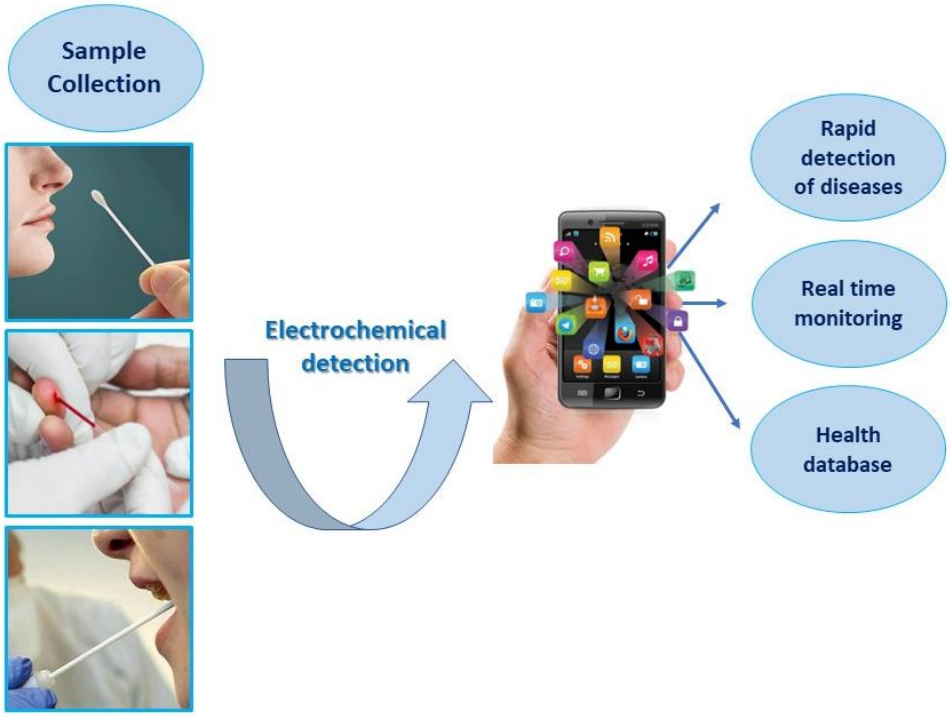

Figure 9. Smartphone-based electrochemical biosensors for health monitoring.

One of the first application of a smartphone-based electrochemical biosensor system was described as amperometric sensing [164]. Amperometry is a type of voltametric technique in which a constant voltage is applied to the working electrode, and the current provided by the oxidation/reduction of an electroactive analyte is then measured as a function of time. Liu et al. reported an amperometric aptasensor integrated with a smartphone assisted portable wireless biochip, for the simultaneous real-time monitoring of insulin and glucose in saliva with the lowest detectable concentration of 0.85 nM and 0.8 nM, respectively. The sensing platform, combined with a Bluetooth transmission system to generate the digital diagnosis by smartphone signal readout, provided a PoC testing tool for the diagnosis of diabetes and other insulin-resistance-associated diseases [165]. Voltammetry is another electrochemical technique which includes different type of measurements such as differential pulse voltammetry (DPV), cyclic voltammetry (CV), square wave voltammetry (SWV), and linear sweep voltammetry (LSV), widely used in smartphone integrated electrochemical sensors [166]. In 2020 Low et al. developed a DPV-based electrochemical sensor combined with smartphone for the detection of circulating miRNA-21 biomarker in saliva. They demonstrated that the smartphone based biosensing system, equipped with a specific Android application, and displayed comparable performances with commercial workstations for the detection of miRNA-21 [167]. Combining a screen-printed immunosensor with a smartphone based electrochemical system, Fan et al. reported the detection of cancer antigen 125 (CA125) by DPV measurements. Data were transmitted to smartphone through Bluetooth, acting as interface for the communication with remote medical center via Internet. The developed system provided a sensitive detection of CA125 with a LOD of 2 mU mL-1 [168].

Electrochemical impedance spectroscopy (EIS), in which the impedance of the system is measured as a function of frequency, is also widely used in the fabrication of EIS-based electrochemical sensors combined with smartphones. For example, Talukder et al. reported a portable system for personalized monitoring of blood cell count consisting of a smartphone-based microfluidic impedance cytometer [169].

The recent advances in this field highlight the advantages of electrochemical sensing systems integrated with smartphone, thanks to their high simplicity of fabrication [170]. Moreover, the

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development of multiplexing smartphone-based electrochemical systems can be used for the simultaneous detection of various biomarkers, thus enabling remote control of diseases by doctors and accelerating the diagnosis and treatment of pathology.

5. Comparison of Liquid Biopsy Electrochemical Methods: Advantages and Limitations

Nowadays cancer is the major disease affecting human health and life and its large diffusion requires the development of simple, practical and facile diagnosis methods for simplifying its treatment and improving its cure rate. Compared with medical imaging and pathological examination, which are the most common cancer diagnosis methods, liquid biopsy represents a promising strategy for cancer biomarker detection, opening the way to direct and rapid diagnostic methods with high efficacy [171,172]. Among the several biomarkers, circulating tumor cells (CTCs) have been well-established as promising targets for the detection of tumor liquid biopsy. Since tumor cells can be shed into the blood before the formation of visible solid tumor lesions, detecting CTCs before the imaging findings or the clinical manifestations, is an efficient method for early diagnosis and monitoring of cancer [173]. However, the content of CTCs in peripheral blood circulation is very little and the techniques used for its detection (fluorescence imaging, magnetic resonance imaging, cytological detection [174–176]) have several shortcomings, such as high cost, long time, low sensitivity, lack of specificity, thus limiting their use in clinical applications [177].

In the last years, electrochemical sensing technology has been widely investigated as a good alternative method for the detection of CTCs because of its advantages of high sensitivity, good selectivity, low cost, easy portability and rapid detection. Compared with traditional detection techniques, electrochemical methods demonstrate competitive results in terms of LOD and selectivity. CTCs can be electrochemically detected by using two common types of approaches. The first one is often related to the impedimetric sensors and exploits the change of electron transfer produced by CTCs capture on the electrode, usually conjugated to various recognitive materials including antibody [178], aptamer [179] and receptors [180]. However, this type of sensor usually needs just one electrode to work, thus resulting in a lack of the capture efficiency. To overcome this limit, modifying electrodes with nanomaterials, can be a good strategy to enhance the capture efficiency for CTCs. For example, Wang et al. conjugated gold nanostars with high surface area, with CTCs specific aptamer. Owing to this designing, the sensor showed a sensitive detection limit of 5 cells/mL [181]. Cai et al. developed a dual recognition electrochemical cytosensor for the detection of CTCs. The sensor based on Cabot carbon black (BP2000)/AuNPs anchoring anti- epithelial cell adhesion molecule (anti-EpCAM) antibodies as capture probes and novel branched PtAuRh trimetallic nanospheres (b-PtAuRh TNS) linked with aptamers targeting mucin1 (MUC1) as signal probes, exhibited a wide linear range of 5 - 1 × 106 cells mL-1 and a low detection limit of 1 cell mL^{-1} [182]. In another work, Zhang et al. exploited the reaction of LiFePO4 with sodium molybdate to generate electrochemical signal for detecting CTCs. In particular, they captured CTCs from sample by using Fe3O4 magnetic nanospheres (MNs) modified with EpCAM antibody, while gold nanoparticles modified LiFePO4 (LiFePO4/Au) were used as electrochemical probe. The assay presented a detection range from 3 to 10,000 cell mL-1 with the detection limit of 1 cell mL^{-1} [183]. Although several studies have demonstrated the advantages of electrochemical sensing technology for the detection of CTCs and the significant advances in biosensing research area thanks to immunotechnology, microfluidic and nanotechnology, the clinical use of such biosensors is still limited. In fact, the number of CTCs in peripheral blood circulation is very little and its detection can be very difficult. Moreover, the existing detection techniques use nucleic acids and antibodies as target molecules which, lacking of specificity for the classification of captured CTCs, cannot be utilized to give precise information about patient-specific tumor biology. Thus, combining advanced technologies such as microfluidic and DNA walker, and exploring more cell-specific targets, could be a significative strategy to improve the sensitivity and specificity of such biosensors [184] .

As the voltammetry is the most commonly used electrochemical technique for liquid biopsies, this review primarily focuses on DPV technique, a subtype of voltammetry because DPV is considered to be an important electrochemical method for liquid biopsy applications because it offers

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high sensitivity, selectivity, wide dynamic range, rapid analysis, and minimal interference from other components in body fluids.

Scientists can determine which electrochemical methods are best suited for particular applications by comparing the various electrochemical techniques used in liquid biopsy biosensing, depending on elements like the type of biomarker being analyzed, the concentration range, and the complexity of the sample matrix.

Such a comparison can help to guide the development of new biosensors for liquid biopsy analysis, as well as to optimize existing methods for improved performance and sensitivity. Additionally, understanding the advantages and limitations of different electrochemical methods can aid in the interpretation of experimental results, and can inform the selection of appropriate analytical methods for a given research question.

Table 2. A comparison of various electrochemical methods employed in biosensing applications for liquid biopsy analysis.

EC Method	Advantages	Limitations
Potentiometric	▪ High selectivity for specific analytes through the use of ion-selective electrodes (ISEs) ▪ Wide range of analytes that can be detected using ISEs, including ions, gases, and molecules ▪ Simple instrumentation, with ISEs often consisting of a single electrode and a reference electrode ▪ Non-destructive, as the sample is not consumed during the analysis	▪ Limited sensitivity compared to other electrochemical methods ▪ Limited dynamic range, as ISEs typically have a limited linear response range ▪ Interference from other ions or molecules in the sample can affect the accuracy of the analysis ▪ Slow response time compared to other electrochemical methods
Impedimetric	▪ High sensitivity for certain analytes ▪ Can measure non-faradaic processes such as adsorption and desorption ▪ Can provide information on both the electron transfer kinetics and the charge transfer resistance of the system	▪ Requires complex instrumentation and data analysis ▪ Limited dynamic range compared to other electrochemical methods ▪ Sensitive to electrode fouling and surface defects

Conductometric	▪ High sensitivity, as changes in conductivity can be highly sensitive to analyte concentration ▪ Wide range of analytes that can be detected, including ions, gases, and molecules ▪ Simple instrumentation, with conductometric biosensors often consisting of a pair of electrodes and a transducer ▪ Non-destructive, as the sample is not consumed during the analysis	▪ Limited selectivity compared to other electrochemical methods ▪ Interference from other ions or molecules in the sample can affect the accuracy of the analysis ▪ May be affected by changes in temperature, humidity, and other environmental factors ▪ May require optimization of electrode and transducer properties to achieve the desired sensitivity and selectivity
Cyclic Voltammetry (CV)	▪ High sensitivity for certain analytes ▪ Simple instrumentation and low cost ▪ Can measure both oxidation and reduction reactions	▪ Limited selectivity; it can be affected by interfering species ▪ Low resolution; the current signal can be difficult to interpret ▪ Slow scan rate that can limit the speed of analysis
Differential PulseVoltammetry (DPV)	▪ High sensitivity and selectivity for certain analytes ▪ Wide dynamic range ▪ Rapid analysis ▪ Minimal interference from other components in the sample	▪ Limited applicability to certain types of analytes (e.g., those with weak redox activity) ▪ Requires careful optimization of parameters such as pulse width and amplitude ▪ High background noise can be a problem in complex samples
Stripping Voltammetry (SV)	▪ High sensitivity and selectivity for analytes, like heavy metals and trace elements ▪ Wide range of analytes that can be detected, including ions, gases, and molecules ▪ Non-destructive, as the sample is not consumed during the analysis ▪ Can be used for both qualitative and quantitative analysis	▪ Requires pre-concentration of the analyte before measurement, which can be time-consuming and may limit the speed of analysis ▪ Can be affected by interference from other species in the sample ▪ Limited dynamic range, particularly for quantitative analysis ▪ May require specialized instrumentation, such as a mercury electrode

Each electrochemical technique has advantages and disadvantages of its own. The choice of method will depend on the specific analyte of interest and the requirements of the analysis.

The desire to manufacture micrototal analysis systems, low-cost point-of-care diagnostics, and environmental monitoring devices has sparked the creation of tiny and portable biosensor devices. So, for the development of such biosensor it is essential to understand the electrochemical method on which this biosensor operates.

The efficient transducer surface or immobilization matrix are the most significant steps in the fabrication of a miniaturized electrochemical biosensor. For optimal biosensor performance, we need to carefully select materials, electrochemical methodology and manufacturing process. POC devices used for liquid biopsy might benefit from wise device design and efficient detection procedures. Research needs to be conducted to create combinatorial electrochemical biosensors with high throughput and low cost for cancer diagnosis, therapy, and monitoring utilizing liquid biopsy. The commercialization of biosensors will increase when an electrochemical-based biosensing platform works effectively in a real-world sample environment with excellent selectivity, sensitivity, and stability.

6. Future Perspectives and Concluding Remarks

This review paper highlights the significance of screening and early diagnosis in disease management, particularly in the context of cancer. It emphasizes the importance of non-invasive analytical methods capable of detecting biomarkers to facilitate successful treatments and improve patient survival rates. The focus of the study is on electrochemical methods used for the development of biosensors in liquid biopsy, owing to their ability to offer rapid response, precise detection, and low detection limits. The review discusses the advancements in electrochemical biosensors, which hold the potential to enhance the specificity and sensitivity of conventional analytical techniques. Electrochemical biosensors demonstrate the ability to detect minute quantities of analytes, including proteins, nucleic acids, and circulating tumor cells, even in complex bodily fluids such as urine, serum, and blood. Among the various detection techniques explored for cancer biomarker detection, voltammetric sensors are extensively discussed due to their advantages and technical characteristics, which have led to their widespread use in the quantitative detection of ions and molecules.

The review also provides a comprehensive comparison of different electrochemical techniques to aid in the selection of appropriate analytical methods based on specific requirements. This comparative analysis helps researchers and clinicians identify the most suitable approach for their intended applications. Looking towards the future, the development and refinement of electrochemical biosensors hold tremendous potential for advancing diagnostic capabilities in the field of liquid biopsy. Further advancements in sensor design, surface modification techniques, and integration with emerging technologies like nanomaterials and microfluidics are expected to enhance the performance, reliability, and multiplexing capabilities of electrochemical biosensors. These developments will likely contribute to improved disease detection and monitoring, enabling personalized and targeted treatment strategies.

However, it is important to acknowledge that while electrochemical biosensors offer great promise, there are still challenges to overcome. Some of these challenges include improving the selectivity and stability of the sensors, standardizing protocols for clinical use, and addressing the complexities associated with analyzing biomarkers in diverse biological matrices. The continuous innovation and optimization of electrochemical biosensing technologies are expected to play a vital role in improving disease diagnosis, monitoring treatment efficacy, and ultimately enhancing patient outcomes.

The potential and ambition of liquid biopsy pose significant challenges and opportunities for personalized medicine and Point-Of-Care diagnostics and follow-ups. Within this context, the utilization of miniaturized and rapid detection tools, such as electrochemical sensors, holds great promise for advancing the field. These devices can be seamlessly integrated into everyday objects like smartphones, smartwatches, and more. Furthermore, as individuals become increasingly health-conscious and emphasize early disease screening, the introduction and widespread use of user-friendly sensors, lab-on-chip devices, and similar tools for manipulating and analyzing biofluids are poised to greatly benefit self-awareness and disease detection.

Despite the significant advances in electrochemical biosensors for liquid biopsy, there are still some challenges and limitations that need to be addressed. These include the optimization of the biosensor design, the selection of the most suitable biomarkers and detection techniques, the validation of the biosensor performance in clinical samples, and the standardization of the biosensor fabrication and operation. Moreover, there is a need for more interdisciplinary collaboration among researchers from different fields, such as chemistry, biology, engineering, and medicine, to develop innovative and effective solutions for liquid biopsy. Furthermore, there is a potential for combining electrochemical biosensors with other analytical methods, such as optical or magnetic sensors, to achieve complementary and synergistic results. Electrochemical biosensors for liquid biopsy have a bright future ahead, as they can revolutionize the approach to diseases and improve the quality of life of patients.

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